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Biologically Active Triterpenoids of Biogenetic Interest from the Fresh Fruit Coats of *Azadirachta indica*

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Abstract—Two new triterpenoids of biogenetic interest, namely, 26,27-dinor-17-oxa-1, 14,20(22)-trien-3, 16-dioxo-7α-acetoxy-17β-methoxy-4,4,8-trimethyl(5α, 13α)-cholane (meliacinin) **1** and 24,25,26,27-tetranor-apotirucalla(-apoeupha)-7α-acetoxy-1,14,20(22)-trien-3-oxo-23-oic acid (azadironic acid) **2** have been isolated from the fresh fruit coats of *Azadirachta indica*. Their structures were established through comparative spectral studies. **1** is the first dinorapoeuphane with seco ring **D** of deoxygedunin type and **2** is the first tetranorapoeuphane with an open side chain representing a biogenetic intermediate between the intact triterpenoids and meliacins. Both **1** and **2** showed toxicity against mosquito (*Anopheles stephensi*) with LC_{50} 13 and 4.5 ppm, respectively.

Introduction

Azadirachta indica (neem) belongs to the family Meliaceae and is widely distributed in Asia and Africa. In view of the attributed therapeutic and pesticidal importance,[1–5] comprehensive chemical and biological investigations on its different parts have been carried out by various groups of workers and a host of new constituents has been reported in literature.[6–13] In one of our programs the fresh fruits were manually separated into their seeds and seed coats, extracted with ethanol and the activity of each was determined against *Musca domestica*. It was observed that the coat extract was 200 times more effective (LC_{50} 1.1 ppm) than that of the seed extract (LC_{50} 195 ppm).[5] Following this observation, in the present studies the two extracts were tested for their activity against *Anopheles stephensi* by WHO method[14] and a similar difference of activity was noted. On the other hand, in a separate program of estimating azadirachtin, which is the most active pesticidal constituent of neem, in different parts of the tree, collected from different areas of Pakistan, it was observed that the coat extract is devoid of any azadirachtin content. These studies prompted us to undertake a bioassay directed study on the seed coats extract and identify the constituents responsible for the observed pesticidal activity against *A. stephensi*. These investigations led to the isolation of seven biologically active triterpenoids, including two new nortriterpenoids of biogenetic interest, meliacinin **1** (LC_{50} 13 ppm) and azadironic acid **2** (LC_{50} 4.5 ppm), besides five known compounds, azadiradione[6] (LC_{50} 15 ppm), epoxyazadiradione[6] (LC_{50} 18 ppm), limocin A & B[10] (LC_{50} 19 ppm) and desfuranoazadiradione[11] (LC_{50} 37 ppm). The methyl ester of azadironic acid **2a** exhibited higher toxicity (LC_{50} 2.8 ppm) than that of **2**.

Results and Discussion

The EIMS and HREIMS of **1** showed a molecular ion peak M^+ at *m/z* 512 and 512.3145, respectively, corresponding to the molecular formula $C_{31}H_{44}O_6$ (calcd for $C_{31}H_{44}O_6$, 512.3138). The IR spectrum exhibited peaks at 3070 (vinylic C–H), 1740 br. (ester carbonyl and α,β-unsaturated lactone), 1685 and 1640 (α,β-unsaturated carbonyl and C=C), 1380 and 1370 (geminal methyls) and 1220 (C–O) cm^{-1}. The UV spectrum displayed maxima at 226 nm. A pair of AB-doublets at δ 7.15 and 5.89 ($J_{1,2}$=10.2 Hz), five quaternary methyls displayed by singlets at δ 1.24 (H-18 and H-19), 1.06 (H-28), 1.08 (H-29) and 1.34 (H-30) in the ^1H NMR (Table 1) and mass fragments at *m/z* 137.0975 ($C_9H_{13}O$; fragment **a**) and 149.0973 ($C_{10}H_{13}O$; fragment **b**) indicated the triterpenoidal nucleus in **1**, with 1-en-3-one system in ring A. A one-proton triplet at δ 5.31 (J=3.5 Hz) was related to H-7 geminal to the acetoxy function (δ $OCOCH_3$ 2.09).[6,7,10,11,13] A sharp singlet at δ 5.84 (H-15) and the significant mass fragment at *m/z* 370.2151 ($C_{23}H_{30}O_4$; fragment **c**) arising from the retro-Diels–Alder cleavage of ring D, and *m/z* 328.2044 ($C_{21}H_{28}O_3$), resulting

Keywords: *Azadirachta indica*; Meliaceae; neem fruit coats; triterpenoids; azadironic acid; meliacinin.

