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Isolation and structure determination of new 4,12-dideoxyphorbol esters from *Euphorbia pannonica* Host.

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ABSTRACT

Phytochemical study of the aerial parts of *Euphorbia pannonica* led to the isolation of two new tigliane diterpenes, 4,12-dideoxy(4β)phorbol-20-benzoate-13-isobutyrate (**1**) and 4,12-dideoxy(4β)phorbol-20-benzoate-13-isovalerianate (**2**). The structures and relative stereochemistry were elucidated on the basis of extensive spectroscopic analyses, including 1D and 2D NMR experiments (^{1}H NMR, JMOD, COSY, NOESY, HSQC and HMBC).

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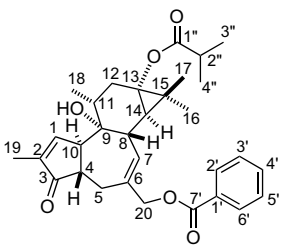
1. Introduction

Ingenane, tigliane and daphnane diterpenes, referred to in common as phorboids, are typical constituents of the families Euphorbiaceae and Thymelaeaceae. Earlier studies on these compounds focused mainly on their extremely skin-irritant and tumour-promoting activities, which are results of their protein kinase C-activating potential.[1] Further investigations have disclosed numerous other wider pharmacological activities, e.g., cytotoxic, antileukaemic, antiviral, sedative and analgesic effects.[2–6] Among the phorboids, ingenol 3-angelate (PEP005) and prostratin are of special interest, since the antitumour agent ingenol 3-angelate, isolated from *Euphorbia peplus*, is currently being investigated in phase II clinical trials for the treatment of basal cell carcinoma and actinic keratosis.[7] Prostratin, a 12-deoxyphorbol derivative from the Samoan medicinal plant *Homalanthus nutans*, and other phorbol 13-monoesters are promising agents for HIV-1 therapy, targeting HIV viral reservoirs.[8,9]

As part of our ongoing search for biologically active compounds from Hungarian Euphorbiaceae, *Euphorbia pannonica* Host. (syn. *Euphorbia nicaeensis* All.) was investigated. *E. pannonica* is a perennial, herbaceous plant with a circum-Mediterranean and Central European distribution. This species grows in dry and sunny places at altitudes between 100 and 1800 m, in degraded stages of Mediterranean *Quercus* forests.[10] Phytochemical investigations of this plant have not been reported previously.

The present paper reports the isolation of two new tigliane-type diterpenes, 4,12-dideoxy(4β)phorbol-20-benzoate-13-isobutyrate (**1**) and 4,12-dideoxy(4β)phorbol-20-benzoate-13-isovalerianate (**2**), from the aerial parts of *E. pannonica*.



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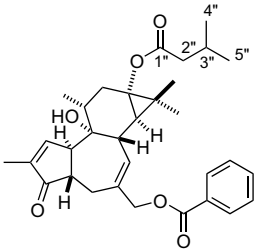

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2. Results and discussion

The aerial parts of *E. pannonica* were extracted with MeOH at room temperature and, after concentration, the extract was partitioned between CH_2Cl_2 and H_2O. The CH_2Cl_2 phase was subjected to a multistep chromatographic separation and purification procedure, including CC, VLC, CPC, preparative TLC and HPLC, to yield pure compounds **1** and **2**. These were elucidated by extensive mass spectral and NMR studies to be 4,12-dideoxyphorbol-20-benzoate-

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13-isobutyrate (**1**) and 4,12-dideoxyphorbol-20-benzoate-13-isovalerianate (**2**).

