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SPECTROSCOPIC AND BIOLOGICAL INVESTIGATION OF NIMBOLIDE AND 28-DEOXONIMBOLIDE FROM *AZADIRACHTA INDICA*

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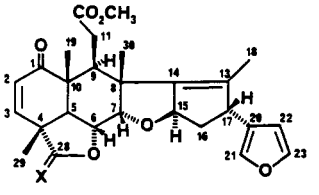
ABSTRACT.—The reisolation of nimbolide [**1**] from *Azadirachta indica* of Tanzanian origin and the isolation and structure elucidation of a new limonoid, 28-deoxonimbolide [**2**], from the same plant source are reported. For the first time, unambiguous ^{1}H- and ^{13}C-nmr assignments of compounds **1** and **2** are presented, as well as their in vitro cytotoxic activity against human tumor cell lines.

Many medicinal uses of the various parts of *Azadirachta indica* A. Juss. (Meliaceae) have been reported in traditional folklore medicine (1,2). Antibacterial and antifungal (1,3,4), antimalarial (5–7), antipyretic (8,9), and anti-inflammatory (8,10) uses are those most frequently cited. Recently, two isolates of *A. indica* have been reported to exert moderate antitumor activity (11). In addition, some polysaccharides from the same plant source have also been evaluated for their antitumor activity (12–14).

In 1967, the isolation of nimbolide [**1**], a new meliacin-type limonoid, was reported from *A. indica* of Nigerian origin (15). The structure of **1** was established on the basis of chemical evidence, because partial hydrogenation of **1** and subsequent hydrolysis gave the previously known dihydronimbic acid (16). Apparently, no ^{1}H- or ^{13}C-nmr data have been reported for nimbolide [**1**]. Biologically, the acute toxicity of nimbolide [**1**] was found to be 600 and 500 mg/kg body wt in rats and hamsters, respectively (17), and no mutagenic potential was demonstrated in preliminary studies conducted with *Salmonella typhimurium* (18). Recently, it was shown that nimbolide [**1**], from *A. indica* var. *siamensis* of Thai origin, inhibited chloroquine-resistant *Plasmodium falciparum* Welch in vitro (19), suggesting that this type of compound could be a potential source of new antimalarial agents; unfortunately, no in vivo activity could be established.

We report here the reisolation of **1** from *A. indica* of Tanzanian origin and the isolation and structure elucidation of a new limonoid, 28-deoxonimbolide [**2**], from the same plant source. For the first time, unambiguous ^{1}H- and ^{13}C-nmr assignments of



1 X=O
2 X=H_2

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compounds **1** and **2** are presented, as well as their in vitro cytotoxic activity against human tumor cell lines.

Nimbolide [**1**] was obtained as colorless plates crystallizing from Me_2CO. The ir spectrum of **1** showed carbonyl absorptions at 1665 ($C{=}O_{conj}$), 1725 ($C{=}O_{ester}$) and 1772 cm^{-1} ($C{=}O_{lactone}$), while the mass spectrum showed a molecular ion at *m/z* 466, analyzing for $C_{27}H_{30}O_7$. These data supported the assumption that the isolate was nimbolide [**1**], a finding that was subsequently confirmed by direct comparison with an authentic sample of **1** obtained from *A. indica* var. *siamensis* collected in Thailand.

The ^{1}H-nmr spectrum of **1** (Table 1) indicated the presence of one methoxy (δ 3.54) and four methyl groups (δ 1.70, 1.48, 1.37, and 1.22), whose detailed assignments were determined subsequently through combined HETCOR and selective INEPT experiments. The 11-methylene group appeared as a geminally coupled doublet of doublets (dd) at δ 3.25 and 2.38, while the 16-methylene resonated at δ 2.21 and 2.12 as a pair of ddd multiplets. Three slightly broadened aromatic singlets at δ 6.26, 7.22, and 7.31 were assigned as the protons of the furan moiety, H-22, H-21, and H-23, respectively. The ^{1}H-^{1}H COSY spectrum of **1** displayed two coupled doublets at δ 5.93 (H-2) and 7.28 (H-3); a triplet resonance at δ 5.53 and a doublet of doublets at δ 3.67 showed couplings with both of the C-16 protons, thereby permitting their assignment

TABLE 1. ^{1}H- and ^{13}C-NMR Spectral Assignments of Nimbolides **1** and **2**.[a]