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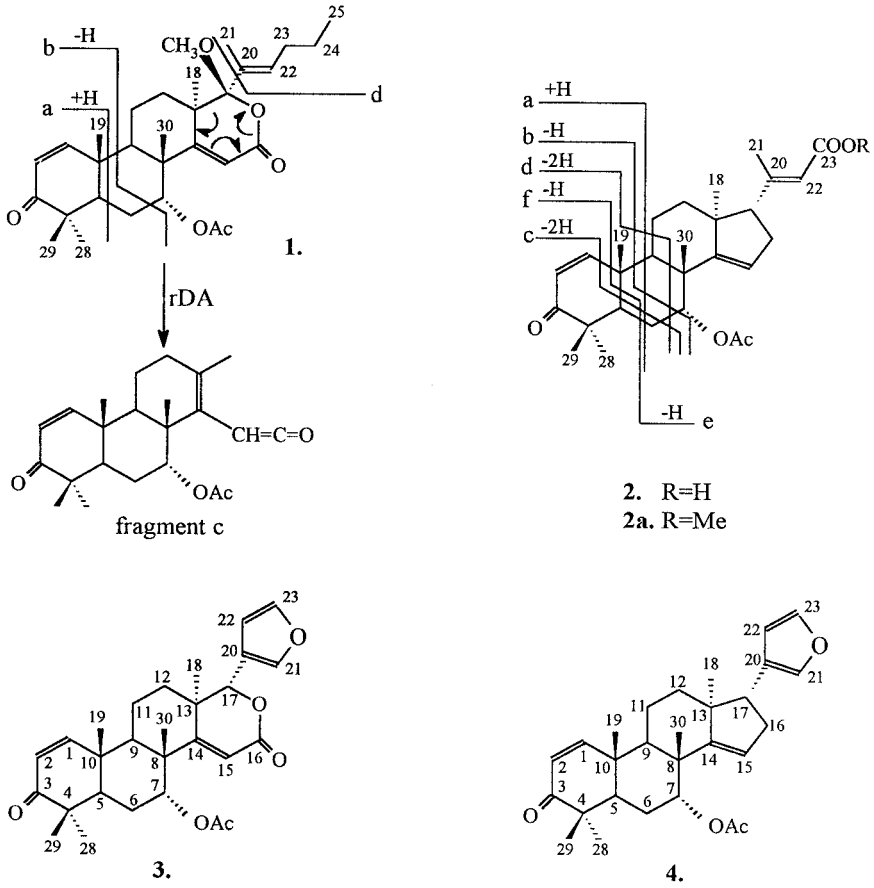
Table 1. [1]H NMR chemical shifts (δH) for **1** to **4**. Multiplicities and coupling constant (Hz) given in parentheses

