Tetracyclic Triterpenoids from the Leaves of *Azadirachta indica* and Their Insecticidal Activities

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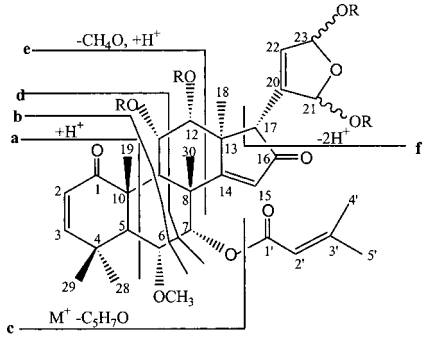
A new tetranortriterpenoid, meliatetraolenone [24,25,26,27-tetranor-*apo*tirucalla-(*apo*eupha)-6α-*O*-methyl, 7α-senecioyl(7-deacetyl)-11α,12α,21,23-tetrahydroxy-21,23-epoxy-2,14,20(22)-trien-1,16-dione] (1) was isolated from the methanolic extract of fresh leaves of *Azadirachta indica* along with the known compound odoratone (3) which was hitherto unreported from this source. Their structures have been elucidated by spectral studies including 2D NMR. The insecticidal activities of 1 as well as those of odoratone (3) are reported. 1 and odoratone both showed mortality on fourth instar larvae of mosquitoes (*Anopheles stephensi*) with LC_{50} values of 16 and 154 ppm, respectively.

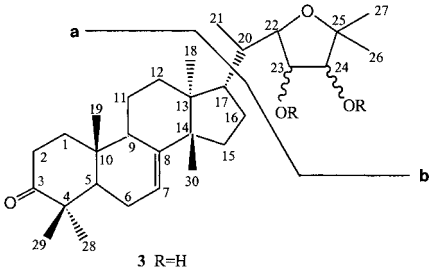
Key words *Azadirachta indica*; Meliaceae; triterpenoid; *Anopheles stephensi*

In view of the attributed therapeutic and pesticidal importance of *Azadirachta indica* (neem), comprehensive investigations of its different parts have been carried out by various groups, leading to the isolation of a series of constituents.[1—6] In a continuation of our studies on its constituents,[7—9] one new tetracyclic triterpenoid, meliatetraolenone (**1**), and the hitherto unreported compound odoratone (**3**) were isolated from the methanolic extract of the fresh *A. indica* leaves. Their structures are based on extensive 1D and 2D NMR studies. The insecticidal properties of **1** and **3** were determined on the fourth instar larvae of mosquitoes (*Anopheles stephensi*), and their LC_{50} values were 16 and 154 ppm, respectively.

The molecular formula $C_{32}H_{42}O_{10}$ of meliatetraolenone (**1**) was established by EI-HR-MS [*m/z* 586.2738, M^+]. The UV spectrum exhibited an absorption maximum at 230 nm and the IR spectrum showed absorption bands at 3450 (OH), 1660—1725 (carbonyls of α,β-unsaturated ketone and ester), 1600 (C=C) and 1375 (geminal methyls) cm^{-1}. The ^{1}H-NMR data were indicative of the terpenoidal nature of meliatetraolenone (**1**) with the presence of five quaternary methyl singlets at δ 0.90, 1.12, 1.24, 1.27, and 1.39 (Table 1). A pair of AB doublets at δ 5.85 and 6.40 (J=10.1 Hz, H-2, H-3) in the ^{1}H-NMR spectrum could be assigned to the olefinic protons of the enone system as in nimbin.[10] This was further confirmed by the mass fragment **a** (*m/z* 137.0903; $C_9H_{13}O$). The ^{1}H-NMR spectrum further showed the presence of signals at δ 4.38 (dd, J=12.6, 2.8 Hz), 5.29 (d, J=2.8 Hz), and 2.19 (d, J=12.6 Hz) attributable to H-6, H-7, and H-5, respectively. The chemical shift and coupling constant of H-7 showed that C-7 carries the ester moiety while C-6 has either a hydroxy or a methoxy substituent (δ_{OCH_3} 3.48).[9] The presence of a methoxy group at C-6 was confirmed by the mass fragment **b** (*vide* Experimental) at *m/z* 180.1075 ($C_{11}H_{16}O_2$). The ^{1}H- and ^{13}C-NMR data showed that the ester is a senecioxy function [δ_H 1.96, H-4′, 2.08, H-5′; 6.01, H-2′; δ_C, 172.0 (C-1′); 115.0 (C-2′); 158.0 (C-3′); 21.5 (C-4′), and 25.2 (C-5′). This ester moiety was further supported by mass fragment **c** at *m/z* 503.2314, ($M^+-C_5H_7O$). In the ^{1}H-NMR spectrum two one-proton doublets were observed at δ 2.59 (J=12.5 Hz) and 4.25 (J=3.0 Hz) and a one-proton double doublet at δ 4.05 (J=12.5, 3.0 Hz) which were attributed to H-9, H-12, and H-11, respectively. The chemical shifts and coupling constants of these protons suggested two oxygen substituents at C-11 and C-12, both with the α-disposition and the molecular formula and IR spectrum indicated the nature of these substituents to be hydroxyl functions. On acetylation (*vide* Experimental), the signals at δ 4.25 and 4.05 shifted to δ 5.28 and 5.19 in the ^{1}H-NMR spectrum of **1**, confirming the presence of two hydroxyl groups. Their location at C-11 and C-12 was also supported by the mass fragments **d** at *m/z* 223.1291 ($C_{13}H_{19}O_3$) and **e** (*m/z* 360.1917, $C_{21}H_{28}O_5$). The ^{1}H-NMR spectrum further showed two one-



