Structure and Spasmolytic Activity of Eucalyptanoic Acid from *Eucalyptus camaldulensis* var. *obtusa* and Synthesis of Its Active Derivative from Oleanolic Acid

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A new triterpenoid acid named eucalyptanoic acid (**1**) has been isolated from the fresh uncrushed leaves of *Eucalyptus camaldulensis* var. *obtusa* along with two known constituents, β-sitosterol (**2**) and betulinic acid (**3**). The structure of **1** has been established as 3β-hydroxyolean-9(11),12-dien-28-oic acid through spectral studies including 1D and 2D NMR. **1** and its acetyl (**1a**) and acetylmethyl (**1b**) derivatives were tested for spasmolytic activity. **1b** was found to be the most active spasmolytic, mediated through blockade of calcium influx at 1 mg/mL. In the present study **1b** was also prepared starting from oleanolic acid (**4**). Acetylation of **4** gave **4a**, which on methylation afforded **4b**. Reaction of **4b** with *N*-bromosuccinimide (NBS) furnished **1b**. Hence **4** may be regarded as the biogenetic precursor of **1**. Compounds **4** and **4a** were found inactive at 1 mg/mL, while **4b** was moderately active in showing spasmolytic activity.

In a previous paper,[1] we reported spasmolytic constituents from *Eucalyptus camaldulensis* var. *obtusa* (Myrtaceae). In a continued study on the chemical constituents of this locally available medicinal tree, we now wish to report the isolation and structure elucidation of a new pentacyclic triterpenoid acid, elucalyptanoic acid (**1**), and two known compounds, β-sitosterol (**2**) and betulinic acid (**3**), from fresh uncrushed leaves of the plant. The structure of **1** has been established through 1D and 2D NMR including COSY-45, NOESY, *J*-resolved, HMQC, and HMBC spectra. Compound **1** and its acetyl (**1a**) and acetylmethyl derivatives (**1b**), prepared during the present studies, were tested for spasmolytic activity. **1b** was found to be the most active, causing its action through a calcium antagonist effect at 1 mg/mL. **1b** was also synthesized through reaction of the acetylmethyl derivative (**4b**) of oleanolic acid (**4**) with NBS in high yield. **4b** was obtained on methylation of the acetyl derivative (**4a**) of **4**. Compounds **4**, **4a,** and **4b** were also tested for spasmolytic activity. This is the first report of isolation of **1** as a free acid, although its methyl ester has been reported earlier.[2] Synthesis of the 9(11):12 diene of methylacetyloleanolate from oleanolic acid is reported for the first time.

The molecular formula of **1**, $C_{30}H_{46}O_3$, was obtained from HREIMS. It showed IR absorption bands at 3450−2600 (br, COOH, OH), 1690 (C=O), and 1620 (C=C). The ^{1}H NMR spectrum of **1** exhibited signals due to seven tertiary methyl groups at δ 0.90, 0.96, 0.98, 1.15, 1.18, 1.24, and 1.26 and a one-proton double doublet at δ 3.25 [J = 13.9, 3.4 Hz, H-18; correlated with δ_C 41.9 (CH, DEPT) in HMQC].[3] These data indicated that **1** belongs to Δ^{12} oleanane type of triterpenoids. A carbinolic methine proton at δ 3.42 [1H, dd, J = 10.2, 5.2 Hz; correlated with δ_C 78.1 (CH, DEPT) in HMQC] indicated the presence of a hydroxy group, which was confirmed by its acetylation to **1a** with Ac_2O/py. It was placed at position 3 on biogenetic grounds. Its β configuration was assigned on the basis of chemical shifts and coupling constants of H-3.[4]

