Two New Triterpenoids from Azadirachta i ndica and Their Insecticidal Activity

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Two New Triterpenoids from *Azadirachta indica* and Their Insecticidal Activity

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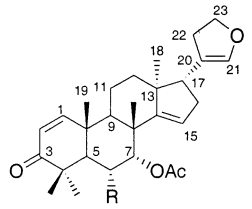
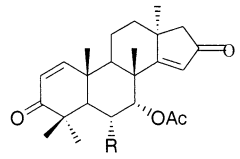
Two new triterpenoids, 22,23-dihydronimocinol (**1**) and desfurano-6α-hydroxyazadiradione (**2**), were isolated from a methanolic extract of the fresh leaves of *Azadirachta indica* (neem) along with a known meliacin, 7α-senecioyl-(7-deacetyl)-23-*O*-methylnimocinolide. The structures of **1** and **2** were elucidated through spectral and chemical studies. Compounds **1** and **2** showed mortality for fourth instar larvae of the mosquito (*Anopheles stephensi*), with LC_{50} values of 60 and 43 ppm, respectively.

The neem tree (*Azadirachta indica* A. Juss.; Meliaceae) is well known in Asia and Africa, and almost every part of the plant is used in indigenous systems of medicine.[1,2] Much interest has been shown in the phytochemistry of this tree due to its medicinal and pesticidal properties.[3−5] A continuing search toward obtaining new bioactive compounds has resulted in the isolation and structure elucidation of the new tetranortriterpenoid 22,23-dihydronimocinol (**1**) and the new octanortriterpenoid desfurano-6α-hydroxyazadiradione (**2**), along with the known meliacin 7α-senecioyl-(7-deacetyl)-23-*O*-methylnimocinolide,[6] from the neutral fraction of a methanolic extract of neem tree leaves. The insecticidal properties of **1** and **2** were determined on the fourth instar larvae of the mosquito (*Anopheles stephensi*).



1 R = OH
1a R = OAc



2 R = OH
2a R = OAc

The two new tetracyclic triterpenoids 22,23-dihydronimocinol (**1**) and desfurano-6α-hydroxyazadiradione (**2**) were obtained from a neutral fraction of the methanolic extract of neem leaves. The molecular formula $C_{28}H_{38}O_5$ of **1** has been deduced with the help of HREIMS. The UV spectrum exhibited an absorption maximum at 225 nm consistent with an α,*β*-unsaturated ketone, while the IR spectrum displayed peaks at 3400 (hydroxyl), 1682, 1725 (carbonyls of α,*β*-unsaturated ketone and ester), 1604 (C=C), and 1375 (geminal dimethyls) cm^{-1}. The ^1H NMR spectrum (Table 1) indicated the probable triterpenoidal nature of this compound from the presence of five quaternary methyls at δ 1.12, 1.24 (× 2), 1.27, and 1.30. In addition, the ^1H NMR spectrum further showed a pair of AB doublets at δ 7.09 (J = 10.1 Hz) and 5.90 (J = 10.1 Hz), while the ^{13}C NMR spectrum exhibited signals at δ 157.0 (C-1), 124.0 (C-2), and 205.0 (C-3), characteristic of the 1-en-3-one system of ring A of the meliacins.[7] This ring substructure was also supported by a mass spectral fragment at *m*/*z* 137.0953 corresponding to $C_9H_{13}O$ (Figure 1), resulting from cleavage of ring B. The presence of a hydroxyl group indicated by the IR absorption at 3400 cm^{-1} was supported by the formation of monoacetyl derivative **1a** (Experimental Section). The respective locations of the α-oriented hydroxy and acetoxy substituents at C-6 and C-7 were revealed by their geminal proton signals at δ 4.38 (dd, J = 11.7, 2.5 Hz, H-6) and 5.35 (d, J = 2.5 Hz, H-7) and a one-proton doublet at δ 2.20 (d, J = 11.7 Hz) for H-5. The ^1H NMR spectrum also showed a one-proton double doublet at δ 5.40 (J = 3.7, 1.5 Hz) due to H-15. A comparison of the ^1H and ^{13}C NMR spectral data (Table 1 and Experimental Section) of **1** with those of nimocinol[7] indicated that both have the same carbocyclic nucleus (A−D), but the signals of the furan ring, a usual feature of the meliacins, were missing in **1**. Instead, a triplet at δ 6.38 (t, J = 1.5 Hz, H-21) and two multiplets between δ 2.01−2.42 (H-22) and 3.52−3.61 (H-23) were observed, indicating a dihydrofuran structure. Additional evidence was obtained from the mass fragment at *m*/*z* 123.0869 [$C_8H_{11}O$] (Figure 1) and the ^{13}C NMR chemical shifts at δ 123.0 (C-20), 142.0 (C-21), 29.5 (C-22), and 67.0 (C-23). These spectral data established that **1** is 22,23-dihydronimocinol [24-norchola-1,14,20-trien-3-one,7(acetoxy)-21,23-epoxy-6-hydroxy,-4,4,8-trimethyl-(5α,-6α,7α,17α)], which is a new compound. Its ^1H and ^{13}C NMR assignments were made through 2D NMR studies including HMQC, HMBC, and $^1H-^1H$ COSY data.