1 R=H
2 R=Ac



3 R=H
4 R=Ac
5 R=H, $\Delta^{9(11)}$

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proton singlets at δ 5.82 and 3.43 attributable to H-15 and H-17, respectively. These signals and their corresponding carbons at δ 125.3 and 57.0 were suggestive of ring D of azadiradione.[11] Moreover, the signals of a furan ring, a characteristic feature of meliacins,[11,12] were missing in the NMR spectra (^{1}H-, ^{13}C-NMR, Table 1), and instead a 21,23-dihydroxy-but-20(22) en-21,23-ether moiety[13] was indicated by the proton signals at δ 5.92 and 5.89 and the vinylic proton signal at δ 6.79 which were assigned to H-21, H-23, and H-22, respectively. Their corresponding carbon signals were discernible from the ^{1}H-detected heteronuclear multiple quantum coherence (HMQC) spectrum at δ_C 97.5 (C-21), 96.0 (C-23), and 126.0 (C-22) along with a signal in the broad band spectrum at δ_C 142.5 (C-20). This side chain was supported by the diagnostic mass fragment **f** at *m*/*z* 112.118 ($C_5H_4O_3$). The assignments of protons and carbons were further supported by the interactions observed in the two dimensional (2D)-Nuclear Overhauser effect (NOE) (NOESY) and heteronuclear multiple bond conectivity (HMBC) plots (Fig. 1, Table 1).

Thus the NOESY spectrum showed spatial connectivities of H-5 with H-28, H-17 with H-30, and H-18 with H-22. The interaction between H-18 and H-22 suggested the α-orientation of the side chain ring at C-17. In the HMBC spectrum, cross-peaks were noted for correlation between C-28/H-6, C-6/H-7, C-7/H-30, C-17/H-15, and C-17/H-18. The NMR assignments (COSY, NOESY HMQC, HMBC) compared well with the shifts reported for compounds with similar partial structures.[10,11]

In the light of the above spectral data, the structure of **1** has been deduced to be 24,25,26,27-tetranor-*apo*tirucalla-(*apo*eupha)-6α-methoxy,7α-senecioxy-11α,12α,21,23-tetrahydroxy-21,23-epoxy-2,14,20(22)-trien-1,16-dione (**1**). Compound **3** was identified as odoratone on the basis of extensive spectroscopic studies including 1D (^{1}H-, ^{13}C-NMR) and 2D (COSY, NOESY, HMQC, HMBC, and *J* resolved)

Table 1. NMR Data of Meliatetraolenone (**1**) ($CDCl_3$)

