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LIMONOIDS FROM *AZADIRACHTA EXCELSA*

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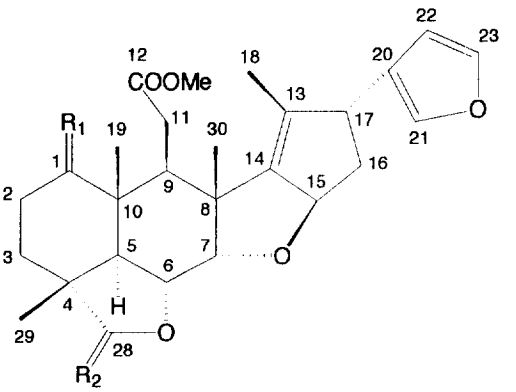
Key Word Index—*Azadirachta excelsa*; Meliaceae; limonoids; 2,3-dihydronimbolide; 3-deoxymethylnimbidate; nimbolide; 28-deoxonimbolide; electrospray mass spectrometry; cytotoxic activity.

Abstract—Activity-directed fractionation of a stem extract of *Azadirachta excelsa* using KB (human oral epidermoid carcinoma) cells led to the isolation of four meliacin-type limonoids. Two of these constituents were novel, namely, 2,3-dihydronimbolide and 3-deoxymethylnimbidate, and these were purified along with the known compounds, nimbolide and 28-deoxonimbolide. The structures of the new compounds were determined by spectroscopic methods. Nimbolide and 28-deoxonimbolide were broadly cytotoxic when evaluated against a panel of human cancer cell lines, while the two novel compounds were inactive in this regard. The defection of nimbolide and 28-deoxonimbolide as cytotoxic constituents was facilitated by an electrospray LC/MS dereplication procedure.

INTRODUCTION

As part of our continued search for novel plant-derived anticancer agents, we have investigated a chloroform-soluble extract of the stems of *Azadirachta excelsa* (Jack) Jacobs (Meliaceae). Many biological effects have been reported for extracts of plants in the genus *Azadirachta*, with estrogenic and antipyretic [1–4], feeding inhibitory [5–8], insect growth inhibitory [9], antibacterial and antifungal [10–12], antiviral [13], antiulcer [14], antimalarial [15–21], anti-inflammatory [3, 22–24], cytotoxic [25–29] and anti-HIV-1 activities [30] being the most frequently cited. Tetranortriterpenoids, specifically meliacin-type limonoids, have been isolated as the major bioactive principles in the genus *Azadirachta* [31].

The species *A. excelsa* has not been studied previously. In the present paper, we report the isolation of two new, albeit non-cytotoxic, meliacin-type limonoid derivatives, 2,3-dihydronimbolide (**1**) and 3-deoxymethylnimbidate (**2**), along with two known cytotoxic compounds, nimbolide (**3**) and 28-deoxonimbolide (**4**), from the stems of *A. excelsa*.



	R_1	R_2	Other
1	O	O	
2	α-OH	H_2	
2a	α-OAc	H_2	
3	O	O	$\triangle^{2,3}$
4	O	H_2	$\triangle^{2,3}$

RESULTS AND DISCUSSION

A chloroform soluble extract (ED_{50} 5.6 μg ml^{-1} against the KB cell line) of the stems of *A. excelsa* was subjected to a previously published LC-MS dereplication procedure [32], in which the column effluent was split and then both passed into an electrospray mass spectrometer and fractionated into a 96-well plate, with the latter subjected to bioassay using KB

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cells [33]. The masses found in those wells with biological activity were at 440, 452, 454, 456, 466, 484, 488 and 498 amu, of which 452 and 466 amu corresponded to the presence of 28-deoxonimbolide (**4**) and nimbolide (**3**), respectively. Subsequent to the work up of the *A. excelsa* stem chloroform-soluble extract by conventional column chromatographic methods, compounds **3** and **4** were isolated and identified as the known compounds, nimbolide and 28-deoxonimbolide, by comparison with published spectroscopic data [28]. Nimbolide (**3**) was first reported from *A. indica* of Nigerian origin [34], and a spectroscopic and cytotoxicity study has appeared on compounds **3** and **4** [28]. The dereplication stage in the present investigation helped to rapidly indicate that nimbolide (**3**) and 28-deoxonimbolide (**4**) could be responsible for some of the observed cytotoxicity of the initial crude extract of *A. excelsa* stems.

