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Pregnane steroids from the heartwood of *Azadirachta indica*

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The heartwood of *Azadirachta indica* (Meliaceae) yielded a new steroid (2α, 3β, 4β-trihydroxypregnan-16-one) and 2 known steroids (2β, 3β, 4β-trihydroxypregnan-16-one and 2α, 3α, 4β-trihydroxypregnan-16-one). Their structures were elucidated through spectral studies including 2D NMR (COSY, NOESY, J-resolved, HMQC, HMBC) experiments.

Key Words: *Azadirachta indica*, Meliaceae, heartwood, steroids, 2α, 3β, 4β-trihydroxypregnan-16-one, 2β, 3β, 4β-trihydroxypregnan-16-one, 2α, 3α, 4β-trihydroxypregnan-16-one.

Introduction

An indigenous tree of the Indo-Pakistan subcontinent *Azadirachta indica* A.Juss (Linn. *Melia indica*), known commonly as *neem*, belongs to the family Meliaceae (order Rutales). It is widely distributed in Asia, Africa, other tropical and subtropical regions, and semiarid to wet tropical regions about 700 m above sea level.[1,2] Various parts of the tree are highly reputed for the treatment of several human ailments including diseases of bacterial and fungal origin, ulcers, eczema, jaundice, and liver complaints.[3,4] A large number of chemical constituents from different parts of *Azadirachta indica* have been reported;[5–12] however, the phytochemistry of the heartwood has not been investigated widely.

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Experimental

General experimental procedures

IR Spectra ($CHCl_3$): *Jasco-A-302* spectrophotometer; ν in cm^{-1}. ^{1}H-NMR, COSY, NOESY, J-resolved and ^{13}C-NMR: *Bruker Avance* 400 spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C nuclei; chemical shifts (δ) in ppm referenced to residual solvent signal, coupling constants J in Hz. EI-MS: *Finnigan-Mat-311A* mass spectrometer; source at 250 °C and 70 eV; m/z (rel.-%). HR-EI-MS: *Jeol-JMS-HX-110* mass spectrometer; EI, source at 250 °C and 70 eV; m/z (rel.-%). Silica gel 60 for gravity column: (*Merck*, 63-200 μm). TLC: aluminum cards precoated (0.2 mm thickness) with silica gel SiF_{254} (*E. Merck*). HPLC (LC-908, JAI) was performed on the size exclusion (column: polystyrene, JAIGEL-1H and JAIGEL-2H make: JAI, attached in series; 100% $CHCl_3$ (HPLC grade) was used as mobile phase. The purity of the sample was checked on TLC, which was visualized under UV and sprayed with I_2 vapors. n-Hexane used was of boiling range 60-80 °C.

Plant material

The trunk of neem (*Azadirachta indica* A.Juss) was collected from the Karachi region (south coast, altitude 13 feet) in March-April 2004 and identified by Dr. S. I. Ali, Department of Botany, University of Karachi. A voucher specimen (NM-1) has been deposited in the Herbarium, Department of Botany, University of Karachi. The heartwood was obtained on removing the bark with the help of an axe.

Extraction and isolation of compounds

The heartwood (8 kg) of *Azadirachta indica* was repeatedly ($\times$ 5) extracted with MeOH (25 L) at room temperature. The combined methanolic extract was freed of the solvent in vacuo to a thick syrup, which was partitioned between chloroform and water. The chloroform layer was dried over Na_2SO_4 (anhyd.), and concentrated under reduced pressure to obtain a gummy residue. It was dissolved in a small quantity of chloroform and poured over excess of n-hexane. A yellowish brown powder separated out, which was filtered, and washed with excess of $n-$hexane to yield a yellowish white residue (6.12 g). It was subjected to a gravity column (silica gel) and eluted with $CHCl_3$ and $CHCl_3$-MeOH (9.5:0.5, 9.0:1.0, 8.5:1.5, 8.0:2.0, 7.0:3.0, 6.5:3.5, 6.0:4.0, 1.0:1.0). As a result 101 fractions were obtained and combined on the basis of TLC to obtain 55 fractions. Fraction # 28 (145.9 mg; $CHCl_3$-MeOH 9.5:0.5; eluated) was further subjected to a gravity column (silica gel) and eluted with benzene and benzene-acetone (9.0:1.0, 8.5:1.5, 8.0:2.0, 7.0:3.0, 6.5:3.5, 6.0:4.0, 1.0:1.0). Ultimately 10 fractions were obtained on combining the fractions on the basis of TLC. Fraction # 7 (30 mg; benzene-acetone; 6:4 eluated) was washed with cold benzene-acetone (1:1) to give a white semi-crystalline powder (**1**).