No.	**1**		
	δ_H (*J*=Hz)	δ_C	HMBC (H→C)
1	—	205.0	—
2	5.85, d (10.1)	126.5	C-1, C-3
3	6.40, d (10.1)	148.5	C-2, C-1
4	—	40.0	—
5	2.19, d (12.6)	49.5	C-4, C-6
6	4.38, dd (12.6, 2.8)	69.5	C-5, C-7
7	5.29, d (2.8)	79.6	C-6, C-8
8	—	44.7	—
9	2.59, d (12.5)	47.3	C-8, C-10, C-11
10	—	48.5	—
11	4.05, dd (12.5, 3.0)	73.5	C-9, C-10, C-12
12	4.25, d (3.0)	71.0	C-11, C-13
13	—	51.0	—
14	—	189.0	—
15	5.82, s	125.3	C-14, C-16, C-17
16		207.0	—
17	3.43, s	57.0	C-13, C-15, C-20
18	0.90, s	20.3	C-13
19	1.12, s	21.9	C-1
20	—	142.5	—
21	5.92, m	97.5	C-20, C-22
22	6.79, t (1.25)	126.0	C-20, C-21, C-23
23	5.89, t (1.25)	96.0	C-20, C-22
28	1.27, s	26.3	C-4, C-5
29	1.24, s	22.8	C-3, C-4, C-5
30	1.39, s	31.6	C-7, C-8, C-9, C-14
1′	—	172.0	—
2′	6.01, s	115.0	C-1′, C-3′
3′	—	158.0	—
4′	1.96, br s	21.5	C-3′
5′	2.08, br s	25.2	C-3′
OCH_3	3.48, s	52.4	C-6

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

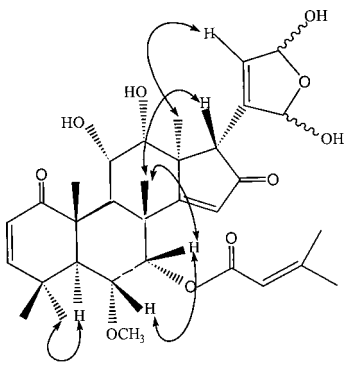

Fig. 1. NOE (NOESY) Interactions of **1**

Table 2. NMR Data of Odoratone (**3**) ($CDCl_3$)

No.	**3**		
	δ_H (*J*=Hz)	δ_C	HMBC (H→C)
1a	1.96, dd (16.0, 8.5)	34.1	C-2, C-10
1b	2.06, dd (16.0, 8.5)		
2a	2.45, ddd (16.0, 8.5, 4.5)	34.9	C-1, C-3
2b	2.01, m		—
3	—	216.0	—
4	—	37.5	—
5	1.49, dd (9.5, 3.0)	49.3	C-4, C-6
6a	2.74, dd (14.5, 5.5)	24.3	C-5, C-7
6b	2.20, dt (14.5, 3.0)		
7	5.30, dd (6.0, 3.0)	117.8	C-6, C-8
8	—	146.6	—
9	1.56, dd (9.0, 3.5)	46.5	C-8, C-11
10	—	35.0	—
11a	1.38, m	27.7	C-12, C-9
11b	1.61, m		
12a	1.72, m	33.8	C-11, C-13
12b	1.6, m		—
13	—	43.5	—
14	—	51.2	—
15a	2.46, ddd (17.0, 8.5, 4.5)	38.5	C-17
15b	2.02—2.30, m		—
16a	1.68, m	18.3	—
16b	1.35, m		—
17	1.73, m	52.4	C-13, C-15, C-16
18	0.98, s	12.4	C-13
19	0.81, s	14.0	C-1, C-10
20	3.84, dd (6.5, 1.5)	48.5	—
21	0.84, d (6.5)	12.8	C-20
22	3.80, dd (6.5, 1.5)	83.7	C-20, C23
23	3.95, t (6.5)	72.9	C-22
24	3.65, d, (6.5)	77.4	—
25	—	80.8	—
26	1.21, s	27.6	C-25
27	1.23, s	28.4	C-25
28	1.03, s	21.8	C-4, C-5
29	1.05, s	24.5	C-3, C-4
30	1.09, s	21.3	C-8, C14

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

NMR and mass spectroscopy, and comparison of the data with those of odoratone reported perivously.[14,15] Odoratone was isolated earlier from *Cedrela odorata* L., but to the best of our knowledge this is the first instance of its isolation from the leaves of *A. indica*.

Both **1** and **3** were tested for their pesticidal activities against fourth instar larvae of *A. stephensi* and exhibited toxicity with LC_{50} values of 16 and 154 ppm, respectively.

