Halosterols A and B, Chymotrypsin Inhibitory Sterols from *Haloxylon recurvum*

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Two new sterols, halosterols A (1) and B (2), have been isolated from the $CHCl_3$ soluble fraction of *Haloxylon recurvum*, and their structures were elucidated by spectroscopic techniques including two dimensional-NMR. Both the compounds displayed chymotrypsin enzyme inhibitory potential.

Key words *Haloxylon recurvum*; sterol; halosterol A; halosterol B; chymotrypsin inhibition

Haloxylon recurvum Bunge ex Boiss belongs to the family Chenopodiaceae which comprises 100 genera and 1200 species.[1)] In Pakistan this family is represented by 35 genera. Only five species of *Haloxylon* are found in Pakistan.[2)] *H. recurvum* is a perennial shrub with glabrous leaves. It is widely distributed in Turkey, Syria, Iraq, Iran, Afghanistan, Kashmir, India, and Central Asia.[3)] The plant is traditionally used as an external application to treat insect stings. The ash of the plant is used to treat internal ulcers.[4,5)] Local physicians use a decoction of this plant to treat viral disease. No phytochemical work has so far been reported on this species. The MeOH extract of *H. recurvum* showed strong toxicity in a brine shrimp lethality test. On fractionation, the major toxicity was observed in the $CHCl_3$ soluble fraction. Further pharmacological screening of the $CHCl_3$ soluble fraction showed strong inhibition against chymotrypsin enzyme. This prompted us to carry out bioassay directed isolation studies of the active constituents of this fraction. As a result we now report the isolation and structural elucidation of two new sterols named halosterols A (**1**) and B (**2**). Both of these showed inhibitory potential against chymotrypsin.

Results and Discussion

The MeOH extract of the whole plant of *H. recurvum* was processed as described in Experimental to afford two new sterols named halosterols A (**1**) and B (**2**). Halosterol A (**1**) was isolated as colorless crystals and gave positive Salkowski and Lieberman Burchard tests for steroids. The high resolution electron impact mass spectrum (HR-EI-MS) of **1** showed a molecular ion peak at *m*/*z* 428.3620, consistent with the molecular formula $C_{29}H_{48}O_2$ (Calcd for $C_{29}H_{48}O_2$, 428.3654) possessing six degrees of unsaturation. The IR spectrum of **1** showed absorption bands for a hydroxyl group (3300 cm^{-1}), α,β-unsaturated cyclic ketone (1686 cm^{-1}) and a conjugated double bond (1605 cm^{-1}). The UV spectrum displayed absorption at 238 nm, which is characteristic of a Δ^4-3-keto steroidal skeleton.[6)] In EI-MS the ion at *m*/*z* 410 was due to the loss of water molecules from the parent molecule, while the ion at *m*/*z* 229 was indicative diagnostic of steroids bearing a side chain at C-17.[7)] The prominent peaks in the EI-MS of **1** are *m*/*z* 386 (M−42; loss of ketene from ring A), *m*/*z* 371 (M−57; loss of ketene plus a methyl radical, *m*/*z* 345 (M−85; loss of C-1, 2, 3, 10 and 19), *m*/*z* 305 (M−123) and *m*/*z* 124. The latter two ions result from fission of the 6–7 and 9–10 bonds of ring B, with the charge remaining on either the hydrocarbon or oxygen containing fragment, respectively.[8)] The peaks at *m*/*z* 271 and 229 corresponded to the loss of the side chain and ring D fission, revealing the presence of second oxygen functionality in the side chain. The ^{1}H-NMR spectrum of **1** showed close resemblance to that of 24-ethyl-3-oxocholesta-4,22-dien-25-ol[9)] with specific differences in the side chain only. A proton singlet at δ 6.11 indicated a Δ^4-unsaturation,[10)] while an oxymethine proton was observed at δ 3.74 (1H, ddd, J=10.4, 3.0, 1.1 Hz). Two singlets at δ 0.89 and δ 0.68 were accounted by Me-19 and Me-18, respectively. The doublets at δ 0.99 (J=7.4 Hz), δ 0.85 (J=6.8 Hz) and δ 0.82 (J=6.8 Hz) corresponded to Me-21, Me-26 and Me-27, while the triplet at δ 0.62 (J=7.1 Hz) was assigned to Me-29. The broad band (BB) and distortionless enhancement by polarization transfer (DEPT) ^{13}C-NMR spectra revealed the presence of six methyl, ten methylene, nine methine and four quaternary carbons (Table 1). The downfield signal at δ 200.1 was due to a carbonyl carbon. Two downfield signals at δ 169.5 and 122.4 were assigned to C-5 and C-4, respectively. The signal at δ 72.2 was assigned to a hydroxyl bearing carbon. These assignments were made on the basis of comparison of chemical shifts with (24*R*)-24-methylcholest-5-en-3β-ol,[11,12)] and were confirmed through heteronuclear multiple quantum coherence (HMQC).

