TETRANORTRITERPENOIDS FROM AZADIRACHTA INDICA A.JUSS (MELIACEAE)

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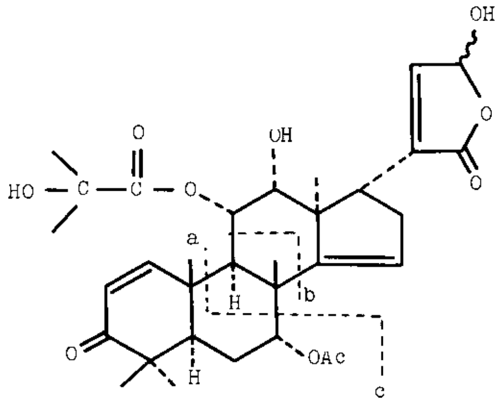
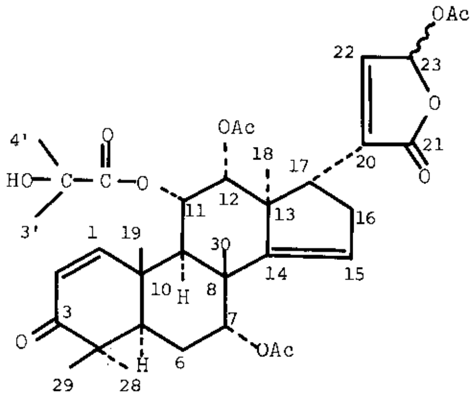
Abstract — Nimbocinolide, a new tetranortriterpenoid has been isolated from the acidic fraction of fresh, undried leaves of A.indica (neem) and its structure was established as 21,23-epoxy-1,14,20-apoeupha(apotirucalla)-trien-3,21-dione-7α,11α,12α,23ξ-tetraol-11-(2'-hydroxy-2'-methyl) propionate-7-acetate (I), through chemical and spectral studies.

As a result of investigations in the constituents of the fruits, leaves, twigs and stem and root bark of neem, a series of terpenoidal constituents have been reported by various groups of workers.[1-5] Present studies in the acidic fraction of the leaves have led to the isolation of a new tetranortriterpenoid γ-hydroxybutenolide, nimbocinolide (I) along with the known terpenoid nimocinolide (III) communicated earlier.[2] (I) is of potential biological importance as preliminary experiments carried out on the acidic fraction have shown that it is capable of disturbing the metamorphic growth of mosquitoes (Aedes aegypti) and produces larval-pupal intermediates. Detailed biological investigations of this fraction, as well as of the uniform constituent nimbocinolide, are in progress and will be reported elsewhere. Further, nimocinolide and its isomer isonimocinolide have also been shown to possess insect growth regulating properties.[2] A complete ^{1}H- and ^{13}C-nmr assignment of the known ring C-secotetranortriterpenoids, nimbin (IV)[6,7] (the first crystalline triterpenoid isolated by S.Siddiqui in 1942 from neem oil),[6] and deacetylnimbin[8,9] (V) has also been made through detailed 2D-nmr studies including noesy, cosy-45, J-resolved and heterocosy.

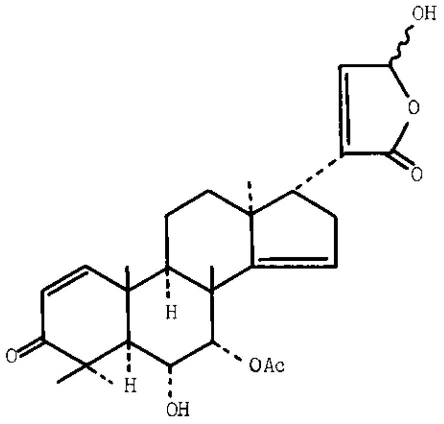
Ethanolic extract of the fresh, undried, uncrushed leaves of neem was divided into acidic and neutral fractions. The residue obtained on usual work up of the acidic fraction ultimately afforded nimbocinolide (I) and nimocinolide (III), following the isolation procedure recorded in the experimental. Nimbocinolide (I) has molecular formula $C_{32}H_{42}O_{10}$ (high resolution mass), its uv spectrum showed absorption at 225 nm and the ir spectrum showed peaks at 3600-3300 (OH), 1760 (α,β-unsaturated γ-lactone), 1740 (ester carbonyl), 1660 (cyclohexenone), 1645 and 820 cm^{-1} (trisubstituted double bonds). These observations along with the nmr spectral data showed