The molecular formula $C_{24}H_{32}O_5$ of **2** was determined with the help of HREIMS. Its UV spectrum exhibited an absorption maximum at 230 nm, and the IR spectrum

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Table 1. NMR Data for **1** and **2**[a]

	1		**2**	
position	δ_H	δ_C	δ_H	δ_C
1	7.09 (1H, d, J = 10.1)	157.0	7.11 (1H, d, J = 10.0)	156.2
2	5.90 (1H, d, J = 10.1)	124.0	5.93 (1H, d, J = 10.0)	125.9
3		205.0		204.5
4		40.0		44.5
5	2.20 (1H, d, J = 11.7)	50.3	2.23 (1H, d, J = 12.0)	46.2
6	4.38 (1H, dd, J = 11.7, 2.5)	67.3	4.35 (1H, dd, J = 12.0, 2.5)	65.0
7	5.35 (1H, d, J = 2.5 Hz)	79.0	5.35 (1H, d, J = 2.5)	78.5
8		46.0		43.2
9	2.48 (1H, dd, J = 12.8, 3.2)	37.3	2.35 (1H, t, J = 7.5)	37.9
10		34.5		39.5
11α	1.84 (1H, m)	16.0	1.91 (1H, m)	16.5
11β	2.50 (1H, m)		2.16 (1H, m)	
12α	1.96 (1H, m)	32.0	1.96 (1H, m)	33.0
12β	2.25 (1H, m)		2.16 (1H, m)	
13		46.0		45.2
14		158.0		193.5
15	5.40 (1H, dd, J = 3.7, 1.5)	119.0	5.74 (1H, dd, J = 3.0, 1.5)	123.0
16α	1.9−2.0 (1H, m)	33.8		208.0
16β	1.9−2.0 (1H, m)			
17α		51.0	2.72 (1H, dd, J = 22.5, 3.5)	57.0
17β	2.3−2.4 (1H, m)		3.12 (1H, dd, J = 22.5, 1.5)	
18	1.12 27 (3H, s)	21.8	1.27 (3H, s)	28.5
19	1.24 (3H, s)	21.8	1.09 (3H, s)	18.3
20		123.0		
21	6.38 (1H, t, J = 1.5)	142.0		
22	2.01−2.42 (2H, m)	29.5		
23	3.52−3.61 (2H, m)	67.0		
28	1.27 (3H, s)	27.0	1.28 (3H, s)	27.0
29	1.24 (3H, s)	24.0	1.30 (3H, s)	21.5
30	1.30 (3H, s)	20.0	1.39 (3H, s)	25.9
$OCOCH_3$		172.0		169.5
$OCOCH_3$	2.04 (3H, s)	21.0	2.06 (3H, s)	21.6

[a] Assignments have been made on the basis of BB, DEPT, HMQC, HMBC, and $^1H-^1H$ COSY experiments. Coupling constants are expressed in Hz. The *C*-methyl shifts have been assigned through comparison with those of related meliacins.[7,8]

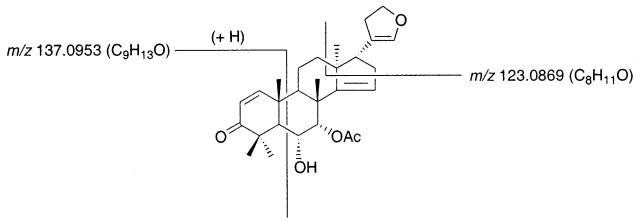

Figure 1. Mass spectral fragmentation pattern for **1**.

