Structurally Novel Diterpenoid Constituents from the Stem Bark of *Azadirachta indica* (Meliaceae)

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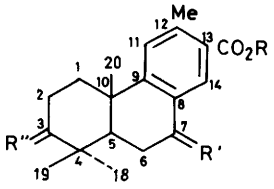
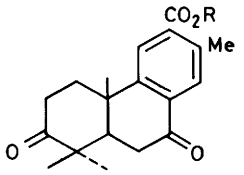
Studies in the neutral fraction of stem bark of *Azadirachta indica* (neem) have resulted in the isolation and structure elucidation of three new diterpenoids, margolone (**1a**), margolonone (**2a**), and isomargolonone (**3a**). The structure of these compounds has been established as 12-methyl-7-oxopodocarpa-8,11,13-triene-13-carboxylic acid, 12-methyl-3,7-dioxopodocarpa-8,11,13-triene-13-carboxylic acid, and 13-methyl-3,7-dioxopodocarpa-8,11,13-triene-12-carboxylic acid respectively, through chemical transformations and spectral studies. These diterpenoids showed antibacterial activity against various gram-positive and gram-negative organisms and represent the first isolation of podocarpane derivatives with carbon substituents at both C-12 and C-13.

Azadirachta indica A. Juss (Meliaceae) commonly known as neem is indigenous to the Indo-Pakistan subcontinent. Almost every part of the tree has been used for the treatment of a variety of human ailments, particularly against diseases of bacterial and fungal origin.[1,2] The bark is regarded as a bitter tonic, astringent, and as being useful in fever, thirst, nausea, vomiting, and skin diseases.[3] It has been found that polysaccharides isolated from the neem bark have strong anti-inflammatory[4] and antitumour action.[4,5] More recently an antineoplastic drug has also been obtained from the bark.[6]

As a result of earlier studies, a series of new terpenoidal constituents has been obtained from the various parts of neem.[7–9] Present work on the stem bark has led to the isolation of the new aromatic tricyclic diterpenoids margolone, margolonone, and isomargolonone. Their structures have been elucidated as 12-methyl-7-oxopodocarpa-8,11,13-triene-13-carboxylic acid (**1a**), 12-methyl-3,7-dioxopodocarpa-8,11,13-triene-13-carboxylic acid (**2a**), and 13-methyl-3,7-dioxopodocarpa-8,11,13-triene-12-carboxylic acid (**3a**). This is the first instance of the isolation of podocarpane derivatives with carbon substituents at both C-12 and C-13.



	R	R'	R''
(1a)	H	O	H_2
(1b)	Me	O	H_2
(1c)	H	H_2	H_2
(2a)	H	O	O
(2b)	Me	O	O



(**3a**) R = H
(**3b**) R = Me

These constituents were tested for their antibacterial activity against various gram-positive and gram-negative organisms and were found effective against a few of them. Thus margolone (**1a**) was found to possess antibacterial activity against *Klebsiella oxytoca*, margolonone (**2a**) was effective against *Staphylococcus epidermidis*, *K. oxytoca*, and *Serratia lutea*, whereas isomargolonone (**3a**) was effective against *K. oxytoca* and *S. lutea*. Details of these studies will be published elsewhere.

Results and Discussion

The ethanolic extract of neem stem bark was divided into acidic and neutral fractions (as described in the Experimental section). After usual work-up, the neutral fraction was subjected to solvent fractionation followed by separation and purification through successive preparative t.l.c. (p.l.c.) on silica gel (chloroform–methanol 9.25:0.75) and precoated t.l.c. plates (silica gel; chloroform–methanol 9.75:0.25) to give three uniform diterpenoids, margolone (**1a**), margolonone (**2a**), and isomargolonone (**3a**).

Margolone (**1a**) has molecular formula $C_{19}H_{24}O_3$ (through peak matching of its molecular ion), showing eight double-bond equivalents. Its i.r. spectrum displayed peaks at 3 400 (CO_2H), 2 850 (C–H), 1 710br (α,β-unsaturated ketone and CO_2H), 1 660 (aromatic double bond), and 1 280 cm^{-1} (C–O), while in the u.v. spectrum maxima were observed at 204, 240, 278, 285, 360, and 383 nm. The diterpenoidal nature of compound (**1a**) was indicated by the molecular formula and presence in the ^1H n.m.r. spectrum of three three-proton upfield singlets at δ 0.87, 0.94, and 1.00. The ^{13}C n.m.r. spectrum (Table) (broad band and spin echo) showed that margolone (**1a**) has two tertiary and four quaternary olefinic carbons (of aromatic ring), four methyls, four methylenes, one methine, two sp^3 quaternary carbons, and two carbonyls. The appearance of only two downfield singlets at δ 6.96 and 8.54 and two tertiary aromatic carbons (δ_C 120.9 and 140.4 respectively) suggested two substituents at C-12 and C-13. The n.m.r. spectrum further indicated that one of these substituents is methyl (δ_H 2.59; δ_C 23.0) while the other is a carboxy function (δ_H 8.47 exchanged with D_2O; δ_C 181.9) which was corroborated through methylation of margolone (**1a**) to ester (**1b**) (δ OMe, 4.06, M^+, 314) on reaction with CH_2N_2 and its failure to form an acetyl derivative. The remaining carbonyl function was placed at C-7 in the light of the chemical shifts of 11-H, 14-H, and C-7 (δ 198.2, see Table) which are comparable with those reported for sugiol,[10,11] and the double doublets of