Two non-cytotoxic compounds were also obtained in this investigation by phytochemical fractionation of the stems of *A. excelsa*. Compound **1** was obtained as colorless plates (MeOH), mp 179–181°C, and its molecular formula of $C_{27}H_{32}O_7$ was determined by HREIMS. The IR spectrum of **1** showed three carbonyl absorbances at 1778 (γ-lactone), 1734 (ester) and 1711 (C=O) cm^{-1}, indicating the presence of non-conjugated ketone group. The ^1H NMR spectrum of **1** exhibited very similar data to those of **3** [28], except for the lack of signals due to two coupled olefinic protons (H-1 and H-2). Two methylene groups were observed at δ_H 2.80 (1H, *m*, H-2a), 2.37 (1H, *m*, H-2b) and 2.10 (2H, *m*, H_2-3), whose assignments were determined subsequently by analysis of HETCOR and selective INEPT NMR experiments. Thus, in a selective INEPT NMR experiment performed on **1**, irradiation of H_3-19 (δ_H 1.29, $^3J_{CH}$ = 5 Hz) led to the enhancement of the carbon signals at δ_C 210.5 (*s*), 49.3 (*d*), and 40.8 (*d*), which were assignable to C-1, C-5 and C-9, respectively. Irradiation of H_3-29 (δ_C 1.50, $^3J_{CH}$ = 5 Hz) led, in turn, to the enhancement of the C-28, C-5, C-4 and C-3 resonances at δ 177.7 (*s*), 49.3 (*d*), 40.8 (*s*) and 33.2 (*t*), giving further evidence for the location of the isolated ketone group at C-1. In the ^1H NMR spectrum of **1**, signals at δ_H 5.53 (1H, *t*, *J* = 6.6 Hz, H-15), 4.57 (1H, *dd*, *J* = 12.2, 3.5 Hz, H-6), 4.21 (1H, *d*, *J* = 3.5 Hz, H-7), 3.67 (1H, *br d*, *J* = 8.4 Hz, H-17), 2.68 (1H, *t*, *J* = 5.3 Hz, H-9), and 2.68 (1H, *d*, *J* = 12.2 Hz, H-5) and their corresponding ^{13}C NMR resonances (Table 1) were comparable to those of **3** [28], indicating the presence of the same stereochemistry at these positions in the two compounds. All of the ^1H and ^{13}C NMR assignments for **1** were achieved by ^1H-^1H COSY, ^1H-^1H ROESY, DEPT, HETCOR, and selective INEPT NMR experiments. As a result of the analysis of the data obtained, compound **1** was identified as the novel compound 2,3-dihydronimbolide.

Compound **2**, having a molecular formula of $C_{27}H_{36}O_6$ as determined by HREIMS, showed a broad hydroxyl absorbance at 3437 cm^{-1}, along with an ester carbonyl absorbance at 1730 cm^{-1} in the IR spectrum. The presence of a hydroxy group was also supported by a fragment ion peak at *m/z* 438 $[M-H_2O]^+$ in the low-resolution EI mass spectrum of **2**. Analysis of the ^1H and ^{13}C NMR spectra of **2** indicated that it was a structurally related limonoid to **1**, with obvious differences being the lack of carbonyl groups at C-1 and C-28 when compared with **1**. The ^{13}C NMR signal at δ_C 83.5 (*t*) was assigned to C-28, indicating the presence of an oxygenated methylene carbon in the molecule of **2**. In addition, two methylene groups were observed at δ_C 25.9 (*t*) and 29.3 (*t*) and were assignable to C-2 and C-3, respectively, and an oxygenated carbon signal appeared at δ_C 70.6 (*d*) in the ^{13}C NMR spectrum of **2** (Table 1). This was assignable to C-1 based on a selective INEPT NMR experiment, in which irradiation of H_3-19 (δ_H 0.90, $^3J_{CH}$ = 6 Hz) enhanced the oxygenated carbon signal at δ_C 70.6 (*s*), along with two signals at δ_C 43.4 (*d*) and 39.5 (*d*), assignable to C-1, C-5 and C-9, respectively. The monoacetate (**2a**) of **2** showed a *ca.* 1.40 ppm downfield shift in the H-1 signal to δ_H 4.79 in the ^1H NMR spectrum, when compared with the underivatized compound. The H-1 signal of **2a** was observed as a triplet with an equatorial-equatorial bond coupling constant (*J* = 2.6 Hz), suggesting that the hydroxy group at C-1 has an α-configuration, by comparison with that of salannol [35]. Thus, the structure of **2** was assigned as 3-deoxymethylnimbidate.