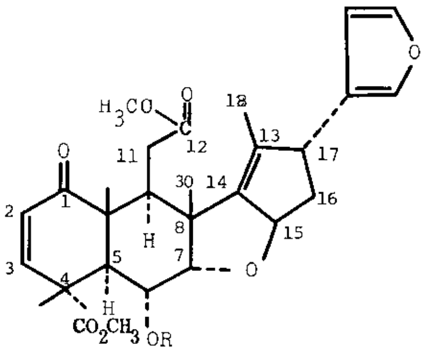
Nimbocinolide (I)
Fragment d=b-AcOH
Fragment e=c-AcOH



II



Nimocinolide (III)



IV R=Ac
V R=H

that nimbocinolide (I) has a meliacane nucleus with 1-en-3-one ring A (δ_{H-1} 7.09, d, J=10.2Hz, δ_{H-2} 5.90, d, J=10.2Hz; δ_{C-1} 157.5, δ_{C-2} 126.0, δ_{C-3} 205.5), C=C at C-14 (δ_{H-15} 5.53; δ_{C-14} 155.5, δ_{C-15} 119.2) and a 23-hydroxy-20(22)-butene(21→23)-γ-lactone side chain (δ_{H-22} 6.95, δ_{H-23} 6.12; δ_{C-20} 136.5, δ_{C-21} 168.7, δ_{C-22} 145.2, δ_{C-23} 97.1). The presence of this side chain was supported by a fragment at m/z 486.2655 ($C_{28}H_{38}O_7$) in the mass spectrum arising from the loss of $C_4H_4O_3$. The ^{1}H-nmr spectrum further showed an acetoxy methyl function resonating as a sharp singlet at δ 2.17. These values are comparable with those observed in nimocinolide,[2] however, H-7β geminal to the ester function appeared as a doublet (J=2.4Hz) at δ 5.34 in nimocinolide whereas, the same proton in I appeared as a multiplet (W h/2 = 6.0 Hz) at δ 5.50 (Table I), indicating the absence of a substituent at C-6 and the α disposition of the acetate function. Apart from these observations, the ^{1}H-nmr spectrum exhibited a one-proton *doublet of doublets* (J=12.3, 2.3Hz) at δ 5.46 and a one-proton doublet at δ 4.06 (J=2.3Hz), suggesting the presence of another ester and a hydroxyl function. The multiplicities and coupling constants of these protons along with a doublet at δ 2.72 (J=12.3Hz) indicated the location of the ester and the hydroxyl function at C-11 and C-12 respectively with α disposition. This was confirmed through ^{1}H-^{1}H homonuclear decoupling experiments, thus irradiation of the signal at δ 4.06 collapsed the double doublet at δ 5.46 to a doublet (J=12.3Hz), while irradiation at δ 5.46 resulted in the collapse of the doublets at δ 4.06 (J=2.3Hz) and 2.72 (J=12.3Hz) to singlets and irradiation at δ 2.72 collapsed the doublet at δ 5.46 to a doublet (J=2.3Hz). The structural features recorded so far and a fragment at m/z 483.2372 ($C_{28}H_{35}O_7$) in the mass spectrum, indicated the composition of the ester function at C-11 as $C_4H_7O_3$. Its structure was established as $(CH_3)_2C(OH)-\overset{O}{\overset{\|}{C}}-O-$ through the absence of any proton geminal to the hydroxyl function in the ^{1}H-nmr spectrum, presence of a quaternary carbon (δ 73.7) in the ^{13}C-nmr spectrum and the formation of only the diacetate derivative (II) (acetic anhydride/pyridine, overnight, room temperature). Finally, the location of the acetate group at C-7 and 2'-hydroxy-2'-methylpropyloxy function at C-11 was corroborated by the significant fragments a-e observed in the mass spectrum of I. The double signals observed for C-15 to C-17 and C-20 to C-23 (Table 2) indicated that I is also epimeric at C-23 as observed in nimocinolide (loc.cit). It may be mentioned in this context that isonimbocinolide, the C-21, C-23 isomer of nimbocinolide (I) has earlier been isolated from neem leaves[10] and a comparison of the spectral data of two compounds clearly revealed that they have identical carbocyclic nuclei and differ only in the nature of C-17 side chain.

