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STEROIDAL CONSTITUENTS OF *HOLARRHENA PUBESCENS*

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ABSTRACT.—Two new compounds, an androstane steroid, kurchinicin (1,4-dien-11α,17β-dihydroxy-3-oxoandrostane) [**1**], and a base of the conanine series, kurchinine (11α-hydroxy-3-oxo-1,4,20-conatriene) [**2**], have been isolated along with a known compound, holadyson (1,4-dien-11α,20α-dihydroxy-18,20-oxido-3-oxopregnane) [**3**], from the bark of *Holarrhena pubescens*. Their structures have been established through spectroscopic studies.

In continuation of studies (1) on the bark of *Holarrhena pubescens* Buch. Ham [syn. *H. antidysenterica* L. (2), (Apocynaceae)], the isolation and structural elucidation of two new compounds and one known compound are reported. The new compounds are kurchinicin, a steroid of the pregnane series, and kurchinine, an alkaloid of the conanine series. The known compound has been identified as holadyson (3). The structural studies are based on ^{1}H- and 2D nmr experiments (COSY-45, NOESY, *J*-resolved and hetero-COSY).

RESULTS AND DISCUSSION

Kurchinicin [**1**] showed a molecular ion peak in the hrms at *m/z* 302.1886 corresponding to the molecular formula $C_{19}H_{26}O_3$ (calcd 302.1881). The molecular formula showed seven double-bond equivalents in the molecule. Compound **1** showed two singlets for tertiary methyls at δ 1.31 and 0.83 in the ^{1}H-nmr spectrum (Table 1). It exhibited strong absorptions in the ir spectrum at 1660, 1620 and 1600 cm^{-1} and uv absorption at 243 nm, indicating the presence of a conjugated carbonyl moiety. The ^{1}H-nmr spectrum showed three deshielded olefinic protons at δ 7.74 (1H, d, *J*=10.23 Hz, H-1), 6.14 (1H, dd, *J*=10.23, 2.04 Hz, H-2) and 6.09 (1H, t, *J*=2.04 Hz, H-4), thus accounting for three double-bond equivalents (2 C=C and 1 C=O). Because no other double bond was indicated by the spectral data, four remaining unsaturations were accounted for by four rings suggestive of a steroidal nucleus. The chemical shifts and coupling constants of the olefinic signals, along with a peak at *m/z* 121.0695, permitted the location of a 1,4-dien-3-one moiety in ring A of the steroidal molecule. This was further confirmed by ^{13}C nmr showing a signal at δ 186.8 characteristic of an α,β-unsaturated ketone, and other peaks at δ 158.7, 125.2, 124.7 and 167.9 for olefinic carbons.

A one-proton doublet of triplets at δ 4.07 (*J*=10.55, 10.55, 5.07 Hz) indicated an axial proton geminal to an OH group followed by a peak at δ 68.1 in the ^{13}C-nmr

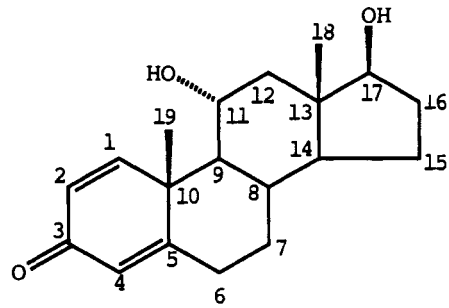

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TABLE 1. ^{1}H- and ^{13}C-Nmr Data of Kurchinicin [**1**].[a]

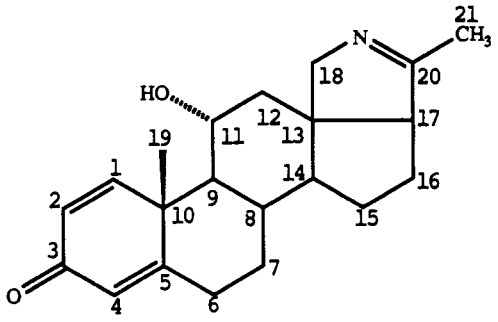
C	δ C	H	δ H	Multiplicity	*J* (Hz)
1	158.7	1	7.74	d	10.23
2	125.2	2	6.14	dd	10.23, 2.04
3	186.8	—	—	—	—
4	124.7	4	6.09	t	2.04
5	167.9	—	—	—	—
6	33.0	6a	2.45	m	—
		6b	1.05	m	—
7	31.9	7	1.70	m	—
8	34.7	8	1.44	m	—
9	60.6	9	1.08	m	—
10	43.5	—	—	—	—
11	68.1	11β	4.07	ddd	10.55, 10.55, 5.07
12	48.0	12a	2.20	m	—
		12b	1.18	m	—
13	44.0	—	—	—	—
14	49.5	14	2.15	m	—
15	23.0	15a	1.60	m	—
		15b	1.22	m	—
16	33.1	16a	1.50	m	—
		16b	1.42	m	—
17	80.8	17α	3.65	t	8.19
18	12.2	18	0.83	s	—
19	18.7	19	1.31	s	—

