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Macrocyclic diterpenes from *Euphorbia nivulia*☆

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Abstract

The latex of *Euphorbia nivulia* afforded two ingol diterpenes 3,12-diacetyl-8-benzoylingol (**4**) and 3,12-diacetyl-7-benzoyl-8-nicotinylingol (**5**) along with three known ingol diterpenes **1**, **2**,and **3**, and two known triterpenes cycloart-25-en-3β-ol and cyclonivulinol. Their structures have been assigned on the basis of their structural data as well as their acetylated products. The diterpenes **1**–**5** were tested for the LPS induced PGE_2 inhibition activity.

Keywords: *Euphorbia nivulia*; Euphorbiaceae; Ingol diterpenes; 3,12-Diacetyl-8-benzoylingol; 3,12-Diacetyl-7-benzoyl-8-nicotinylingol; In vitro PGE_2 assay

1. Introduction

The genus *Euphorbia* is the largest in the spurge family with over 1000 species and is subdivided into many subgenera and sections, a number of which have been treated as distinct genera (Morgenstern et al., 1996). Modern studies have highlighted the wide spread use of several of these plants to treat cancerous conditions in the traditional medicine of many areas of the world (Hecker, 1971; Uemura et al., 1975; Appendino et al., 1998; Alberto et al., 1999). The plant *Euphorbia nivulia* (Euphorbiaceae) is a succulent Euphorbiaceae found in tropics. Several triterpenes have previously been reported from this plant (Rao et al., 1986; Satyanarayana et al., 1991, 1992). The leaves and the latex of this plant are used in the Ayurvedic system of medicine for bronchitis and rheumatism (Chopra et al., 1956).

Ingols are derivatives of the macrocyclic lathyrane diterpenes. The naturally occurring esters of ingol lack the irritant and toxic properties of the daphnanes or tiglianes to which they are biogenetically related (Fatope et al., 1996). All these diterpenes have been frequently reported from the plants of the families' Euphorbiaceae and Thymelaeaceae and claim intrinsic interest because of their diverse biological activities (Khan and Malik, 1990). However, ingols have attracted considerable interest as antineoplastic agents (Sahai et al., 1981; Fatope et al., 1996). In our search for new bioactive molecules from natural sources (Reddy et al., 2001), we have investigated the latex of the plant *Euphorbia nivulia*. Since this plant is known to possess the therapeutic properties against diseases like bronchitis and rheumatism (Chopra et al., 1956), the isolated compounds were tested for their activity against LPS induced PGE_2 inhibition using an in vitro enzyme immunoassay method. This paper describes the isolation and structural elucidation of the two new ingol esters and the biological activity for all the diterpenes.

2. Results and discussion

In the context of our search for new chemical entities, we further isolated five components structurally related to ingol skeleton from the latex of *E. nivulia* along with two known triterpenes cycloart-25-en-3β-ol and cyclonivulinol. The triterpene structures were confirmed from the reported data (Rao et al., 1986; Satyanarayana et al., 1992). Compounds **1**, **2** and **3** exhibited spectral properties (UV, IR, ^{1}H NMR, ^{13}C, DEPT, COSY, NOESY, HMQC, EIMS and HRMS) that suggested