Compound **1** was isolated as a colourless oil with $[\alpha]_D^{26}$ −20 ($CHCl_3$, *c* 0.04). Its ESIMS spectrum displayed quasi-molecular peaks at *m*/*z* 529 $[M+Na]^+$ and 507 $[M+H]^+$, indicating a molecular mass of 506 Da. The HREIMS spectrum revealed a fragment ion at *m*/*z* 384.2304 $[M+H-C_6H_5COOH]^+$ (calcd for $C_{24}H_{32}O_4$, 384.2301), indicating the loss of a benzoic acid moiety from the molecule, which accordingly has the composition $C_{31}H_{38}O_6$. The ^1H NMR and JMOD spectra exhibited typical signals for one benzoyl group (δ_H 8.02 dd, 7.55 dt and 7.43 t; δ_C 166.2, 130.1, 129.7, 128.3 and 133.0) and one isobutanoyl group (δ_H 2.55 sept, 1.17 d and 1.16 d; δ_C 179.0, 34.3, 18.6 and 18.7). A diterpene skeleton containing 20 carbon atoms (4 methyl, 3 methylene, 7 methine groups and 6 quaternary carbons) (Table 1) was identified via ^1H NMR, JMOD and HSQC spectra. The carbon signals at δ_C 160.2, 136.8, 136.6 and 131.6, together with the proton signals at δ_H 7.57 and 5.66, revealed the presence of two trisubstituted olefin groups (C-1, C-2 and C-6, C-7). One keto group (C-3) was apparent from the signal at δ_C 210.0, and one isolated *O*-substituted methylene (C-20) from the signals at δ_H 4.72 s (2H) and δ_C 69.8. A broad singlet at δ_H 5.5, which showed no correlation in the HSQC spectrum to any carbon, was assigned to a hydroxy group (9-OH). The gradient COSY spectrum indicated three spin systems of correlated protons: =CH–CH–CH–CH_2– (A) (δ_H 7.57, 3.32, 2.46, 2.93 and 2.22), =CH–CH–CH– (B) (δ_H 5.66, 2.16 and 0.83) and –CH(CH_3)–CH_2– (C) (δ_H 1.43, 0.92 and 2.13/1.55). These structural parts, tertiary methyls and quaternary carbons were connected by inspection of the long-range C–H correlations observed in the HMBC spectrum. The two and three-bond correlations of the keto group (C-3) with the H-1, H-5 and H-19 signals, and of the quaternary C-2 with the H-1 and H-19 signals revealed that structural fragment A is the C-1–C-10–C-4–C-5 part of the molecule, i.e., compound **1** must be a 4-deoxy derivative. HMBC cross-peaks between C-6 and H-5, C-6 and H-20, C-7 and H-20 and C-9 and H-8 proved the presence of a 6,7-unsaturated seven-membered ring B bearing one O-functionality on C-9 (δ_C 75.2) and one –CH_2OR group ($\delta_{C\text{-}20}$ 69.8). The structure was further elucidated with the aid of the HMBC correlations observed between C-9 and H-12 and H-18; C-13 and H-12, H-14, H-16 and H-17; and C-15 and H-14, H-16 and H-17, indicating that structural element C corresponds to the C-12–C-11–C-18 part of the molecule and the whole structure is based on 4,12-dideoxyphorbol skeleton. The chemical shifts δ_C 62.8 and 69.8 pointed to O-substitutions (two ester groups) on C-13 and C-20. The attachment of the benzoyl group at C-20 was evident from the HMBC correlation of the carbonyl signal at δ_C 166.2 (benzoyl CO) with the proton signal at δ_H 4.72 (H-20). The positions of the isobutanoyl and hydroxy groups were concluded on the basis of biogenetic considerations and literature data on structurally related compounds.[1,11,12] All tigliane diterpenoids discovered in nature so far contain a free 9-OH group, suggesting the location of the isobutanoyl group on C-13. Accordingly, the chemical shifts of C-9 (δ_C 75.2) and C-13 (δ_C 62.8) of compound **1** are in the ranges 75.5–77.1 and 62.7–63.6 ppm, respectively, characteristic of 9-hydroxy-13-acyl-substituted phorbol esters. Therefore, a 9-hydroxy-13-isobutanoyl structure was deduced for compound **1**.