Table-1. ^{1}H-Nmr Spectral Data (δ_H ppm and J/Hz) of Triterpenoids

Proton	I	II	IV	V	Nimbandiol[15]
1	7.09,d $J_{1,2}$ 10.2	7.14,d $J_{1,2}$ 10.1	-	-	-
2	5.90,d $J_{2,1}$ 10.2	5.88,d $J_{2,1}$ 10.1	5.86,d $J_{2,3}$ 10.1	5.85,d $J_{2,3}$ 10.1	5.75,d $J_{2,3}$ 10.2
3	-	-	6.32,d $J_{3,2}$ 10.1	6.40,d $J_{3,2}$ 10.1	6.53,d $J_{3,2}$ 10.2
5	2.05,dd $J_{5,6\alpha}$ 2.2 $J_{5,6\beta}$ 9.2	1.91-1.80,m	3.66,d $J_{5,6}$ 12.4	3.40,d $J_{5,6}$ 11.6	2.66,d $J_{5,6}$ 11.5
6	1.77,m	1.91-1.80,m	5.20,dd $J_{6,5}$ 12.4 $J_{6,7}$ 3.0	3.93,ddd $J_{6,5}$ 11.6 $J_{6,OH}$ 11.6 $J_{6,7}$ 3.3	4.24,ddd $J_{6,5}$ 11.5 $J_{6,OH}$ 7.5 $J_{6,7}$ 3.5
7	5.50,m $W_{1/2}$ 6.0	5.40-5.29,m	4.03,d $J_{7,6}$ 3.0	4.01,d $J_{7,6}$ 3.3	3.99,d $J_{7,6}$ 3.5
9	2.72,d $J_{9,11}$ 12.3	2.66,d $J_{9,11}$ 10.1	2.83,t $J_{9,11\alpha}$ 5.4 $J_{9,11\beta}$ 5.4	2.76,dd $J_{9,11\alpha}$ 5.6 $J_{9,11\beta}$ 3.8	2.70,dd $J_{9,11\alpha}$ 6.0 $J_{9,11\beta}$ 4.0
11α	-	-	2.90,dd J_{gem} 16.0 $J_{11\alpha,9}$ 5.4	2.88,dd J_{gem} 16.3 $J_{11\alpha,9}$ 5.6	2.91,dd J_{gem} 16.5 $J_{11\alpha,9}$ 6.0
11β	5.46,dd $J_{11,9}$ 12.3 $J_{11,12}$ 2.3	5.40-5.29,m	2.21,dd J_{gem} 16.0 $J_{11\beta,9}$ 5.4	2.21,dd J_{gem} 16.3 $J_{11\beta,9}$ 3.8	2.18,dd J_{gem} 16.5 $J_{11\beta,9}$ 4.0
12β	4.06,d $J_{12,11}$ 2.3	5.40-5.29,m	-	-	-
15	5.53,dd $J_{15,16\beta}$ 3.9 $J_{15,16\alpha}$ 1.8	5.57,m	5.54,dddq $J_{15,16\alpha}$ 6.6 $J_{15,16\beta}$ 6.6 $J_{15,18}$ 1.7 $J_{15,17}$ 0.8	5.54,dddq $J_{15,16\alpha}$ 6.6 $J_{15,16\beta}$ 6.6 $J_{15,18}$ 1.7 $J_{15,17}$ 1.2	5.48,dddq $J_{15,16\alpha}$ 7.0 $J_{15,16\beta}$ 7.0 $J_{15,18}$ 1.8 $J_{15,17}$ 1.0
16α	2.64,ddd J_{gem} 15.0 $J_{16\alpha,17}$ 9.5 $J_{16\alpha,15}$ 1.8	2.35,m	2.18,dd J_{gem} 12.0 $J_{16\alpha,15}$ 6.6	2.18,dd J_{gem} 12.2 $J_{16\alpha,15}$ 6.6	2.16,dd br J_{gem} 12.0 $J_{16\alpha,15}$ 7.0
16β	2.40,ddd J_{gem} 15.0 $J_{16\beta,17}$ 7.2 $J_{16\beta,15}$ 3.9	2.35,m	2.02,ddd J_{gem} 12.0 $J_{16\beta,17}$ 8.2 $J_{16\beta,15}$ 6.6	2.02,ddd J_{gem} 12.2 $J_{16\beta,17}$ 7.7 $J_{16\beta,15}$ 6.6	2.02,ddd J_{gem} 12.0 $J_{16\beta,17}$ 8.0 $J_{16\beta,15}$ 7.0
17	2.89,ddd $J_{17,16\alpha}$ 9.5 $J_{17,16\beta}$ 7.2 $J_{17,22}$ 1.4	2.79,m	3.62,brdd $J_{17,16\beta}$ 8.2 $J_{17,15}$ 0.8	3.66,brdd $J_{17,16\beta}$ 7.7 $J_{17,15}$ 1.2	3.65,brd $J_{17,16\beta}$ 8.0
18	0.93,s	0.96,s	1.66,br.d $J_{18,15}$ 1.7	1.69,br.d $J_{18,15}$ 1.7	1.67,d $J_{18,15}$ 1.8
19	1.17,s	1.08,s	1.27,s	1.22,s	1.16,s

