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Euphosalicin, a new diterpene polyester with multidrug resistance reversing activity from *Euphorbia salicifolia*

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Abstract—A novel diterpene polyester, named euphosalicin (**1**), the first representative of a new class of bicyclic diterpenes, was isolated from the dichloromethane extract of the whole plant of *Euphorbia salicifolia*, together with one known (**2**) and two new jatrophane diterpenes (**3**, **4**). The new carbon skeleton is formally derived from the jatrophane framework by incorporation of a geminal methyl group in the ring system. Compound **1** was found to be more active than verapamil in reversing multidrug resistance in mouse lymphoma cells. © 2000 Elsevier Science Ltd. All rights reserved.

1. Introduction

The Euphorbiaceae (spurge) family is known to produce various highly functionalized, structurally unique macrocyclic diterpenes. In recent years, considerable attention has been paid to the jatrophane, lathyrane, cembrane and casbane diterpenoids because of their structural complexity, biogenetic relevance and marked biological activity, e.g. antitumour, cytotoxic, antibacterial, platelet aggregation inhibiting and vasoconstrictor effects.[1–6]

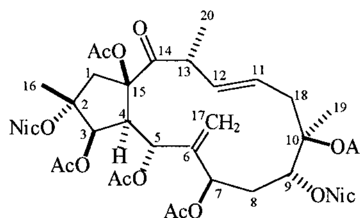
As an area of our current interest in the chemistry and pharmacology of Hungarian Euphorbiaceae species, we have investigated the secondary metabolites of *Euphorbia salicifolia* Host., which is a perennial herb distributed throughout Central and South-East Europe.[7] In the present paper, we describe the isolation and structural characterization of three new (**1**, **3**, **4**) and one known (**2**) diterpenes isolated from the dichloromethane extract of the fresh plants of *E. salicifolia*. This is the first report of the diterpene constituents of the title plant.

2. Results and discussion

The MeOH extract of the fresh plants of *E. salicifolia* was concentrated and subjected to solvent partitioning to furnish CH_2Cl_2 and H_2O-soluble fractions. The CH_2Cl_2 fraction was chromatographed on a polyamide open column, with various MeOH–H_2O mixtures (2:3, 3:2 and 4:1) as eluents. The fractions obtained with MeOH–H_2O (2:3) were separated by VLC (vacuum liquid chromatography) on Si gel, by CC on RP-18 Si gel, by preparative TLC and by crystallization to afford diterpene polyesters **1**–**4**.

2.1. Structure of euphosalicin (1)

A molecular weight of 820 (*m/z* 821.3121, $[M+H]^+$, Δ=1.2 ppm) was determined by HRFABMS, indicating the molecular formula $C_{42}H_{49}O_{15}N_2$ ($[M+H]^+$: 821.3133). Compound **1** exhibited an IR absorption band at 1736 cm^{-1} and UV maxima at 219 and 263.5 nm, characteristic of ester and nicotinoyl groups. Its ^1H NMR spectra in $CDCl_3$ and in C_6D_6 at ambient temperature contained several broad and overlapped signals, which became sharper on heating at 340 K. In the JMOD (*J*-Modulated Spin Echo Experiment) spectrum of **1** at 340 K, three signals appeared which were not present in the spectra recorded in the two solvents at



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(Nic = nicotinoyl, iBu = isobutanoyl)

Keywords: terpenes and terpenoids; esters; natural products; NMR.

