Antimycobacterial Triterpenes from *Melia volkensii*

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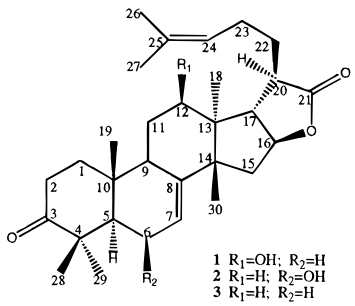
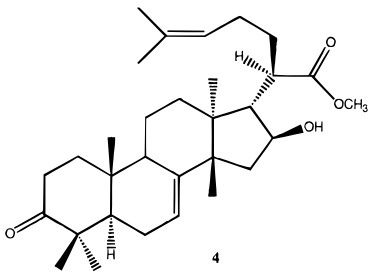
Received June 30, 1998

In a bioassay-guided search for antimycobacterial compounds from higher plants, we have chemically investigated methanolic extracts of seeds of *Melia volkensii*. Chromatographic fractions provided two new euphane (20*R*)-type triterpenoids. The structures of the new compounds, 12β-hydroxykulactone (**1**) and 6β-hydroxykulactone (**2**), were elucidated by 1D and 2D NMR (^{13}C, ^{1}H, ^{1}H−^{1}H COSY, HMQC, HMBC, and NOESY spectra) and FABMS studies and shown to be hydroxyl derivatives of kulactone (**3**). Also isolated was the known kulonate (**4**). In a radiorespirometric bioassay against *Mycobacterium tuberculosis*, compounds **1**, **2**, and **4** exhibited minimum inhibitory concentrations of 16, 4, and 16 μg/mL, respectively.

Melia volkensii Gürke (Meliaceae) is a subtropical tree found in dry areas of East Africa. A tea prepared from the bark is used in local folk medicine to alleviate pain, but it is poisonous at higher dose levels.[1] Extracts of seed kernels possess potent antifeedent activity against the desert locust, *Schistocerca gregaria*,[2,3] and fruit kernel extracts have also demonstrated growth-inhibitory activity against larvae of the mosquitoes *Aedes aegypti* and *Anopheles arabiensis*.[4,5] Chemical investigations of the fruits have resulted in the isolation of the insect antifeedants volkensin; salannin; and 1-cinnamoyl-, 1-tigloyl-, and 1-acetyltrichilinin.[6,7] More recent chemical investigations of *M. volkensii* have focused on the activity against the human breast-tumor cell line (MCF-7), studies which resulted in the isolation of meliavolin; meliavolkin; meliavolen; melianinone; 3-episapelin A; nimbolin B; meliavolkensins A and B; melianins A, B, and C; meliavolkinin, 1,3-diacetylvilasinin; and meliavolkenin.[8−12]

Described below is the chemical investigation of active fractions of the methanolic extract of the seeds of *M. volkensii*, which resulted in the isolation of two new and one known euphane-type triterpenes. Their structure elucidation by spectroscopic characterization as well as their minimum inhibitory concentrations (MICs) against *Mycobacterium tuberculosis* (H_{37}Rv) are presented.





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Results and Discussion

In a broad screening of crude plant extracts for inhibitory activity against *M. tuberculosis*,[13] the methanol extract of the seeds of *M. volkensii* demonstrated 99% inhibition at 100 μg/mL. Standard fractionation by vacuum−liquid chromatography (VLC)[14] using Si gel and solvents of increasing polarity provided the known kulactone (**3**), and kulonate (**4**), and the new 12β-hydroxykulactone (**1**) and 6β-hydroxykulactone (**2**). Kulonate (**4**) had been previously obtained from *Melia azedarach*.[15,16]

