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Tetracyclic triterpenoids from the leaves of *Azadirachta indica*

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Abstract

Two new tetracyclic triterpenoids zafaral [24,25,26,27-tetranor*apo*tirucalla-(*apo*eupha)-6α-methoxy-7α-acetoxy-1,14-dien-3,16-dione-21-al] (**1**) and meliacinanhydride [24,25,26,27-tetranor*apo*tirucalla-(*apo*eupha)-6α-hydroxy,11α-methoxy-7α,12α-diacetoxy,1,14,20(22)-trien-3-one] (**2**) have been isolated from the methanolic extract of neem leaves along with two known constituents nimocinol and isomeldenin. Their structures and the relative configurations were determined by spectroscopic methods (^{1}H and ^{13}C NMR, IR, and MS) and 2D NMR experiments.

Keywords: *Azadirachta indica*; Meliaceae; Fresh leaves; Triterpenoids; Zafaral; Meliacinanhydride

1. Introduction

In view of enormous therapeutic and economical importance (Fujiwara et al., 1982, Okpanyi and Ezeukwu, 1981. Kraus, 1995) attributed to *Azadirachta indica* (neem), studies undertaken by several groups of workers on its various parts have led to the isolation of a host of new constituents (Lavie et al., 1971, Kraus et al., 1981, Siddiqui et al., 1986, Akhila and Rani, 1999). In continuation of our investigations on the terpenoidal constituents of the leaves of neem (Siddiqui et al., 1999, 2001), two new tetracyclic triterpenoids zafaral (**1**) and meliacinanhydride (**2**) along with two known constituents nimocinol and isomeldenin have been isolated from the methanolic leaves extract. Their structures have been elucidated as 24,25,26,27-tetranor *apo*tirucalla-(*apo*eupha)-6α-methoxy-7α-acetoxy-1,14 dien-3,16-dione-21-al (**1**) and 24,25,26,27-tetranor-*apo*tirucalla-(*apo*eupha)-6α-hydroxy,11α-methoxy,7α,12 α-diacetoxy,1,14,20(22)-trien-3-one (**2**).

2. Results and discussion

Zafaral **(1)** showed the molecular ion peak at m/z 484 in the EIMS and at m/z 484.2795 in the HREIMS, corresponding to the molecular formula $C_{29}H_{40}O_6$ requiring ten degrees of unsaturation. The IR spectrum showed characteristic absorption bands at 2850 cm^{-1} (aldehydic C–H stretching), 1735, 1725, 1720, 1680 cm^{-1} (ester, aldehyde and α,β-unsaturated carbonyl moieties) and 1370 cm^{-1} (geminal methyls), while the UV spectrum exhibited maximum at 225.0 nm. The ^{1}H NMR spectrum (300 MHz, $CDCl_3$ Table 1) showed a pair of AB doublets at δ 7.05 (H-1) and 5.90 (H-2) with coupling constant $J = 10.2$ Hz indicating the presence of 1-en-3-one system (Siddiqui et al., 1984). It was also supported by the ^{13}C NMR spectral values for C-1, C-2, and C-3 (Table 1) and significant mass fragment **a** at m/z 137.0919 ($C_9H_{13}O$) (vide experimental and structure) in the EI-HRMS spectrum. The ^{1}H NMR spectrum further showed two doublets at δ 5.29 ($J = 2.8$ Hz) and 2.11 ($J = 11.3$ Hz) attributable to H-7 and H-5, respectively, while a double doublet at δ 4.24 ($J = 11.3$, 2.8 Hz) was due to H-6. Their crossed peaks in the HMQC spectrum appeared at δ_c 82.0 (C-7), 48.5 (C-5), and 67.5 (C-6). These NMR signals were suggestive of two α-oriented oxygen substituents at C-6 and C-7.