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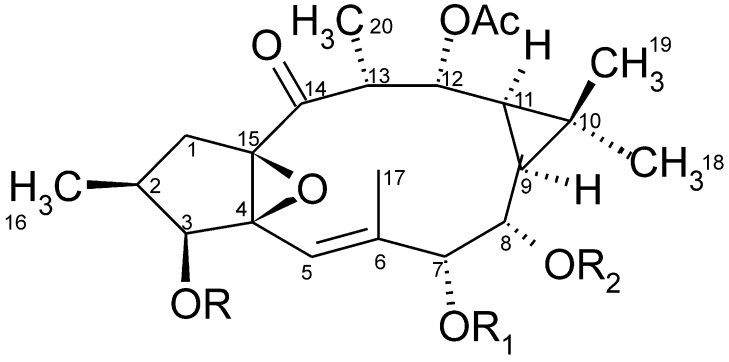
they were the esters of the macrocyclic diterpene ingol corresponding to those isolated in previous research from other species of the genus (Lin and Kinghorn, 1983; Connolly et al., 1984; Opferkuch and Hecker, 1973). However, compounds **4** and **5** are new natural products. The structures of these compounds were established through 1D and 2D (COSY, NOESY, HMQC, and HMBC) NMR measurements. All these compounds were identified as ingol esters bearing various acyl groups such as acetyl, benzoyl, nicotinyl and angeloyl moieties. The configurations at the stereogenic centers were deduced from coupling constant values, NOE measurements and comparison with literature data. In all cases the values of $J_{2,3}$ is approximately equal to 8.4 Hz and chemical shifts of 1α and 1β indicated that the configuration at C-2 and C-3 are as in true ingol derivatives (Marco et al., 1997, 1998).

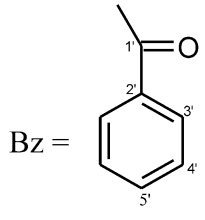
Compounds **1**, **2** and **3** identified as macrocyclic diterpene ingol esters as 3,7,12-triacetyl-8-benzoylingol (**1**) ($[\alpha]_D^{25}$ −62° (*c* 0.5, $CHCl_3$), lit. $[\alpha]_D^{25}$ −62.9° (*c* 0.08, $CHCl_3$) (Opferkuch and Hecker 1973; Lin and Kinghorn, 1983), 3,12-diacetyl-7-angeloyl-8-methoxyingol (**2**) ($[\alpha]_D^{25}$ −8° (*c* 0.5, $CHCl_3$), lit. $[\alpha]_D^{25}$ −6.5° (*c* 0.09, $CHCl_3$) (Lin and Kinghorn 1983) and compound **3**, 7-angeloyl-12-acetyl-8-methoxyingol, $[\alpha]_D^{25}$ +8° (*c* 0.09, $CHCl_3$) was earlier reported as a hydrolyzed product of its corresponding triacetate (Connolly et al, 1984).

Compound **4** was obtained as a white powder mp 95–97 °C and $[\alpha]_D^{25}$ −27° (*c* 0.5, $CHCl_3$). High-resolution mass 554.2501 suggested a molecular formula of $C_{31}H_{38}O_9$, which was consistent with the carbon and hydrogen numbers counted in the NMR spectra. Its ^1H NMR spectra showed the presence of the two acetate groups (δ 2.12 and 2.11) and one benzoate group (δ 8.06, 7.59, and 7.46). Further signals were observed for the presence of a trisubstituted double bond at δ 5.84, four methine protons connected to ester carbonyls at δ 5.22, 4.92, 4.77and 4.39, a vinylic methyl at δ 2.09, two tertiary methyls at δ 0.85, 1.13 and two secondary methyl groups at δ 0.92 and 1.10. The ^{13}C NMR spectrum showed the presence of 31 carbons. Their multiplicity assignments were determined by carrying out multipulse 1D DEPT experiments, showed the presence of 13 CH, 1 CH_2 and 7 CH_3 carbons.

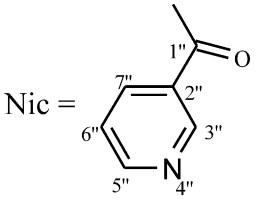
This foregoing spectral data together with the tracing of cross peaks in the $^1H-^1H$ cosy and HMQC spectra led to an ingol structure for **4**. In its ^1H NMR spectrum the proton signals at δ 5.22, 4.92, 4.77 and 4.39 are shifted down field with respect to normal hydroxy methine geminal protons except for the signal at δ 4.39 suggesting the sites for ester linkages. The ^1H NMR spectral data of compound **4** is closely related to the compound **1** with the absence of one acetylated group at 3 or 7 or 12. From the reported spectral data of compound **1** (Lin and Kinghorn, 1983), it was observed that the chemical shifts of the protons at 3, 8 and 12 are unchanged except for the signal at δ 4.39 which may be due to the presence of hydroxy group at C-7. This was further confirmed by the superimposible ^1H NMR spectral data of acetylated (Py/Ac_2O) compound **4** with that of the isolated compound **1**. Thus the structure of compound **4** is established as 3,12-diacetyl-8-benzoylingol (**4**).