Experimental

General Experimental Procedures Ultraviolet spectra were recorded in MeOH on a Shimadzu 240 spectrophotometer, while infrared spectra were measured in $CHCl_3$ on a JASCO IRA-1 spectrophotometer. Mass spectra were measured on a Finnigan MAT 112 11/34 computer system. NMR spectra (both 1D and 2D) were recorded in $CDCl_3$ on Bruker AM 300 and Bruker AM 400 NMR spectrometers operating at 300 and 400 MHz, respectively, for ^{1}H-NMR and at 75 and 100 MHz, respectively, for ^{13}C-NMR. The chemical shifts are recorded in ppm (δ) and coupling constants (J) are in Hz. TLC was performed on precoated alumina (Riedel-de Haen Dccards ALF). Plates were visualized under UV light (254 and 366)/I_2 vapors.

Plant Material *A. indica* leaves were collected in spring from the Karachi region and identified by Professor S. I. Ali, Department of Botany, University of Karachi, and a voucher specimen (No. NM-1) has been deposited in the herbarium of the Department of Botany, University of Karachi.

Extraction and Isolation The fresh and uncrushed leaves (20 kg) of *A. indica* were collected in March 1997 from the Karachi region and repeatedly extracted with methanol at room temperature. The combined methanolic extract was partitioned between ethyl acetate and water. The ethyl acetate layer was dried (Na_2SO_4, anhydrous), treated with charcoal, and filtered. The charcoal bed was successively eluted with ethyl acetate and benzene–methanol (1 : 1 v/v). The ethyl acetate filtrate and eluate and the benzene–methanol filtrate (1 : 1 v/v) were combined and solvent removed at reduced pressure. The residue thus obtained was divided into petroleum ether-soluble and -insoluble fractions. The latter fraction was collected in ethyl acetate and treated with 4% aqueous Na_2CO_3 solution to separate the acidic (A) and neutral (N) fractions. The ethyl acetate layer containing the N fraction was washed with water and dried (Na_2SO_4, anhydrous). The residue left after removal of the solvent *in vacuo* was successively treated with different percentages (10 to 100%) of methanol in water. Several fractions were obtained and combined on the basis of their TLC spectra. The 70% and 80% methanol fractions were combined to form fraction NA which was subjected to VLC (silica gel-60 $GF_{60—254}$; 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 (7.0 : 3.0; F-7) eluate of VLC afforded fraction NA1, showing two major components along with some minor spots. On purification over preparative TLC (petroleum ether–EtOAc; 6.5 : 3.5), two components were obtained; one was identified as a new constituent, meliatetraolenone (**1**), while the other was identified as odoratone (**3**), a known compound, isolated for the first time from the leaves of *A. indica*.

Meliatetraolenone (**1**): Fine colorless needles (27 mg) mp 80—82 °C; $[\alpha]_D^{27}$ +7.6° (c=0.12, $CHCl_3$); UV λ_{max}^{MeOH} nm (log ε): 230 (3.86); IR $\nu_{max}^{CHCl_3}$ 3450, 1660—1725, 1600 cm^{-1}; ^{1}H- and ^{13}C-NMR, see Table 1; EI-MS (70 eV) (rel. int. %) *m/z* 586 [M^+] (12); EI-HR-MS *m/z* 586.2738 [$C_{32}H_{42}O_{10}$, Calcd for $C_{32}H_{42}O_{10}$ 586.2779] (12), 503.2314 [**c**, $C_{27}H_{35}O_9$] (8), 454.2408 [$C_{27}H_{34}O_6$] (11), 360.1917 [**e**, $C_{21}H_{28}O_5$] (6), 319.1733 [$C_{22}H_{23}O_2$] (12), 301.1762 [$C_{19}H_{25}O_3$] (12), 286.1552 [$C_{18}H_{22}O_3$] (9), 269.1581 [$C_{18}H_{21}O_2$] (16), 233.1291 [**d**, $C_{13}H_{19}O_3$] (16), 180.1075 [**b**, $C_{11}H_{16}O_2$] (25), 165.0870 [$C_{10}H_{13}O_2$] (24), 137.0903 [**a**, $C_9H_{13}O$] (49), 124.0457 [$C_7H_8O_2$] (13), 112.118 [**f**, $C_5H_4O_3$] (6).