The ^{1}H NMR spectrum of **1** exhibited the typical mutually coupled (J = 5.8 Hz) vinylic signals at δ 5.70 and 5.75 correlated with δ_C 115.4 (C-11) and δ_C 123.4 (C-12),[2,5] respectively, in the HMQC spectrum, assigned to H-11 and H-12, respectively (Table 1). The interactions of these protons in the ^{1}H, ^{1}H COSY spectrum supported this assignment. The interactions of H-11 with C-8 (δ_C 42.3), C-10 (δ_C 39.2), and C-12 (δ_C123.4) and H-12 with C-9 (δ_C 155.3), C-11 (δ_C 115.4), and C-18 (δ_C 41.9) in the HMBC spectrum (Table 1) confirmed the presence of a cisoid diene at C-9 (11):12 in **1**. Its position was further supported by significant fragments at *m*/*z* 285.1840 and 271.2051 in the HREIMS due to cleavage of ring B and ring D[6] (Figure 1) and UV absorption at λ 280 nm, characteristic of a homoannular diene system at ring C.[7]

The presence of a quaternary carbon in the ^{13}C NMR spectrum (broad band) at δ_C 179.7 supported the presence of a carboxyl group (IR: 3450−2600, 1690 cm^{-1}; acetylmethyl derivative **1b**), and a prominent peak in the HREIMS at *m*/*z* 239.1765 obtained by decarboxylation of fragment *m*/*z* 285.1840 (Figure 1) suggested its presence in ring D or E. It was located at C-17 on the basis of comparable chemical shift values of rings D and E with those of triterpenes of similar structures.[8−10] HMBC interaction of H-18 with C-28 confirmed the location of COOH. In light of the above data the structure of **1** has been established as 3β-hydroxyolean-9(11),12-dien-28-oic acid.

1b was also synthesized from oleanolic acid (**4**) by acetylation (Ac_2O/py, **4a**) and methylation (CH_2N_2, **4b**) followed by reaction with NBS in high yield following the procedure reported earlier[11] for the preparation of 3β-acetoxyoleana-9(11)-12-diene from β-amyrin acetate.

Compounds **1**, **1a**, **1b**, **4**, **4a**, and **4b** were tested for possible spasmolytic activity via their effects on the spontaneous movements of isolated rabbit jejunum, and the results are shown in Table 2. Compounds **1a**, **4**, and **4a** were found inactive up to the dose of 1 mg/mL, while **1** and **4b**, with relatively moderate spasmolytic action, exhibited about 58−68% inhibition of spontaneous contractions at the highest dose tested (1 mg/mL). Compound **1b** was found to be the most active and caused almost complete

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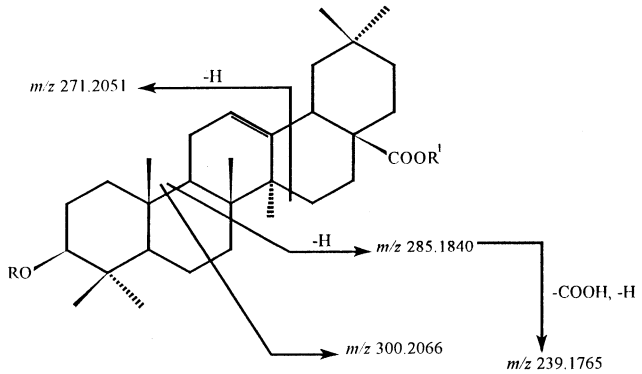
Table 1. ^{1}H and ^{13}C NMR Data and HMBC Correlations of **1** in C_5D_5N[a]

		correlated protons				
C	δ_C	H	δ_H	multiplicity	*J* (Hz)	HMBC
1	37.7	1a	1.98	m		
		1b	1.89	m		
2	28.5	2a	2.15	m		
		2b	2.05	m		
3	78.1	3	3.42	dd	10.2,5.2	2,4,23,24
4	39.6					
5	51.8	5	0.93	m		
6	18.6	6a	1.58	m		
		6b	1.50	m		
7	32.5	7	1.30	m		
8	42.3					
9	155.3					
10	39.2					
11	115.4	11	5.70	d	5.8	8,10,12
12	123.4	12	5.75	d	5.8	9,11,18
13	144.6					
14	43.0					
15	27.8	15a	1.85	m		
		15b	1.70	m		
16	23.7	16a	2.10	m		
		16b	1.90	m		
17	46.5					
18	41.9	18	3.25	dd	13.9,3.4	12,13,14,16,17,19,28
19	46.4	19a	1.75	m		
		19b	1.26	m		
20	29.4					
21	34.1	21a	1.15	m		
		21b	1.12	m		
22	33.5	22a	1.52	m		
		22b	1.45			
23	28.6	23	1.18	s		3,5,4,24
24	16.4	24	0.98	s		3,4,5,23
25	21.2	25	1.15	s		1,5,9,10
26	21.3	26	1.26	s		7,8,14
27	26.0	27	1.24	s		8,14,15
28	179.7	-				
29	33.1	29	0.90	s		19,21,30
30	23.6	30	0.96	s		19,20,21,29