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Table 1
NMR spectral data (broad band, DEPT and HMQC) and long range correlations (HMBC) of zafaral (**1**) ($CDCl_3$)

1			
No.	δ_H (J = Hz)	δ_C	C/H long range correlations
1	7.05 *d* (10.2)	157.0	C-2, C-19
2	5.90 *d* (10.2)	126.5	C-1, C-3
3	–	204.0	–
4	–	43.8	–
5	2.11, *d* (11.3)	48.5	C-4, C-6
6	4.24, *dd* (11.3, 2.8)	67.5	C-5, C-7
7	5.29, *d* (2.8)	82.0	C-6, C-8
8	–	45.4	–
9	2.23, *dd* (14.3, 5.3)	38.5	C-8, C-10, C-11
10	–	42.0	–
11a	2.40–2.55, *m*	16.5	C-9, C-10, C-12
11b	2.10–2.20, *m*		
12a	2.46, *m*	32.8	C-11, C-13
12b	2.82, *m*		
13	–	59.5	–
14	–	193.5	–
15	5.76, *s*	123.9	C-14, C-16, C-17
16	–	208.0	–
17	3.21, *dd*, (8.3, 1.6)	56.5	C-13, C-15, C-20
18	1.08, *s*	18.3	C-13
19	1.16, *s*	16.0	C-1
20	3.32, *m*	143.6	C-17, C-22
21	9.80, *d* (7.6)	203.0	–
22	2.00–2.30, *m*	32.0	C-20, C-21, C-23
23	1.24, *t* (8.2)	22.0	C-20, C-22
28	1.22, *s*	24.9	C-4, C-5
29	1.24, *s*	21.2	C-3, C-4, C-5
30	1.27, *s*	25.7	C-7, C-8, C-9, C-14
OAc	1.93	19.8, 169.0	–
OCH_3	3.48, *s*	53.1	C-6

The assignments are based on COSY-45, *J*-resolved, and HMQC spectra.

These were attributed to an acetoxy group (δ_H 1.93; δ_C 169.0,19.8) and a methoxy group (δ_H 3.48; δ_C 53.1). a broad singlet at δ 5.76 and a double doublet at δ 3.21 (J = 8.3, 1.6 Hz) were attributable to H-15 and H-17, respectively, suggesting the 14-en-16-one system of ring D as observed in azadiradione and other meliacins containing this type of ring D (Lavie et al., 1971). In case of **1**, however H-17 appeared as a double doublet and the larger coupling of 8.3 Hz suggested a proton at C-20. The smaller J value (1.6 Hz) may be attributed to its long range coupling with H-15. Moreover, the IR and NMR spectral data (Table 1) indicated the absence of furan ring signals, a characteristic feature of the *meliacins* (Siddiqui et al., 1984) and showed the presence of an aldehydic group (ν_{max}: 2850; δ_H: δ 9.8, *d*, J = 7.6 Hz; δ_c 203.0). The molecular formula and the mass spectral data revealed a four-carbon side chain showing a doublet at δ 9.8 with coupling constant of 7.6 Hz attributable to aldehydic proton (H-21) along with a three-proton triplet at δ 1.24 (J = 8.2 Hz) attributed to H-23 favored the aldehydic group at C-21 (vide fragment **c**, see Fig. 1).

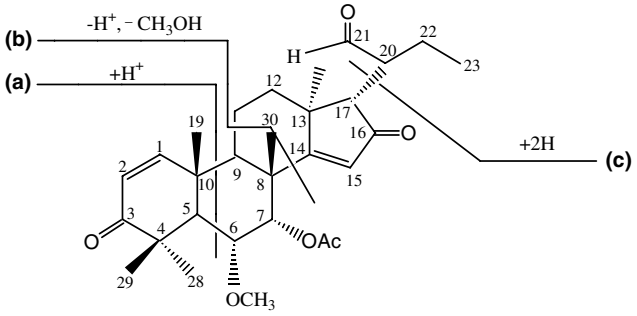
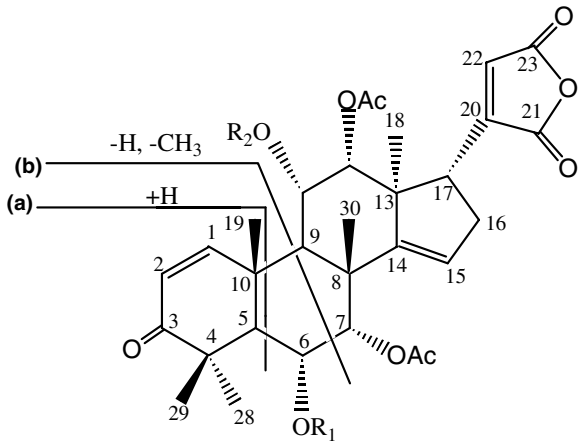


Fig. 1. Significant HREIMS fragments of **1**.