[a]These assignments were confirmed by COSY-45, *J*-resolved and hetero-COSY nmr techniques.

spectrum. The multiplicities and coupling constants of this signal suggested the location of the OH group at either C-7 or C-11. The downfield chemical shift (δ 7.74) of H-1 which is comparable with other 11α-OH compounds (4) located this group at position 11.

The remaining oxygen function was placed at C-17 with β-disposition on the basis of a triplet of $J=8.19$ Hz at δ 3.65, a characteristic of the 17β-OH androstane nucleus (5). A peak at δ 80.8 in the ^{13}C-nmr spectrum further supported an OH group in the five-membered ring D of the molecule. The multiplicity of various protons described above was also indicated by the 2D *J*-resolved spectrum, while the connectivities of various protons and protons/carbons were evident from COSY-45 and hetero-COSY nmr spectra (Table 1), respectively. Finally, the orientation of the 11α-OH group was manifested by the NOESY spectrum which showed connectivities of Me-19 with H-11β along with other expected interactions. These observations led to the assignment of structure **1** to kurchinicin.

The molecular formula ($C_{21}H_{27}NO_2$) of **2** was obtained through accurate mass measurement (found 325.2055, calcd 325.2041) determined for the molecular ion and showed nine double-bond equivalents. Its ir (1650, 1620 and 1600 cm^{-1}) and uv (245 nm) absorptions and the signals in the ^{1}H-nmr spectrum at δ 7.85 (1H, d, $J=10.26$ Hz), 6.13 (1H, dd, $J=10.26$, 1.97 Hz) and 6.08 (1H, t, $J=1.97$ Hz) (Table 2) and in the ^{13}C-nmr at δ 159.2, 125.1, 186.9, 124.7, and 168.1 were suggestive of the 1,4-dien-3-one system in ring A, which was supported by a strong peak at *m/z* 121.0675 (C_8H_9O, calcd 121.0653) in the hrms. The ir spectrum also showed the presence of an OH group (3400 cm^{-1}) which was placed at C-11 with α-disposition on the basis of the above discussion for compound **1**. However, in contrast to **1**, **2** showed only one tertiary Me group at δ 1.23 (Me-19). On the other hand, a three-proton singlet was observed at δ 2.02 due to



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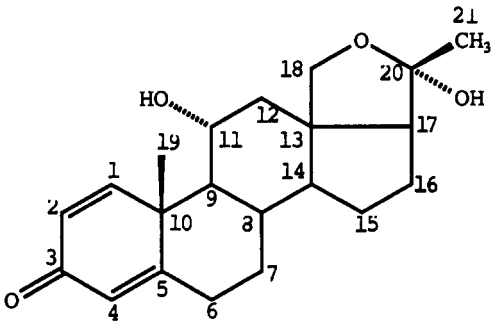
a vinylic Me which, together with an abundant ion at *m/z* 55.0418 (C_3H_5N, calcd 55.0421) indicated a -CH_2-N=C-Me moiety, commonly present as an 18,20-epimino ring in the conanine skeleton of the pregnane series of steroidal alkaloids (6). A triplet at δ 2.24 (J=7.47 Hz) for H-17 supported a double bond between C-20 and the N atom. By analogy with harmaline (7), an immediate collapse of the C-21 Me and H-17 signals present at δ 2.02 and δ 2.24, respectively, was observed due to exchange of these protons with deuterium when the ^{1}H-nmr spectrum was recorded in deuterated MeOH. Further, the deuterated compound gave an M^+ at *m/z* 330 in the fdms showing an increase of 5 mass units due to 21-CD_3, 17-CD and 11-OD. These experiments unambiguously proved the existence of an imine-enamine equilibrium and hence the carbon-nitrogen double bond between C-20 and the nitrogen in **2**. It was, therefore, concluded that kurchinine has structure **2**.

