Steroidal Alkaloids from *Holarrhena antidysenterica* (L.) Wall.[1)]

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Chemical investigations on the stem bark of *Holarrhena antidysenterica* resulted in the isolation of a new steroidal alkaloid designated as holadysenterine (1), together with three known steroidal alkaloids, conessine (2), isoconessimine (3) and kurchessine (4). Their structures were elucidated on the basis of 1D- and 2D-NMR techniques and high-resolution mass spectrometry.

Key words *Holarrhena antidysenterica*; Apocynaceae; steroidal alkaloid; holadysenterine; conessine; isoconessimine; kurchessine

Holarrhena antidysenterica (L.) Wall. (Apocynaceae) is a medicinal plant, found throughout the Indian subcontinent. Stem bark of the plant, commercially known as "kurchi", has been extensively investigsted due to its traditional use in the treatment of amoebic dysentery, diarrhea, asthma, bronchopneumonia and malaria.[2)] Stem bark and seeds of the plant are reported to contain a number of steroidal alkaloids, such as conanines, 3-aminoconanines, 20-aminoconanines, 3-aminopregnans, 3,20-diaminopregnanes and their derivatives.[3)] Being the principle alkaloid, conessine is mostly studied for its antidiarrhoel properties.[3)] Recently, the antiplasmodial activity of conessine and isoconessine has been reported against the chloroquine-resistant strain FcB1 of *Plasmodium falciparum*.[4)] In continuation to our previous attempt on the isolation of novel molecules from the Himalayan bioresource,[5—8)] a new steroidal alkaloid was isolated and characterized, designated as holadysenterine (**1**), together with three known steroidal alkaloids, conessine (**2**), isoconessimine (**3**) and kurchessine (**4**) from the stem bark of *H. antidysenterica*.

Results and Discussion

Alkaloidal fraction (1.19%) isolated from the stem bark (1 kg) of the plant was chromatographed over basic alumina, led to the isolation of compounds **1**—**4**.

Compound **1** was obtained as an amorphous powder. Its high-resolution mass spectrum in positive-ion FAB-MS showed a $[M+H]^+$ at m/z 391.2892 (Calcd for $C_{23}H_{39}N_2O_3$ 391.2961) corresponded to the molecular formula $C_{23}H_{38}N_2O_3$, indicating six degrees of unsaturation in the molecule. Four of these were accounted for a tetracyclic structure of pregnane-type skeleton and two were due to endocyclic double bond and acetamide functionality at the C-20 position. The IR spectrum showed absorption at 1618 (C=C), 1680 (amide carbonyl), 3410 (OH) and 3540 (NH) cm^{-1}.

The ^{1}H-NMR spectrum of **1** (Table 1) included two doublets at δ 3.41 (1H, d, J=11.7 Hz) and 3.75 (1H, d, J=11.7 Hz) and were assigned to the C-13 hydroxymethylene protons. The vinylic proton was recorded at δ 5.46 as a broad singlet. Two wide multiplets at δ 2.92 and 3.53 were assigned to H-3α- and H-20β-, respectively. The β-orientation of H-20 was deduced by comparing the spectral values with published data for steroidal alkaloids[9—19)] and biogenetic considerations keeping in view of the fact that all pregnane-type steroidal alkloids are biosynthesized from cholesterol *via* pregnenolone.[10,20)] The H-21 (3H, d, J=6.6 Hz) and H-19 (3H, s) methyl groups appeared at δ 1.38 and 1.09, respectively. A three proton singlet at δ 1.97 was due to the presence of acetamide methyl protons. A D_2O exchangeable proton singlet due to *N*-hydroxyl at δ 4.95 appeared to be overlapped with solvent (CD_3OD) signal.[21)] However, on D_2O exchange the C-21 methyl proton signal shielded to δ 1.11 which suggested the position of hydroxyl group at amine functional of side chain.[21)]

The ^{13}C-NMR spectrum of **1** (Table 1) showed 22 resonances, while the DEPT spectrum indicated the presence of three methyls, eight methylenes and seven methines. Signals easily identified were that of the carbonyls of the acetamide at δ 178.5 as well as those of the two vinylic carbons at δ 138.8 for C-5 and 122.2 for C-6. The downfield shift of 9.7 ppm[9)] in acetamide carbonyl resonance suggested the presence of a hydroxyl group attached to amine function of acetamide group. The attachment of hydroxyl group at amine center was further confirmed by the analysis of mass fragmentation data which showed the peaks at m/z 317 $[M+H-CH_3CONOH]^+$ (–C–N– bond cleavage), 289

Table 1. ^{1}H- and ^{13}C-NMR Spectral Data of Compound **1** (300, 75 MHz in CD_3OD)