In the light of these spectral data, the structure of **1** was elucidated as [24,25,26,27-tetranor*apo*tirucalla-(*apo*eupha)-6α-methoxy-7α-acetoxy-1,14-dien-3,16-dione-21-al].

The stereochemistry of various centers of **1** was established on the basis of coupling constants of various protons and ^1H–^1H interactions in the 2D-NOE spectrum. Thus it showed spatial connectivities of H-1 with H-2; H-2 with H-19; H-5 with H-28, H-17 with H-30. The last connectivity manifested that the side chain at C-17 is α-oriented.

Meliacinanhydride (**2**) was obtained from the neutral fraction of the methanolic leaves extract. It was assigned the molecular formula $C_{31}H_{38}O_{10}$ on the basis of HREIMS, which showed the molecular ion peak at m/z 570.2134. The peaks at m/z 510 $[M–CH_3COOH]^+$ and 450 $[M–2CH_3COOH]^+$ indicated the presence of two acetate groups in the molecule. The UV spectrum showed absorption maximum at 227.0 nm consistent with an α,β-unsaturated ketone system while the IR spectrum displayed peaks at 3450 cm^{-1} (OH), 1810, 1770, 1720 cm^{-1} (anhydride and ester carbonyls), 1660 cm^{-1} (cyclohexenone), 1600, 820 cm^{-1} (C=C str. and C–H bend. of R_2C=CHR) and 1375 cm^{-1} (geminal methyls). The molecular formula of **2** showed the presence of thirteen double bond equivalents, three of which were accounted for by an α,β-unsaturated cyclohexenone system in ring A, four by the α,β-unsaturated cyclic anhydride side chain, two by two acetoxy moieties displayed by the ^1H NMR spectrum (δ 2.03 and 2.06 each three-proton singlet), one by an isolated C=C double bond ($\delta_{C\text{-}14}$ 158.2, 119.5) and three by the remaining three rings (B–D) of the tetracyclic nucleus. The triterpenoidal nature of **2** was indicated by the presence of five quaternary methyl singlets at δ 1.11, 1.24, 1.25, 1.29, and 1.39 in the ^1H NMR spectrum (vide Experimental). The 1-en-3-one system was supported by the mass fragment **a** at m/z 137.0992 ($C_9H_{13}O$) (vide structure, Fig. 2) and two one-proton AB doublets at δ 7.10 and 5.88 (J = 10.0 Hz) attributed to H-1 and H-2, respectively. The corresponding carbon signals were observed at δ 156.8 (C-1) and 125.9 (C-2) in the HMQC spectrum along with a signal at 203.5 (C-3) in the broad



2 R_1=H
R_2=CH_3
3 R_1=Ac
R_2=CH_3

Fig. 2. Significant HR-EI-MS fragments of **2**.

