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Triterpenoidal constituents from *Eucalyptus camaldulensis* var. *obtusa* leaves

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Abstract

An investigation on the constituents of the fresh, uncrushed leaves of *Eucalyptus camaldulensis* var. *obtusa* has led to the isolation of the triterpenoid amirinic acid and four known triterpenoids ursolic acid lactone, betulinic acid, oleanolic acid and ursolic acid. Amirinic acid transformed into amirolide in deuterated chloroform at room temperature. The new products were characterized by exhaustive spectroscopic studies as 2α,3β,7β-trihydroxy-11α-methoxyurs-12-en-28-oic acid and 2α,3β,7β-trihydroxyurs-11-en-28,13β-olide.

Keywords: *Eucalyptus camaldulensis* var. *obtusa*; Myrtaceae; Triterpenoids; Amirinic acid; Amirolide

1. Introduction

Eucalyptus belonging to the family Myrtaceae is a large genus of aromatic trees comprising more than 500 species indigenous to Australia, Tasmania and neighbouring islands. Various species of *Eucalyptus* are cultivated particularly in subtropical and warm temperate regions on account of their economic and medicinal value. The leaves of some species are used for the production of essential oil. The major constituent of the oil is eucalyptol (cineol), which has diaphoretic, disinfectant, expectorant and antimalarial properties. As an antiseptic it is specially used in the treatment of infection of upper respiratory tract and in certain skin diseases. The gum is used in diarrhoea and as astringent in dentistry, cuts, etc. It is also used as an astringent in haemorrhage (Sastri, 1952; Penfold and Willis, 1961). Phytochemical studies undertaken by different groups of workers on different parts of the plant have resulted in the isolation of various terpenoids (Wang and Fujimoto, 1993), flavonoids (Okamura et al., 1993) and phenolic acids (Boukef et al., 1976). The present chemical investigation on the fresh and uncrushed leaves of the plant led to the isolation of a new pentacyclic triterpenenoid (**1**) along with four known compounds ursolic acid lactone (Wang and Fujimoto, 1993; Dayal, 1987), betulinic acid (Siddiqui et al., 1988), oleanolic acid (Movsumov and Aliev, 1985) and ursolic acid (Dayal, 1987; Mathuram et al., 1998). Compound **1** is transformed into the new compound **2** in deuterated chloroform at room temperature.

2. Results and discussion

Compound **1** had the molecular formula $C_{31}H_{50}O_6$ as evidenced by HR-EIMS. Its IR spectrum showed hydroxy (3460 cm^{-1}), carboxyl (3420–2650, 1710 cm^{-1}), olefinic (1630 cm^{-1}) and C–O (1150 cm^{-1}) absorption bands, while UV spectrum showed only a terminal absorption (202 nm). The ^{1}H-NMR spectrum exhibited five tertiary methyl signals at δ 0.77, 0.88, 0.98, 1.00 and 1.15 and two secondary methyl signals at δ 0.78 (3H, *d*, J = 7.0 Hz) and 0.93 (3H, *d*, J = 7.0

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Hz). It further showed trisubstituted double bond at δ 5.49 (1H, *d*, J = 3.5 Hz, H-12) and a one-proton doublet at δ 2.22 (J = 11.5 Hz, H-18). These data and molecular formula suggested that **1** belongs to an ursane type triterpene. Its molecular formula indicated that it contain three oxygen atoms more than ursolic acid. The appearance of fragment at m/z 240.1721 showed that two additional oxygen atoms are present in ring A or B as hydroxyl groups. The ^{1}H-NMR spectrum of **1** showed signals at δ 3.67 (1H, ddd, J = 11.2, 9.5, 4.6 Hz) and δ 2.98 (1H, *d*, J = 9.5 Hz) ascribable, respectively, to the 2β- and 3α-protons on the carbons bearing hydroxyl function (Menezes et al., 1998). These data located the two hydroxyl groups at positions 2 and 3 with α and β configuration, respectively. The third hydroxyl group could be placed at C-7 in an equatorial disposition since the proton at C-7 resonated as doublets of doublet centred at δ 3.78 (1H, J = 10.0, 5.6 Hz) (Atta-ur-Rahman et al., 1973; Anaya et al., 1989). Location of the hydroxyl group at C-6 instead of C-7 would have caused an additional splitting of the carbinylic proton due to H-5.