Fraction # 33 (132 mg; $CHCl_3$-MeOH; 9.4:0.6; eluated) was purified through size exclusion columns JAIGEL-1H and JAIGEL-2H attached in series installed on a JAI preparative HPLC model LC-908 using 100% $CHCl_3$ (HPLC grade) as a mobile phase. The UV detector was set at 256 nm. Peaks eluting at 68 min and 70 min at a flow rate of 3 mL/min were identified as **2** (10 mg) and **3** (3 mg), respectively.

2α, 3β, 4β - Trihydroxypregnan-16-one (**1**): white semi-crystalline powder (30 mg); Mp 235-237 °C; $[\alpha]_D^{20}$ -82.5°(*c*= 0.05, MeOH); IR: ν_{max} ($CHCl_3$) cm^{-1}: 3407 (O-H stretching), 1742 (>C=O stretching), 1043 (-C-O stretching). EIMS (probe) 70 eV, *m/z*(rel. int.): 350 [M^+] (48%), 332 (17), 314 (10), 307 (22), 289 (13), 264 (100), 246 (20), 229 (40), 121 (21). HREIMS *m/z*, 350.2459 (Calc. for $C_{21}H_{34}O_4$ 350.2457). ^{1}H- and ^{13}C-NMR data are presented in the Table.

Results and discussion

The methanolic heartwood extract yielded a new pregnane steroid, namely 2α, 3β, 4β-trihydroxypregnan-16-one (**1**), and 2 known steroids 2β, 3β, 4β-trihydroxypregnan-16-one (**2**) and 2α, 3α, 4β-trihydroxypregnan-16-one (**3**), which are reported for the first time from heartwood of *A. indica*. The molecular formula of compound **1** was assigned as $C_{21}H_{34}O_4$ with the help of HREIMS, which showed M^+ at *m/z* 350.2459 (calc for $C_{21}H_{34}O_4$ 350.2457). The IR spectrum showed signals for a hydroxyl group (3407 cm^{-1}) and 5-member ring ketone (1742 cm^{-1}).