Compounds **1**–**4** were evaluated against a panel of human cancer cell lines as summarized in Table 2. Nimbolide (**3**) demonstrated a broad cytotoxic activity against the cell lines tested in the present investigation, and exhibited similar levels of cytotoxic potency to previously published data [28, 29]. 28-Deoxonimbolide (**4**) was in general less active than **3**, and showed weak cytotoxic activity against the BC1, KB, KB-V1$^+$ and KB-V1$^-$ cell lines (ED_{50} values around 4 $\mu g\ ml^{-1}$), consistent with a previous observation [28]. The novel compounds **1** and **2** were found to be non-cytotoxic against the cell lines tested in the panel. As judged by the compounds obtained in the present study, the ring-A enone unit of nimbolide (**3**) seems to be important for cytotoxic activity.

EXPERIMENTAL

MPs, uncorr. IR: film. ^1H and ^{13}C NMR spectra were recorded on either a 300 or 360 MHz NMR instrument with TMS as an int. standard. HMBC and ROESY data were obtained using a 500 MHz NMR instrument. Low- and high-resolution MS spectra were measured with a Finnigan MAT-90 instrument (70 eV). CC was carried out with the Merck silica gel (70-230 and 230-400) and Whatman reversed-phase C_{18} silica gel.

Plant material. The stems of *A. excelsa* were collected in Khao Chong, Trang, Thailand in May, 1993, and identified by one of us (T.S.). A voucher specimen

(A2590) has been deposited at the Field Museum of Natural History, Chicago, Illinois, USA.

Extraction and isolation. The air-dried stems (9 kg) of *A. excelsa* were extracted with three changes of MeOH (1 × 5 l., 2 × 4 l.). The resultant extracts were combined, concentrated under a vacuum, and then dissolved in 400 ml of MeOH:H_2O (4:1), which was washed with hexanes (3 × 200 ml). The lower layer was concentrated under red. pres. and partitioned between 10% MeOH (300 ml) and $CHCl_3$ (3 × 200 ml). The $CHCl_3$-soluble extract (2.2 g, ED_{50} 5.6 μg ml^{-1} against the KB cell line) was subjected to silica gel CC and eluted with hexanes-Me_2CO-MeOH mixtures in a gradient. Fractions 14–18 were combined and purified by silica gel CC using mixtures of hexanes-EtOAc-MeOH (6:1:0.1) to afford **3** which was then crystallized from MeOH (colorless needles, 82 mg). Fraction 20 was purified over a silica gel column using mixtures of hexanes-EtOAc-MeOH (5:1:0.1) to afford **1** which was again crystallized from MeOH (colorless plates, 102 mg). Fractions 7–11 were combined and chromatographed over a silica gel column and eluted using mixtures of hexanes-Me_2CO (10:1–6:1) to yield **2** (8 mg). Combined subfractions 7–10, a mixture of **2** and **4**, were purified by reversed-phase C_{18} silica gel CC and eluted with MeOH-CH_3CN-H_2O mixtures (45:5:50 → 60:5:35) to provide **2** (6 mg) and **4** (2 mg).

LC-MS dereplication procedure. The $CHCl_3$-soluble extract of *A. excelsa* stems, prepared as indicated above, was submitted for dereplication analysis, employing a previously published protocol, with the published chromatographic conditions used, and the KB cytotoxicity assay used to monitor activity [32, 33].