[a] Assignments are based on ^{1}H, ^{13}C (broad band, DEPT), ^{1}H, ^{1}H COSY, HMQC, and HMBC experiments and comparison with similar compounds.[5,8−10]



1 R, R¹ = H; 9,11Δ
1a R = CH_3CO; R = H; 9,11Δ
1b R = CH_3CO; R¹ = CH_3; 9,11Δ
4 R, R¹ = H
4a R = CH_3CO; R¹ = H
4b R = CH_3CO; R¹ = CH_3

Figure 1. Significant mass fragmentation of **1**.

inhibition at a dose of 1 mg/mL (Table 2). When tested against K^+(50 mM)-induced contractions, compounds **1**, **1b**, and **4b** caused inhibitory action, **1b** being the most active (Table 2). The results indicated that the −COOMe group at C-17 is responsible for spasmolytic activity in acetylmethyloleanolic acid (**4b**), as the parent compound **4** and its acetyl derivative **4a** are inactive. In diene **1**, which showed moderate activity, the presence of the −COOMe functionality at C-17 enhances the activity, while the acetoxy group at C-3 deactivates it. In conclusion, the presence of −COOMe at C-17 and a C=C bond at C-9(11) in **1b** is responsible for greater activity in this series of triterpenoids.

Table 2. Effect of Compounds **1**, **1a**, **1b**, **4**, **4a**, and **4b** on Spontaneous and K^+-Induced Contractions in Rabbit Jejunum[a]

	effect (% inhibition)	
compound	spontaneous contractions	K^+-induced contractions
1	58.2 ± 5.8	31.1 ± 3.8
1a	no effect	no effect
1b	95.3 ± 3.6	85.9 ± 5.4
4	no effect	no effect
4a	no effect	no effect
4b	67.7 ± 3.9	35.5 ± 4.3

[a] The values shown represent mean ± SEM of 3−5 determinations obtained at the dose of 1 mg/mL.

The contractions of smooth muscle preparations, including rabbit jejunum, are dependent upon an increase in the cytoplasmic-free Ca^{2+}, which activates the contractile elements.[12]

The contractions induced by high K^+ (>30 mM) are dependent upon ingress of Ca^{2+} into the cells through VDCs,[13] and a substance that inhibits K^+-induced contractions is considered a calcium channel blocker. K^+ channel openers also inhibit K^+-induced contractions, but the spasmolytic effect of the test compounds is not mediated through this mechanism because chromokalin and other K^+ channel openers do not inhibit contractions induced by K^+ concentrations greater than 30 mM.[14,15] Thus, inhibition of high K^+(50 mM)-induced contraction of rabbit jejunum by these compounds may be visualized as an outcome of restricted Ca^{2+} entry via VDCs.

These data indicate that compounds **1**, **1b**, and **4b** exhibit spasmolytic action through blockade of calcium influx. The plant *E. camaldulensis* var. *obtusa* has been used in traditional medicine for the treatment of diarrhea, a hyperactive state of the gut. Calcium antagonists have been considered useful in diarrhea,[16] and it is likely that the presence of these compounds in the plant along with previously reported compounds[1] with similar action may be responsible for its folkloric use in the control of diarrhea.

