Two New Triterpene Esters from the Twigs of *Brachylaena ramiflora* from the Madagascar Rainforest[1]

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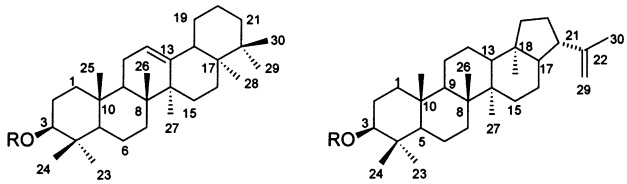
Bioassay-guided fractionation of a CH_2Cl_2/MeOH extract of the small twigs of *Brachylaena ramiflora* var. *ramiflora* resulted in the isolation of the two new triterpene esters **1** and **2** and five known triterpenoids, α-amyrin palmitate (**3**), *β*-amyrin palmitate (**4**), *β*-amyrin acetate (**5**), lupeyl acetate (**6**), and lupeol (**7**). The structures of the two new compounds were established as kairatenyl palmitate (**1**) and hopenyl palmitate (**2**) on the basis of 1D and 2D NMR spectroscopic data interpretation and chemical conversions. All the isolated compounds showed weak cytotoxicity against the A2780 human ovarian cancer cell line.

In our continuing research on the isolation of bioactive compounds from the Suriname and Madagascar rainforests as part of an International Cooperative Biodiversity Group (ICBG) program,[2] a twig sample of the plant *Brachylaena ramiflora* (DC.) Humbert var. *ramiflora* (Asteraceae) was collected in Madagascar and was found to give a cytotoxic CH_2Cl_2/MeOH extract. *Brachylaena*, a member of the subfamily *Inulae*, is a genus of 15−20 species that occurs in tropical Africa and Madagascar and is questionably native in the Mascarenes.[3] Five species occur in Madagascar, and *B. ramiflora* is widespread and common at elevations from 800 to 2000 m and often persists and is common in highly disturbed areas.[4] Like all of the Malagasy species of *Brachylaena*, *B. ramiflora* may reach 20 m in height and yields very durable timbers known to be termite resistant.[4]

The genus *Brachylaena* is a rich source of a number of sesquiterpenoids, some of which exhibit antibacterial activity.[5−8] The initial crude extract of *B. ramiflora* was selected for bioassay-guided fractionation on the basis of its cytotoxicity, with an IC_{50} value of 10.8 *μ*g/mL against the A2780 ovarian cancer cell line. The crude extract after extensive chromatography followed by HPLC yielded the two new triterpene esters **1** and **2**, in addition to the five known triterpenes **3**−**7**.

Liquid−liquid partition of the crude extract of *B. ramiflora* indicated that the activity was concentrated in the *n*-hexane-soluble fraction (IC_{50} 10.4 *μ*g/mL) of the *n*-hexane/aqueous MeOH partition. Chromatography of this fraction on a Sephadex LH-20 column and then by HPLC furnished the two new triterpene esters kairatenyl palmitate (**1**) and hopenyl palmitate (**2**), in addition to the five known triterpenes **3**−**7**. The structures of the five known compounds were identified as α-amyrin palmitate (**3**), *β*-amyrin palmitate (**4**), *β*-amyrin acetate (**5**), lupeyl acetate (**6**), and lupeol (**7**), by comparison of their spectral data with values reported in the literature.[9−11] In this paper we report the structure elucidation of the two new triterpene fatty esters, kairatenyl palmitate (**1**) and hopenyl palmitate (**2**).