The hydroxyl group in the side chain could be assigned to C-22 on the basis of hetero nuclear multiple bond correlation

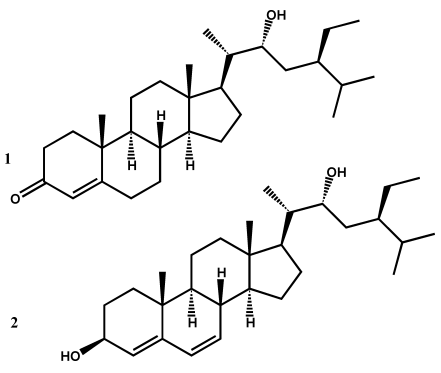

Fig. 1. Structures of Halosterols A (**1**) and B (**2**)

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Table 1. ^{13}C-NMR Data of Compounds **1** and **2** at 100 MHz in $CDCl_3$

Position	**1**	**2**
1	37.9	36.5
2	33.1	32.0
3	200.1	71.8
4	122.4	119.1
5	169.5	146.0
6	33.0	132.5
7	31.5	135.9
8	34.6	36.2
9	52.2	43.1
10	36.9	40.9
11	20.9	21.5
12	38.0	39.0
13	42.6	41.4
14	56.1	55.1
15	24.4	25.5
16	28.1	29.0
17	55.9	56.9
18	12.9	12.9
19	18.1	17.9
20	42.9	43.1
21	12.9	12.7
22	72.2	72.4
23	29.9	29.9
24	40.3	40.2
25	28.8	28.9
26	19.9	19.5
27	18.0	18.6
28	23.5	23.8
29	11.9	11.8

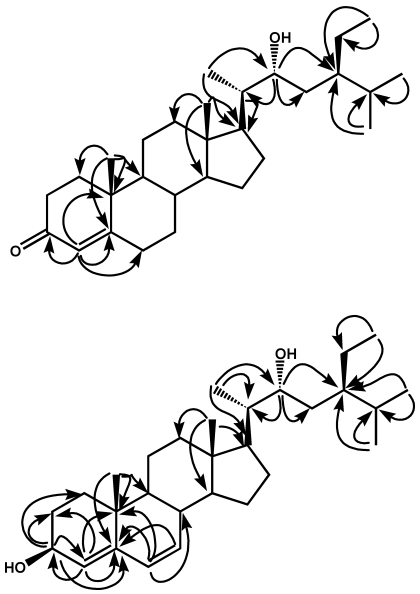

Fig. 2. Important HMBC Correlations of Halosterols A (**1**) and B (**2**)

Table 2. *In Vitro* Quantitative Inhibition of Chymotrypsin

Compound	$IC_{50}\pm$S.E.M.[a] (mm)
1	47.11±1.62
2	21.57±1.02
Chymostatin[b]	8.01±0.11

a) Standard mean error (S.E.M.) of three experimental determinations. *b*) Positive control used in assays.

(HMBC); the proton at δ 3.74 showed ^2J correlations with C-20 (δ 42.9) and C-23 (δ 29.9), and ^3J correlations with C-21 (δ 12.6) and C-17 (δ 55.9). The β configuration of this proton was suggested by the NOEs from H-21 to H-12α, H-17, H-18, and OH-22; from H-20 to H-18; and from H-22 to H-16α, and H-20. The utility of the ^{13}C-NMR chemical shift difference of C-20 has been reported to discriminate between (22*R*)- and (22*S*)-hydroxycholesterols, where the observed chemical shifts of C-20 were δ 42.6 and 40.3, respectively.[12] The application of this chemical shift rule to C-20 (δ 42.9) in **1** provided conclusive evidence for the *R* configuration of C-22. The configuration at C-24 was suggested to be *R* by comparing the ^{13}C-NMR data of C-20 to C-25 of **1** with those of scheicherastatin,[13] whose stereochemistry at C-24 has been established by X-ray crystal structure determination.[13] Thus, halosterol A (**1**) was assigned the structure (22*R*,24*R*)-24-ethyl-3-oxocholest-4-en-22-ol.