The IR data for **1** indicated the presence of hydroxyl(s) (3516 cm^{-1}) and carbonyls (1781 and 1705 cm^{-1}), the peak at 1781 cm^{-1} strongly suggesting the presence of a γ-lactone moiety. HRFABMS data indicated a molecular ion consistent with a molecular formula of $C_{30}H_{44}O_4$. Inspection of the ^{1}H NMR spectral data of **1** (Table 1) showed seven methyl singlets, two one-proton olefinic signals at δ 5.39 and 5.11, and two deshielded one-proton signals at δ 4.19 and 4.03. The ^{1}H NMR, IR, and MS data suggested that **1** represents a hydroxyl derivative of **3**.[15,16] This was supported by the presence of a peak at δ 4.03 (dd, J = 4.6, 9.3 Hz) corresponding to H-12 in **1**, which was the only proton absorption missing in the spectrum of **3**. ^{13}C NMR experiments confirmed the presence of seven methyls, a ketone (δ 216.3 s), and lactone (δ 180.6 s, 82.3 d) and hydroxyl (δ 72.2 d) groups. ^{13}C NMR assignments were based on DEPT, HMQC, and HMBC spectral data (Tables 1 and 2). The location of the hydroxyl group was established by HMBC experiments, which showed a ^{3}J-coupling between C-12 and H-18 in the HMBC experiment (Figure 1). Additional spectral assignments and HMBC couplings are listed in Table 1 and are summarized by arrows in Figure 1. An NOE correlation between H-12 and H-18 suggested that, by analogy with an α-methyl at C-13 in **3**, the relative configuration of the hydroxyl group at C-12 of **1** had to be β. Additional NOE correlations confirmed that the relative stereochemistry in **1** is the same as in **3**. Similar specific optical rotation values of **1** and **3** suggest that they have the same absolute configuration.

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10.1021/np980288u CCC: $18.00
Published on Web 03/12/1999

Table 1. ^{1}H NMR (400 MHz, $CDCl_3$) and HMBC (300 MHz, $CDCl_3$) Spectral Data of Compounds **1** and **2**

	1		**2**	
proton	δ_H multi, *J* (Hz)[a]	HMBC	δ_H multi, *J* (Hz)[a]	HMBC
1b	1.97 m			
2β	2.78 ddd (5.5, 14.6, 14.6)	1, 3	2.86 ddd (5.5, 14.1, 14.1)	1, 3
5	1.72 m	6, 9, 10, 19, 28, 29	1.49 d (4.4)	4, 6, 10
6			4.49 br s	
7	5.39 br d (2.4)		5.46 dd (3.5, 3.5)	
12α	4.03 dd (4.6, 9.3)			
15a	1.75 m	13, 14, 16, 30	1.76 dd (7.2, 13.6)	13, 14, 16, 30
15b			2.34 dd (10.2, 13.6)	
16α	4.19 ddd (7.3, 10.4, 12.0)	20	4.15 ddd (7.2, 10.2, 12.0)	
17	2.53 dd (12.0, 12.0)	14, 16, 18, 20	2.14 dd (12.0, 12.0)	13, 16, 18
18	0.83 s	12, 13, 14, 17	0.91 s	12, 13, 14, 17
19	1.04 s	1, 5, 9, 10	1.26 s	1, 5, 9, 10
23	2.18 m	20, 22, 24, 25		
24	5.11 dddd (1.1, 1.1, 7.2, 7.2)		5.10 dddd (1.3, 1.3, 6.9, 6.9)	
26	1.69 s	24, 25, 27	1.69 s	24, 25, 27
27	1.63 s	24, 25, 26	1.62 s	24, 25, 26
28	1.13 s	3, 4, 5, 29	1.52 s	3, 4, 5, 29
29	1.05 s	3, 4, 5, 28	1.23 s	3, 4, 5, 28
30	1.39 s	8, 13, 14, 15	1.31 s	8, 13, 14, 15

[a] Expressed as δ values in ppm, with *J* values in Hz in parentheses.

Table 2. ^{13}C NMR Spectral Data of Compounds **1** and **2** (75.4 MHz, $CDCl_3$)[a]

	compound[b]	
carbon	**1**	**2**
C-1	38.6 t	40.2 t
C-2	34.9 t	34.9 t
C-3	216.3 s	216.0 s
C-4	48.1 s	49.2 s
C-5	52.6 d	56.9 d
C-6	24.6 t	67.3 d
C-7	119.4 d	122.2 d
C-8	143.2 s	146.3 s
C-9	48.2 d	49.0 d
C-10	35.5 s	35.7 s
C-11	30.3 t	17.0 t
C-12	72.2 d	29.5 t
C-13	55.2 s ***	39.6 s
C-14	44.9 s ***	55.4 s
C-15	36.5 t	35.7 t
C-16	82.3 d	82.4 d
C-17	53.5 d	58.3 d
C-18	20.1 q	21.6 q
C-19	12.8 q	15.3 q
C-20	45.7 d	45.6 d
C-21	180.6 s	180.6 s
C-22	29.3 t	29.7 t *
C-23	26.2 t	26.3 t *
C-24	123.8 d	123.5 d
C-25	133.0 s	133.0 s
C-26	26.0 q *	25.9 q
C-27	18.1 q *	18.1 q
C-28	21.6 q **	24.9 q
C-29	24.6 q **	24.1 q
C-30	34.0 q	31.6 q

[a] Peak multiplicities were determined by heteronuclear multipulse programs (DEPT); s = singlet, d = doublet, t = triplet, q = quartet. [b] Interchangeable peaks are represented by *, **, or ***.