Carbon	Nimbolide [**1**]		28-Deoxonimbolide [**2**]	
	^{1}H	^{13}C	^{1}H	^{13}C
1	—	200.55	—	202.38
2	5.93 (d, 9.6)	130.76	5.84 (d, 9.4)	130.28
3	7.28 (d, 9.6)	149.34	7.01 (d, 9.4)	151.99
4	—	43.47	—	42.00
5	3.19 (d, 12.6)	47.55	2.76 (d, 12.6)	48.99
6	4.63 (dd, 3.6, 12.6)	73.24	4.06 (dd, 3.4, 12.6)	72.51
7	4.27 (d, 3.6)	82.65	4.16 (d, 3.4)	85.32
8	—	50.10	—	50.65
9	2.27 (t, 5.4)	40.89	2.60 (t, 5.4)	41.25
10	—	45.05	—	46.07
11-H_a	3.25 (dd, 5.4, 16.2)	31.93	3.20 (dd, 5.4, 16.1)	32.40
11-H_b	2.38 (dd, 5.7, 16.2)		2.31 (dd, 5.7, 16.1)	
12	—	172.76	—	173.29
13	—	136.16	—	135.12
14	—	144.54	—	145.88
15	5.53 (t, 6.6)	88.17	5.45 (m)	87.81
16-Hα	2.21 (ddd, 1.0, 6.6, 8.4)	41.03	2.15 (ddd, 1.0, 6.4, 8.3)	41.45
16-Hβ	2.10 (ddd, 1.2, 6.8, 8.4)		2.06 (ddd, 1.2, 6.6, 8.3)	
17	3.67 (dd, 1.2, 8.4)	49.23	3.59 (dd, 1.2, 8.3)	49.39
18	1.70 (s)	12.72	1.67 (s)	12.90
19	1.22 (s)	14.95	1.17 (s)	14.63
20	—	126.33	—	126.80
21	7.22 (s)	138.64	7.16 (s)	138.80
22	6.26 (s)	110.12	6.22 (s)	110.41
23	7.31 (s)	142.87	7.27 (s)	142.98
28-H_α	—	174.78	3.75 (d, 7.2)	79.30
28-H_β			3.68 (d, 7.2)	
29	1.48 (s)	18.33	1.35 (s)	20.56
30	1.37 (s)	16.93	1.31 (s)	17.35
OMe	3.54 (s)	51.55	3.49 (s)	51.65

[a]Recorded in $CDCl_3$.

as H-15 and H-17, respectively; and a second triplet at δ 2.27 was coupled only with the C-11 methylene protons and consequently could be assigned as H-9. The doublet of doublets at δ 4.43 exhibited coupling with the doublets at δ 4.21 and 3.19, suggesting their respective assignment as H-6, H-7, and H-5. Unambiguous assignment of the ^{13}C-nmr spectrum of nimbolide [**1**] was performed using APT, HETCOR, and selective INEPT (20) pulse programming sequences. All of the protonated carbon atoms could be assigned by the APT and HETCOR spectra, while the *J*-modulated selective INEPT technique, in which a particular proton is irradiated with a soft proton pulse resulting in magnetization transfer and selective enhancement of carbon atoms three bonds away from the irradiated proton, established the assignment of the quaternary carbon atoms. Details of the selective INEPT experiments on **1** are summarized in Table 2. The complete assignments of the ^{1}H- and ^{13}C-nmr spectra of nimbolide [**1**] are shown in Table 1.

TABLE 2. Selective INEPT Data for Nimbolide [**1**].

Proton irradiated		Carbons observed							
δ_H		δ_C							
H-3	(7.28)	43.47	(C-4)[a]	47.55	(C-5)	174.78	(C-28)	200.55	(C-1)
H-2	(5.93)	43.47	(C-4)	45.05	(C-10)				
H-11$_a$	(3.25)	45.05	(C-10)	50.10	(C-8)	172.76	(C-12)[a]		
H-15	(5.53)	49.23	(C-17)	50.10	(C-8)	136.16	(C-13)	144.54	(C-14)[a]
H-7	(4.27)	40.89	(C-9)	47.55	(C-5)	73.24	(C-6)[a]	144.54	(C-14)
H-17	(3.67)	88.17	(C-15)	110.12	(C-22)	138.64	(C-21)	144.54	(C-14)
10-Me	(1.22)	40.89	(C-9)	45.05	(C-10)[a]	47.55	(C-5)	200.55	(C-1)
8-Me	(1.37)	40.89	(C-9)	50.10	(C-8)[a]	82.65	(C-7)	144.54	(C-14)
4-Me	(1.48)	43.47	(C-4)[a]	47.55	(C-5)	149.34	(C-3)	174.78	(C-28)
13-Me	(1.70)	49.23	(C-17)	136.16	(C-13)[a]	144.54	(C-14)		

[a]Enhancement observed by two-bond coupling.