2,3-Dihydronimbolide (**1**). Colorless plates (MeOH), mp 179–181 C. $[\alpha]_D^{25}$ +122.2 (MeOH; *c* 0.1). IR ν_{max}^{film} cm^{-1}: 2953, 2874, 1778 (γ-lactone), 1734 (COOMe), 1711 (C=O), 1437, 1294, 1167, 1069, 1032, 972, 938, 756. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.33 (1H, *d*, *J* = 1.0 Hz, H-23), 7.25 (1H, *s*, H-21), 6.33 (1H, *d*, *J* = 1.0 Hz, H-22), 5.53 (1H, *t*, *J* = 6.6 Hz, H-15), 4.57 (1H, *dd*, *J* = 12.2, 3.5 Hz, H-6), 4.21 (1H, *d*, *J* = 3.5 Hz, H-7), 3.67 (1H, *br d*, *J* = 8.4 Hz, H-17), 3.55 (3H, *s*, COOMe), 2.86 (1H, *dd*, *J* = 15.6, 5.3 Hz, H-11a), 2.80 (1H, *m*, H-2a), 2.68 (1H, *t*, *J* = 5.3 Hz, H-9), 2.68 (1H, *d*, *J* = 12.2 Hz, H-5), 2.37 (1H, *m*, H-2b), 2.32 (1H, *dd*, *J* = 15.6, 5.7 Hz, H-11b), 2.21 (1H, *dd*, *J* = 12.0, 6.6 Hz, H-16a), 2.10 (3H, *m*, H_2-3, 16b), 1.70 (3H, *s*, Me-18), 1.50 (3H, *s*, Me-29), 1.33 (3H, *s*, Me-30), 1.29 (3H, *s*, Me-19). ^{13}C NMR: Table 1. EI-MS (70 eV) *m/z* (rel. int.): $[M]^+$ 468 (100), 453 (4), 437 (9), 415 (2), 339 (2), 267 (2), 259 (11), 231 (10), 215 (19), 201 (39), 197 (13), 185 (10), 182 (10), 173 (14), 159 (7), 147 (7), 131 (6), 121 (8), 105 (8). HREIMS *m/z*: 468.2151 (calcd for $C_{27}H_{32}O_7$, 468.2148).

3-Deoxymethylnimbidate (**2**). White amorphous powder $[\alpha]_D^{25}$ +62.2° (MeOH; *c* 0.2). IR ν_{max}^{film} cm^{-1}: 3437 (OH), 2924, 2859, 1730 (COOMe), 1447, 1385, 1157, 1034, 666. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.32 (1H, *d*, *J* = 1.0 Hz, H-23), 7.16 (1H, *s*, H-21), 6.16 (1H, *d*, *J* = 1.0 Hz, H-22), 5.46 (1H, *t*, *J* = 6.6 Hz, H-15), 4.22 (1H, *d*, *J* = 3.6 Hz, H-7), 3.93 (1H, *dd*, *J* = 12.6, 3.6 Hz, H-6), 3.68 (1H, *d*, *J* = 7.2 Hz, H-28a), 3.65 (2H, *br d*, *J* = 6.8 Hz, H-17, 28b), 3.55 (3H, *s*, COOMe), 3.39 (1H, *br s*, H-1), 2.53-2.07 (5H, *m*, H-9, H_2-11, H_2-16), 2.33 (1H, *d*, *J* = 12.6 Hz, H-5), 1.91-1.46 (4H, *m*, H_2-2, H_2-3), 1.71 (3H, *d*, *J* = 1.5 Hz, Me-18), 1.26 (3H, *s*, Me-30), 1.11 (3H, *s*, Me-29), 0.90 (3H, *s*, Me-19). ^{13}C NMR: Table 1. EIMS (70 eV) *m/z* (rel. int.): $[M]^+$ 456 (100), 441 (6), 438 (5), 397 (11), 365 (2), 335 (3), 309 (2), 283 (10), 259 (16), 237 (5), 231 (19), 215 (10), 202 (21), 173 (15), 159 (7), 147 (12), 107 (9). HREIMS *m/z*: 456.2507 (calcd for $C_{27}H_{36}O_6$, 456.2512).

Table 1. ^{13}C NMR spectral data of compounds **1** and **2** (90.8 MHz, $CDCl_3$).