Compound **2** gave a parent peak of *m*/*z* 469.5 $[M + H]^+$ in the HRFABMS, corresponding to a molecular formula of $C_{30}H_{44}O_4$. IR spectral data and ^{1}H NMR chemical shifts and splitting patterns of **2** (Table 1) were very similar to those of **1**. However, instead of the doublet of doublets at δ 4.03 (H-12) in **1**, a broad singlet appeared at δ 4.49 in **2**. Examination of the COSY spectrum of **2** indicated a strong coupling between the olefinic proton signal H-7 and the broad singlet at δ 4.49, which suggested that **1** and **2** are constitutional isomers differing only in the position of the hydroxyl group, which must be attached to C-6 in **2**. This

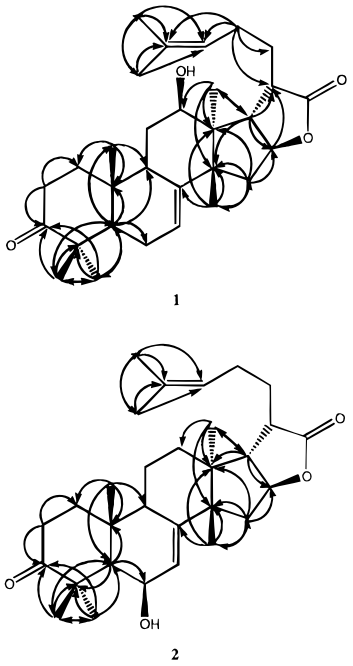

Figure 1. ^{1}H−^{13}C HMBC correlations for **1** and **2**.

was confirmed by examination of its HMQC and HMBC spectra, which showed a strong coupling between H-5 (δ 1.49) and C-6 (δ 67.3). Based on chemical shift considerations, the C-6 absorption had to be due to an oxygen-bearing carbon. A strong correlation between H-6 and H-29 in the NOESY spectrum revealed that the relative configuration of the hydroxyl group at C-6 had to be β. Further NOE correlations confirmed that the relative configuration of **2** is the same as in **3** and similar specific optical rotations suggest the same absolute configuration in **2** and **3**. All ^{1}H and ^{13}C NMR spectral assignments were based on DEPT, HMQC, and HMBC experiments, and the data are summarized in Tables 1 and 2, and a summary of HMBC correlations of **2** is also presented in Figure 1.

Kulonate (**4**) was identified by spectral comparison (IR, ^{1}H and ^{13}C NMR) with previously reported values.[15] It had been previously obtained from *M. azedarach*[15] and represents the methyl ester solvolysis product of kulactone (**3**). In a radiorespirometric bioassay [14,17] against *M. tubercu-*

losis ($H_{37}Rv$), triterpenes **1** and **4** showed MICs of 16 μg/mL, while **2** had a value of 4 μg/mL.

Experimental Section

General Experimental Procedures. MS were obtained on a Hewlett−Packard 5971A GC−MS or a TSQ70 FAB mass spectrometer. IR spectra were run on a Perkin−Elmer 1760X spectrometer as a film on KBr plates. VLC separations were carried out on TLC grade Si gel (MN Kieselgel).[18] ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on either a Bruker AM 400 MHz spectrometer or a Bruker ARX 300 MHz spectrometer. 2D NMR data, including HMBC[19] and HMQC[20] experiments, were obtained on a Bruker ARX 300 MHz spectrometer utilizing Bruker's standard pulse programs.

Plant Material. Seeds of *M. volkensii* were obtained from ripening fruits collected in November 1995, in Voi, Kenya. A voucher specimen (voucher no. MU/BOT/75M) has been deposited at the Department of Botany Herbarium, Moi University, Kenya.

Extraction and Isolation. Crushed seeds (1 kg) were allowed to stand in 2 L of MeOH for 1 week. The extract was decanted and the residual pulp re-extracted under similar conditions. The combined extracts were evaporated under vacuum to yield 18.6 g of a thick brown oil. Part of the crude MeOH extract (8.0 g) was adsorbed onto 8.0 g of Si gel and packed onto a 6.5 cm i.d. column containing 160.0 g of Si gel. The crude extract was separated into 10 fractions of 200 mL each, using an increasing polarity gradient of hexane (H), EtOAc (E), Me_2CO (A), and MeOH (M) [fraction 1, H; fraction 2, H:E (19:1); fraction 3, H:E (4:1); fraction 4, H:E (1:1); fraction 5, H:E (1:4); fraction 6, E; fraction 7, E:A (1:1); fraction 8, A; fraction 9, A:M (1:1); fraction 10, M]. Activities of the 10 fractions against *M. tuberculosis* indicated that fractions 3−5 were the only active fractions with percent inhibitions of 82, 96, and 95 at 33 μg/mL, respectively. Therefore, fraction 4 was chemically analyzed.