The relative stereochemistries of the centers C-6, C-7, C-9, C-15, and C-17 were established through a series of nOe experiments. Irradiation of H-15 enhanced both H-21 and H-22, establishing the configurations at C-15 and C-17, as well as H-9 and H-16α. The cis B-C ring junction was also evident from the irradiation of H_3-30 which produced nOe enhancement in the H-7 resonance. Additional nOe experiments served to reaffirm the stereochemical assignments.

The second compound isolated from *A. indica*, mp 142–144°, exhibited ir and eims spectral data suggesting a structure analogous to nimbolide except for the absence of the C-28 carbonyl group. The lack of the lactone carbonyl absorption from the ir spectra, and the observed molecular ion at *m/z* 542 corresponding to $C_{27}H_{32}O_6$, suggested 28-deoxonimbolide [**2**] as the structure for the isolate.

The ^{1}H-nmr and ^{1}H-^{1}H COSY spectrum of 28-deoxonimbolide [**2**] displayed characteristic resonances and coupling patterns (Table 1) similar to those of nimbolide [**1**]. The only significant difference was the appearance of an additional geminally coupled pair of doublets at δ 3.68 and 3.75 in the ^{1}H-nmr spectrum of **2**, due to the presence of two C-28 methylene protons (21). Unambiguous assignment of the ^{13}C-nmr spectrum and final confirmation of the structure of 28-deoxonimbolide [**2**] were performed by APT, HETCOR, and selective INEPT spectroscopy using a strategy of irradiation experiments similar to those used for nimbolide [**1**]. Results of the selective INEPT experiments on 28-deoxonimbolide [**2**] are summarized in Table 3, and the complete assignments of the ^{1}H- and ^{13}C-nmr spectra of **2** are reported in Table 1.

TABLE 3. Selective INEPT Data for 28-Deoxonimbolide [**2**].

Proton irradiated		Carbons observed							
δH		δC							
H-3	(7.01)	42.00	(C-4)[a]	48.99	(C-5)	79.30	(C-28)	202.38	(C-1)
H-2	(5.84)	42.00	(C-4)	46.07	(C-10)				
H_a-11	(3.20)	46.07	(C-10)	50.65	(C-8)	173.29	(C-12)[a]		
H-15	(5.45)	49.39	(C-17)	50.65	(C-8)	135.12	(C-13)	145.88	(C-14)[a]
H-7	(4.16)	41.25	(C-9)	48.99	(C-5)	145.88	(C-14)		
H-17	(3.59)	87.81	(C-15)	110.41	(C-22)	138.80	(C-21)	145.88	(C-14)
10-Me	(1.17)	41.25	(C-9)	46.07	(C-10)[a]	48.99	(C-5)	202.38	(C-1)
8-Me	(1.31)	41.25	(C-9)	50.65	(C-8)[a]	85.35	(C-7)	145.88	(C-14)
4-Me	(1.35)	42.00	(C-4)[a]	48.99	(C-5)	79.39	(C-28)	151.99	(C-3)
13-Me	(1.67)	49.39	(C-17)	135.12	(C-13)[a]	145.88	(C-14)		

[a]Enhancement observed by two-bond coupling.