No.	**1**	**3**[16]	**2**	**4**[9]
1	7.15 (d, 10.2)	7.05 (d, 10.0)	7.16 (d, 10.2)	7.13 (d, 10.2)
2	5.89 (d, 10.2)	5.85 (d, 10.0)	5.88 (d, 10.2)	5.83 (d, 10.2)
5	2.11 (dd, 12.0, 3.0)	–	1.95 (dd, 12.1, 2.9)	2.18 (dd, 12.2, 2.7)
6α	1.89–1.96 (m)	–	1.69–2.03 (m)	1.90 (ddd, 17.0, 3.4, 2.7)
6β	1.89–1.96 (m)	–	1.69–2.03 (m)	1.78 (ddd, 17.0, 12.2, 3.4)
7	5.31 (t, 3.5)	5.24 (m)	5.29 (m)	5.25 (t, 3.4, 3.4)
9	2.34 (dd, 12.0, 5.2)	–	2.36 (dd, 11.9, 5.3)	2.21 (dd, 11.5, 2.8)
11α	1.89–1.96 (m)	–	1.69–2.03 (m)	1.75 (m)
11β	1.89–1.96 (m)	–	1.69–2.03 (m)	1.95 (m)
12α	1.89–1.96 (m)	–	1.69–2.03 (m)	1.65 (m)
12β	1.89–1.96 (m)	–	1.69–2.03 (m)	1.85 (m)
15	5.84 (s)	5.68 (s)	5.23 (m)	5.35 (dd, 3.4, 2.1)
16α	–	–	2.21–2.38 (m)	2.41 (ddd, 16.3, 10.8, 2.1)
16β	–	–	2.21–2.38 (m)	2.31 (ddd, 16.3, 7.4, 3.4)
17	–	4.97 (s)	1.94 (t, 8.0)	2.78 (dd, 10.8, 7.4)
18	1.24 (s)	1.20 (s)	1.28 (s)	0.77 (s)
19	1.24 (s)	1.25 (s)	1.24 (s)	1.20 (s)
21	1.93 (s)	7.45 (m)	2.06 (br.s)	7.21 (s)
22	5.31 (t, 3.6)	6.42 (m)	5.90 (s)	6.59 (dd, 1.7, 0.7)
23a/b	0.84–1.90 (m)	7.45 (m)	–	7.34 (t, 1.7)
24a/b	0.84–1.90 (m)	–	–	–
25	0.89 (t, 7.6)	–	–	–
28	1.06 (s)	1.10 (s)	1.07 (s)	1.05 (s)
29	1.08 (s)	1.10 (s)	1.08 (s)	1.06 (s)
30	1.34 (s)	1.35 (s)	1.38 (s)	1.23 (s)
OAc	2.09 (s)	2.00 (s)	2.04 (s)	1.93 (s)
OMe	3.48 (s)	–	–	–
COOH	–	–	10.20 (s)	–



Scheme 1.

Table 2. ^{13}C NMR chemical shifts (δC) of **2** and **4**[9]

No.	**2**	**4**[9]	No.	**2**	**4**[9]	No.	**2**	**4**[9]
1	157.0	158.20	11.	17.4	16.56	21.	22.4	139.74
2	126.1	125.53	12.	29.7	33.09	22.	125.0	111.09
3	203.5	204.58	13.	45.5	47.22	23.	190.8	142.60
4	44.4	44.18	14.	158.8	158.95	28.	22.7	21.34
5	45.4	46.21	15.	118.6	119.16	29.	27.0[a]	27.10
6	23.6	23.85	16.	34.0	34.46	30.	27.3[a]	27.36
7	73.6	74.61	17.	54.8	51.72	$OCOCH_3$	170.0	170.11
8	42.0	42.88	18.	21.1[a]	20.69	$OCOCH_3$	21.6[a]	21.16
9	38.0	38.76	19.	18.0	19.07			
10	40.1	40.01	20.	140.0	124.62			

[a] Assignments may be interchanged.

from the loss of ketene from fragment **c** (Scheme 1) were indicative of deoxygedunin (**3**) nucleus.[8,16] A three-proton sharp singlet at δ 3.48 and the fragment at *m/z* 480.2881 $[C_{30}H_{40}O_5]^+$ due to loss of CH_3OH from the molecular ion in HREIMS indicated the presence of a methoxy group. Absence of a carbinylic signal suggested that the methoxy group is located at a quaternary carbon and the absence of characteristic H-17 signal of the gedunin type of meliacins[6,8,16] indicated this to be C-17. The molecular formula showed ten double bond equivalents, the tetracyclic deoxygedunin nucleus and its functionalities accounted for nine of these. The remaining double bond had to be incorporated in the side chain unit (C_6H_{11}) which was identified as $-C(CH_3)=CH-CH_2-CH_2-CH_3$ on the basis of the following observations. The ^1H NMR of **1** showed a three-proton singlet at δ 1.93 assigned to the vinylic methyl and a vinylic proton resonating at δ 5.31 (dd, J=3.6 Hz). Furthermore, it had a triplet at δ 0.89 (J=7.6 Hz) indicating the presence of a terminal ethyl group. The structure of the side chain was also supported by the fragment in the HREIMS at *m/z* 83.0868 (C_6H_{11}; fragment **d**). Hence meliacinin was assigned the structure as 26,27-dinor-17-oxa-1,14,20(22)-trien-3, 16-dioxo-7α-(acetoxy)-17β-methoxy-4,4,8-trimethyl-(5α,13α)-cholane (**1**).