Experimental Section

General Experimental Procedures. Melting points were determined using a Büchi 535 melting point apparatus and are uncorrected. IR and UV spectra were recorded on Jasco A-302 and Hitachi-U-3200 spectrophotometers, respectively. Mass spectra were recorded on a Finnigan MAT 312 double focusing mass spectrometer connected to a PDP 11/34 computer system. The ^{1}H NMR spectra were recorded on a Bruker AMX 400 NMR spectrometer operating at 400 MHz, while the ^{13}C NMR spectra were obtained on the same instrument operating at 100 MHz. The spectra were referenced to the residual solvent signals. The chemical shifts are reported in δ (ppm), and the coupling constants are in Hz. All thin-layer chromatography (TLC) and preparative TLC (PLC) were performed on Merck silica gel 60 F_{254} plates or layers of Merck Kieselgel 60 G_{254} (0.1 × 20 × 20 cm) made up on glass plates. Zones were detected under ultraviolet light (254 or 366 nm). Vacuum liquid chromatography (VLC)[17] was performed on Merck silica gel 60 G_{254}. The ^{13}C NMR spectral assignments have been made partly through a comparison of the chemical shifts with the published data for similar compounds[5,8−10] and partly through an analysis of DEPT, HMQC, and HMBC spectra.

Plant Material. The leaves of the plant were collected from the Karachi region. The plant was identified by Mr. MIH Brooker, eucalypt botanist, Centre for Plant Biodiversity Research, Australian National Herbarium (CANB.), Canberra, Australia, and a voucher specimen has been deposited in the herbarium.

Extraction and Isolation. The extraction and solvent partitioning of the freshly collected leaves of the plant were conducted as described previously.[1] The residue (58 g) of the petroleum ether phase obtained after partitioning of the petroleum ether-soluble fraction with 90% methanol (aqueous) was subjected to VLC (petroleum ether−EtOAc, in order of increasing polarity). A total of 18 fractions were obtained through combining the eluates on the basis of TLC.

Fractions 10 (petroleum ether−EtOAc, 8.75:1.25 eluate), 11 (petroleum ether−EtOAc, 8.5:1.5 eluate), and 12 (petroleum ether−EtOAc, 8:2 eluate) on concentration and keeping overnight at room temperature afforded *β*-sitosterol (**2**) as a colorless crystallizate (388 mg). Similarly, fractions 13 (petroleum ether−EtOAc, 7.5:2.5 eluate) and 14 (petroleum ether−EtOAc, 7:3 eluate) on concentration and keeping overnight at room temperature afforded betulinic acid (**3**) as a colorless crystallizate (100 mg). The mother liquour of fraction 12 (8 g) was subjected to flash column chromatography (Model Aldrich, silica gel E. Merck, 9385; $CHCl_3$−MeOH in order of increasing polarity). A total of nine fractions were obtained on combining the eluates on the basis of TLC. Fraction 4 (800 mg), which eluted with $CHCl_3$, was further subjected to flash column chromatography (Model Eyela EF 10, silica gel E. Merck, 9385; $CHCl_3$−MeOH in order of increasing polarity). Various fractions were obtained on combining the eluates on the basis of TLC. Fraction 2, eluted with $CHCl_3$, afforded **1** (66 mg), which formed flowers of needles after repeated crystallization from $CHCl_3$−MeOH (1:1).

3*β*-Hydroxyolean-9(11),12-dien-28-oic acid (1): colorless flowers of needles ($CHCl_3$−MeOH, 1:1); mp 219−220 °C; UV λ_{max} (MeOH) 280 nm; IR ν_{max} (KBr) 3450−2600 br, 2950, 2840, 1690, 1620, and 1060 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m*/*z* 454 $[M]^+$ (100), 436 (12), 410 (6), 300 (18), 285 (30), 271 (10), 239 (19); HREIMS *m*/*z* 454.3452 [$C_{30}H_{46}O_3$, M^+; calcd for $C_{30}H_{46}O_3$, 454.3446], 436.3309 [$C_{30}H_{44}O_2$, $M^+ - H_2O$], 300.2066 [$C_{20}H_{28}O_2$], 285.1840 [$C_{19}H_{25}O_2$], 271.2051 [$C_{19}H_{27}O$], 239.1765 [$C_{18}H_{23}$].