Halosterol B (**2**) was also obtained as colorless crystals which gave positive color tests for a sterol. The HR-EI-MS showed an M^+ peak at *m*/*z* 428.3612 corresponding to the molecular formula $C_{29}H_{48}O_2$ (Calcd for $C_{29}H_{48}O_2$, 428.3654). The IR spectrum showed absorption for a hydroxyl group (3300—3350 cm^{-1}) and conjugated double bond (1620 cm^{-1}). The UV spectrum showed absorptions at 232, 240 and 248 nm, which is characteristic of stigmasta-4,6-dien-3-β-ol.[14] The EI-MS showed an intense peak at *m*/*z* 271 due to the loss of the side chain, indicating the presence of one hydroxyl and two olefinic bonds in the steroidal nucleus. The ^{1}H-NMR spectrum showed hydroxymethine protons at δ 3.71 and δ 3.68, respectively. Other features were similar to those of **1** with notable differences in the signals of the steroidal nucleus. It showed the signals of a trisubsituted double bond at δ 5.85 as a doublet (J=4.4 Hz) and two further signals of a disubstituted double bond at δ 5.81 (d, J=10.2 Hz) and 5.45 (dd, J=10.2, 2.5 Hz). The hydroxyl group was assigned to the usual C-3 position on biogenetic analogy, ^{1}H–^{1}H correlation spectroscopy (COSY), HMBC correlation (Fig. 2) and comparison of NMR spectral data with 24β-ethylcholest-4-en-3β-ol.[14,15] Its stereochemistry was assigned as β and pseudo-equatorial based upon the coupling constants of H-3 (J=10.9, 4.4 Hz). The relative position of the double bond was confirmed through ^{1}H–^{1}H COSY correlation; H-3 showed connectivity to H-4 at δ 5.85 while H-7 at δ 5.45 showed connectivity to both H-6 at δ 5.81 and H-8 at δ 2.09. The HMBC correlations of the steroidal nucleus were in complete agreement with the presence of a double bond at C-4 and C-6 with the hydroxyl group at C-3. The ^{1}H- and ^{13}C-NMR chemical shifts of the side chain showed very close resemblance to those of **1**. This led us to deduce the structure of halosterol B (**2**) as (22*R*,24*R*)-24-ethylcholesta-4, 6-dien-3β,22-diol.

The importance of enzyme inhibitors as drugs is enormous since these molecules have been used for treating a number of physiological conditions.[16] Serine protease inhibitors have been proposed to be part of the plant's natural defense system against insect predation, and functions by inhibiting insect proteinases.[17—19] Hence, these inhibitors have gained attention as possible sources of engineered resistance against pests and pathogens in transgenic plants expressing heterologous inhibitors.[20] Chronic infection by hepatitis C virus can

lead to progressive liver injury, cirrhosis, and liver cancer. A chymotrypsin like serine protease known as NS3 protease, is thought to be essential for viral replication and has become a target for anti-HCV drugs.[21] The search for new and effective inhibitors of serine proteases is an urgent need in drug development, and identifying new chymotrypsin inhibitors appears to be a promising approach. Compounds **1** and **2** showed significant inhibition against chymotrypsin (Table 2).

Experimental

Experimental General Experimental Procedure UV and IR spectra were recorded on Hitachi-UV-3200 and Jasco-320-A spectrometers, respectively. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AM-400 spectrometer with tetramethylsilane (TMS) as an external standard. The 2D-NMR spectra were recorded on a Bruker AMX 500 NMR spectrometer. Optical rotations were measured on a Jasco DIP-360 digital polarimeter using a 10 cm tube. Mass spectra (EI and HR-EI-MS) were measured in an electron impact mode on Finnigan MAT 12 and MAT 312 spectrometers, and ions are given in *m*/*z* (%). TLC was performed on precoated silica gel F_{254} plates; the detection was done at 254 nm and by spraying with ceric sulphate reagent. Silica gel (E. Merck, 230—400 mesh) was used for column chromatography. Melting points were determined on a Gallenkemp apparatus and are uncorrected. For enzyme inhibition assays all the chemicals and chymotrypsin were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Plant Material The whole plant *Haloxylon recurvum* Bunge ex Boiss was collected from the Cholistan desert near the Bahawalpur district, Pakistan in October, 2001 and identified by Dr. Muhammad Arshad, Plant Taxonomist, Cholistan Institute of Desert Studies, Islamia University Bahawalpur, where a voucher specimen (020/CIDS/IUB/PK) has been deposited.