The ^{1}H-NMR spectrum of **1** further showed a singlet at δ 3.26 and a doublet of doublets at δ 3.72 (1H, *dd*, J = 8.5, 3.5 Hz, H-11β) which indicate the presence of methoxyl group at C-11. The position and the stereochemistry of methoxyl group was determined by the coupling constant of doublet of doublets.The larger coupling constant (J = 8.5 Hz) could be seen as the result of trans diaxial coupling with the α-axial proton at C-9 (δ_H 1.62) and the smaller one (J = 3.5 Hz) as the interaction of the same proton with the vinylic hydrogen atom at C-12. Accordingly, the methoxyl geminal proton H-11 is β-axial and therefore methoxyl group at that position is α-equatorial (Amagaya et al., 1979; Hota and Bapuji, 1993). The strong mass fragment at m/z 246.1618 ($C_{16}H_{22}O_2$) obtained through cleavage of ring C after removal of MeOH further supported this assignment and located the carboxylic group (3420–2650, 1710 cm^{-1}) at C-17 (Budzikiewicz et al., 1963). Thus, the structure of **1** was established as 2α,3β,7β-trihydroxy-11α-methoxyurs-12-en-28-oic acid. It is important to mention here that triterpenoids with β-oriented hydroxyl group at C-7 are very rare in nature.

The solution of **1** in deuterated chloroform from ^{1}H-NMR was allowed to dry at room temperature and the dried sample kept at room temperature awaiting its turn of 2D NMR techniques which were recorded after 6 months. However, the spectra showed that the downfield signals were quite different from those expected for **1** suggesting transformation of **1** into a new compound. Hence, a detailed spectral analysis of **2** was performed which led to decide its structure as 2α,3β,7β-trihyroxyurs-11-en-28,13β-olide as discussed below.

Compound **2** did not show molecular ion peak in the EIMS and HREIMS. Its molecular formula $C_{30}H_{46}O_5$ was confirmed by combined application of ^{1}H and ^{13}C-NMR (DEPT). It showed pseudomolecular ion peak at m/z 468.3199 $[C_{30}H_{44}O_4]^+$ corresponding to M^+-H_2O along with a prominent peak at m/z 424.3357 $[C_{29}H_{44}O_2]^+$ indicating the loss of CO_2 from peak at m/z 468.3199. Its UV spectrum exhibited absorption band at 205 nm indicating the lack of conjugation in the molecule. Its IR showed a γ-lactone (1755 cm^{-1}) with olefinic (1635 cm^{-1}) and hydroxyl (3440 cm^{-1}) groups. The ^{1}H and ^{13}C-NMR data (Table 1) of **2** indicated that it belongs to α-amyrin type of triterpenoids. Its ^{1}H-NMR spectrum showed five tertiary methyl signals at δ 0.86, 0.88, 0.94, 1.05 and 1.17 and two secondary methyls at δ 0.92 (*d*, J = 6.2 Hz) and 0.99 (*d*, J = 6.0 Hz). It further showed

Table 1
^{1}H- and ^{13}C-NMR spectral data for compound **2**[a]