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 connectivities between H-5α/H-9α, H-9α/H-18α and between H-7β/H-30β, and H-30β/-OCH_3. Hence $-OCH_3$ was given β disposition at C-17.

The EIMS and HREIMS of **2** showed a molecular ion peak M^+ at *m/z* 454 and 454.2712, respectively, suggesting the molecular formula as $C_{28}H_{38}O_5$ (calcd for $C_{28}H_{38}O_5$, 454.2719). The presence of five quaternary methyls in the ^1H NMR spectrum at δ 1.28 (H-18), 1.24 (H-19), 1.07 (H-28), 1.08 (H-29) and 1.38 (H-30) indicated its triterpenoidal nature. The IR spectrum exhibited peaks at 3100 (vinylic C–H), 3000–2700 br. (OH), 1705 br. (ester carbonyl and α,β-unsaturated acid), 1660 (α,β-unsaturated cyclohexenone), 1375 and 1365 (geminal methyls) and 1210 (C–O) cm^{-1}. The UV spectrum displayed maxima at 226 nm. The spectral data (IR, UV, ^1H and ^{13}C NMR) revealed that **2** has the same tetracyclic skeleton as that of azadirone (**4**).[6,9,10,13] Thus a pair of AB doublets at δ 7.16 and 5.88 ($J_{1,2}$=10.2 Hz) in the ^1H NMR spectrum (Table 1) and the signals at δ 157.0 (C-1), 126.1 (C-2) and 203.5 (C-3) in the ^{13}C NMR spectrum (Table 2) indicated ring A 1-ene-3-one system[6,7,9–11,13] which was also supported by mass fragments at *m/z* 137.0969 ($C_9H_{13}O$; fragment **a**) and 149.0973 ($C_{10}H_{13}O$; fragment **b**). H-7 and H-15 were observed as one proton multiplets at δ 5.29 and 5.23, respectively, while two one-proton double doublets related to H-5 and H-9 resonated at δ 1.95 ($J_{5,6\beta}$=12.1, $J_{5,6\alpha}$=2.9 Hz) and δ 2.36 ($J_{9,11\beta}$=11.9, $J_{9,11\alpha}$=5.3 Hz), respectively. The acetoxy methyl at C-7 was noted at δ 2.04 in the ^1H NMR spectrum as a three-proton singlet. The molecular formula and the ^{13}C NMR spectrum demonstrated that **2** has 28 carbons, 22 of which are the part of the triterpenoidal skeleton while two are accounted for by the acetyl group. The ^1H NMR spectrum further showed a vinylic methyl at δ 2.06 (br. s), a one-proton sharp singlet for an olefinic proton at δ 5.90, and a one-proton singlet at δ 10.20 due to the COOH proton. In the ^{13}C NMR spectrum the respective resonances were observed at δ 22.4, 125.0 and 190.8 along with a quaternary carbon at δ 140.0. These features indicated that C-23 forms the carboxyl function and the double bond should be between C-20 and C-22. The chemical shifts of the vinylic methyl and H-22 points to the configuration of the double bond as being *E*.[17] The carboxyl moiety of **2** was confirmed by methylation with diazomethane. The mass spectrum of the methylated compound, **2a**, showed the molecular ion peak at *m/z* 468 corresponding to the molecular formula $C_{29}H_{40}O_5$ while the ^1H NMR spectrum exhibited a three-proton singlet at δ 3.51 for -OMe in addition to other signals of **2**. In the light of the spectral studies described above, the structure of azadironic acid has been assigned as 24,25,26,27-tetranor-apotirucalla(-apoeupha) acetoxy, 1, 14, 20 (22)-trien-3-oxo-23-oic acid (**2**), which was substantiated by various mass fragments **a**–**f** (vide structure and Experimental).

The stereochemistry as depicted in the structure of **2** was arrived at on the basis of coupling constants of various protons and $^1H-^1H$ interactions observed in the 2D NOE spectrum. Thus connectivities were observed between H-5α/H-9α, H-9α/H-18α and between H-7β/H-30β.