Extraction and Purification The air dried whole plant (20 kg) was exhaustively extracted with methanol (3×35 l) at room temperature. The extract was evaporated to yield a residue (650 g), which was partitioned between *n*-hexane (60 g), $CHCl_3$ (75 g), EtOAc (40.5 g), *n*-BuOH (55 g) and water (25 g). The $CHCl_3$ soluble fraction, which showed strong cytotoxicity and chymotrypsin inhibition, was subjected to column chromatography over silica gel and eluted with mixtures of *n*-hexane–$CHCl_3$, $CHCl_3$, and $CHCl_3$–MeOH, in increasing order of polarity. The fractions which were obtained from *n*-hexane : $CHCl_3$ (1.0 : 9.0) were combined, rechromatographed over silica gel and eluted with *n*-hexane : $CHCl_3$ in increasing order of polarity. The fractions obtained from *n*-hexane : $CHCl_3$ (1.5 : 8.5) were subjected to preparative TLC (*n*-hexane : $CHCl_3$: 0.5 : 9.5) to afford the pure compounds **1** (15 mg) and **2** (12 mg), respectively.

Halosterol A (**1**): Colorless crystals. mp 210 °C. ^{1}H-NMR ($CDCl_3$, 400 MHz), δ: 6.11 (1H, s, H-4), 3.74 (1H, ddd, J=10.4, 3.0, 1.1 Hz, H-22), 0.99 (3H, d, J=7.4 Hz, Me-21), 0.89 (3H, s, Me-19), 0.85 (3H, d, J=6.8 Hz, Me-26), 0.82 (3H, d, J=6.8 Hz, Me-27), 0.68 (3H, s, Me-18), 0.62 (3H, t, J=7.1 Hz, Me-29). ^{13}C-NMR ($CDCl_3$, 100 MHz) see Table 1. IR (KBr) ν_{max} cm^{-1}: 3300, 1686, 1605 1261, 1033, 805. UV (MeOH) λ_{max} (log ε): 238 nm (4.2). EI-MS *m*/*z* (rel int %) 428 (M^+, 18), 410 (29), 386 (15), 371 (48), 345 (28), 305 (39), 271 (32), 229 (100), 124 (50), 85 (22), 43 (48). HR-EI-MS Found *m*/*z* 428.3620 (Calcd for $C_{29}H_{48}O_2$, 428.3654). $[\alpha]_D^{20}$ −35.5° (c=1.0, $CHCl_3$).

Halosterol B (**2**): Colorless crystals. mp 195—197 °C. ^{1}H-NMR ($CDCl_3$, 400 MHz), δ: 5.85 (1H, d, J=4.4 Hz, H-4), 5.81 (1H, d, J=10.2 Hz, H-6), 5.45 (1H, dd, J=10.2, 2.5 Hz, H-7), 3.71 (1H, dd, J=10.9, 4.4 Hz, H-3α), 3.68 (1H, ddd, J=10.1, 3.6, 1.7 Hz, H-22), 0.98 (3H, d, J=7.1 Hz, Me-21), 0.95 (3H, s, Me-19), 0.85 (3H, d, J=6.2 Hz, Me-26), 0.81 (3H, d, J=6.2 Hz, Me-27), 0.66 (3H, s, Me-18), 0.62 (3H, t, J=7.0 Hz, Me-29). ^{13}C-NMR ($CDCl_3$, 100 MHz) see Table 1. IR (KBr) ν_{max} cm^{-1} 3300—3350, 2960, 2855, 1620 and 1380. UV (MeOH) λ_{max} (log ε) 232 (3.8), 240 (3.1) and 248 (4.1). EI-MS *m*/*z* (rel int %) 428 (M^+ 20), 395 (28), 392 (33), 271 (51), 269 (48), 230 (35), 198 (45), 157 (65), 81 (38), 70 (26), 55 (100), 43 (40). HR-EI-MS *m*/*z* 428.3612 (Calcd for $C_{29}H_{48}O_2$, 428.3654). $[\alpha]_D^{20}$ −72° (c=1.0, $CHCl_3$).

Chymotrypsin Inhibition Activities Chymotrypsin inhibitory activities of compounds **1** and **2** were determined by the method of Cannell *et al.*[22] (Table 2). Chymotrypsin (9 units/ml of 50 μM tris–HCl buffer, pH 7.6) was preincubated with compounds **1** and **2** for 20 min at 25 °C. One hundred microliters of substrate solution (*N*-succinyl-phenylalanine-*p*-nitroanilide, 1 mg/ml of 50 mM tris–HCl buffer, pH 7.6) was added to start the enzyme reaction. The absorbance of released *p*-nitroaniline was continuously monitored at 410 nm until a significant color change was observed. The final DMSO concentration in the reaction mixture was 7%.

Estimation of IC_{50} Values: The concentrations of test compounds **1** and **2** that inhibited the hydrolysis of substrates (chymotrypsin) 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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