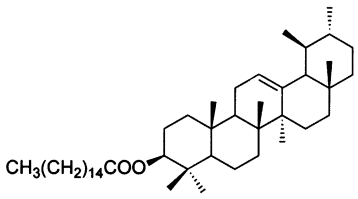


1 R = $CH_3(CH_2)_{14}CO$

8 R = H

2 R = $CH_3(CH_2)_{14}CO$

9 R = H



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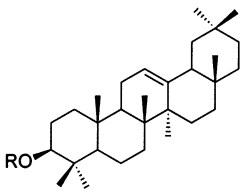
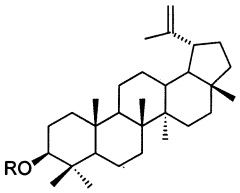


4 R = $CH_3(CH_2)_{14}CO$

5 R = Ac



6 R = Ac

7 R = H

Kairatenyl palmitate (**1**) was obtained as a colorless viscous liquid and was shown to have the molecular formula $C_{46}H_{80}O_2$ by HRFABMS, ^{13}C NMR, and DEPT spectra; this composition indicated seven degrees of unsaturation. It gave a positive Liebermann-Burchard (LB) test for terpenoids and steroids. The only characteristic IR absorption band was observed at 1725 cm^{-1}, indicating the presence of a carbonyl group in its structure. The ^1H NMR spectrum showed the presence of eight methyl singlets at *δ* 0.73, 0.81, 0.86, 0.88, 0.91, 0.93, 0.99, and 1.07, a doublet

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Table 1. NMR Data for Compounds **1** and **2** ($CDCl_3$)[a]

	1		2	
position	δ_H	δ_C	δ_H	δ_C
1	1.84 m	37.5	1.78 m	38.5
	1.25 m		1.22 m	
2	1.76 m	23.6	1.68 m	23.8
	1.54 m		1.46 m	
3	4.52 dd 8.6, 7.2	80.6	4.49 dd 8.1, 7.2	80.7
4		37.9		37.8
5	1.22 m	55.6	1.18 m	55.5
6	1.42 m	18.6	1.38 m	18.4
7	1.52 m	32.5	1.46 m	34.2
8		39.6		42.0
9	1.38 m	48.5	1.34 m	50.6
10		36.6		37.2
11	1.48 m	23.3	1.28 m	21.1
12	5.17 ddd 2.4, 2.6, 4.4	117.6	1.46 m	23.9
13		146.7	1.30 m	50.4
14		41.8		40.9
15	1.42 m	24.8	1.42 m	34.2
16	1.38 m	29.4	1.30 m	22.7
17		37.5	1.08 m	55.4
18	1.36 m	41.6		44.8
19	1.03 m	23.9	1.38 m	42.1
20	1.18 m	22.1	1.46 m	28.0
21	1.34 m	36.2	2.61 m	47.3
22		37.7		148.9
23	0.86 s	28.1	0.82 s	28.2
24	0.88 s	16.6[b]	0.86 s	16.3[b]
25	0.91 s	15.4	0.98 s	16.0[b]
26	0.73 s	16.2[b]	0.73 s	16.7
27	1.07 s	21.6[c]	0.94 s	16.7
28	0.99 s	21.3[c]	0.99 s	16.0[b]
29	0.93 s	25.6	4.76 s	109.5
30	0.81 s	22.2	1.76 s	25.2
1′		173.7		173.8
2′	2.28 t 6.6	34.8	2.27 t 6.6	34.9
3′	1.74 m	25.2	1.74 m	25.3
4′−13′	1.28 br s	29.2−29.8	1.28 br s	29.2−29.9
14′	1.28 br s	31.9	1.28 br s	32.0
15′	1.28 br s	22.7	1.28 br s	22.7
16′	0.89 t 7.0	14.1	0.89 t 7.0	14.1

[a] Assignments made on the basis of COSY, HMQC, and HMBC and comparison with the literature data.[12,14−16] [b,c] Values having the same superscript in the respective columns are interchangeable.