Contd...

Table-1. (Contd.)

Proton	I	II	IV	V	Nimbandiol[15]
21	-	-	7.21,dd $J_{21,23}$ 1.7 $J_{21,22}$ 0.8	7.23,dd $J_{21,23}$ 1.8 $J_{21,22}$ 0.8	7.33,m
22	6.95,t $J_{22,17}$ 1.4 $J_{22,23}$ 1.4	6.90,m	6.32,dd $J_{22,23}$ 1.7 $J_{22,21}$ 0.8	6.32,dd $J_{22,23}$ 1.8 $J_{22,21}$ 0.8	6.26,m
23	6.12,m	6.90,m	7.30,t $J_{23,22}$ 1.7 $J_{23,21}$ 1.7	7.31,t $J_{23,22}$ 1.8 $J_{23,21}$ 1.8	7.21,m
28	1.28,s	1.25,s	-	-	-
29	1.19,s	1.22,s	1.33,s	1.28,s	1.58,s
30	1.26,s	1.25,s	1.35,s	1.28,s	1.30,s
OH	3.45,m 1.74,m 1.74,m	1.63,m	-	2.00,d $J_{OH,6}$ 11.6	4.29,s 3.14,d
CO_2CH_3	-	-	3.72,s 3.63,s	3.69,s 3.64,s	3.63,s
$OCOCH_3$	2.17,s	2.16,s 2.04,s 1.98,s	2.03,s	-	-
H-3'	1.32,s	1.28,s	-	-	-
H-4'	1.32,s	1.28,s	-	-	-

Table-2. ^{13}C-Nmr Chemical Shifts (δ_C/ppm) of Triterpenoids

Carbons	I*	IV**	V**	Carbons	I	IV	V
1	157.5	201.5	202.1	18	20.7[b]	12.8	12.9
2	126.0	125.9	126.5	19	21.0[b]	17.1	16.4
3	205.5	147.5	148.1	20	136.5 137.0	126.7	126.9
4	45.5[a]	47.0[d]	47.4[e]	21	168.7 169.0	138.9	139.1
5	36.0	41.4	43.0	22	145.2 145.6	110.4	110.5
6	26.8	68.6	66.3	23	97.1 97.6	142.9	143.1
7	71.6	84.5	87.6	28	21.9[b]	173.5	173.6
8	45.0[a]	47.8[d]	47.9[e]	29	24.5[b]	16.5	17.2
9	46.6	38.5	39.1	30	31.7[c]	16.6	17.5
10	35.0	47.9[d]	47.5[e]	1'	174.5	-	-
11	76.6	34.2	34.4	2'	73.7	-	-
12	69.0	174.5	175.6	3'	30.1[c]	-	-
13	50.0	134.9	134.9	4'	31.7[c]	-	-
14	155.5	146.0	146.9	$\mathrm{CO_2\underline{Me}}$	-	51.5	51.7
15	119.2 119.8	87.0	87.0	$\mathrm{CO_2\underline{Me}}$	-	52.8	53.0
16	33.0 33.3	41.5	41.5	$\mathrm{O\overset{O}{\overset{\Vert}{\underline{C}}}CH_3}$	169.5	170.4	-
17	51.8 52.1	49.4	49.7	$\mathrm{O\overset{O}{\overset{\Vert}{C}}\underline{CH}_3}$	20.7	20.8	-