TABLE 2. ^{1}H- and ^{13}C-Nmr Data of Kurchinine [**2**].[a]

C	δ C	H	δ H	Multiplicity	J (Hz)
1	159.2	1	7.85	d	10.26
2	125.1	2	6.13	dd	10.26, 1.97
3	186.9	—	—	—	—
4	124.7	4	6.08	t	1.97
5	168.1	—	—	—	—
6	33.0	6α	2.49	ddd	13.28, 5.0, 5.0
		6β	2.39	ddd	13.28, 10.0, 5.0
7	29.7	7a	1.40	m	—
		7b	1.35	m	—
8	36.2	8	1.90	m	—
9	53.3	9	1.40	m	—
10	44.1	—	—	—	—
11	68.7	11β	4.08	ddd	11.42, 11.42, 5.19
12	43.6	12α	2.20	m	—
		12β	1.70	dd	14.00, 5.00
13	44.1	—	—	—	—
14	59.8	14	1.30	m	—
15	24.2	15	1.80	m	—
16	34.1	16a	2.30	m	—
		16b	2.05	m	—
17	36.2	17	2.24	t	7.47
18	65.6	18	4.10	m	—
19	18.6	19	1.23	s	—
20	168.1	—	—	—	—
21	18.6	21	2.02	s	—

[a]These assignments were confirmed by COSY-45, J-resolved and hetero-COSY nmr techniques.



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Compound **3**, molecular formula $C_{21}H_{28}O_4$, (M^+ 344.2009, calcd 344.1987) was identified as holadyson, on the basis of detailed ir, uv, ^{1}H-nmr and ^{13}C-nmr data (see Experimental); this compound has been reported previously from the same source. Its physical data is comparable with reported values (3). Exhaustive 2D nmr (COSY-45, NOESY, *J*-resolved and hetero-COSY) and ^{13}C-nmr (DEPT) studies have been undertaken with **3** for the first time, which resulted in the complete assignment of all the carbons and hydrogens. A connectivity of the Me-19 (β-orientation) resonance at δ 1.26 and the Me-21 signal at δ 1.48 was observed in the NOESY spectrum which led to the suggestion of the β-orientation of Me-21 and, hence, α-disposition of the OH group at C-20.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Mps are uncorrected. Ms were recorded on a Finnigan MAT 112 and 312 double-focussing mass spectrometer connected to a PDP 11/34 computer system; nmr spectra ($CDCl_3$; 400 MHz for ^{1}H and 75 MHz for ^{13}C), were recorded on a Bruker AM 300 and Bruker AM 400 FT-NMR spectrometer. The ^{13}C-nmr spectral assignments (Tables 1 and 2) have been made partly through a comparison of the chemical shifts with the published data for similar compounds (6) and partly through the appearance of signals in the DEPT and hetero-COSY spectra (Tables 1 and 2). Precoated thin-layer cards (DC-karten SiF) were used for tlc. The petroleum ether used was of the boiling range 60–70°.

PLANT MATERIAL.—The bark of *Holarrhena pubescens* (syn. *H. antidysenterica*) was supplied by the courtesy of the Hamdard Foundation Pakistan Ltd. It was identified by Miss Ashreen Jahan, botanist, Hamdard Foundation Pakistan Ltd.

EXTRACTION AND ISOLATION.—Uncrushed bark (10 kg) was macerated with 10% methanolic NaOH (10 liters) for 48 h at 28°, and repeatedly percolated with MeOH for 48 h (five times) at the same temperature following the reported procedure (8) in order to hydrolyze the tannates. Each extract was neutralized with 30% aq. HOAc. The pH of the syrupy concentrate (2 liters), obtained upon removal of the solvent from the combined extracts under reduced pressure, was reduced by adding 10% aqueous HOAc at 28°, and the solution was extracted with EtOAc. The aqueous phase was then basified with 20% ammonia and again shaken out with EtOAc. The second moist EtOAc phase was treated with a vigorous stream of CO_2. The precipitate containing the carbonate bases was filtered, and the filtrate was dried over anhydrous Na_2SO_4 and freed of the solvent under reduced pressure. The residue (20 g) was divided into petroleum ether-soluble and petroleum ether-insoluble fractions.

The petroleum ether-soluble fraction yielded conessine (9 g) according to the reported isolation procedure (8). The petroleum ether-insoluble portion (11 g), when dissolved in 10% aqueous AcOH and treated with $(NH_4)_2SO_4$, furnished a colorless precipitate of sulfates which was filtered. The sulfate mother liquor was made alkaline with 10% aqueous NaOH and extracted with EtOAc. The EtOAc phase was washed with H_2O to neutral pH, dried (Na_2SO_4) and evaporated under reduced pressure to give a colorless residue (9.5 g). This residue was subjected to flash column chromatography (Al_2O_3, Merck 90; petroleum ether, petroleum ether-EtOAc, in order of increasing polarity).