Table. ^{13}C N.m.r. spectral data of margolone (**1a**) and sugiol

Carbon	(**1a**)	Sugiol[16]
C-1	38.1	37.3
C-2	18.6	18.4
C-3	40.9	40.9
C-4	33.3	32.7
C-5	47.6	49.1
C-6	33.5	35.4
C-7	198.2	199.2
C-8	127.6	122.4
C-9	164.1	156.3
C-10	35.4	37.4
C-11	120.9	109.0
C-12	136.9	160.3
C-13	136.9	133.0
C-14	140.4	125.6
C-15		26.1
C-16		21.8
C-17		21.6
C-18	21.2	20.7
C-19	32.4	31.9
C-20	22.2	22.5
ArMe	23.0	
CO_2H	181.9	

6-H_α (δ 2.55, *J* 19.0 and 6.5 Hz) and 6-H_β (δ 2.42, *J* 19.0 and 14.0 Hz) observed in the 2D *J*-resolved spectrum.

The placement of the carboxy group at C-13 was decided from analysis of a 2D n.O.e. (NOESY) spectrum which showed the spatial connectivity of 14-H (δ 8.54) with the carboxy proton (δ 8.47) along with the interaction of various upfield protons.

The molecular formula $C_{19}H_{22}O_4$ (314.1517) of margolonone (**2a**) was obtained through the exact mass observed in the high-resolution mass spectrum. The u.v. spectrum showed maximum absorptions at 204, 242, 275, 282, 363, and 380 nm and the i.r. spectrum displayed peaks at 3 400 (CO_2H), 2 850 (C–H), 1 720br (six-membered, α,β-unsaturated ketones and carboxy group), 1 660 (aromatic double bond), and 1 280 cm^{-1} (C–O). It formed the methyl derivative (**2b**) on reaction with CH_2N_2 but failed to give an acetyl derivative. The ^1H n.m.r. spectrum showed three three-proton singlets at δ 1.16, 1.21, and 1.50 attributable to 4-Me_α, 4-Me_β, and 10-Me. A three-proton singlet at δ 2.47 and two sharp singlets at δ 6.98 and 8.61 were due to ArMe, 11-H, and 14-H respectively.

The data recorded so far suggested that margolonone had the same skeleton as margolone by an increment of 14 mass units. The chemical shifts of the 11-H and 14-H singlets at δ 6.98 and 8.61 in the ^1H n.m.r. spectrum of margolonone (**2a**) further indicated that the aromatic ring and the carbonyl substituent at C-7 are identical in both (**1a**) and (**2a**). The NOESY spectrum showed that the respective positions of Me and CO_2H groups were also the same as observed in margolone (**1a**). The increment in mass could be justified by a carbonyl function at C-3 which was corroborated by the downfield chemical shift of the angular methyl group[10,12] (δ 1.50) and a significant fragment at *m/z* 125.0980 ($C_8H_{13}O$) in the mass spectrum.[13] Final correlation of structures (**1a**) and (**2a**) was obtained through their Clemmenson reduction to a common reduction product (**1c**).

Isomargolonone (**3a**) has molecular formula ($C_{19}H_{22}O_4$) (through peak matching of its molecular ion). The u.v. spectrum exhibited absorptions at 208, 240, 280, and 310 nm while the i.r. spectrum displayed peaks at 3 400 (CO_2H), 2 850 (C–H), 1 720—1 700 (carbonyls), 1 600 (aromatic double bond), and 1 100 cm^{-1} (C–O). The nine double-bond equivalents indicated by the molecular formula could be justified by the three rings, three double bonds of the aromatic ring, and three carbonyl functions. In the ^1H n.m.r. spectrum, two downfield one-proton singlets observed at δ 7.84 and 7.92 again showed two substituents at C-12 and C-13 as observed in the case of compounds (**1a**) and (**2a**). However, the chemical shifts of these protons are not comparable with those of (**1a**) and (**2a**) and indicated that both C-11 and C-14 have electron-withdrawing *ortho*-substituents. That one of these is a carboxy group could be arrived at through the formation of the methyl derivative (**3b**) on reaction of isomargolonone (**3a**) with CH_2N_2 and its failure to yield an acetyl derivative on reaction with Ac_2O–pyridine. The placement of this group at C-12 left two carbonyls to be accounted for, one of which was placed at C-7 keeping in view the above discussion while the second could be placed at C-3 in the light of the downfield chemical shift of 10-Me (δ 1.41)[10,12] and a significant fragment at *m/z* 125.0980 ($C_8H_{13}O$) in the high-resolution mass spectrum.[13] Finally, a three-proton singlet at δ 2.24 showed the presence of an aromatic methyl group which was placed at C-13. These observations led to the structure of isomargolonone as (**3a**).