band spectrum. The ^{1}H NMR spectrum further showed signals at δ 4.35 (*dd*, $J = 12.0$, 3.0 Hz, H-6), 5.33 (*d*, $J = 3.0$ Hz, H-7) and 2.23 (*d*, $J = 12.0$ Hz, H-5). The chemical shifts and coupling constants of these protons suggested an acetoxy substituent at C-7 and a hydroxy (or methoxy) substituent at C-6 both with α-dispositions. Two one-proton doublets were also observed in the ^{1}H NMR spectrum at δ 2.35 ($J = 12.5$ Hz) and 5.28 ($J = 2.5$ Hz) and a one-proton double doublet at δ 4.23 ($J = 12.5$, 2.5 Hz) attributable to H-9, H-12, and H-11, respectively, manifesting two oxygen substituents, a hydroxyl (or methoxy) substituent at C-11 and an acetoxy group at C-12, both with α-dispositions. acetylation of **2** afforded the monoacetylated product (**3**) favoring only one hydroxyl group in the molecule. The position of hydroxyl group at C-6 of **2** was supported by the mass fragment **b** (m/z 148.0998; $C_{10}H_{12}O$) and the comparatively downfield shift of H-6 from that of **1,** which has methoxy substituent at C-6. Further, a cross peak in the NOESY plot for interaction methoxy with H-1 provided conclusive evidence in favor of methoxy at C-11. A multiplet at δ 5.37 (H-15) in the ^{1}H NMR and CH and C carbon shifts at δ 119.5 (C-15) and 158.2 (C-14) in the ^{13}C NMR spectrum were in agreement with a double bond at C-14 (Lavie and Jain, 1967). The signals for β-substituted furan ring, a characteristic feature of the meliacins (Siddiqui et al., 1984, Lavie et al., 1971) were again missing in the ^{1}H NMR spectrum and instead a cyclic anhydride was indicated by a singlet at δ 6.81 assigned to H-22 having a connected carbon in the HMQC spectrum at δ 134.0 which is comparable with the reported value for similar moiety (Koer et al., 1975) and two additional carbonyls at δ_C 171.3 and 169.6. These features together with five methyl singlets in the ^{1}H NMR spectrum, suggested that meliacinanhydride is 24,25,26,27-tetranor*apo*tirucalla-(*apo*eupha)-6α-hydroxy,11α-methoxy,7α,12α-diacetoxy-,1,14,20-(22)-trien-3-one (**2**).

The assignments of protons and carbons were further supported by the interactions observed in the 2D-NOE (NOESY) and HMBC plots. Thus NOESY spectrum showed spatial connectivities of H-1 with H-2; H-2 with H-19; H-5 with H-28; H-15 with H-17 and H-18 with H-22. The interaction of H-5 with H-28 in the NOESY and its COSY interaction with H-6 as well as that of H-6 with H-7 helped in exact assignment of these protons. Similarly H-9 was correlated with H-11 and H-11 with H-12 in the COSY plot. The NOESY interaction of H-22 with H-18 showed the α-disposition of anhydride ring at C-17.

3. Experimental

3.1. General experimental procedures

Silica gel *60 PF_{254}*; *Aldrich* column, 100 ml; silica gel *9385* (*Merck,* 0.040-0.063 mm). Preparation of TLC: precoated alumina (Riedel-de-Haen DC-cards ALF) sheets; detection at 254 and 366 nm with *UV KL* UV lamps, *H. Jurgens & Co.* and I_2 spray. Melting points: *Gallenkamp apparatus*. Optical rotations: *Jasco DIP-360* digital polarimeter. UV Spectra: *Hitachi-3200* λ_{max} (log ξ) in nm. IR spectra: *Jasco-A302* spectrophotometer; ν_{max} in cm^{-1}. ^{1}H NMR, COSY, NOESY, and *J*-resolved: *Bruker* spectrometers at 300 and 400 MHz; chemical shifts δ in ppm rel. to $SiMe_4$ as an internal standard, coupling constants *J* in Hz. ^{13}C NMR: *Bruker* spectrometer at 125 MHz. The EI-MS and HR-EI-MS: *Finnigan Mat*-311A and *Jeol JMS HX-110* mass spectrometers. EI source at 250° and 70 eV; m/z (rel.%). HPLC (LC-**6A**, Shimadzu) was performed in the reverse phase (column: C_{18}, Techspher, 50 DS, 30 cm × 10 mm; mobile phase 60% CH_3CN–H_2O (v/v); loop 20 μl flow rate 1 ml/min.)

3.2. Plant material

The leaves of *Azadirachta indica* were collected in March 1996 from Karachi, Pakistan and identified by Prof. Dr. S.I. Ali, Department of Botany, University of Karachi. A voucher specimen (No. NM-1) has been deposited in the Herbarium of Botany Department, University of Karachi.