Acetylation of Meliatetraolenone (1) To a solution of **1** (10 mg) in pyridine (1 ml) acetic anhydride (1 ml) was added and the reaction mixture was allowed to stand at room temperature overnight. After the usual work-up the tetraacetyl derivative (**2**) was obtained showing a single spot on TLC. UV λ_{max}^{MeOH} nm (log ε): 230 (3.91); IR $\nu_{max}^{CHCl_3}$ 1720—1735 cm^{-1}. EI-MS *m/z* 754 [M^+]; ^{1}H-NMR δ 5.28 (1H, dd, 12.5, 3.0 Hz; H-11), 5.19 (1H, d, 3.0 Hz; H-12), 2.03 (6H, s, $OCOCH_3$), 6.68 (1H, m; H-21), 6.83 (1H, t, 1.5 Hz; H-23), 2.10 (6H, s, $OCOC\underline{H}_3$).

Odoratone (**3**): Colorless needles mp 78—79 °C; $[\alpha]_D^{27}$ −30.0° (c=0.02, $CHCl_3$); UV λ_{max}^{MeOH} nm (log ε): 230 (4.06); IR $\nu_{max}^{CHCl_3}$ 3600, 1710, 1600, and 1375 cm^{-1}; ^{1}H- and ^{13}C-NMR, see Table 2; EI-HR-MS *m/z* (rel. int. %) 472.3253 (M^+, $C_{30}H_{48}O_4$, Calcd for $C_{30}H_{48}O_4$, 472.3346] (85), 313.2474 (**a**, $C_{22}H_{33}O$] (13), 299.2352 ($C_{21}H_{31}O$] (38), 159.1094 (**b**, $C_8H_{15}O_3$] (10), 121.0986 (C_9H_{13}] (9).

Acetylation of Odoratone (3) Acetic anhydride (0.5 ml) was added to a solution of **3** (12 mg) in pyridine (1 ml) and the reaction mixture was allowed to stand overnight at room temperature. The acetyl derivative (**4**) was obtained after the usual work-up as a white powder showing a single spot on TLC. UV λ_{max}^{MeOH} nm (log ε): 230 (4.20); IR $\nu_{max}^{CHCl_3}$ 1735—1720 cm^{-1}; EI-MS *m/z* 556 [M^+]; ^{1}H-NMR δ 5.20 (1H, t, 6.5 Hz, H-23), 5.05 (1H, d, 6.5 Hz, H-24), 2.04 (6H, s, $OCOC\underline{H}_3$).

Dehydrogenation of Odoratone (3) To a solution of odoratone (**3**) (10 mg) in chloroform (1 ml) a solution of mercurric acetate (30 mg) in glacial acetic acid (1 ml) was added and the reaction mixture was stirred at 20 °C. After 24 h, the mercurous acetate formed was filtered off and the solvent from the filtrate was removed *in vacuo*, affording the diene (**5**). UV λ_{max}^{MeOH} nm (log ε): 205 (4.08), (log ε): 249 (4.31); IR $\nu_{max}^{CHCl_3}$ 3600, 1710, 1600, and 1375 cm^{-1}; EI-MS *m/z* 470 [M^+]; ^{1}H-NMR δ 5.07 (1H, dd, 6.2, 3.5 Hz, H-11), δ 4.85 (1H, dd, 6.0, 3.0 Hz, H-7).

Insecticidal Activity. Insects *A. stephensi* larvae (P.C.S.I.R. strain) were reared in the laboratory of the Zoology Department, Karachi University, under controlled temperature (28±1 °C). They were fed with sterilized powder of dried prawns.

Biological Tests (Screening Procedure) Ten early fourth instar mosquito larvae were collected in 5 ml of the rearing tap water and transferred to 100-ml glass beakers containing 45 ml of distilled water. The compounds were tested at 28±1 °C at 5 final concentrations. Controls consisted of water alone. Each concentration and control was run in duplicate and mortality was recorded after 24 h.

Accurate Tests The WHO method[16] was modified for the testing. A batch of 10 insects (fourth instar larvae) was released into a 100-ml beaker containing 50 ml of filtered tap water. The concentrations selected in the preliminary screening of each compound were tested at 28±1 °C. A group of seven beakers was set up, five for different concentrations and one each for control and checking. Each experiment was repeated five times. The experiment was discarded if mortality exceeded 10% in the controls. Mortality was recorded after 24 h and data were corrected using Abbott's formula.[17]

Calculation of LC_{50} Values The lethal concentrations (LC_{50}) were calculated using PROBIT analysis,[17] placing the average mortalities on the y-axis and the dose in ppm on the x-axis.

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