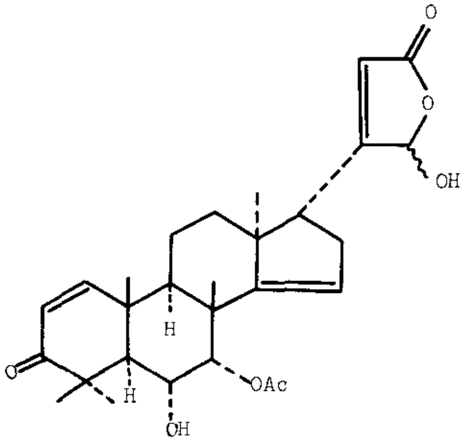
*Carbons identified through GASPE

**Carbons identified through DEPT

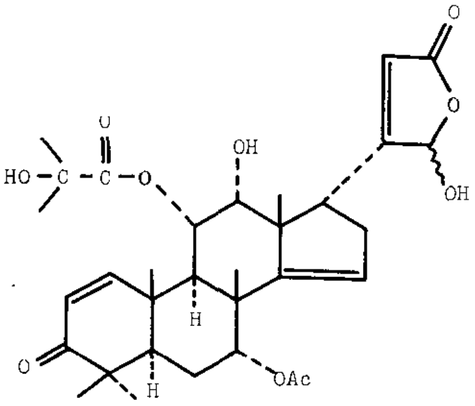
a-e Assignments may be reversed.

Table-3. 2D ^{1}H-^{13}C Heteronuclear correlation spectral data of Nimbin and Deacetylnimbin

	IV		V	
No. H/C	δ_H	δ_C	δ_H	δ_C
2	5.86	125.9	5.85	126.5
3	6.32	147.5	6.40	148.1
5	3.66	41.4	3.40	43.0
6	5.20	68.6	3.93	66.3
7	4.03	84.5	4.01	87.6
9	2.83	38.5	2.76	39.1
11	2.90,2.21	34.2	2.88,2.21	34.4
15	5.54	87.0	5.54	87.0
16	2.18,2.02	41.5	2.18,2.02	41.5
17	3.62	49.4	3.66	49.7
18	1.66	12.8	1.69	12.9
19	1.27	17.1	1.22	16.4
21	7.21	138.9	7.23	139.1
22	6.32	110.4	6.32	110.5
23	7.30	142.9	7.31	143.1
29	1.33	16.5	1.28	17.2
30	1.35	16.6	1.28	17.5
OCH_3	3.63	51.5	3.64	51.7
OCH_3	3.72	52.8	3.69	53.0
$O-\overset{O}{\overset{\|}{C}}-CH_3$	2.03	20.8	-	-



Isonimocinolide



Isonimbocinolide

Stereochemistry of various centres of nimbocinolide (I) has been established through noesy experiment, which showed the spatial connectivities of H-9 with H-3' and H-4'; H-11 with H-12; H-12 with H-30; and H-22 with H-3', H-4' and H-18. The spatial proximity of H-22 with H-18 showed that the side chain at C-17 has α disposition.

Nimbin (IV) and deacetylnimbin (V) were obtained from the 90% methanol soluble fraction of petroleum ether extract of neem kernel through flask chromatography (see experimental). A complete assignment of ^{1}H-nmr and ^{13}C-nmr chemical shifts (Table 1-3) has been carried out through nmr spectral data and 2D studies including cosy-45, noesy, heterocosy (Table-3) and J-resolved, as well as ^{1}H-^{1}H-double resonance experiments. It is interesting to note that H-6 and OH-6 in V resonated as a doublet of double doublet (^{1}H-nmr and 2D-J resolved, $J_{6,7}$ 3.3, $J_{6,5}$ 11.6, $J_{6,OH}$ 11.6) and a doublet ($J_{OH,6}$ 11.6) respectively, but when the ^{1}H-nmr spectrum of the same sample was run again for decoupling experiments H-6 appeared as a doublet of doublet ($J_{6,7}$ 3.2, $J_{6.5}$ 11.6).