Compound **1** represents the first dinortriterpenoid of apoeuphane (apotirucallane) skeleton with seco ring **D** of deoxygedunin type although a few dinortriterpenoids with intact ring **D** have been reported earlier.[13] Compound **2** is a tetranortriterpenoid with open side chain and may be regarded as a degradative intermediate between the intact eight carbons side chain of triterpenes and the furan or hemiacetal ring in meliacins or butenolides during biosynthesis.[15]

The insecticidal activity was determined on *A. stephensi* (4th instar larvae). Compounds **1**, **2** and **2a** and the known constituents azadiradione, epoxyazadiradione, limocin A & B and desfuranoazadiradione showed insect growth regulating (IGR) effect on these larvae. **1**, **2**, and **2a** had LC_{50} 13, 4.5 and 2.8 ppm, respectively, while azadiradione, epoxyazadiradione, limocin A & B and desfuranoazadiradione showed LC_{50} 15, 18, 19 and 37 ppm, respectively.

Experimental

IR and UV spectra were measured in $CHCl_3$ and MeOH on JASCO IR-Spectrophotometer A-302, and Hitachi

UV-spectrophotometer U-3200, respectively. Optical rotations were measured on JASCO digital polarimeter DIP-360. ^{1}H NMR spectra were recorded in $CDCl_3$ with Me_4Si as internal standard on a Bruker Aspect AM 300 instrument operating at 300 MHz while ^{13}C NMR spectra were recorded in $CDCl_3$ at 75 MHz. The chemical shifts (δ) are in ppm and coupling constant (J) are in Hz. Mass spectra were recorded on double focussing Finnigan MAT-112 spectrometer with ei energy 70 eV and ion source temperature 250°C. Assignments of the proton and carbon chemical shifts are based on a comparison of chemical shifts of compounds with related partial structures.[6–11,13,16] E. Merck Kieselgel 60 PF_{254}, coated on glass plates was used for analytical (TLC), preparative (thick layer) chromatography and for VLC. For flash column chromatography E. Merck Kieselgel 60 (0.040–0.063 mm) was used.