Both nimbolide [**1**] and 28-deoxonimbolide [**2**] were evaluated in the P-388 (lymphocytic leukemia), KB (carcinoma of the nasopharynx), HT-1080 (human fibrosarcoma), LU-1 (lung cancer), COL-2 (colon cancer), MEL-2 (melanoma), and BC-1 (breast cancer) cell lines in vitro according to established protocols (22–25). Cytotoxicity data (ED_{50} values) are shown in Table 4. Each of the cell lines examined was more sensitive to nimbolide [**1**] than to 28-deoxonimbolide [**2**]. The difference in susceptibility ranged from approximately 2-fold (lung cancer) to approximately 10-fold (P-388). Nonetheless, both compounds demonstrated significant cytotoxic activity with all of the cell lines tested; this is likely due to the presence of the α,β-unsaturated ketone structural element (26). As judged by the data currently reported, however, the δ-lactone moiety also contributes to the cytotoxic potential. Comparative testing of **1** and **2** against *P. falciparum* in vitro will be described subsequently.

TABLE 4. Cytotoxicity Data of the Nimbolides.

Cell line	Compound	
	ED_{50} (μg/ml)	
	1	**2**
BC-1	0.39	1.34
COL-2	0.41	1.81
HT-1080	0.31	1.04
LU-1	0.42	0.84
MEL-2	0.53	2.05
KB	0.25	1.30
P-388	0.065	0.66

EXPERIMENTAL

GENERAL EXPERIMENTAL METHODS.—All solvents were redistilled. Melting points were determined on a Kofler-type hot stage apparatus and are uncorrected. Adsorption cc was performed with Si gel 60 (70–230 mesh) (E. Merck, Darmstadt, FRG). Tlc and preparative tlc were performed with Si gel 60 F_{254} and GF Uniplates from Analtech. Optical rotations were measured with a Perkin-Elmer Model 241 polarimeter. Uv spectra were recorded by employing a Beckman Model DU-7 spectrometer, and ir spectra were obtained with a Nicolet MX-1 interferometer. Mass spectra were determined with a Varian MAT 112S double focusing spectrometer operating at 80 eV. The ^{1}H-nmr spectra ($CDCl_3$ solutions with TMS as

internal standard) were obtained with Nicolet NMC 360 (360 MHz) and Varian XL-300 (300 MHz) spectrometers. ^{13}C-nmr spectra were measured at 90.8 MHz using a Nicolet NMC 360 spectrometer. Chemical shift values are reported in ppm downfield from TMS. ^{1}H-^{1}H COSY and ^{1}H-^{13}C HETCOR spectra were recorded on a Varian XL-300 spectrometer using standard Varian pulse programs. Selective INEPT experiments were performed on a Nicolet NMC 360 spectrometer. Proton pulse widths were calibrated by using a sample of HOAc in 10% C_6D_6 ($^{1r}J = 6.7$ Hz) in a 5-mm nmr tube. For the methine protons, 7 Hz was used as the ^{3}J value, and 5 Hz was used for the irradiation of the methyl protons. In each irradiation experiment 5000 acquisitions were accumulated.

PLANT MATERIAL.—Fresh leaves of *A. indica* were collected in Dar es Salaam, Tanzania, in May 1988. Preliminary identification was done by the Pharmacognosy Department, Faculty of Medicine, Muhimbili Medical Center, where a herbarium specimen is deposited. The identity of the plant material was authenticated by the Department of Botany, University of Dar es Salaam, Tanzania.

EXTRACTION AND ISOLATION.—The leaves were separated from the stalks, dried at 60° for 12 h, and ground to a powder. The powder (10 kg) was moistened with NH_3 solution (10%) and air-dried at 25° for 6 h and then 60° for 2 h. The dried powder was extracted (in batches of 500 g) with $CHCl_3$ in a Soxhlet apparatus for 8 h, the extract was concentrated to dryness under reduced pressure, and the residues were pooled to give a crude extract (520 g). Most of the pigments were removed by flash chromatography using a Si gel column and a $CHCl_3$-petroleum ether (9:1) eluent. Fractions were collected (11 × 100 ml), the first three of which contained chlorophyll. From color reactions observed under uv and Dragendorff's spray reagent, fractions 4–8 and 9–11 were pooled to give two main fractions, A and B.