The stereochemistry of nimbin (IV) and deacetylnimbin (V) as depicted in the structure was confirmed through 2D-nOe experiments (noesy). In IV the noesy showed spatial connectivities of H-21 with H-18 and H-17; H-22 with H-15, H-17, and H-18; H-15 with H-16α and H-9; H-6 with H-29, H-30, H-7 and H-17; H-7 with H-19, H-30, H-16β and H-17; H-17 with H-30, H-18, and H-16β; H-5 with H-9; and also of H-23 with H-22; and H-3 with H-2. In case of V the noesy exhibited spatial proximity between H-21 with H-17, H-18, H-30 and H-9; H-22 with H-15, H-17 and H-18; H-15 with H-9 and H-16α; H-6 with H-29/H-30; H-17 with H-16β, H-18, and H-30; H-5 with H-9; and also of H-23 with H-22; and H-3 with H-2.

EXPERIMENTAL

Melting points were recorded in glass capillary tubes and are uncorrected. Ir (in $CHCl_3$) and uv (in MeOH) spectra were measured on JASCO-IR-I and Pye-Unicam SP-800 spectrometers respectively. Mass spectra were recorded on Finnigan MAT 112 and 312 double focussing mass spectrometers; exact masses have been measured through peak matching. Nmr spectra were recorded in $CDCl_3$ on a Bruker Aspect AM 300 spectrometer in $CDCl_3$ operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C nuclei. Chemical shifts are recorded in ppm (δ) and coupling constants (J) are in Hz. Delay time for heterocosy was 36 ms, while for noesy experiment the pulse delay and mixing time were 2 sec. and 0.5 sec. respectively. ^{13}C-nmr spectral assignments have been made partly through GASPE and DEPT spectra and partly through a comparison of chemical shifts with the published data for similar compounds.[2,9-15] Assignments of various protons particularly in the high field region, have been made through homonuclear proton decoupling and two dimensional experiments (cosy, noesy, J-resolved and heterocosy). The purity of samples was checked on tlc (silica gel and aluminium oxide).

Isolation of Nimbocinolide (I): The ethanolic extract of the fresh, undried, uncrushed neem leaves (40 kg) collected in winter from Karachi region, was evaporated to a dark green thickish mass, which was partitioned between ethyl acetate and water. The ethyl acetate layer was repeatedly extracted with 4% Na_2CO_3 which was acidified and shaken out with ethyl acetate. After usual work up, the ethyl acetate layer was charcoaled and the charcoal was successively eluted with ethyl acetate and methanol-benzene (1:1) (yield of total acidic fraction = 80g 0.4% on dry wt. basis). The residue obtained on removal of the solvent from the ethyl acetate eluate, was divided into petroleum ether soluble and insoluble fraction. The experimental procedure of the latter fraction has been described earlier which ultimately led to the isolation of isoazadirolide,[14] scopoletin,[14] isonimocinolide[2] and isonimbocinolide,[10] along with a fraction A-3". In the present working A-3" was resolved into two bands A and B (570 mg) on plates coated with aluminium oxide (ethyl acetate - methanol 99:1). Studies on the structure elucidation of A could not be persued due to its insufficient quantity. B(20 mg)was acetylated (acetic anhydride/pyridine, overnight, room temperature) and resolved into two constituents, II (12 mg) and the acetyl derivative of nimocinolide[2] (4 mg) through preparative tlc (aluminium oxide, chloroform). B (550 mg) could be eventually separated into nimbocinolide (I, 380 mg) and nimocinolide (III, 154 mg), prior to acetylation through chromatography on plates coated with silica gel (benzene-ethyl acetate 2:8). I, on recrystallization from methanol-benzene (1:1) formed rods (total 392 mg, 0.50% on the wt. of total acidic fraction), mp 115-118°, $[\alpha]_D^{22} = 40.0°$ ($CHCl_3$). Hrms m/z (%): 586.2770 (M^+, calc. for $C_{32}H_{42}O_{10}$: 586.2777) (2), 486.2655 (calc. for $C_{29}H_{38}O_7$: 486.2617) (2), 483.2372 (calc. for $C_{28}H_{35}O_7$: 483.2382) (3), 468.2569 (calc. for $C_{28}H_{36}O_6$: 468.2512 (1), 466.2370 (calc for $C_{28}H_{34}O_6$: 466.2355) (8), 424.2239 (calc. for $C_{26}H_{32}O_5$: 424.2249) (6), 406.2140 (calc. for $C_{26}H_{30}O_4$: 406.2144) (10), 364.1553 (calc. for $C_{19}H_{24}O_7$: 364.1551, *fragment a) (5), 262.1530 (calc. for* $C_{16}H_{22}O_3$: *262.1569, fragment b) (10)*, 222.1290 (calc. for $C_{13}H_{18}O_3$: 222.1255 fragment c) (12), 202.1346 (calc. for $C_{14}H_{18}O$:202.1357, fragment d) (15), 162.1032 (calc. for $C_{11}H_{14}O$: 162.1044, fragment e) (30) and 137.0969 (calc. for $C_9H_{13}O$: 137.0966, ring A) (95).