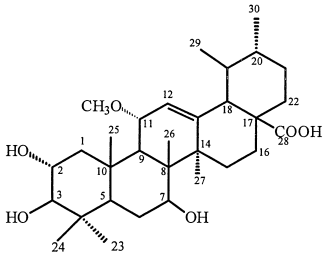
C	δC	Correlated protons			
		H	δH	Multiplicity	J (Hz)
1	46.9	1e	2.05	*dd*	11.8, 4.6
		1a	0.85	*m*	–
2	68.6	2	3.67	*ddd*	11.2, 9.4, 4.6
3	83.9	3	2.97	*d*	9.4
4	39.3	–	–	–	–
5	55.5	5	0.73	*dd*	12.8, 3.2
6	28.3	6	N.O.[c]	–	–
7	75.0	7	3.79	*dd*	10.0, 5.5
8	48.1	–	–	–	–
9	53.1	9	1.97	*dd*	3.1[b], 1.4[b]
10	36.4	–	–	–	–
11	133.2	11	5.95	*dd*	10.2, 1.4
12	129.2	12	5.52	*dd*	10.2, 3.1
13	89.5	–	–	–	–
14	42.6	–	–	–	–
15	25.6	15	1.72	*m*	
			1.20	*m*	–
16	22.7	16	2.10	*m*	–
			1.40	*m*	–
17	45.2	–	–	–	–
18	60.7	18	1.62	*d*	11.7
19	38.2	19	1.78	*m*	–
20	40.3	20	0.88	*m*	–
21	30.6	21	1.58	*m*	–
22	31.4	22	1.84	*m*	–
23	27.9	23	0.88	*s*	–
24	16.1	24	0.86	*s*	–
25	19.4	25	0.94	*s*	–
26	19.1	26	1.05	*s*	–
27	16.1	27	1.17	*s*	–
28	179.1	–	–	–	–
29	17.8	29	0.99	*d*	6.0
30	17.9	30	0.92	*d*	6.2

[a] Assignments are based on DEPT, ^{1}H, ^{1}H- COSY, HMQC and HMBC experiments.
[b] From 2D *J*-resolved experiment.
[c] N.O. = not observed.

two olefinic resonances centred at δ 5.95 (1H, *dd*, J = 10.2, 1.4 Hz) and δ 5.52 (1H, *dd*, J = 10.2, 3.1 Hz) correlated with δ_C 133.2 (C-11) and δ_C 129.2 (C-12), respectively, in the HMQC spectrum. The chemical shift and the splitting pattern of these protons were similar to those reported for urs-11-en-28, 13β-olide (Katai et al., 1983; Pereda-Miranda and Delgado, 1990). These protons showed connectivities with H-9 in the COSY-45 spectrum. It may be noted that H-12 showed large coupling (J = 3.1 Hz) with allylic H-9 as this C–H bond is perpendicular to the C═C plane, whereas coupling between H-9 and H-11 is small (J = 1.4 Hz) as the dihedral angle between them is about 90° in the Drieding model of the molecule (Pavia et al., 1979).

The presence of quaternary carbon signals in the ^{13}C-NMR spectrum (broad band) at δ 89.5 (C-13) and 179.1 (C-28) along with a prominent peak at m/z 424.3357 (468-CO_2) in the HREIMS supported a lactone moiety between C-13 and C-28 (Cheung and Wong, 1972). The peak at m/z 285.1834 and m/z 233.1488 in the HREIMS confirmed these assignments. These data and a comparison of the ^{13}C-NMR chemical shifts of **2** with those of δ-lactones (Pereda-Miranda and Delgado, 1990) of the ursane series particularly of ring C, D and E confirmed that **2** possesses an ursane type skeleton with a disubstituted double bond at C-11 position and δ-lactone moiety at 28,13β position.