PURIFICATION OF NIMBOLIDE.—Main fraction A was subjected to repeated Si gel cc eluting with the following solvent mixtures: EtOAc-petroleum ether-MeOH (1:8.5:0.5), EtOAc-petroleum ether (7:3), and EtOAc-petroleum ether (6:4) to afford two compounds. The first compound was collected and washed with Et_2O followed by recrystallization from Me_2CO to give nimbolide [**1**] (133 mg): colorless plates; mp 228–230° [lit. (15) 245–247°]; $[\alpha]^{25}D$ +211° ($c = 0.10$, $CHCl_3$) [lit. (19) 206°]; uv (MeOH) λ max 220 (log ε 2.448) nm; ir (KBr) ν max 2947, 2880, 1772, 1735, 1685, 1665, 1153, 1067, 1047, 1037, 940 cm^{-1}; ^{1}H nmr (360 MHz, $CDCl_3$) and ^{13}C nmr (90.8 MHz, $CDCl_3$) see Table 1; eims (80 eV) *m/z* (rel. int.) $[M]^+$ 466 (59), 435 (90), 422 (10), 335 (22), 215 (44), 202 (29), 201 (53), 199 (38), 197 (54), 187 (26), 185 (43), 173 (47), 159 (28), 155 (11), 147 (32), 146 (19), 145 (32), 135 (24), 131 (27), 129 (28), 128 (28), 121 (22), 119 (52), 117 (24), 115 (35), 109 (37), 107 (30), 105 (57), 95 (28), 91 (87).

PURIFICATION OF 28-DEOXONIMBOLIDE.—The second compound from the above process was passed through a Si gel column eluting with $CHCl_3$-petroleum ether (8:2) to give 28-deoxonimbolide [**2**] (1.99 g): colorless needles (from Et_2O/petroleum ether); mp 142–144°; $[\alpha]^{25}D$ +228° ($c = 0.103$, $CHCl_3$); uv (MeOH) λ max 221.5 (log ε 2.581) nm; ir (KBr) ν max 2880, 1725, 1676, 1395, 1156, 1148, 1041, 953, 809 cm^{-1}; ^{1}H nmr (300 MHz, $CDCl_3$) and ^{13}C-nmr (90.8 MHz, $CDCl_3$) see Table 1; eims (80 eV) *m/z* (rel. int.) $[M]^+$ 452 (55), 335 (16), 259 (45), 231 (9), 230 (21), 219 (16), 215 (27), 202 (41), 201 (29), 199 (40), 187 (22), 185 (44), 173 (35), 159 (24), 147 (21), 145 (28), 135 (22), 131 (21), 129 (20), 119 (23), 117 (18), 115 (25), 107 (20), 105 (36), 95 (100), 93 (16), 91 (67); hrms observed 452.22036, calcd 452.21988.

CYTOTOXICITY TESTING.—P-388, KB, and HT-1080 (human fibrosarcoma) were purchased from ATCC. LU-1 (lung cancer), COL-2 (colon cancer), MEL-2 (melanoma) and BC-1 (breast cancer) were established from primary human tumors in the University of Illinois College of Medicine, Division of Surgical Oncology.

The P-388 cells were cultured in Fisher's medium supplemented with 10% heat-inactivated (56° for 30 min) fetal bovine serum (FBS). The KB cells were maintained in BME containing 10% heat-inactivated FBS. The HT-1080 and LU-1 cell lines were cultured in minimal essential medium (MEM) containing Earle's salts and supplemented with 1% of nonessential amino acids (NAA) and 10% heat-inactivated FBS. The COL-2 and BC-1 cell lines were maintained in MEM containing Earle's salts, supplemented with 1% NAA and 15% heat-inactivated FBS, and the MEL-2 cell line was grown in MEM containing Hank's salts supplemented with 1% NAA and 15% heat-inactivated FBS. All the cell lines except MEL-2 were cultured at 37° in a humidified atmosphere of 5% CO_2 in air. The MEL-2 cell line was maintained at 37° in closed tissue culture flasks.

Nimbolides **1** and **2** were evaluated for cytotoxicity basically by the procedures established by the National Cancer Institute (22) as described previously (23–25). The cultured cells (at log growth-phase) were treated in duplicate with 5 concentrations (0.0125–2.0 μg/ml) of nimbolides and incubated for periods of 48 (P-388, HT-1080) or 72 h (BC-1, COL-2, KB, and MEL-2) at 37° in humidified atmosphere of 5% CO_2 in air, with the exception of MEL-2 which was incubated at 37° without CO_2. The quantity of cells in each tube was then determined by counting (P-388) or protein analysis (all other cell lines), and the aver-

aged data were expressed as a percentage relative to controls treated only with solvent (DMSO). The dose that inhibited cell growth by 50% (ED_{50}) was calculated. Results are given in Table 4.

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