of doublets a δ 4.52 (1H, J = 8.6, 7.2 Hz), and a doublet of doublets of doublets at δ 5.17 (1H, J = 2.4, 2.6, 4.4 Hz). These observations suggested the basic skeleton of a 3β-substituted triterpenoid. The ^{13}C NMR values for all the carbons in compound **1** were assigned on the basis of DEPT, HMQC, and HMBC spectra (Table 1), which indicated the presence of eight sp^3 methyls, 10 sp^3 methylenes, four sp^3 methines, six sp^3 quaternary carbons, one sp^2 methine carbon, and one sp^2 quaternary carbon in its structure. A search of the literature found that the ^{1}H and ^{13}C NMR values of **1** were almost identical to those of kairatenyl acetate,[12] except for the replacement of the signals corresponding to the acetoxy group at the C-3 position with those for a palmitoyloxy group. The basic skeleton of the kairatenyl derivative in **1** was supported by the following key HMBC correlations: H-3/C-1, C-2, C-4, C-5, C-23, C-24; H-6/C-5, C-7, C-8, C-10; H-11/C-8, C-9, C-10, C-12, C-13; H-18/C-13, C-14, C-17, C-19, C-22, C-28. The presence of the palmitoyloxy group was inferred from the ^{1}H NMR signals of **1,** which showed a triplet at δ 2.28 (2H, J = 6.6 Hz), a multiplet centered at δ 1.74 (2H), a broad singlet at δ 1.28 corresponding to 24 protons, and a methyl singlet at δ 0.89 (J = 7.0 Hz). In the absence of any other assignable oxymethine proton in the ^{1}H NMR spectrum of **1**, the palmitoyloxy group was placed at the C-3 position and was supported by COSY (H-16′/H-15′; H-15′/H-14′; H-4′/H-3′; H-3′/H-2′) and HMBC (H-16′/C-14′, C-15′; H-4′/C-2′, C-3′; H-2′/C-1′, C-3′, C-4′; H-3/C-1′, C-1, C-2′, C-2, C-4, C-5) correlations. The presence of the palmitoyloxy group was further supported by the mass spectral fragment observed in the EIMS of **1** at m/z 409 ($M - C_{16}H_{31}O_2^+$) formed by the loss of the palmitoyloxy side chain from the molecular ion and from the HMBC correlations as shown in Figure 1. Alkaline hydrolysis of **1** furnished kairatenol (**8**)[12] and palmitic acid (m/z 256)[13] and confirmed the structure completely. On the basis of the above spectral data, compound **1** was assigned as kairatenyl 3β-*O*-palmitate.

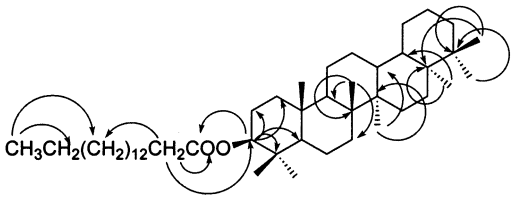

Figure 1. Selected HMBC correlations for **1**.