The molecular formula of **2** indicated that it contains two oxygen atoms more than ursolic acid lactone. The appearance of signal at m/z 201.1544 and m/z 147.1097 (201-3H_2O) and the absence of ion at m/z 207 showed that the additional oxygen atoms are present in ring A or B as hydroxyl groups. The NMR spectrum of **2** showed signals at δ 3.67 (1H, *ddd*, J = 11.2, 9.4, 4.6 Hz; δ_C = 68.6 in DEPT and HMQC) and δ 2.97 (1H, *d*, J = 9.4 Hz; δ_C = 83.9 in DEPT and HMQC) ascribable to 2β- and 3α-protons, respectively, as observed in case of **1**. These data located the two hydroxyl groups at position 2 and 3 with α and β configuration, respectively, in **2** also. The interaction between δ 3.67 and δ 2.97 in COSY-45 supported these assignments. The third hydroxyl group was placed at C-7 with an equatorial disposition since the proton at C-7 resonated as doublet of doublets centred at δ 3.79 (J = 10.0, 5.5 Hz) as observed in **1** and correlated with δ75.0 in HMQC spectrum. Its β stereochemistry was also confirmed by interactions of H-7 with H-5, H-9 and Me-27 in the NOESY plot. In the light of above data the structure of **2** has been established as 2α,3β,7β-trihydroxyurs-11-en-28,13β-olide. Formation of **2** may be envisaged through removal of the methoxyl group due to its protonation by traces of acid in deuterated chloroform, migration of double bond from C-12 to C-11 and lactonization at C-13. Such transformations have earlier been proposed in the case of isolation of triterpenoids with 11-en-28,13β-olide unit under different conditions (Cheung and Tokes, 1968; Ikuta et al., 1995).



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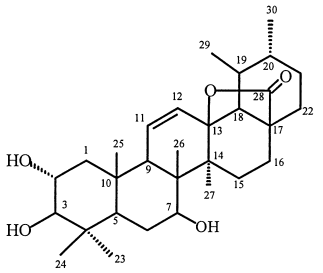

2

3. Experimental

3.1. General

Melting point was determined using a Gallenkamp melting point apparatus and is uncorrected. IR and UV spectra were recorded on JASCO A-302 and HITACHI-U-3200 specrtrophotometers, respectively. Mass spectra were recorded on a Finnigan MAT 312 double focussing 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. The ^{13}C-NMR spectral assignments have been made partly through a comparison of the chemical shifts with the published data for similar compounds (Wang and Fujimoto, 1993; Menezes et al., 1998; Anaya et al., 1989; Katai et al., 1983) and partly through an analysis of DEPT, HMQC and HMBC spectra.

3.2. Plant material

The leaves of the plant were collected from the Kar-

achi 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.

3.3. Extraction and isolation

Fresh and uncrushed leaves (20 kg) of *E. camaldulensis* var. *obtusa* were repeatedly extracted with EtOH at room temperature. The concentrated syrupy residue, obtained on removal of the solvent from the ethanolic extract in vacuo, was partitioned between EtOAc and water. The former layer was then dried (Na_2SO_4), treated with charcoal, filtered, and washed with EtOAc. The charcoal bed was further washed with MeOH–benzene (1:1). The residue left on removal of the solvent from the EtOAc filtrate and washings was divided into petrol-soluble and petrol-insoluble fractions. The petrol-insoluble fraction (90 g) was subjected to VLC (silica gel GF_{254}, $CHCl_3$, $CHCl_3$-MeOH, in order of increasing polarity). Various fractions were obtained on combining the eluates on the basis of TLC. The major fraction (17 g) eluted with $CHCl_3$–MeOH (9.9:0.1) was rechromatographed (VLC, petrol, petrol–EtOAc, in order of increasing polarity). The eluates were combined on the basis of TLC, furnishing total 10 fractions (F-1 to F-10). Fraction F-7 (6.1 g) obtained on combining the eluates with petrol–EtOAc (8:2) was again subjected to VLC (silica gel GF_{254}, petrol, petrol–EtOAc followed by $CHCl_3$, $CHCl_3$-MeOH in order of increasing polarity). Various fractions were obtained on combining the eluates on the basis of TLC. Fraction 2 eluated with petrol–EtOAc, 9:1 showed four spots on TLC which were separated through thick layer chromatography (silica gel GF_{254}, $CHCl_3$–MeOH, 9.5:0.5). As a result pure ursolic acid lactone (8 mg), betulinic acid (3.5 mg), oleanolic acid (6.8 mg) and ursolic acid (2 mg) were obtained in order of polarity. The fraction 3 (petrol–EtOAc, 8.5:1.5 eluate) of fraction F-7 was pure ursolic acid (300 mg) while fraction 4 (petrol–EtOAc, 8:2 eluate) was subjected to flash column chromatography (silica gel E. Merck, 9385; petrol–EtOAc in order of increasing polarity). The petrol–EtOAc 8.5:1.5 eluate afforded **1** (16.6 mg) which transformed into **2** in $CDCl_3$ at room temperature.