It may be of interest to note the effect of the polar substituents on the chemical shifts of the methyl protons, particularly 10-Me. Thus in compounds (**2a**) and (**3a**) the 10-Me protons resonate downfield (δ 1.50 and 1.41 respectively) due to the presence of the carbonyl function at C-3. The more downfield shift of these protons in margolonone (**2a**) as compared with that of isomargolonone (**3a**) is in agreement with the earlier observations that polar *para* substituents cause a downfield shift of the angular methyl protons.[14]

Experimental

I.r. (in $CHCl_3$) and u.v. (in MeOH) spectra were measured on a JASCO IRA-I and Pye-Unicam SP-800 spectrometer respectively. Mass spectra were recorded on a Finnigan MAT 311A double-focussing mass spectrometer. N.m.r. spectra were recorded in $CDCl_3$ on a Bruker Aspect AM 400 spectrometer operating at 400 MHz for ^1H and 100 MHz for ^{13}C nuclei. The assignments of ^{13}C n.m.r. chemical shifts are based on chemical shift rules[15] and comparison with sugiol and other similar compounds.[11,16] Optical rotations were measured at 24 °C in $CHCl_3$ on a Polartronic-D polarimeter. Merck Kieselgel 60 PF_{254} coated glass plates were used for analytical t.l.c. and p.l.c. Light petroleum refers to that fraction boiling in the range 60—70 °C.

Extraction and Isolation.—The ethanolic extract of neem stem bark (20 kg) collected in May 1987 from the Karachi region was freed of the solvent and partitioned between ethyl acetate and water. The former was shaken with 4% aqueous Na_2CO_3 to separate the acidic and neutral fractions. The residue obtained on usual work-up of the ethyl acetate phase containing the neutral fraction was divided into light petroleum-soluble and light petroleum-insoluble portions. The latter fraction was taken up in ethyl acetate and treated with an excess of light petroleum to give a light yellow insoluble powder (total 30 g) which was filtered off; a portion (4 g) of this powder was subjected to p.l.c. (chloroform–methanol 9.25:0.75), as a result of which compounds (**1a**), (**2a**), and (**3a**) were obtained with some allied impurities. They were purified through chromatography on precoated t.l.c. plates (chloroform–methanol, 9.75:0.25), when margolone (**1a**), margolonone (**2a**), and isomargolonone (**3a**) were isolated as uniform constituents.

Margolone (**1a**). Yellow amorphous powder (50 mg), $[\alpha]_D^{25}$ −6°; e.i.m.s. *m/z* (%) 300.1725 (M^+, 38. $C_{19}H_{24}O_3$ requires *M*, 300.1725), 285.1490 (*M* − CH_3, 4), 272.1776 (*M* − CO, 4), 215.0708 ($C_{13}H_{11}O_3$, 12), 177.0551 ($C_{10}H_9O_3$, 24), and 55 (C_4H_7, 100); δ_H 0.87, 0.94, and 1.00 (9 H, each s, 4-Me_α, 4-Me_β,

10-Me), 2.42 (dd, J_{gem} 19.0, $J_{6\beta,5}$ 14.0 Hz, 6-H$_\beta$), 2.55 (dd, J_{gem} 19.0, $J_{6\alpha,5}$ 6.5 Hz, 6-H$_\alpha$), 2.59 (ArMe), 6.96 (11-H), 8.47 (s, exchangeable with D_2O, CO_2H), and 8.54 (s, 14-H).

Methylation of Margolone (**1a**).—A solution of margolone (**1a**) (7.5 mg) in ether (5 ml) was treated with freshly prepared diazomethane at room temperature for 4 h. The reaction mixture was evaporated to dryness, when the methylated product (**1b**) was obtained as an amorphous powder (7.8 mg); $\lambda_{max.}$ 208, 233, 288, and 360 nm; $\nu_{max.}$ 2 850, 1 690, 1 610, and 1 350 cm^{-1}; e.i.m.s. *m/z* (%) 314 (M^+, 4), 299 (M − 15, 2), and 115 (100); δ_H 0.94, 0.99, and 1.09 (9 H, each s, 4-Me$_\alpha$, 4-Me$_\beta$, 10-Me), 4.06 (s, CO_2Me), 6.95 (s, 11-H), and 8.30 (s, 14-H).