3.3. Extraction and isolation

The fresh and uncrushed leaves (20 kg) were repeatedly (×5) extracted with MeOH at room temperature. The combined extracts, after removal of solvent, were partitioned between EtOAc and H_2O. The EtOAc layer was washed, dried (anhydrous Na_2SO_4), treated with

charcoal, and filtered. The charcoal bed was washed successively with EtOAc and a mixture of methanol–benzene (1:1). The filtrates and washings were combined and the solvent evaporated under reduced pressure. The residue thereby obtained was taken up in EtOAc and treated with 4% aqueous Na_2CO_3 to separate the acidic fraction from the neutral fraction. The EtOAc phase containing the neutral fraction was washed, dried (Na_2SO_4), and evaporated under a vacuum. The residue was divided into petroleum ether soluble and insoluble fractions, and the latter was successively treated with different percentages of aqueous MeOH (10%, 20% up to 100%). As a result, several fractions were obtained and combined on the basis of their TLC patterns. The 40%, 50% and 60% aqueous MeOH fractions were pooled and extracted with EtOAc after adding NaCl solution. The EtOAc extract was dried (anhydrous Na_2SO_4) and solvent removed under reduced pressure to yield a residue (5.74 g), which was subjected to vacuum-liquid chromatography (VLC) (silica gel GF_{60-254}; $CHCl_3$, $CHCl_3$–MeOH, in mixtures of increasing polarity). the $CHCl_3$–MeOH (9.9:0.1 and 9.85:0.15) elutes were combined on the basis of TLC and freed of the solvent to give A (3.9 gm) which was further subjected to VLC (silica gel, GF_{60-254}, petroleum ether, petroleum ether–EtOAc, $CHCl_3$, $CHCl_3$–MeOH in order of increasing polarity). The petroleum ether–EtOAc, 7.0:3.0 and 6.5:3.5) elutes were also combined (139 mg) and subjected to preparative TLC (silica gel GF60-254, petroleum ether–EtOAc, 6.5:3.5) affording one major component showing single spot on TLC. However, its ^1H NMR spectrum indicated that it was still a mixture of several constituents with one major band, which after a number of trials, could ultimately be purified on precoated alumina cards (Riedel-de-Haen 37364 Dc-cards ALF, petroleum ether–EtOAc; 6.5:3.5) to afford zafaral (**1**) (10 mg).

The 70% and 80% methanol fractions referred to at the outset were combined together to form a fraction B, which was subjected to VLC (silica gel-60 GF60-254; petroleum ether, petroleum ether–ethyl acetate in order of increasing polarity up to a ratio of 7:3 and then $CHCl_3$, $CHCl_3$–MeOH in order of increasing polarity). The petroleum ether–EtOAc (7.5:2.5; f-6) eluate furnished fraction B1 (1.05 gm), which after thick layer chromatography, furnished two sub-fractions, B1–1 and B1–2. Fraction #B1–2 also showed two major bands with some minor constituents on TLC. On preparative TLC over precoated alumina-cards (Riedel-de-Haen 37364 DC-cards ALF, petrol–EtOAc; 7.0:3.0) it afforded two components, one in a pure state which was characterized as meliacinanhydride (**2**) (9 mg) while the other band could be resolved further through HPLC. the HPLC was performed in the reverse phase (column; C_{18}, Techsphere, mobile phase 60% CH_3CN–H_2O; v/v) affording two known compounds identified as nimocinol and isomeldenin. Both these have earlier been reported from this source.

3.3.1. Zafaral (*1*)

Crystalline, m.p. 71–72 °C $[\alpha]_D^{27} = +41.0°$ ($CHCl_3$; $c = 0.02$,). UV λ_{max}^{MeOH} nm ($\log \varepsilon$): 225.0 (3.59). IR ν_{max}^{CHCl3} cm^{-1}: 2850, 1735, 1725, 1720, 1680, 1370. ^1H NMR, ^{13}C NMR and HMBC, Table 1. HREIMS: 484.2795 (68, $C_{29}H_{40}O_6^+$, M^+; calc. 484.2834), 259.1362 (14, $C_{16}H_{19}O_3^+$, **b**), 165.0882 (12, $C_{10}H_{13}O_2^+$), 137.0919 (21, $C_9H_{13}O^+$, **a**), 124.0457 (13, $C_7H_8O_2^+$), 71.0699 (12, $C_4H_7O^+$, **c**).