Compound **5** was obtained as a white powder mp 90–92 °C and $[\alpha]_D^{25}$ −61° (*c* 0.5, $CHCl_3$). The high-resolution mass 659.2722 suggested a molecular formula of $C_{37}H_{41}O_{10}N$, which was consistent with the carbon and hydrogen numbers counted in the NMR spectra. Its ^1H





1 R=R_1=Ac; R_2=Bz
2 R=Ac; R_1=Ang; R_2=Me
3 R=H; R_1=Ang; R_2=Me;
4 R=Ac; R_1=H; R_2=Bz
5 R=Ac; R_1=Bz; R_2=Nic



NMR spectrum showed the presence of two acetate groups (δ 2.25 and 2.11), one benzoate group (δ 8.06, 7.59 and 7.49) and one nicotinate group (δ 9.14, 8.77, 8.25 and 7.38). Further signals were observed for the presence of a trisubstituted double bond proton at δ 5.78, three methine protons at δ 5.21, 4.98 and 4.96 connected to ester carbonyls, two tertiary methyls at δ 0.87 and 1.17, a vinylic methyl at δ 2.06 and two secondary methyls at δ 0.93 and 1.12. The ^{13}C NMR spectrum showed the presence of 37 carbon atoms, which includes benzoate and nicotinate groups. Their multiplicity assignments were determined by carrying out multipulse 1D DEPT experiments showing the presence of 19 CH, 1 CH_2 and 7 CH_3 carbons.

The ^1H NMR spectrum of compound **5** (Table 1) clearly indicated that the compound **5** is an ingol tetraester bearing a benzoate and a nicotinate residue along with two acetates. The foregoing spectral data is closely related to 3,12-diacetyl-8-nicotinyl-7-phenylacetyl-19-acetoxyingol (Fatope et al., 1996) except for the devoid of the signals due to the 19-methyl oxygenation. Chemical shift considerations further indicated that the ester moieties are located at the hydroxyl groups connected to C-3, C-7, C-8 and C-12 positions. The nicotinate and benzoate groups were found to be attached to C-8 and C-7 respectively by HMBC correlations. In the HMBC spectrum, the aromatic protons (3′) of the benzoate group at δ 8.06 showed correlations with carbonyl carbon at δ 165.16 and the methine proton at δ 5.58 (H-7) showed correlations with C-6 (139.24), C-8 (73.55), C-5 (117.62), C-17 (17.60) and three bond connectivity to benzoate carbonyl at δ 165.16. Further the nicotinate aromatic protons at δ 9.14 (3″) and 8.25 (7″) showed correlations with carbonyl carbon at δ 164.05 and the methine proton at δ 4.96 (H-8) showed correlations with C-9 (31.07), C-11 (25.10), C-7 (77.00) and nicotinate carbonyl group at 164.05. Consequently the remaining two acetates could only be attached to oxymethine carbons C-3 and C-12. Thus the structure of compound **5** was established as 3,12-diacetyl-7-benzoyl-8-nicotinylingol.