The molecular formula of compound **2** was also determined as $C_{46}H_{80}O_2$ by HRFABMS. It also gave a positive LB test for terpenoids, and its IR spectrum showed the presence of a carbonyl group (1732 cm^{-1}), similar to that of **1**. The mass spectral fragment observed at m/z 409 and in the EIMS showed the presence of a palmitoyloxy group in **2** as in **1**. The presence of a palmitoyloxy group in **2** was supported by the ^{1}H NMR values at δ 2.27 (2H, J = 6.6 Hz, H-2′), δ 1.74 (2H, H-3′), δ 1.28 (24H, br s, H-4′ to H-15′), and δ 0.89 (3H, t, J = 7.0 Hz, H-16′) and from the EIMS fragment at m/z 409, similar to **1**. Although compounds **1** and **2** both had palmitoyloxy side chains at the C-3 position, their ^{1}H NMR values for the basic skeleton of the two terpenoids differed significantly (Table 1). The ^{1}H NMR spectrum of **2** showed the presence of six methyl singlets at δ 0.73, 0.82, 0.86, 0.94, 0.98, and 0.99, an oxymethine at δ 4.46, and an isopropenyl group inferred by the presence of a methyl singlet at δ 1.76 and a broad singlet at δ 4.76 (2H). The ^{13}C NMR values for all the carbons were assigned from the DEPT, HMQC, and HMBC spectra and are given in Table 1. The ^{1}H NMR spectra, and especially the presence of an isopropenyl group, suggested that compound **2** is a pentacyclic triterpene of the lup-22(29)-en-3β-ol or hop-22(29)-3β-ol type.[11,14] The basic skeleton of a lup-22-(29)-en-3β-ol triterpenoid could be ruled out for compound **2** on the basis of the differences in the ^{13}C NMR values of **2** with lupeol derivatives.[15] A close comparison of the ^{1}H and ^{13}C NMR values of **2** with those of **1** and of 3β-methoxyhop-22(29)-ene, isolated from *Chionochloa cheesemanii*,[16] suggested that compound **2** is a hop-22(29)-ene terpenoid having a palmitoyloxy group at the C-3β position. The HMBC spectra of **2**, which showed the correlations H-3/C-1, C-2, C-4, C-5, C-23, C-24; H-6/C-5, C-7, C-8, C-10; H-9/C-8, C-10, C-11, C-12, C-26; H-13/C-12, C-14, C-17, C-18, C-27; H-21/C-17, C-18, C-20, C-22, C-29, C-30, supported the basic skeleton of a hopenyl derivative further. The presence of the palmitoyloxy group at the C-3 position was suggested by the COSY and HMBC correlations H-16′/H-15′; H-15′/H-14′; H-4′/H-3′; H-3′/H-2′, and H-16′/C-14′ or C-15′; H-4′/C-12′, C-3′; H-2′/C-1′, C-3′, C-4′; H-3/C-1′, C-1, C-2′, C-2, C-4, C-5, respectively, identical to **1**. On alkaline hydrolysis, compound **2** yielded hopenol (**9**)[14] and palmitic acid (m/z 256),[13] confirming the structure completely. Thus, compound **2** was established as hopenyl 3β-*O*-palmitate.

All the isolated compounds **1**−**7** were tested for cytotoxicity against A2780 ovarian cancer cells, and all were found to be marginally cytotoxic, with IC_{50} values of 10.0, 11.6,

15.4, 13.4, 12.1, 15.0, and 16.2 μg/mL, respectively. Actinomycin D at 2 μg/mL and an IC_{50} value of 2−5 ng/mL was used as the positive control.

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter. IR and UV spectra were measured on MIDAC M-series FTIR and Shimadzu UV-1201 spectrophotometers, respectively. NMR spectra were obtained on a JEOL Eclipse 500 spectrometer. The chemical shifts are given in δ (ppm) with TMS (tetramethylsilane) as internal reference, and coupling constants are reported in Hz. Mass spectra were obtained on a JEOL HX-110 instrument. Sephadex LH-20 was used for column chromatography. HPLC was performed on a Shimadzu LC-10AT instrument with an ODS A323 column (250 × 10 mm). Preparative TLC was performed on silica gel GF254 (1000 μm) plates from Analtech, Inc., Newark, DE.

Cytotoxicity Bioassays. The A2780 ovarian cancer cell line assay was performed at Virginia Polytechnic Institute and State University as previously reported.[2,17]

Plant Material. *Brachylaena ramiflora* (Asteraceae) was collected by C. Birkinshaw, P. Antilahimena, S. Randrianasolo, and S. Rakotonandrasana on December 15, 1999. The plant is an 8 m tall tree and was collected in the province of Toamasina in a community forest outside of Zahamena National Park, 3 km east of Andranomalaza Atsimo in an evergreen humid forest fragment at 1200 m in elevation.

Extract Preparation. The small twigs of *B. ramiflora* were dried, ground, and extracted with EtOH to give the dried methanolic extract MG120.