Fraction 4 (410 mg) was adsorbed on 360 mg of reversed-phase C_{18} Si gel (40−63 μm) and placed onto a VLC column (2.3 cm i.d.) packed with 8.1 g of C_{18} Si gel and chromatographed using 20 × 50 mL H_2O−MeOH mixtures of decreasing polarity, 61 fractions (20 mL each) being collected. Subfractions 46−50 (88 mg) were combined for further separation, as were subfractions 51−54 (53 mg) due to similar TLC and NMR patterns.

The mixture of compounds in subfractions 46−50 were adsorbed onto 112 mg of Si gel and placed onto a VLC column (2.3 cm i. d.) packed with 7.5 g of Si gel and chromatographed using 18 × 50 mL hexane−EtOAc mixtures of increasing polarity, 58 fractions (20 mL each) being collected. Subfractions 19−22 contained 8 mg of pure **1** and fractions 23−27 provided 12 mg of **2**.

The mixture of compounds in subfractions 51−54 were separated in a manner similar to that described above for subfractions 46−50, resulting in 69 fractions (20 mL each) with fractions 27−29 providing 6 mg of pure **4**.

12β-Hydroxykulactone (1): colorless oil; $[\alpha]^{25}_D$ −33.6° (*c* 0.0015, $CHCl_3$); IR (KBr) ν_{max} 3516 (OH), 2979, 1781 (γ-lactone), 1705 (ketone), 1452, 1365 cm^{-1}; ^{1}H NMR spectral data, see Table 1; ^{13}C NMR spectral data, see Table 2; EIMS (70 eV) *m*/*z* 444 (5), 296 (9), 148 (72), 120 (28), 107 (100), 91 (17), 77 (11), 65 (6), 55 (6); HRFABMS *m*/*z* 469.3317 $[M + H]^+$, calcd for $C_{30}H_{45}O_4$, 469.3318 (Δmmu = −0.1).

6β-Hydroxykulactone (2): colorless oil; $[\alpha]^{25}_D$ −47.1° (*c* 0.0015, $CHCl_3$); IR (KBr) ν_{max} 3504 (OH), 1781 (γ-lactone), 1705 (ketone), 1452, 1365 cm^{-1}; ^{1}H NMR spectral data, see Table 1; ^{13}C NMR spectral data, see Table 2; EIMS (70 eV) *m*/*z* $[M - H_2O]^+$ 444 (11), 393 (4), 227 (28), 185 (18), 171 (16), 157 (16), 145 (18), 119 (18), 107 (28), 91 (26), 81 (26), 69 (100), 55 (54); HRFABMS *m*/*z* 469.3334, $[M + H]^+$, calcd for $C_{30}H_{45}O_4$, 469.3318 (Δmmu = +1.6).

Radiorespirometric Bioassays. Fractions or pure compounds were added to 4 mL BACTEC 12B media (which contains 1 μCi/mL of [1−^{14}C] palmitic acid) and were then inoculated with *Mycobacterium tuberculosis* H37Rv (ATCC 27294). Cultures were incubated at 37 °C and evolved $^{14}CO_2$ measured daily in the BACTEC 460 instrument (expressed as growth index, or GI, units). Fractions were evaluated at two concentrations and activity expressed as a percent inhibition of the GI relative to control cultures receiving only DMSO.[14] This determination was made on the first day when the control cultures achieved a GI of 999 (the maximum measurable GI). The advantage of this method is the requirement for only one or two testing concentrations in order to determine the relative activity of multiple fractions. Pure compounds were tested at multiple twofold concentrations in the BACTEC system, and MICs were determined by the conventional method.[17] The MIC was defined as the lowest concentration that yielded a daily change in GI less than control vials (which had received a 1:100 diluted inoculum) on the day after the control vials achieved a GI of 30. This represents a 99% inhibition of the inoculum.

Acknowledgment. Financial support from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, under intra-agency agreement Y1-AI-5016, is gratefully acknowledged. We thank Anita N. Biswas and Ken E. White for technical assistance and Lewis Parnell for mass spectral data. This study was supported in part by a Fulbright Fellowship Grant to M.S.R.

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NP980288U