Table 1
^1H NMR and ^{13}C NMR data for the ingol diterpenes **4** and **5**

	4		**5**		
H	^1H NMR	^{13}C	^1H NMR	^{13}C	HMBC[a]
1a	1.69 *d* (14.9)	31.65	1.71 *d* (15.1)[b]	31.59	C3,C4, C15, C2, C16
1b	2.83 *dd* (15.1, 9.2)		2.81 *dd* (15.1, 9.2)		C15, C2, C16
2	2.59 *m*	29.66	2.53 *m*	29.55	C4,C3, C15, C16, C1
3	5.22 *d* (8.4)	77.59	5.21 *d* (8.4)	76.78	C2, C16
4	–	73.68		73.24	
5	5.84 *s*	116.59	5.78 *s*	117.62	C6,C4,C3,C7,C8, C17
6	–	141.38		139.24	
7	4.39 *s*	76.14	5.58 *s*	77.00	C6, C5, C8, C17, C1′
8	4.77 *dd* (10.8, 1.3)	70.71	4.96 *d* (1.9)	73.35	C9, C11, C7, C1″
9	1.55 *dd* 10.5, 9.4)	23.55	1.55 *t* (10.8, 9.4)	31.07	C8,C12,C10,C18, C19
10	–	19.15		19.63	
11	1.19 *d* (9.2)	30.92	1.29 *dd* (9.2, 4.6)	25.10	C12, C10, C18, C19
12	4.92 *dd* (11.1, 4.1)	74.94	4.99 *d* (1.9)	70.46	C11, C14, C9
13	2.96 *m*	43.23	3.05 *m*	43.26	C14, C20
14	–	207.46		207.41	
15	–	71.36		71.27	
16	0.9 *d* (7.4)	16.30	0.93 *d* (3H,7.4)	16.89	C1, C2, C3, C4
17	2.09 *s*	17.37	2.06 *s* (3H)	17.60	C6, C5, C7
18	1.13 *s*	29.05	1.17 *s* (3H)	29.24	C9, C10, C11, C19
19	0.85 *s*	16.88	0.87 *s* (3H)	16.19	C18, C11, C10, C9
20	1.10 *d* (7.4)	13.22	1.12 *d* (3H, 7.5)	13.30	C12, C13, C14
OAc	2.12 *s* (3H)	170.59, 170.34	2.25 *s* (3H)	170.41, 170.36	
	2.11 *s* (3H)	20.88, 20.53	2.11 *s* (3H)	20.93, 20.44	
OBz	8.06 *d* (2H, 8.2)	165.95	8.06 *dd* (2H, 8.4, 1.3)	165.16 133.39, 130.04	C1′
	7.59 *tt* (1H, 8.6, 1.3)	133.17, 129.82	7.59 *dd* (1H, 7.4, 1.8)	129.54, 128.62 129.75, 128.34	
	7.46 *t* (2H, 7.8)	129.57, 128.44	7.49 *t* (2H, 7.7)		
ONic	–	–	9.14 *bs*	164.05	C1″
			8.77 *d* (3.6)	153.36, 150.58	
			8.25 *ddd* (7.9, 1.9, 1.6)	137.37, 125.84 123.51	C1″
			7.38 *dd* (8.0, 4.9)		

[a] HMBC correlations for the ingol diterpene **5**.
[b] δ in ppm and *J* (parentheses) in Hz (500 MHz, $CDCl_3$).

All the ingol diterpenes (**1**–**5**) were evaluated for their PGE_2 inhibition property using in vitro assay method employing Enzyme Immunoassay Kits (Cayman, Ann Arbor. MI, Cat. No. 514010). Among them, diterpene **3** has shown significant PGE_2 inhibition.

The IC_{50} value for compound **3** was found to be 0.003 μM compared to that of celecoxib (0.050 μM). The remaining diterpenes have shown insignificant PGE_2 inhibition even at the concentration of 0.02 μM.

3. Experimental

3.1. General

The ^1H NMR and ^{13}C NMR spectra were recorded on a Varian unity INOVA 500 MHz spectrometer with standard pulse sequences, operating at 500 and 125 MHz, respectively and all 1D and 2D spectra (^1H–^1H COSY, NOED, HMQC, and HMBC) were recorded using tetramethylsilane (TMS) as an internal standard. The ^1H and ^{13} C NMR chemical shifts refer to $CDCl_3$ at 7.26 ppm and $CDCl_3$ at 77.0 ppm, respectively. Chemical shifts are reported in parts per million, and coupling constants (*J*) are expressed in Hz. Silica gel (60–100 mesh, ACME) was used for open column chromatography. UV and IR spectra were recorded on Shimadzu 240 and Perkin-Elmer RXI FT–IR spectro photometers, respectively. Mass spectra were recorded on a Finnigan-MAT 1020 instrument. Optical rotations were measured on a JASCO DIP-370 polarimeter.