Position	Group	δ_H (ppm)	δ_C (ppm)
1	CH_2	1.19 (m), 1.23 (m)	36.9
2	CH_2	1.38 (m), 1.63 (m)	26.7
3	CH	2.92 (m)	50.9
4	CH_2	2.02 (m), 2.34 (m)	36.7
5	C	—	138.8
6	CH	5.46 (br s)	122.2
7	CH_2	2.42 (m)	32.1
8	CH	1.54 (m)	31.6
9	CH	1.09 (m)	50.1
10	C	—	36.3
11	CH_2	1.18 (m), 1.60 (m)	19.8
12	CH_2	1.54 (m), 1.69 (m)	31.5
13	C	—	46.1
14	CH	1.60 (m)	54.7
15	CH_2	1.18 (m), 1.60 (m)	23.1
16	CH_2	1.63 (m)	22.5
17	CH	1.23 (m)	55.2
18	CH_2	3.41 (d, J=11.7), 3.75 (d, J=11.7)	57.0
19	CH_3	1.09 (s)	18.1
20	CH	3.53 (m)	48.4
21	CH_3	1.38 (d, J=6.6)	18.8
N–CO	C	—	178.5
CO–Me	CH_3	1.97 (s)	22.5

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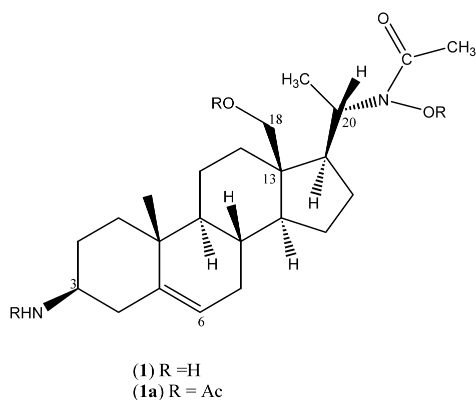

Fig. 1. Chemical Structure of Holadysenterine (**1**) and Triacetyl Holadysenterine (**1a**)

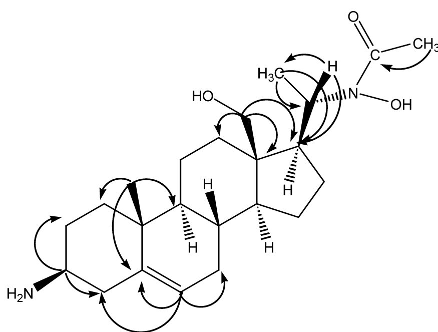

Fig. 2. Key HMBC Correlations of Holadysenterine (**1**)

$[M+H-C_4H_8NO_2]^+$ (C_{17}–C_{20} bond cleavage) and 278 $[M+H-C_5H_8NO_2]^+$ (C_{13}–C_{17} and C_{16}–C_{17} bonds cleavage). Two methyl signals at δ 18.1, 18.8 and at δ 22.5 were assigned to C-21, C-19 and methyl of acetamide group, respectively. Other signals at δ 57.0, 48.4 and 50.9 were accounted for C-13 hydroxymethylene, C-20 and C-3, respectively. The ^{1}H- and ^{13}C-NMR chemical shifts of **1** suggested it as 18-hydroxy derivative of 3,20-diaminopregnane.[21)]

Acetylation of **1** gave triacetyl derivative (**1a**) which displayed protonated molecular ion peak $[M+H]^+$ at *m/z* 517.3234. Its ^{1}H-NMR spectrum showed overlapped methyl singlet integrated for nine protons for acetyl groups at δ 1.93—1.96 (*N*-acetoxy, NH-Ac and N-Ac) and 2.04 (18-OAc). HMQC spectrum was used to establish the direct ^{1}H/^{13}C one-bond shift correlations of protonated carbons. The H-3 (δ 2.92) and H-20 (δ 3.53) methine protons showed direct ^{1}H/^{13}C connectivities with C-3 (δ 50.9) and C-20 (δ 48.4), respectively. The H-6 (δ 5.46) showed HMQC interaction with the C-6 (δ 122.2). Two doublet signals at δ 3.41 and 3.75 showed shift correlation with the C-18 (δ 57.0). The HMBC spectrum was very informative for accurate ^{13}C-NMR chemical shift assignments of the quaternary carbons and for locating the exact position of hydroxymethylene group. The proton of C-20 (δ 3.53) showed HMBC interactions with C-21 (δ 18.8) and C-17 (δ 55.2), while H-21 (δ 1.38) methyl protons exhibited long-range interaction with C-20 (δ 48.4) and C-17 (δ 55.2). The protons of hydroxymethylene (δ 3.41, 3.75) showed HMBC correlation with C-13 (δ 46.1), C-12 (δ 31.5), C-14 (δ 54.7) and C-17 (δ 55.2). The stereochemical assignments of stereogenic centers were made on the basis of chemical shifts comparisons with reported compounds and biogenetic considerations.[9—20)] Based on critical spectroscopic studies, the structure of compound **1** was established as (20*S*)-20-acetylhydroxylamino,3β-amino,13β-hydroxymethylenepregn-5-ene and was designated as holadysenterine.