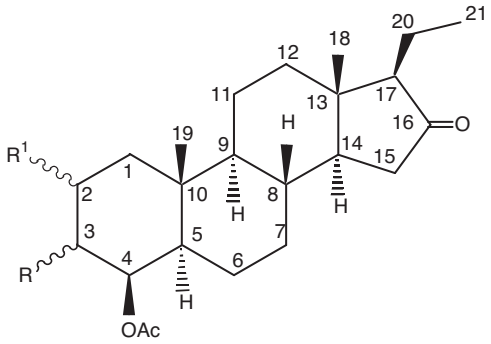
The steroidal nature of **1** was indicated by a methyl triplet in the ^{1}H-NMR spectrum at δ 1.05 (*J*=7.4 Hz) and 2 methyl singlets at δ 0.57 and 1.38 attributable to H-21, H-18, and H-19, respectively.[13–16] The ^{1}H-NMR spectrum further showed 3 oxymethine protons at δ 1.55 ddd (*J*=9.4, 9.4, 4.3 Hz, H-2), 3.84 dd (*J*=9.4, 3.6 Hz, H-3), and 4.22 dd (*J*=3.6, 2.7 Hz, H-4) connected with carbons at δ 68.7, 78.8, and 75.9, respectively, in the HMQC spectrum. The H-3 methine showed coupling in the COSY spectrum with both H-2 and H-4 and H-4 proton with H-5, which demonstrated that all the 3 hydroxyl groups are located in ring A at C-2, C-3, and C-4. Further, the coupling constants of these protons favored axial orientation of H-2 and H-3 and equatorial orientation of H-4. These observations led to formulate 2α, 3β, 4β-trihydroxy arrangement in ring A of the steroidal nucleus. This was corroborated by NOESY interactions of H-5 with both H-3 and H-4, which also supported the *trans* junction of ring A and B. The NOESY spectrum also showed interactions between H-18 and H-19, and between H-19 and H-2. A triplet at δ 1.05 for a terminal methyl group suggested an ethyl group side chain at C-17, which was supported by the molecular formula. The remaining oxygen of the molecule was decided to be a ketonic function at C-16 considering the degree of unsaturation in the molecule, IR absorption (ν_{max} 1742 cm^{-1}), relatively downfield chemical shift of C-16 (δ_C 218.4) in comparison to 6-membered and acylic ketones and the chemical shifts for H-17 (δ 1. 61) and C-17 (δ 65.1), which match well with the reported values of similar partial structures and other isomers of 2,3,4-trihydroxy pregnanes.[13–16] However, this is the first instance of the isolation of a trihydroxy derivative with this relative configuration, which could be conclusively decided in the light of ^{13}C-NMR shifts of ring A and B carbons and comparison with the shifts of other isomers. The chemical shift of C-2 of **1** matched well with the value of the same carbon in other 2α-hydroxy series. It may be noted that in the 2α hydroxy compounds C-2 appears upfield from that of 2β hydroxy analogues due to the γ-gauche effect of 19-Me on H-2β, which causes an upfield shift of C-2. Furthermore, it was observed that C-5 appears downfield (δ ca ∼5.0 ppm) in **1** and other 3β-hydroxy compounds compared to 3α-hydroxy compounds in which this hydroxyl group exerts a γ-gauche effect on H-5 pushing this carbon upfield.[17,18]

Table. NMR data of **1**, **2** and **3** (C_5D_5N, 400 MHz).