3.2. Plant material

The plant material of *Euphorbia nivulia* (Euphorbiaceae) was collected from Bhadrachalam forest, Andhra Pradesh, India in January 2000 and identified by Professor M. Prabhakar Rao, Department of Botany, Osmania University, Hyderabad, India. The voucher specimen (No. 001662) is kept in the herbarium of the Department of Botany, Osmania University.

3.3. Extraction and isolation

The freezed latex of the plant *Euphorbia nivulia* was dried under vacuum at 35 °C for 24 h to get a white powder (750 g) and extracted with methanol (3×1.5 l) at room temperature. The methanol extract was filtered and evaporated under reduced pressure to yield a brown gummy residue (18 g), which was subjected to silica gel chromatography eluting with hexane through hexane-ethyl acetate mixtures to ethyl acetate. According to differences in composition indicated by TLC, three crude fractions were obtained. Fraction A contained triterpenoids cycloart-25-en-3β-ol (1.2 g) and cyclonivulinol (300 mg). Fraction B was further separated by column chromatography (hexane–acetone, 85:15) to afford compounds **1** (100 mg), **2** (2.4 g) and **3** (30 mg). Fraction C was further separated by column chromatography (hexane–acetone, 80:20) to afford compounds **4** (150 mg) and **5** (40 mg).

3.4. 3,12-Diacetyl-8-benzoylingol (*4*)

White powder, mp 95–97 °C; $[\alpha]_D^{25}$ −27° (*c* 0.5, $CHCl_3$); IR (KBr) ν_{max} 3514, 2937, 1737, 1603, 1452, 1375, 1241, 1174, 1110, 1071, 1022 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 1; MS *m*/*z* 554.2501 $[M]^+$ (calc. for $C_{31}H_{38}O_9$, 554.2515), 494 $[M–AcOH]^+$, 434 $[M–AcOH]^+$, 312 $[M–PhCOOH]^+$, 294 $[M–H_2O]^+$, 105 $[PhCO]^+$.

3.5. 3,12-Diacetyl-7-benzoyl-8-nicotinylingol (5)

White powder, mp 90–92 °C; $[\alpha]_D^{25}$ −61° (*c* 0.5, $CHCl_3$); IR (KBr)ν_{max} ; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 1; MS *m*/*z* 659.2722 $[M]^+$ (calc. for $C_{37}H_{41}O_{10}N$, 659.2730), 599 $[M–AcOH]^+$, 539 $[M–AcOH]^+$, 417 $[M–PhCOOH]^+$, 105 $[PhCO]^+$.

3.6. In vitro PGE_2 assay

Fresh blood was collected in heparinized tubes by venipuncture from healthy human volunteers with written consent. The subjects had no apparent inflammatory conditions and had not taken any NSAID for at least 7 days prior to blood collection. Five hundred microliter aliquots of blood were incubated with either 2 μl of vehicle (DMSO) or 2 μl of test compounds at final concentrations ranging from 0.001 to 0.02 μM for 15 min at 37 °C. For comparison, Celecoxib, a known PGE_2 inhibitor, was used at a final concentration of 50 nM (IC_{50} value). This was followed by incubation of blood with 10 μl of lipopolysaccharide (LPS) (Sigma, St Louis, from *E. coli*, serotype 0127:B8) 100 μg/ml final concentration, diluted in phosphate buffered saline (PBS) for 24 h at 37 °C. Appropriate controls (no LPS) were used as blanks. At the end of the incubation, the blood was centrifuged at 12 000×*g* for 5 min to obtain plasma. The plasma was appropriately diluted with EIA buffer and the pH was adjusted to 4.0 using dilute HCl. The plasma samples were assayed for PGE_2 content using an Enzyme Immuno Assay kit (Cayman, Ann Arbor, USA, Cat. No. 514010) according to the manufacturer's instructions.

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