Nine fractions were ultimately obtained on combining the eluates on the basis of tlc. The major fraction eluted, starting with petroleum ether-EtOAc (4.5:5.5) up to pure EtOAc, was re-chromatographed on a flash column (Eyela, EF-10 Si gel, E. Merck 9385, petroleum ether, petroleum ether-EtOAc, in order of

increasing polarity). The petroleum ether-EtOAc (6:4) eluates furnished a crude fraction (A; 42 mg), along with a pure compound **2** (22 mg), in order of polarity. Similarly the petroleum ether-EtOAc (4.5:5.5) eluate afforded pure **1** (17 mg). The crude fraction A was purified through thick-layer chromatography, over Si gel with solvent system of $CHCl_3$-MeOH (9:1), affording **3** (13 mg).

1,4-Dien-11α,17β-dihydroxy-3-oxoandrostane [**1**].—Plates (MeOH); mp 182–184°, uv λ max (MeOH) 202, 243 nm; ir ν max ($CHCl_3$) 3500, 3450, 2950, 1660, 1620, 1600, 1440, 1150 cm^{-1}; eims *m/z* $[M]^+$ 302.1886 ($C_{19}H_{26}O_3$ requires $[M]^+$ 302.1881) (100), 284.1800 (34), 181.1236 (79), 163.1106 (16), 149.0220 (17), 131.0816 (15), 121.0695 (84), 98.0768 (9); ^1H nmr and ^{13}C nmr; Table 1.

11α-Hydroxy-3-oxo-1,4,20-conatriene [**2**].—Plates (MeOH); mp 202–204°, uv λ max (MeOH) 203, 245 nm; ir ν max ($CHCl_3$) 3650, 3400, 1650, 1620, 1600, 1150 cm^{-1}; eims *m/z* $[M]^+$ 325.2055 ($C_{21}H_{27}NO_2$ requires $[M]^+$ 325.2041) (100), 310.1814 (44), 204.1387 (62), 191.1299 (22), 161.1014 (10), 121.0697 (33), 108.0847 (8), 55.0418 (11); ^1H nmr and ^{13}C nmr; Table 2.

1,4-Dien-11α,20α-dihydroxy-18,20-oxido-3-oxopregnane [**3**].—Plates (MeOH); mp 138–140°, uv λ max (MeOH) 203, 246 nm; ir ν max ($CHCl_3$) 3500, 3450, 2900, 2650, 1650, 1620, 1600, 1590, 1150 cm^{-1}; eims *m/z* $[M]^+$ 344.2009 ($C_{21}H_{28}O_4$ requires $[M]^+$ 344.1987) (20), 326.1892 (100), 284.1776 (5), 266.1650 (8), 205.1266 (55), 161.0996 (14), 147.0880 (21), 134.0789 (11), 121.0693 (37); ^1H nmr δ 7.81 (d, J=10.29 Hz, 1H, H-1), 6.15 (dd, J=10.29, 1.99 Hz, 1H, H-2), 6.09 (t, J=1.99 Hz, 1H, H-4), 2.42 (m, 1H, H-6a) 1.15 (m, 1H, H-6b), 1.60 (m, 2H, H-7), 1.42 (m, 1H, H-8), 1.12 (m, 1H, H-9), 3.68 (m, 1H, H-11β), 2.62 (m, 1H, H-12a), 1.58 (m, 1H, H-12b), 2.10 (m, 1H, H-14), 1.48 (m, 2H, H-15), 2.28 (m, 1H, H-16a), 2.02 (m, 1H, H-16b), 1.30 (m, 1H, H-17), 3.78 (d, J=8.82 Hz, H-18a), 3.69 (d, J=8.82 Hz, H-18b), 1.26 (s, 3H, Me-19), 1.48 (s, 3H, Me-21); ^{13}C nmr δ 158.8 (C-1), 125.5 (C-2), 186.5 (C-3), 124.8 (C-4), 168.1 (C-5), 33.7 (C-6), 27.3 (C-7), 33.6 (C-8), 59.0 (C-9), 44.1 (C-10), 68.2 (C-11), 49.0 (C-12), 50.1 (C-13), 53.9 (C-14), 24.4 (C-15), 35.7 (C-16), 55.8 (C-17), 72.8 (C-18), 18.6 (C-19), 115.5 (C-20), 18.6 (C-21).

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