3.3.2. Meliacinanhydride (*2*)

Crystalline, m.p. 114–115 °C. $[\alpha]_D^{27} = -30.0$ ($c = 0.02$, $CHCl_3$). UV λ_{max}^{MeOH} nm ($\log \varepsilon$): 227.0 (4.53). IR ν_{max}^{CHCl3} cm^{-1}: 3450, 1810, 1770, 1720, 1660, 1600, 1375. ^1H NMR and ^{13}C NMR, Table 2. HREIMS m/z 570.2134 (calc. for $C_{31}H_{38}O_{10}$, 570.2139), 436.2476 (12, $C_{24}H_{36}O_7^+$, **c**), 286.1630 (9, $C_{18}H_{22}O_3^+$), 270.1413 (13, $C_{14}H_{22}O_5^+$), 148.0998 (57, $C_{10}H_{12}O^+$, **b**), 137.0992 (17, $C_9H_{13}O^+$, **a**).

Table 2
NMR spectral data (broad band, DEPT and HMQC) and long range correlations (HMBC) meliacinanhydride (**2**) ($CDCl_3$)

2			
No.	δ_H (J = Hz)	δ_C	C/H long range correlations
1	7.10 *d* (10.0)	156.8	C-2, C19
2	5.88, *d* (10.0)	125.9	C-1, C-3
3	–	203.5	–
4	–	44.2	–
5	2.23, *d* (12.0)	49.0	C-4, C-6
6	4.35, *dd* (12.0, 3.0)	68.5	C-5, C-7
7	5.33, *d* (3.0)	81.4	C-6, C-8
8	–	43.6	–
9	2.35, *d* (12.5)	37.7	C-8, C-10, C-11
10	–	40.8	–
11	4.23, *dd* (12.5, 2.5)	77.8	C-9, C-10, C-12
12	5.28, *d* (2.5)	65.5	C-11, C-13
13	–	54.8	–
14	–	158.2	–
15	5.37, *m*	119.5	C-14, C-16, C-17
16	2.32, *m*	32.9	C-14, C-17
17	2.25, *m*	51.4	C-15, C-20
18	1.11, *s*	21.1	C-13
19	1.24, *s*	21.8	C-1
20	–	153.0	–
21	–	171.3	–
22	6.81, *s*	134.0	C-20, C-21, C-23
23	–	169.6	–
28	1.25, *s*	22.3	C-4, C-5
29	1.29, *s*	19.5	C-3, C-4, C-5
30	1.39, *s*	28.2	C-7, C-9, C-14
OAc	2.03, *s*	18.9, 170.0	–
OAc	2.06, *s*	20.0, 169.6	–
OCH_3	3.47, *s*	52.8	C-11

The assignments are based on COSY-45, *J*-resolved, and HMQC spectra.

*3.3.3. Acetylation of **2***

To a solution of **2** (5 mg) in pyridine (0.5 ml) Ac_2O (0.5 ml) was added and the reaction mixture kept over night at room temperature. The reactants were poured over crushed ice and extracted with EtOAc. EtOAc layer was washed with H_2O, dried (anhydrous Na_2SO_4) and its solvent removed under vacuo. The acetyl derivative (**3**) was thereby obtained as white powder showing a single spot on TLC. UV (MeOH): 227.0 (4.59). IR ($CHCl_3$): 1810, 1770, 1720, 1660, 1600, 1375.^{1}H NMR: δ 5.32 (1H, *dd*, 12.0, 3.0 Hz; H-6), 2.08, (3H, *s*, AcO–C), 2.03, (3H, *s*, AcO–C), 2.06, (3H, *s*, AcO–C). EIMS: 612 [M^+].

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