Acetylation of Eucalyptanoic Acid (1). To a solution of **1** (10 mg) in pyridine (1 mL) was added Ac_2O (1 mL) and the reaction mixture kept at room temperature overnight. Usual workup gave **1a** (9.6 mg) as a colorless crystalline solid: mp 190−191 °C; UV λ_{max} 282 nm; IR ν_{max} 3450−2600 br, 2940, 2835, 1730, 1690, 1625, and 1060 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.58 (1H, d, J = 5.7 Hz, H-12), 5.56 (1H, d, J = 5.7 Hz, H-11), 4.50 (1H, dd, J = 11.0, 5.0 Hz, H-3α), 2.02 (3H, s, C*H*$_3$COO), 1.18 (3H, s, C*H*$_3$), 0.98 (3H, s, C*H*$_3$), 0.93 (3H, s, C*H*$_3$), 0.92 (3H, s, C*H*$_3$), 0.89 (3H, s, C*H*$_3$), 0.87 (3H, s, C*H*$_3$), 0.86 (3H, s, C*H*$_3$); EIMS *m*/*z* 496 $[M^+]$ (100), 452 (10), 436 (11), 313 (10), 285 (19), 253 (8), 239 (10); HREIMS *m*/*z* 496.3560 [$C_{32}H_{48}O_4$, M^+; calcd for $C_{32}H_{48}O_4$, 496.3552].

Methylation of 1a. 1a (5 mg) was dissolved in MeOH, treated with an excess of an ethereal solution of CH_2N_2, and kept at room temperature overnight. On usual workup, methylacetyleucalyptanoate (**1b**) (4.8 mg) was obtained: mp 120−121 °C; UV λ_{max} (MeOH) 282 nm; IR ν_{max} (KBr) 2950, 2855, 1730 br, 1630 and 1050 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.57 (1H, d, J = 5.7 Hz, H-12), 5.55 (1H, d, J = 5.7 Hz, H-11), 4.50 (1H, dd, J = 11.1, 5.0 Hz, H-3α), 3.63 (3H, s, C*H*$_3$OCO), 2.04 (3H, s, C*H*$_3$COO), 1.18 (3H, s, C*H*$_3$), 0.99 (3H, s, C*H*$_3$), 0.94 (3H, s, C*H*$_3$), 0.93 (3H, s, C*H*$_3$), 0.89 (3H, s, C*H*$_3$), 0.88 (3H, s, C*H*$_3$), 0.86 (3H, s, C*H*$_3$); EIMS *m*/*z* 510 $[M^+]$ (100), 451 (20), 450 (15), 313 (12), 299 (27), 253 (10), 239 (17); HREIMS *m*/*z* 510.3717 [$C_{33}H_{50}O_4$, M^+; calcd for $C_{33}H_{50}O_4$, 510.3708].

Acetylation of Oleanolic Acid (4). Acetylation of oleanolic acid (**1**) (200 mg), obtained from *Lantana camara* through a procedure described earlier,[18] with Ac_2O and pyridine (each 1 mL) gave oleanolic acid acetate (**4a**) (198 mg) as a colorless crystalline solid: mp 257−258 °C (lit.[19] mp 256−258 °C); EIMS *m*/*z* 498 $[M]^+$. It was identified by direct comparison (mmp, IR, ^1H NMR, MS) with an authentic sample.

Methylation of 4a. Methylation of **4a** (150 mg) was carried out with a freshly prepared ethereal solution of CH_2N_2 as described for **1a**, which gave methyl acetyl oleanolate (**4b**) (148 mg) as a colorless shiny crystalline solid: mp 223−224 °C (lit.[19] mp 224−225 °C); EIMS *m*/*z* 512 $[M]^+$. It was identified by direct comparison (mmp, IR, ^1H NMR, MS) with an authentic sample.

Synthesis of Methyl 3*β*-Acetoxyolean-9(11),12-dien-28-oate (1b). To a solution of **4b** (100 mg) in dried CCl_4 (10 mL) was added *N*-bromosuccinimide (97%, 20 mg), and the mixture was refluxed on a steam bath for 2.5 h. Filtration and removal of CCl_4 under reduced pressure gave a pale yellow residue, which was dissolved in Et_2O. The ethereal solution was washed with water and dried over Na_2SO_4 (anhydrous). Evaporation of the solvent gave colorless flowers of needles (92 mg) identical in all respects (mmp, IR, UV, ^1H NMR, MS) with **1b**.

Spasmolytic Activity. Spasmolytic activity of the test compounds was studied by using isolated rabbit jejunum preparations, as described previously.[1,20]

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