The relative configurations of the stereogenic centres of **1** were assigned on the basis of a NOESY experiment (Fig. 1). In the NOESY spectrum, correlations were detected between H-10 and H-5α, and between H-4 and H-5β, from which a trans A/B ring junction was deduced. The nuclear *Overhauser* effects between H-5β and H-8, H-8 and H-11, H-11 and H-12β, H-12β and 17-CH_3 and H-8 and 17-CH_3 indicated the β position of all these protons and the methyl group. On the other hand, the NOE interactions between 16-CH_3 and H-14,

Table 1
NMR spectral data for **1** and **2** [$CDCl_3$, 600 MHz (^1H), 150 MHz (^{13}C), δ ppm (*J*=Hz)]

Atom	**1**				**2**	
	^1H	^{13}C	COSY	HMBC (H to C)	^1H	^{13}C
1	7.57 m	160.2	10, 19	2, 3	7.56 m	160.1
2	—	136.8[a]	—	—	—	136.8[a]
3	—	210.0	—	—	—	209.8
4	2.46 dt (4.8, 10.4)	44.1	5α, 5β, 10		2.45 dt (10.4, 4.8)	44.2
5β	2.93 dd (18.6, 4.8)	30.4	20, 5α, 4	4	2.94 dd (18.2, 4.8)	29.7
5α	2.22 dd (18.6, 10.4)		5β, 4	3, 4, 6	2.25 m	
6	—	136.6[a]	—	—	—	136.6[a]
7	5.66 dd (5.4, 2.4)	131.6	20		5.65 br s	131.6
8	2.16 m	42.3	7, 14	9, 11, 13	2.17 m	42.3
9	—	75.2	—	—	—	75.3
10	3.32 m	53.8	1, 4, 19		3.31 br s	53.9
11	1.43 ddq (10.2, 7.2, 6.6)	35.5	18, 12α, 12β		1.43 m	35.5
12β	2.13 dd (15.3, 7.2)	31.8	11, 12α	9, 11, 13	2.15 m	31.9
12α	1.55 dd (15.3, 10.2)		11, 12β	11, 13	1.57 m	
13	—	62.8	—		—	63.0
14	0.83 d (5.4)	31.8	8	7, 13, 15, 17	0.83 d (5.1)	31.8
15	—	22.8	—		—	22.7
16	1.20 s (3H)	23.1	—	13, 14, 15, 17	1.33 s (3H)	23.1
17	1.04 s (3H)	15.3	—	13, 15, 16	1.04 s (3H)	15.2
18	0.92 d (6.6) (3H)	19.2	11,	9, 11	0.93 d (6.2) (3H)	19.2
19	1.75 dd (2.7, 1.5) (3H)	10.2	1, 10	1, 2, 3	1.75 s (3H)	10.2
20	4.72 s (2H)	69.8	7, 5β	5, 6, 7, 7′	4.72 s (2H)	69.8
1′	—	130.1	—	—	—	130.1
2′/6′	8.02 m	129.7	3′, 5′	7′	8.02 m	129.7
3′/5′	7.43 m	128.3	2′, 6′, 4′		7.43 m	128.3
4′	7.55 m	133.0	3′, 5′		7.56 m	133.0
7′	—	166.2	—	—		166.3
1″	—	179.0		—	—	175.2
2″	2.55 sept (6.6)	34.3	3″, 4″	1″, 3″, 4″	2.19 m	43.5
3′	1.17 d (6.6) (3H)	18.6	2″	1″, 2″	2.17 m	25.7
4″	1.16 d (6.6) (3H)	18.7	2″	1″, 2″	0.96 d (6.4) (3H)	22.4
5″	—	—	—	—	0.96 d (6.4) (3H)	22.4
OH-9	5.5 br s	—	—	—	5.46 br s	—

[a] Assignments may be interchanged.

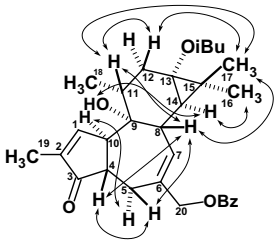

Figure 1. Diagnostic NOESY correlations for compound **1**.

and H-14 and 9-OH were consistent with the α arrangement of H-14 and 16-CH_3. All of the above data are compatible with the structure of **1** being 4,12-dideoxy(4β)phorbol-20-benzoate-13-isobutyrate.