Reduction of Margolone (**1a**).—A mixture of zinc powder (100 mg) and mercury(II) chloride (10 mg) was stirred with conc. hydrochloric acid (2 ml) and water (50 ml) for 10 min. The aqueous solution was decanted and the amalgamated zinc was covered with water (50 ml) and conc. hydrochloric acid (75 ml). A solution of margolone (**1a**) (9 mg) in toluene (2 ml) was added immediately and the reaction mixture was refluxed under a slow stream of hydrogen chloride gas until the zinc amalgam dissolved. The reaction mixture was worked up in the usual manner and subjected to purification on p.l.c. (chloroform) to afford the reduction product (**1c**) (6.8 mg); $\lambda_{max.}$ 208, 250, and 280 nm; $\nu_{max.}$ 3 400 (CO_2H), 2 850 (C–H), 1 660 (aromatic double bond), and 1 100 cm^{-1} (C–O); e.i.m.s. *m/z* 286.1935 (M^+, 80) ($C_{19}H_{26}O_2$), 273.1854 (M − CH_3, 54), and 55 (100).

Margolonone (**2a**).—This was obtained as a yellow amorphous powder (50 mg), $[\alpha]_D^{25}$ −20°; e.i.m.s. *m/z* (%) 314.1548 (M^+, 8. $C_{19}H_{22}O_4$ requires M, 314.1517), 299.3470 (M − Me, 2), 257.1491 (M − C_2HO_2, 4), 255.1384 (M − CH_3–CO_2, 3). 125.0980 ($C_8H_{13}O$, 40), and 55 (C_4H_7, 100); δ_H 1.16, 1.21, and 1.50 (9 H, each s, 4-Me$_\alpha$, 4-Me$_\beta$, 10-Me), 2.47 (ArMe), 6.98 (s, 11-H), 8.49 (s, exchangeable with D_2O, CO_2H), and 8.61 (s, 14-H).

Methylation of Margolonone (**2a**).—To a solution of margolonone (6.5 mg) in ether (5 ml) was added an ethereal solution of freshly prepared diazomethane and the reaction mixture was kept for 4 h at room temperature. Usual work-up gave compound (**2b**) with some allied impurities. It was purified by p.l.c. (chloroform–methanol 9.5:0.5) to yield the methylated product (**2b**) (6.7 mg); $\lambda_{max.}$ 208, 240, 280, 290, and 360 nm; $\nu_{max.}$ 2 850 (C–H), 1 720 (C=O), 1 600 (C=C), and 1 100 cm^{-1} (C–O); e.i.m.s. *m/z* (%) 328.4088 (M^+, 3) ($C_{20}H_{24}O_4$), 282 (M − 46, 2), 254 (M − 74, 4), and 71 (C_4H_7O, 100).

Reduction of Margolonone (**2a**).—Compound (**2a**) (12.5 mg) was reduced in the same manner as described for (**1a**) to yield a product (8.8 mg) identical in all respects with compound (**1c**).

Isomargolonone (**3a**).—This was obtained as a yellow amorphous powder (15 mg), $[\alpha]_D^{25}$ −2.0°; e.i.m.s. *m/z* (%) (M^+, 52, $C_{19}H_{22}O_4$ requires M, 314.1517), 299 (M − CH_3, 30), 281 (M − CH_3 − H_2O, 3), 271 (M − CO − CH_3, 4), and 125 ($C_8H_{13}O$, 100); $\lambda_{max.}$ 208, 240, 280, and 310 nm; δ_H 1.12, 1.19, and 1.41 (9-H, each s, 4-Me$_\alpha$, 4-Me$_\beta$, 10-Me), 2.24 (s, ArMe), 7.84 (11-H), and 7.92 (14-H).

Methylation of Isomargolonone (**3a**).—Isomargolonone (**3a**) (8.4 mg) was methylated in the same manner as described for compounds (**1a**) and (**2a**) to yield the ester (**3b**); $\lambda_{max.}$ 208, 230, and 320 nm; $\nu_{max.}$ 3 400 (OH), 2 850 (C–H), 1 700 (C=O), 1 600 (C=C), and 1 150 cm^{-1} (C–O); e.i.m.s. *m/z* (%) 328.4088 (M^+, 3. $C_{20}H_{24}O_4$), 297 (M − 31, 2), 254 (M − 74, 4), and 69 (100).

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