Fresh, undried, ripe fruits (50 kg) of *A. indica* were collected from Karachi region in the month of July. A voucher specimen (No. NM-1) of the plant material identified by Prof. Dr S. I. Ali, Department of Botany, University of Karachi, has been deposited in the herbarium of Botany Department, University of Karachi. Seeds and coatings of the fruits were separated manually, and each of them extracted repeatedly (×5) with EtOH at room temperature, and the solvent of the extracts was removed under reduced pressure. The ethanolic extract of the uncrushed fruit coats (23 kg) was designated as RB-b and that of seeds as RB-a. RB-b and RB-a showed LC_{50} 290 and 784 ppm, respectively, against *A. stephensi*. RB-b was partitioned between EtOAc and H_2O and a small portion of the EtOAc phase was washed, dried over Na_2SO_4, and freed of the solvent to give a gummy syrup which was kept for determination of biological activity (LC_{50} 165 ppm). The main quantity of the EtOAc phase was treated with 4% aq. Na_2CO_3 solution to ultimately furnish acidic (RB-b 'A', LC_{50} 144 ppm) and neutral (RB-b 'N', LC_{50} 43 ppm) fractions. The neutral fraction which was more effective, was divided into petrol ether soluble (LC_{50} 159 ppm) and petrol ether insoluble fractions (LC_{50} 154 ppm). The petrol ether insoluble fraction was again divided into ether soluble (RB-b 'ES', LC_{50} 106 ppm) and ether insoluble portions (RB-b 'EI'). RB-b 'EI' mainly consisted of a single compound which on recrystalization from MeOH afforded azadiradione[6] (82 g, LC_{50} 15 ppm). Rb-b 'ES' (189 g) was subjected to VLC in a sintered funnel (13 cm diameter) charged with silica gel in three portions and eluted with petrol ether (1 l), petrol ether/EtOAc 90:10 to 10:90 with 10% gradient (2 l each), EtOAc (5 l) and MeOH (4 l) and the eluates (250 ml each) were combined on the basis of TLC [silica gel, petrol ether/EtOAc 98:2 to 85:15; detection UV (254 nm)] into thirteen fractions marked as A through M. Of these, fraction 'D' which eluted with petrol ether/EtOAc 70:30 (2 l) was identified as epoxyazadiradione[6] (18 g, LC_{50} 18 ppm); fraction E, petrol ether/EtOAc 60:40 (2 l) eluate was a mixture of azadiradione and epoxyazadiradione (nearly 1:1); fractions F, petrol ether/EtOAc 50:50 (2 l) eluate and fraction G, petrol ether/EtOAc 40:60 (2 l) eluate consisted of azadiradione with traces of epoxyazadiradione. The rest of the fractions (A-C) and (H-M) were tested for their activity against *A. Stephensi*. Fraction L (1.53 g), which eluted with EtOAc 100% (5 l), was the most active fraction (LC_{50} 66 ppm) among these and was subjected to flash column chromatography in a glass column (5 cm diameter) packed with silica gel at a flow rate of 3 ml with the following eluents; $CHCl_3$ (14 fractions of 25 ml each), $CHCl_3$/MeOH 99:1 to 85:15 with 1% gradient (10 fractions of 25 ml each), 85:15 to 50:50 with 5% gradient (10 fractions of 25 ml each) and MeOH (80 fractions of 25 ml each). The eluates were combined on the basis of TLC [silica gel, $CHCl_3$/MeOH 98:2 to 90:10, detective UV (254 nm)], to afford sixteen fractions (LFC-1 through LFC-16). LFC-2 (159 mg; LC_{50} 36 ppm) eluted with $CHCl_3$ and was further separated through preparative thick layer chromatography on E. Merck kieselgel 60 PF_{254}, $CHCl_3$, detection UV (254 nm), affording eleven bands (LFC-201 through LFC-211). LFC-210 was characterized as meliacinin **1** (R_f 0.13), and LFC-207 as desfuranoazadiradione[11] (R_f 0.46). Limocin A & B[10] were obtained as a (1:1) mixture from LFC-204 (R_f 0.75). The broad band of LFC-205 (R_f 0.6) was further purified by preparative thick layer chromatography (silica gel), $CHCl_3$ to yield azadironic acid **2** (R_f 0.58).

26,27-Dinor-17-oxa-1,14,20(22)-trien-3,16-dioxo-7α-acetoxy-17β-methoxy-4,4,8-trimethyl (5α,13α)-cholane (meliacinin) 1. Compound **1** (6.2 mg); was purified as white powder $[\alpha]_D^{30}=+0.68$ (c, 0.09, MeOH); IR: 3070 (vinylic C–H), 1740 br. (ester carbonyl and α,β-unsaturated lactone), 1685 br. and 1640 (α,β-unsaturated cyclohexenone and C=C), 1380 and 1370 (geminal methyls), 1220 (C–O) cm^{-1}; UV: 226 nm; HR-EI-MS *m/z* (%) Found: 512.3145 (62) $C_{31}H_{44}O_6$: requires M^+, 512.3138, 497.2909 $[C_{30}H_{41}O_6]^+$, $[M-CH_3]^+$, (24), 482.2675 $[C_{29}H_{38}O_6]^+$, $[M-C_2H_6]^+$, (14), 480.2881 $[C_{30}H_{40}O_5]^+$, $[M-MeOH]^+$, (8), 468.2519 $[C_{28}H_{36}O_6]^+$, $[M-C_3H_8]^+$, (39), 453.3012 $[C_{29}H_{41}O_4]^+$, $[M-OAc]^+$, (17), 370.2151 $[C_{23}H_{30}O_4$, fragment **c**$]^+$, (7), 328.2044 $[C_{21}H_{28}O_3]^+$, [fragment **c**-$C_2H_2O]^+$, (100), 149.0973 $[C_{10}H_{13}O$, fragment **b**$]^+$, (13), 137.0975 $[C_9H_{13}O$, fragment **a**$]^+$, (21), 111.0816 $[C_7H_{11}O]^+$, (12), 83.0868 $[C_6H_{11}$, fragment **d**$]^+$, (14), 69.0710 $(C_5H_9)^+$, (16), 55.0554 $[C_4H_7]^+$, (15); δH (Table 1).