Extraction and Isolation. The crude extract MG120 (0.7 g) was suspended in aqueous MeOH (MeOH/H_2O, 9:1, 100 mL) and extracted with *n*-hexane (3 × 100 mL). The aqueous layer was then diluted to 60% MeOH (v/v) with H_2O and extracted with $CHCl_3$ (3 × 100 mL). The aqueous layer was concentrated, and the residue obtained was suspended in H_2O (25 mL) and extracted with *n*-BuOH (3 × 25 mL). The *n*-hexane extract was found to be the most active of all three extracts. The *n*-hexane extract (0.4 g) was fractionated over Sephadex LH-20 using *n*-hexane/EtOAc (100:0 to 0:100) to furnish nine fractions (A−I), of which fractions A, B, and G were found to be more active. Fractions A and B were combined on the basis of their similar TLC and ^{1}H NMR spectrum patterns, which on preparative TLC using *n*-hexane/EtOAc (95:5) yielded two fractions, AB-1 and AB-2. Fraction AB-1 on HPLC (ODS A323, 250 × 10 mm; flow rate 2 mL/min; diode array UV detection) with the mobile phase *n*-hexane yielded the new two triterpene esters **1** (2.6 mg, t_R 9.4 min) and **2** (2.3 mg, t_R 9.8 min) in addition to the two known compounds α-amyrin palmitate (**3**, 1.2 mg) and β-amyrin palmitate (**4**, 2.1 mg). Similarly fraction AB-2 on HPLC with the mobile phase *n*-hexane/EtOAc (98:2) yielded the two known triterpenoids β-amyrin acetate (**5**, 1.2 mg) and lupeyl acetate (**6**, 2.6 mg). Fraction G on preparative TLC using *n*-hexane/EtOAc (75:25) yielded the known compound lupeol (**7**, 2.8 mg). The five known compounds **3**−**7** were identified by comparison of their spectral data with literature values.[9−11]

Kairatenyl Palmitate (1): colorless viscous liquid; $[\alpha]_D$ +17.6° (*c* 0.42, $CHCl_3$); UV (MeOH) λ_{max} 216 nm (ϵ 12 630); IR ν_{max} 2960, 1725, 1450, 1375, 1150, 1050 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; EIMS (positive mode) *m*/*z* 665 [M + H$^+$] (16), 650 (12), 554 (7), 445 (5), 411 (8), 410 (21), 409 (18), 396 (28), 393 (18), 297 (8), 249 (8), 218 (100), 204 (24), 203 (51), 189 (93), 161 (31), 107 (52); HRFABMS *m*/*z* 664.6149 [M]$^+$ (calcd for $C_{46}H_{80}O_2$, 664.6150).

Alkaline Hydrolysis of Kairatenyl Palmitate A (1). To a solution of compound **1** (2.0 mg) in MeOH (1 mL) was added 5% methanolic KOH (3 mL), and the reaction mixture was refluxed for 2 h. The mixture was concentrated, and water (10 mL) was added. The aqueous layer was extracted with CH_2Cl_2 (3 × 10 mL), and the combined organic layer was concentrated to yield a brown semisolid. The solid was purified by preparative TLC (*n*-hexane/EtOAc = 80:20), furnishing a colorless solid (0.6 mg), which was identified as kairetenol (**8**)[12] by spectral data (^{1}H NMR and EIMS). The aqueous layer was acidified with 1 N HCl and extracted with EtOAc (2 × 10 mL) to yield a brown gum, which on preparative TLC (*n*-hexane/EtOAc = 50:50) furnished palmitic acid.[13]

Hopenyl Palmitate (2): colorless viscous liquid; $[\alpha]_D$ +37.8° (*c* 0.56, $CHCl_3$); UV (MeOH) λ_{max} 214 nm (ϵ 14 210); IR ν_{max} 2960, 1732, 1440, 1155, 1055 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; EIMS (positive mode) *m*/*z* 665 [M + H$^+$] (24), 650 (9), 586 (14), 554 (12), 445 (8), 411 (11), 410 (26), 409 (28), 396 (28), 393 (18), 297 (8), 245 (8), 218 (32), 204 (84), 189 (100), 161 (31), 107 (32), 69 (70), 55 (54); HRFABMS *m*/*z* 664.6153 [M]$^+$ (calcd for $C_{46}H_{80}O_2$, 664.6150).

Alkaline Hydrolysis of Hopenyl Palmitate (2). Hydrolysis of **2** (1.5 mg) as previously reported furnished hopenol (**9**)[14] and palmitic acid (*m*/*z* 256).[13]

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