3.4. 2α,3β,7β-Trihydroxy-11α-methoxyurs-12-en-28-oic acid (*1*)

IR ν_{max} (KBr) cm^{-1}: 3460, 3420–2650, 1710, 1630, 1150; UV λ_{max} (MeOH) nm: 202; ^{1}H-NMR ($CDCl_3$): δ 5.49 (1H, *d*, *J* = 3.5Hz, H-12), 3.78 (1H, *dd*, *J* = 10.0, 5.6 Hz, H-7α), 3.72 (1H, *dd*, *J* = 8.5, 3.5 Hz, H-11β), 3.67 (1H, *ddd*, *J* = 11.2, 9.5, 4.6 Hz, H-2β), 3.26 (3H, *s*, OCH_3), 2.98 (1H, *d*, *J* = 9.5 Hz, H-3α), 2.22 (1H, *d*, *J* = 11.5 Hz, H-18), 1.62 (1H, *d*, *J* = 8.5 Hz, H-9), 1.15 (3H, *s*, Me), 1.00 (3H, *s*, Me), 0.98 (3H, *s*, Me), 0.93 (3H, *d*, *J* = 7.0 Hz, H-29/H-30), 0.88 (3H, s, Me), 0.78 (3H, *d*, *J* = 7.0 Hz, H-29/H-30), 0.77 (3H, *s*, Me); HREIMS *m/z* (rel. int.%): 518.3602 [$C_{31}H_{50}O_6$, M^+; calcd. for $C_{31}H_{50}O_6$, 518.3607] (2), 486.3343 [$C_{30}H_{46}O_5$, M^+–MeOH]$^+$ (38), 468.3210 [$C_{30}H_{44}O_4$, 486-H_2O]$^+$ (20), 246.1618 [$C_{16}H_{22}O_2$]$^+$ (12), 240.1721 [$C_{14}H_{24}O_3$]$^+$ (12), 202.1719 [$C_{15}H_{22}$]$^+$ (10), 186.1406 [$C_{14}H_{18}$]$^+$ (15), 119.0836 [C_9H_{11}]$^+$ (50).

3.5. 2α,3β,7β-Trihydroxyurs-11-en-28,13β-olide (*2*)

Fine needles ($CHCl_3$/MeOH), mp. 296–298°C; IR ν_{max} (KBr) cm^{-1}: 3440, 1755, 1635; UV λ_{max} (MeOH) nm: 205; ^{1}H and ^{13}C-NMR ($CDCl_3$): Table 1; HREIMS *m/z* (rel.int. %): 468.3199 [$C_{30}H_{44}O_4$, M^+–H_2O]$^+$ (15), 424.3357 [$C_{29}H_{44}O_2$, 468-CO_2]$^+$ (100), 285.1834 [$C_{19}H_{25}O_2$]$^+$ (12), 233.1488 [$C_{15}H_{21}O_2$]$^+$ (12), 217.1539 [$C_{15}H_{21}O$]$^+$ (11), 201.1544 [$C_{11}H_{21}O_3$]$^+$ (30), 147.1097 [$C_{11}H_{15}$]$^+$ (15), 119.0830 [C_9H_{11}]$^+$ (60).

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