Compounds **2**—**4**, having both conanine and pregnane type stereoidal skeleton, were identified as conessine (**2**), isoconessimine (**3**) and kurchessine (**4**) by the comparison of spectral data with those reported in literature.[4,22)]

Experimental

General Melting points were determined on Mettler FP 800 (Centeral Processor) and were uncorrected. IR spectra were recorded in KBr disks on a Perkin-Elmer 1760 FT-IR, ESI mass spectra were determined in QTOF-micro from Water's micromass and FAB mass spectrum was performed on Jeol 5×102 mass spectrometer with DA-6000 data system using argon (6 kV, 10 mA) as the FAB gas and *m*-nitrobenzyl alcohol as the matrix. ^{1}H- and ^{13}C-NMR spectra were recorded using a Bruker Avance-300 machine probes operated at 300 and 75 MHz, respectively. Column chromatography was carried out with basic alumina. All the chemicals used were purchased from Merck India Ltd.

Plant Material The plant material was collected in April 2004 during flowering stage from Pragpur area (Distt. Kangra), Himachal Pradesh, India.

Extraction and Isolation The air-dried and powdered stem bark of *H. antidysenterica* (1 kg) was extracted with MeOH (5×2.5 l) at room temperature. Combined percolations were dried under reduced pressure to yield 307.8 g as MeOH extract. The MeOH extract (307.8 g) was treated with 2 M HCl for 24 h (200 ml×3). The 2 M HCl soluble portion was partitioned with $CHCl_3$ (400 ml×4) to remove neutral impurities. The aqueous acidic layer was then made alkaline (pH 8.5) with liq. ammonia (30%) and repeatedly extracted with $CHCl_3$ (400 ml×5). The combined $CHCl_3$ fractions were washed with H_2O, dried and evaporated under vacuum to yield crude total alkaloids (11.9 g, 1.19%) as a dark brown sticky mass. The crude extract (11.9 g) was chromatographed over basic alumina, eluting with petroleum benzene (60—80 °C)–EtOAc mixtures with increasing proportion of EtOAc. Fractions 19—23, eluted with petroleum benzene–EtOAc (95 : 5) afforded a pure compound (**2**, 41 mg), which was positive to dragendorff's reagent. Fractions 30—33, eluted with petroleum benzene–EtOAc (92 : 8) gave compound **3** (19 mg). Similarly, fractions 35—36 in petroleum benzene–EtOAc (92 : 8) yielded compound **4** (15 mg). Fractions 221—225, eluted with EtOAc : MeOH (70 : 30) on crystallization with MeOH afforded compound **1** (20 mg).

Holadysenterine (**1**): White amorphous powder, $[\alpha]_D^{25}$ −14.4° (c=0.23, MeOH). mp 218—220 °C. IR (KBr) cm^{-1}: 3540, 3410, 1680, 1618, 1055, 980. ^{1}H- and ^{13}C-NMR (300, 75 MHz) spectral data: see Table 1. HR-FAB-MS *m/z*: 391.2892 $[M+H]^+$, (Calcd for $C_{23}H_{40}N_2O_3$ 391.2961), 317, 354, 307, 289, 278, 154, 136, 115, 107, 85.

Triacetyl Holadysenterine (**1a**): Acetylation of **1** with acetic anhydride and anhydrous pyridine at room temperature for 48 h resulted in white amorphous powder (**1a**) after purification by preparative TLC (EtOAc : hexane, 2 : 8, v/v) of ethyl extract of reaction product. IR (KBr) cm^{-1}: 1755, 1740, 1680, 1618, 980. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.08 (3H, s, H-19), 1.24 (3H, d, J=6.5 Hz, H-21), 1.93—1.96 (N-OAc, 2×N-Ac), 2.04 (18-OAc), 3.01 (1H, m, H-3), 4.01 (1H, d, J=11.2 Hz, Ha-18), 3.42 (1H, m, H-20), 4.33 (1H, d, J=11.2 Hz, Hb-18). HR-ESI-QTOF-MS *m/z*: 517.3234 $[M+H]^+$ (Calcd for $C_{29}H_{45}N_2O_6$ 517.3278).

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