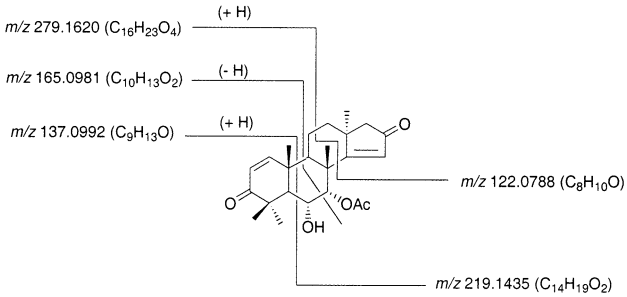

Figure 2. Mass spectral fragmentation pattern for **2**.

displayed peaks at 3400 (hydroxyl), 1725 (α,β-unsaturated five-membered ring ketone and ester carbonyl), 1660 and 1620 (cyclohexenone), and 1375 (geminal dimethyls) cm^{-1}. Five quaternary methyl singlets at δ 1.09, 1.27, 1.28, 1.30, and 1.39 in the ^1H NMR spectrum were supportive of its triterpenoidal nature. The spectral data indicated that **2** has the same tetracyclic skeleton as that of desfuranoazadiradione,[8] but a distinguishing feature of **2** was the hydroxy group at C-6, the presence of which was confirmed by its transformation to the monoacetyl derivative **2a**. The NMR signals of the ring A and B protons of **2** were also comparable with those of **1**. However, it was evident from a double doublet at δ 5.74 (J = 3.0, 1.5 Hz, H-15) that **2** has a carbonyl group at C-16 as in desfuranoazadiradione.[8] The molecular formula and spectral data further demonstrated that **2** has 24 carbons, which were accounted for by the skeleton and the acetoxy carbons. Moreover, the characteristic signals of the furan ring and H-17, present in azadiradione[9] and other meliacins,[10] were not observed, and instead the ^1H NMR spectrum showed two double doublets at δ 2.72 (J = 22.5, 3.0 Hz) and 3.12 (J = 22.5, 1.5 Hz), attributable to the C-17 methylene protons, which exhibited long-range coupling with H-15. These features established that **2** is an octanortriterpenoid, desfurano-6α-hydroxyazadiradione [androsta-1,14-dien-3,16-dione, 6-hydroxy,7(-acetoxy)-4,4,8-trimethyl-(5α,6α,7α,13α,)-]. Further support for this structure came from mass spectral fragments (Figure 2).

Both **1** and **2** were tested for their pesticidal activities against fourth instar larvae of *Anopheles stephensi* and exhibited mortality with LC_{50} values of 62 and 43 ppm, respectively.

Experimental Section

General Experimental Procedures. Melting points were recorded in glass capillary tubes on a Gallenkamp apparatus. Optical rotations were measured on a JASCO DIP-360 polarimeter at 27 °C and are given in units of deg cm^2 g^{-1}. UV (MeOH) and IR ($CHCl_3$) spectra were measured on Hitachi-3200 and JASCO-A302 spectrophotometers, respectively. The ^1H NMR spectra were recorded in $CDCl_3$ on a Bruker Aspect AM instrument operating at 300 MHz, while ^{13}C and 2D NMR spectra (BB, DEPT, COSY 45°, HMQC, and HMBC) were recorded on a Bruker Aspect AM-500 spectrometer operating

at 125 MHz with TMS as internal reference and $CDCl_3$ as solvent. The chemical shifts are recorded in ppm (δ), and coupling constants (J) are in Hz. The EIMS and HREIMS data were carried out with Finnigan MAT-112 and JMS HX-110 spectrometers. Elemental analysis was performed on a Carlo Erba elemental analyzer MOD-1106. All thin-layer chromatography (TLC) was performed on precoated alumina (Riedel-de-Haen DC-cards ALF) sheets. Plates were visualized under UV light (254 and 366 nm) and with iodine vapor.

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

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 was 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 $CHCl_3$−MeOH (9.85:0.15) eluates were combined on the basis of TLC and freed of the solvent to give fraction A (3.9 g), which was again subjected to VLC (silica gel GF_{60-254}, petroleum ether, petroleum ether−EtOAc mixtures of increasing polarity up to 1:1 and then $CHCl_3$ and $CHCl_3$−MeOH mixtures of increasing polarity). The petroleum ether−EtOAc (7:3) eluate furnished fraction B (70 mg), containing three major and two minor constituents by TLC. The residue of this eluate was finally subjected to separation on alumina-coated preparative TLC sheets (petroleum ether−EtOAc, 7:3) affording **2** (15 mg), showing a single spot on TLC.