Compound **2**, a colourless oil, was assigned the molecular formula $C_{32}H_{40}O_6$ from the pseudomolecular ion peak at *m/z* 520.2829 $[M]^+$ (calcd for $C_{32}H_{40}O_6$, 520.2825) in the HRESIMS. The ^1H NMR and JMOD spectra of **2** revealed close similarity to those of compound **1**, the only difference being due to exchange of the isobutyrate group for an isovalerianate group [δ_H 2.19 (2H, m), 2.17 (1H, m) and 0.96 (6H, d, J=6.4 Hz); δ_C 175.2, 43.5, 25.7, 22.4 and 22.4] in **2**.[13] The proton and carbon resonances were fully assigned from COSY, HSQC and HMBC experiments as listed in Table 1. The chemical shifts and coupling constants suggested that **2** shared the same relative configuration as **1**. The HMBC correlations between the signals at δ_C 166.3 (BzCO) and δ_H 4.72 (H-20) confirmed the attachment of a benzoyl group at C-20. The chemical shifts of C-9 (δ_C 75.3) and C-13 (δ_C 63.0) indicated a hydroxy group at C-9 and an ester group at C-13, similarly as in compound **1**. The structure of compound **2** was therefore assigned as 4,12-dideoxy(4β)phorbol-20-benzoate-13-isovalerianate.

3. Conclusion

From the aerial parts of *E. pannonica*, two new diterpenes based on the tigliane skeleton were isolated: 4,12-dideoxy(4β)phorbol-20-benzoate-13-isobutyrate (**1**) and 4,12-dideoxy(4β)phorbol-20-benzoate-13-isovalerianate (**2**). To the best of our knowledge, this is the first report on the secondary metabolites of *E. pannonica*. Our investigation demonstrated a relatively low diterpene content of this species; the isolated compounds were obtained in yields of only 8.2×10^{-5}% (**1**) and 7.1×10^{-5}% (**2**).

The isolated compounds are derivatives of 4,12-dideoxyphorbol, a diterpene alcohol that is very rare in the plant kingdom. Although phorbol esters and their 4- or 12-deoxy derivatives are characteristic metabolites of the Euphorbiaceae family and frequently occur in the species of Crotonoideae and Euphorbioideae subfamilies, other oxygenation patterns, such as 16-hydroxy, 12-deoxy-16-hydroxy, 12-deoxy-5-hydroxy, 12,20-dideoxy and 6,7-epoxy-5-hydroxy, have limited distribution.[1] 4,12-Dideoxyphorbol and three of its esters have been isolated previously only from *Excoecaria bicolor* and *Euphorbia guyoniana* (Euphorbiaceae).[12,14] Furthermore, compound **2** is substituted with an isovalerianate group, which was found for the first time in this group of tigliane diterpenes.

Phorbol esters occur naturally as 13-monoesters, 12,13- or 13,16- or 12,20-diesters and 12,13,20- or 13,16,20-triesters esterified with acetic, isobutyric, tiglic, 2-methylbutyric, benzoic, 2-methylaminobenzoic or saturated and unsaturated long-chain aliphatic fatty acids.[5] Structure–activity relationships, including the roles of the diterpene core, hydrophilic functionalities and ester groups, and the mechanisms of action of phorbol derivatives, have been studied extensively as concerns their skin-irritant, tumour-promoting and HIV-1-inhibitory activities.[9,15,16] However, the pharmacological potential of 4,12-dideoxyphorbol esters has not been investigated to date.