24,25,26,27-Tetranor-apotirucalla(-apoeupha)-7α-acetoxy-1,14,20(22)-trien-3-oxo-23-oic acid (azadironic acid) 2. The title compound (7.3 mg) was obtained as amorphous powder $[\alpha]_D^{30}=+0.25$ (c, 0.04, MeOH); IR: 3100 (vinylic C–H), 3000–2700 br. (OH), 1705 br. (ester carbonyl and α,β-unsaturated acid), 1660 (α, β-unsaturated cyclohexenone), 1375 and 1365 (geminal methyls) 1210 (C–O) cm^{-1}; UV: 226 nm; HR-EI-MS *m/z* (%) Found: 454.2712 (38) $C_{28}H_{38}O_5$: requires M^+ 454.2719, 394.2516 $[C_{26}H_{34}O_3]^+$, $[M-AcOH]^+$ (41), 344.1987 $[C_{21}H_{28}O_4$, fragment **e**$]^+$ (32), 329.1757 $[C_{20}H_{25}O_4]^+$ (35), 309.1857 $[C_{21}H_{25}O_2]^+$ (17), 285.1857 $[C_{19}H_{25}O_2]^+$, [fragment e-AcO$]^+$ (23), 214.1365 $[C_{15}H_{18}O]^+$ (14), 207.1391 $[C_{13}H_{19}O_2]^+$ (52), 201.1284 $[C_{14}H_{17}O]^+$ (41), 189.1285 $[C_{13}H_{17}O]^+$ (41) 161.0973 $[C_{11}H_{13}O$, fragment **d**$]^+$ (50), 149.0973 $[C_{10}H_{13}O$, fragment **b**$]^+$ (61), 137.0969 $[C_9H_{13}O$, fragment **a**$]^+$ (100), 121.0661 $[C_8H_9O$, fragment **c**$]^+$ (76), 108.0582 $[C_7H_8O$, fragment **f**$]^+$ (60), 83.0504 $(C_5H_7O]^+$ (45); δH (Table 1); δC (Table 2).

Methylation of 2

Treatment of **2** (5 mg) with freshly prepared diazomethane in ether and keeping the contents overnight at room

temperature, followed by usual workup and thick layer chromatography afforded the methyl ester **2a** (~50% yield) as amorphous powder; IR: 3100 (vinylic H), 1700 br. (ester carbonyl and α,β-unsaturated ester), 1660 (α,β-unsaturated cyclohexenone), 1375 and 1365 (geminal methyls), 1230 (C–O) cm^{-1}; EI-MS *m/z* (%) Found: M^+, 468 (19), 299 (11), 215 (13), 201 (18), 149 (32), 137 (100), 121 (97), 99 (14); ^1H NMR data showed a singlet of carbomethoxy protons resonating at δ 3.51, along with other signals as observed in case of **2**.

Activity of all the pure compounds was determined. In case of **2**, the activity of its methyl derivative **2a** was also assayed. The observed LC_{50} were: **1** (13 ppm), **2** (4.5 ppm), **2a** (2.8 ppm), desfuranoazadiradione (37 ppm), limocin A & B (19 ppm), azadiradione (15 ppm) and epoxyazadiradione (18 ppm).

Biological Activity

Biological test (Screening Procedure). Ten young 4th instar larvae of *A. stephensi* Aurangi Town wild strain were collected in 5 ml of tap water and transferred in a glass beaker of 100 ml, containing 45 ml of distilled water. The compounds were tested at 28±1°C at five final concentrations. The controls were also set. Each concentration and control was run as duplicate set and mortality was recorded after 24 h.

Accurate tests. The WHO method[14] was modified for the application. A batch of 10 insects (4th instar larvae) was released in 100 ml beaker, containing 50 ml filtered tap water. The concentrations selected in the preliminary screening of each compound were tested at 28±1°C. A group of 7 beakers was set up, five for different concentrations and one each for control and check. Each experiment was repeated five times. The experiment was discarded if the mortality was found more than 10% in control. The mortality was recorded after 24 h and readings were subjected to Abbot's formula.

Calculations of LC_{50}. The lethal concentrations (LC_{50}) were calculated using PROBIT analysis[18] taking the average mortalities on *y*-axis while the dose in ppm on *x*-axis.

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