Diacetylnimbocinolide (II): Rods ($CHCl_3$), mp 98°, ir $\nu_{max}(CHCl_3)$ cm^{-1}: 3400, 1765, 1740 br., 1670, 1640 and 820. Hrms m/z (%): 670.3019 (M^+, clac. for $C_{36}H_{46}O_{12}$: 670.2987) (2), 550.2559 (calc. for $C_{32}H_{38}O_8$: 550.2566) (2), 468.2477 (calc for $C_{28}H_{36}O_6$: 468.2511) (6), 447.2170 (calc. for $C_{28}H_{31}O_5$: 447.2171 (8), and 408.2279 (calc for $C_{26}H_{32}O_4$: 408.2300 (10).

Isolation of nimbin and deacetylnimbin: Neem kernel (4.5 Kg) was extracted twice with petroleum ether at room temperature, and the combined extracts were freed of the solvent under reduced pressure. The oily residue obtained was partitioned between petroleum ether and 90% methanol, the latter was saturated with saline and extracted with ethyl acetate. The residue obtained on usual work up of the ethyl acetate phase was divided into petroleum ether soluble and insoluble fractions. The petroleum ether insoluble fraction was taken in benzene and the benzene solution was successively passed through a battery of flasks containing silica gel (Merck silica gel 60, 0.063-0.200 nm) and the adsorbed material was eluted with benzene, benzene: methanol (99:1, 90:1) and methanol. The residue obtained from the combined benzene and benzene-methanol (99:1) eluates, was divided into petroleum ether soluble and insoluble fractions. On keeping overnight at room temperature, petroleum ether soluble fraction deposited fine needles, which were filtered. The crystalline residue (180 mg) showing two major spots on tlc was subjected to thin layer chromatography (Al_2O_3, $CHCl_3$) when nimbin (110 mg, 0.0025% on the wt. of kernel) mp 204-206° and deacetylnimbin (55 mg, 0.0012% on the wt. of kernel) mp 208-210° were obtained as fine needles.

Nimbin IV: Hrms m/z (%): 540.2355 (M^+ calc. for $C_{30}H_{46}O_9$: 540.2359) (40), 510.2203 (calc. for $C_{29}H_{34}O_8$: 510.2253) (20), 498.2198 (calc. for $C_{28}H_{34}O_8$: 498.2253) (30), 480.2146 (calc. for $C_{28}H_{32}O_7$: 480.2148) (40), and 230.0956 (calc for $C_{14}H_{14}O_3$: 230.0943) (100).

Deacetylnimbin V: Ms m/z (%) 498 M^+ (92), 480(12), 467(16), 389(36), 268(48) and 231.1021 (calc for $C_{14}H_{15}O_3$: 231.1021) (100).

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