	Compound			Compound	
Carbon	**1**	**2**	Carbon	**1**	**2**
1	210.5 *s*	70.6 *d*	15	88.3 *d*	87.7 *d*
2	34.4 *t*	25.9 *t*	16	41.2 *t*	41.5 *t*
3	33.2 *t*	29.3 *t*	17	49.5 *d*	49.4 *d*
4	40.8 *s*	39.5 *s*	18	12.9 *q*	12.8 *q*
5	49.3 *d*	43.4 *d*	19	15.1 *q*	17.1 *q*
6	72.8 *d*	72.8 *d*	20	126.5 *s*	127.0 *s*
7	82.7 *d*	86.1 *d*	21	138.9 *d*	138.5 *d*
8	50.0 *s*	49.3 *s*	22	110.4 *d*	110.2 *d*
9	40.8 *d*	39.5 *d*	23	143.0 *d*	143.0 *d*
10	49.6 *s*	41.3 *s*	28	177.7 *s*	83.5 *t*
11	32.9 *t*	30.7 *t*	29	15.8 *q*	15.3 *q*
12	172.9 *s*	176.3 *s*	30	17.1 *q*	18.8 *q*
13	136.0 *s*	134.3 *s*	COO*Me*	51.7 *q*	52.3 *q*
14	144.9 *s*	146.9 *s*			

Acetylation of **2**. A solution of compound **2** (2 mg) in a mixture of pyridine-Ac_2O (1:1, 1 ml) was allowed to stand at room temp. for 14 hr. After the usual work-up, the resultant product was purified by silica gel CC eluted with hexanes-Me_2CO (4:1) to yield **2a** (1 mg). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.33 (1H, *d*, *J* = 1.0 Hz, H-23), 7.25 (1H, *s*, H-21), 6.33 (1H, *d*, *J* = 1.0 Hz, H-22), 5.45 (1H, *t*, *J* = 6.6 Hz, H-15), 4.79 (1H, *t*, *J* = 2.6 Hz, H-1), 4.14 (1H, *d*, *J* = 3.4 Hz, H-7), 3.93 (1H, *dd*, *J* = 12.4, 3.4 Hz, H-6), 3.71 (1H, *d*, *J* = 7.2 Hz, H-28a), 3.62 (1H, *dd*, *J* = 8.2, 1.4 Hz, H-17), 3.56 (1H, *d*, *J* = 7.2 Hz, H-28b), 3.29 (3H, *s*, COOMe), 2.29 (1H, *d*, *J* = 12.6 Hz, H-5), 2.17 (3H, *s*, OAc), 164 (3H, *d*, *J* = 1.5 Hz, Me-18), 1.28 (3H, *s*, Me-30), 1.12 (3H, *s*, Me-29), 0.92 (3H, *s*, Me-19).

Nimbolide (**3**). Colorless needles (MeOH), mp 227–229 C. $[\alpha]_D^{25}$ +209.2° ($CHCl_3$; *c* 0.1), which exhibited comparable spectral (UV, IR, ^{1}H-NMR, ^{13}C-NMR, EIMS) data to reported values [28].

28-Deoxonimbolide (**4**). Amorphous gum. $[\alpha]_D^{25}$ −9.2° ($CHCl_3$; *c* 0.2), which exhibited comparable

Table 2. Evaluation of the cytotoxic potential of isolates obtained from *A. excelsa**

Compound	Cell line†							
	BC1	Lu1	Col2	KB	KB-V1$^+$	KB-V1$^-$	LNCaP	ASK‡
3	3.1	3.3	4.2	1.7	1.3	1.9	0.9	—
4	3.2	8.5	9.0	4.1	4.0	3.6	1.9	—

* Results are expressed as ED_{50} values ($\mu g\ ml^{-1}$) [33].

† Key: BC1 = human breast cancer; Lu1 = human lung cancer; Col2 = human colon cancer; KB = human epidermoid carcinoma of the mouth; KB-V1$^+$ = drug-resistant KB assessed in presence of vinblastine ($1\ \mu g\ ml^{-1}$); KB-V1$^-$ = drug-resistant KB assessed in absence of vinblastine; LNCaP = hormone-dependent human prostate cancer; ASK = human astrocytoma.

‡ — = Not active when tested at a concentration of 20 $\mu g\ ml^{-1}$ for the ASK cell line.

spectral (UV, IR, ^{1}H-NMR, ^{13}C-NMR, EIMS) data to reported values [28].

Bioassay evaluation. Compounds **1**–**4** were evaluated for cytotoxic activity against a panel of human cancer cell lines according to an established protocol [33], with ED_{50} values of $< 5\ \mu g\ ml^{-1}$ for pure compounds being regarded as significantly active. Antimitotic activity was assessed using cultured astrocytoma cells [36]. The results for **3** and **4** are given in Table 2; compounds **1** and **2** were inactive in all of the assays used.

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