4. Experimental section

4.1. General procedures

Column chromatography (CC) was carried out on polyamide (ICN); vacuum liquid chromatography (VLC) on silica gel G (15 μm, Merck); preparative thin layer chromatography (preparative TLC) on silica gel 60 F_{254} and RP-18 F_{254} plates (Merck); centrifugal planar chromatography (CPC) on silica gel 60 GF_{254} with a Chromatotron instrument (Harrison Research); and HPLC on LiChrospher RP-18 (5 μm, 250×4 mm, Merck) column with a Waters instrument. NMR spectra were recorded on a Bruker Ultrashield Plus 600 spectrometer at 600 MHz (^1H) and 150 MHz (^{13}C), with TMS as internal standard. Two-dimensional data were acquired and processed with standard Bruker software. In the COSY, HSQC and HMBC experiments, gradient-enhanced versions were used. ESIMS was performed on an Applied Biosystems 3200 QTrap instrument in ion trap mode. The sample was injected into an acetonitrile flow at a flow rate of 200 μl/min. HREIMS measurements were made on a VG-ZAB-SEQ sector instrument equipped with an electron impact ion source. The resolution was 10,000 and perfluorokerosene was used as a reference compound, with measurements in peak matching mode.

4.2. Plant material

Aerial parts of *E. pannonica* were collected in the flowering period from wild stock near Kiscsala-Császártöltés, Hungary, in June 2005. The plant material was identified by Dr. Tamás Rédei (Institute of Ecology and Botany of the Hungarian Academy of Sciences, Vácrátót, Hungary). A voucher specimen (No. 763) has been deposited at the Herbarium of the Department of Pharmacognosy, University of Szeged, Szeged, Hungary.

4.3. Extraction and isolation

The frozen and crushed plant material (5100 g) was percolated with 30 l MeOH at room temperature. The crude extract was concentrated in vacuo and exhaustively extracted with CH_2Cl_2 (5×1 l). The organic phase (56.6 g) was chromatographed on a polyamide column (113 g) with mixtures of MeOH and H_2O (2:3, 3:2 and 4:1, each 4 l) as eluents. The fraction (3.2 g) obtained with MeOH–H_2O (3:2) was subjected to silica gel VLC using a gradient system of cyclohexane–$CHCl_3$–Me_2CO (3:2:0, 1:1:0, 2:3:0, 3:7:0, 1:4:0, 10:40:1, 4:16:1 and 1:4:1). Combined fractions 17–33 (0.25 g) were then separated by CPC, using a gradient system of *n*-hexane–EtOAc mixtures (19:1, 9:1, 85:15, 4:1, 3:1 and 7:3). The fractions containing diterpenes (frs. I and II) were separated on CPC, using cyclohexane–EtOAc gradient system (100:0, 49:1, 19:1, 93:7, 9:1 and 17:3). Subfractions 74–76 of fr. I were further purified by preparative TLC on silica gel with cyclohexane–EtOAc (4:1). Final purification was carried out by reverse-phase preparative TLC with a solvent system of MeCN–H_2O (4:1) to afford 4.2 mg of **1**. Subfractions 29–37 from the CPC separation of fr. II were subjected to preparative TLC with *n*-hexane–$CHCl_3$–Me_2CO (50:50:3), and finally purified by RP-HPLC, using MeCN–H_2O (13:7) as mobile phase (flow=1.2 ml/min; t_R=8.3 min) to yield 3.6 mg of **2**.

*4.3.1. 4,12-Dideoxy(4β)phorbol-20-benzoate-13-isobutyrate (**1**)*

Colourless oil; $[\alpha]_D^{26}$ −20 ($CHCl_3$, *c* 0.04); for ^1H NMR and ^{13}C NMR spectroscopic data, see Table 1; ESIMS *m/z* 529 $[M+Na]^+$, 507 $[M+H]^+$, 385 $[M+H-C_6H_5COOH]^+$, 297 $[M+H-C_3H_7COOH]^+$; HREIMS: *m/z* 384.2304 $[M-C_3H_7COOH]^+$ (calcd for $C_{24}H_{32}O_4$, 384.2301).

*4.3.2. 4,12-Dideoxy(4β)phorbol-20-benzoate-13-isovalerianate (**2**)*

Colourless oil; for ^{1}H NMR and ^{13}C NMR spectroscopic data, see Table 1; HREIMS: m/z 520.2829 $[M]^{+}$ (calcd for $C_{32}H_{40}O_6$, 520.2825).

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