The petroleum ether−EtOAc (7:3) and petroleum ether−EtOAc (7.5:2.5) eluates were combined (139 mg) and subjected to TLC on alumina-coated preparative sheets (petroleum ether−EtOAc, 6.5:3.5), affording a major component (69 mg), showing a single spot by TLC. The ^1H NMR spectrum indicated that it was still a mixture of several constituents with two major bands, which after a number of trials could ultimately be separated on precoated alumina sheets (petroleum ether−EtOAc, 6:5:3.5) into **1** (12 mg) and 7α-senecioyl-(7-deacetyl)-23-*O*-methylnimocinolide[6] (15 mg).

22,23-Dihydronimocinol (1): mp 84−85 °C; $[\alpha]^{27}_D$ −34.1° (*c* 0.088, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 225 (3.68) nm; IR ($CHCl_3$) ν_{max} 3400, 1725, 1682, 1604, 1375 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1; HREIMS *m/z* 454.2639 (calcd for $C_{28}H_{38}O_5$, 454.2641) [M^+] (15), 223.1304 ($C_{13}H_{19}O_3$), (8), 149.0952 ($C_{10}H_{13}O$) (15), 137.0953 ($C_9H_{13}O$) (93), 123.0869 ($C_8H_{11}O$) (13); *anal.* C 73.96%, H 8.43%, calcd for $C_{28}H_{38}O_5$, C 74.00%, H 8.37%.

Acetylation of 1. To a solution of **1** (8 mg) in pyridine (1 mL) was added acetic anhydride (1 mL) and the solution kept at room temperature overnight. On the usual workup, the monoacetylated product (**1a**) was obtained, showing a single spot on TLC: UV (MeOH) λ_{max} (log ϵ) 225 (3.72) nm; IR ($CHCl_3$) ν_{max} 3400, 1725, 1682, 1604, 1375 cm^{-1}; EIMS *m/z* 496 [M^+] (4); *anal.* C 72.58%, H 8.13%, calcd for $C_{30}H_{40}O_6$, C 72.53%, H 8.12%.

Desfurano-6α-hydroxyazadiradione (2): mp 113−115 °C; $[\alpha]^{27}_D$ −30.0° (*c* 0.02 $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 230 (4.08) nm; IR ($CHCl_3$) ν_{max} 3400, 1725, 1660, 1620, 1375 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1; HREIMS *m/z* 400.2300 (calcd for $C_{24}H_{32}O_5$, 400.2311) [M^+] (12), 279.1620 ($C_{16}H_{23}O_4$) (6), 219.1435 ($C_{14}H_{19}O_2$) (8), 165.0981 ($C_{10}H_{13}O_2$) (8), 149.0997 (11), 137.0992 ($C_9H_{13}O$) (17), 122.0788 ($C_8H_{10}O$) (12); *anal.* C 71.95%, H 8.00%, calcd for $C_{24}H_{32}O_5$, C 72.00%, H 8.00%.

Acetylation of 2. To a solution of **2** (5 mg) in pyridine (0.5 mL) was added acetic anhydride (0.5 mL) and the reaction mixture kept overnight at room temperature. The acetylated product (**2a**) was obtained after the usual workup, showing a single spot on TLC: UV (MeOH) λ_{max} (log ϵ) 230 (4.12) nm; IR ($CHCl_3$) ν_{max} 1725, 1665, 1610, 1375 cm^{-1}; EIMS *m/z* 442 [M^+] (3); *anal.* C 70.56%, H 7.73%, calcd for $C_{26}H_{34}O_6$, C 70.59%, H 7.75%.

Insecticidal Activity. *Anopheles stephensi* (Aurangi Town strain) was used for the toxicity test. Ten young fourth instar mosquito larvae were collected in 5 mL of the rearing tap water and transferred in 250 mL glass beakers containing 200 mL of distilled water. The compounds were tested at 28 ± 1 °C at five final concentrations. The controls were run in the same way with water containing no sample. Each concentration and control was run as a duplicate set, and mortality was recorded after 24 h. The concentrations were selected on the basis of preliminary screening of each compound. The mortality was recorded after 24 h, and readings were subjected to Abbott's formula.[11] The experiment was discarded if more than 10% mortality was found in the control.

The lethal concentrations (LC_{50}) were calculated using probit analysis,[12] taking the average mortalities on the *y*-axis with the dose in ppm on the *x*-axis.

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