No.	1 H	1 ^{13}C	2 ^{1}H	2 ^{13}C	3 ^{1}H	3 ^{13}C
1α	1.37 (1H, m)	46.8 (CH_2)	1.25 (1H, m)	44.5 (CH_2)	1.94 (1H, m)	41.8 (CH_2)
1β	2.38 (1H, m)		2.38 (1H, m)		2.11 (1H, m)	
2	4.55 (1H,ddd, J=9.4,9.4,4.3)	68.7 (CH_2)	4.54 (1H,ddd, J=6.5,3.5,3.1)	72.7 (CH_2)	4.80 (1H,ddd, J=11.9,4.2,3.2)	66.4 (CH_2)
3	3.84 (1H,dd,J=9.4,3.6)	78.8 (CH)	3.84 (1H,dd,J=3.5,3.3)	72.8 (CH)	4.62 (1H,dd,J=3.2,2.7)	74.9 (CH)
4	4.22 (1H,dd,J=3.6, 2.7)	75.9 (CH) (CH)	4.18 (1H,dd,J=3.3,2.2)	77.2 (CH)	4.39 (1H,dd,J=2.7,2.5)	77.3 (CH)
5	1.28 (1H, m)	49.6 (CH)	1.20 (1H, m)	49.1 (CH)	1.98 (1H, m)	44.0 (CH)
6α	2.08, (1H, m)	26.0 (CH_2)	2.13 (1H, m)	25.6 (CH_2)	2.06 (1H, m)	25.5 (CH_2)
6β	1.52 (1H, m)		1.45 (1H, m)		1.54 (1H, m)	
7α	0.95 (1H, m)	32.8 (CH_2)	0.95 (1H, m)	32.4 (CH_2)	0.99 (1H, m)	32.9 (CH_2)
7β	1.62 (1H, m)		1.59 (1H, m)		1.62 (1H, m)	
8	1.45 (1H, m)	34.0 (CH)	1.51 (1H, m)	34.1 (CH)	1.48 (1H, m)	34.0 (CH)
9	0.83 (1H, m)	55.5 (CH)	0.71 (1H, m)	56.7 (CH)	0.94 (1H, m)	55.6 (CH)
10	-	37.5 (C)	-	34.9 (C)	-	37.0 (C)
11α	1.57 (1H, m)	20.5 (CH_2)	1.52 (1H, m)	20.1 (CH_2)	1.59 (1H, m)	19.8 (CH_2)
11β	1.30 (1H, m)		1.33 (1H, m)		1.31 (1H, m)	
12α	1.32 (1H, m)	38.1 (CH_2)	1.22 (1H, m)	38.1 (CH_2)	1.17 (1H, m)	38.4 CH_2)
12β	1.95 (1H, m)		1.74 (1H, m)		1.69 (1H, m)	
13	-	42.1 (C)	-	42.2 (C)	-	42.1 (C)
14	1.33 (1H, m)	50.5 (CH)	1.30 (1H, m)	50.5 (CH)	1.27 (1H, m)	50.4 (CH)
15α	2.21 (1H, m)	38.5 (CH_2)	2.18 (1H, m)	38.5 (CH_2)	2.16 (1H, m)	38.0 (CH_2)
15β	1.77 (1H, m)		1.74 (1H, m)		1.71 (1H, m)	
16	-	218.4 (C)	-	219.0 (C)	-	219.4 (C)
17	1.61 (1H, m)	65.1 (CH)	1.63 (1H, m)	65.3 (CH)	1.61 (1H, m)	65.2 (CH)
18	0.57 (3H, s)	13.4 (CH_3)	0.57 (3H, s)	13.5 (CH_3)	0.56 (3H, s)	13.4 (CH_3)
19	1.38 (3H, s)	16.3 (CH_3)	1.60 (3H, s)	17.2 (CH_3)	1.42 (3H, s)	15.3 (CH_3)
20a	1.02 (1H, m)	18.0 (CH_2)	1.23 (1H, m)	17.6 (CH_2)	1.22 (1H, m)	17.6 (CH_2)
20b	1.62 (1H, m)		1.70 (1H, m)		1.68 (1H, m)	
21	1.05 (3H, t, J=7.4)	13.6 (CH_3)	1.03 (3H, J=7.4)	13.5 (CH_3)	1.01 (3H, J=7.4)	13.4 (CH_3)

On acetylation with acetic anhydride in the presence of pyridine **1** afforded the triacetyl derivative (**1a**) as evident from 3 OAc singlets in the ^{1}H-NMR spectrum at δ 2.06, 2.00, and 1.95. Acetylation caused the respective geminal protons H-2, H-3, and H-4 to appear downfield at δ 5.28 (J= br.m), 4.90 dd (J= 11.1, 4.0 Hz, H-3), and 5.32 (J= 4.0, 3.3.Hz, H-4).

Compounds **2** and **3** were identified through comparison of their spectral data including ^{1}H- and ^{13}C-NMR data with those reported earlier.[13−17]



1. R = β-OH, R^1 = α-OH
1a. R = β-OAc, R^1 = α-OAc
2. R = R^1 = β-OH
3. R = R^1 = α-OH

Figure. Compounds isolated from *Azadirachta indica*.

Acetylation of 1

To a solution **1** (10 mg) in pyridine (0.5 mL) was added Ac_2O (1 mL) and the mixture was left overnight at room temperature. The mixture was poured over crushed ice and extracted with EtOAc. After the usual workup of the EtOAc phase and purification by TLC (silica gel, $CHCl_3$) the triacetyl derivative, 2α, 3β, 4β- triacetoxypregnan-16-one (**1a**) (3.8 mg) was obtained as an amorphous powder. IR ν_{max} ($CHCl_3$): 1742 (C=O), 1725 (br., ester carbonyls) cm^{-1}; EIMS m/z: 476 [M^+]. ^{1}H-NMR (400MHz, $CDCl_3$): δ 5.32 (1H, dd, J=4.0, 3.3 Hz, H-4), 5.28 (1H, br. m, H-2), 4.90 (1H, dd, J= 11.1, 4.0 Hz, H-3), 2.06, 2.00, 1.95 (each 3H, s, 3 x OAc).

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