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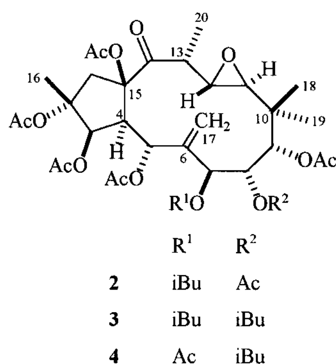
Table 1. NMR spectral data on euphosalicin (**1**) [C_6D_6, 340 K, TMS, δ (ppm), (J=Hz)] ($^{+4}J_{C-H}$ couplings)

Atom	^{1}H	^{13}C	HMBC H no.	NOESY H no.
1a	4.27 dd (16.4, 1.3)	49.7	3, 16	1b, 16
1b	2.24 d (16.4)			1a, 16
2	–	88.7	1a, 3, 16	–
3	6.12 d (3.1)	79.0	1a, 4, 5, 16, 3-OAc$^+$	4, 16
4	3.25 dd (3.1, 1.3)	52.6	1a, 5	3, 7, 6′, 6″
5	5.94 s	69.5	4, 17a, 17b, 5-OAc$^+$	13, 15-OAc
6	–	148.6	5, 17a, 17b	–
7	5.12 dd (10.4, 2.5)	70.0	5, 7-OAc$^+$	4, 8a
8a	2.35 m	35.7	–	7, 11, 19
8b	2.14 ddd (16.8, 10.5, 6.8)			19
9	5.69 brs	74.5	19	19
10	–	85.9	19, 10-OAc$^+$	–
11	5.87 ddd (15.4, 9.6, 4.8)	127.1	12, 13	8a, 13, 18a, 19, 20
12	5.78 dd (15.4, 9.1)	137.4	11, 13, 20	13, 18a, 18b, 19, 20, 4″
13	3.75 dq (9.1, 6.7)	45.4	11, 12, 20	5, 11, 12, 20, 15-OAc
14	–	212.9	1a, 1b, 4, 13, 20	–
15	–	94.1	1a, 1b, 3, 5, 15-OAc$^+$, 16$^+$	–
16	1.57 s	19.1	1b	1a, 1b, 3
17a	5.39 s	112.2	5	3-OAc, 5-OAc
17b	5.16 s			7-OAc, 10-OAc
18a	2.80 dd (14.5, 9.6)	40.7	19	12, 19
18b	2.35 m			19
19	1.59 s	19.1	–	8a, 8b, 9, 11, 12, 18a, 18b
20	1.24 d (6.7)	21.5	12, 13	11, 12, 13
Acetyls				
3-CO	–	168.8	3, 3-OAc	
3-COMe	1.78 s	21.6	–	17a
5-CO	–	169.8	5, 5-OAc	
5-COMe	1.78 s	21.6	–	17a
7-CO	–	170.3	7, 7-OAc	
7-COMe	1.48 s	20.7	–	17b
10-CO	–	170.4	10-OAc	
10-COMe	1.62 s	22.6	–	17b
15-CO	–	170.4	15-OAc	
15-COMe	1.69 s	21.3	–	5, 13
2-ONic	–			
CO		165.2	16$^+$, 2′, 4′	
2′	9.70 d (1.5)	152.7	4′, 6′	2″
3′	–	128.3	2′, 5′	
4′	8.39 dt (7.9, 1.9)	138.0	2′, 6′	
5′	6.70 dd (7.9, 4.8)	124.0	6′	
6′	8.46 dd (4.8, 1.5)	154.0	2′, 4′	4
9-ONic				
CO		165.1	2″, 4″	
2″	9.45 dd (1.5)	152.1	4″, 6″	2′
3″	–	126.8	2″, 5″	
4″	8.03 dt (7.9, 1.9)	137.1	2″, 6″	12
5″	6.69 dd (7.9, 4.8)	123.6	6″	
6″	8.45 dd (4.8, 1.5)	154.7	2″, 4″	4

300 K. These observations demonstrated the conformational flexibility of the molecule.



From the ^{1}H NMR and JMOD spectra of **1**, seven ester residues were easily identified as two nicotinoate and five acetate groups (Table 1). Additionally, the JMOD spectrum revealed the presence of a 20 carbon-containing diterpene core, consisting of five quaternary carbons, eight methines, four methylenes and three methyl groups. After the association of all carbon signals with the corresponding signals for directly bonded protons via an HSQC experiment, proton-proton connectivities were studied by means of ^{1}H–^{1}H COSY measurements. The ^3J and ^4J couplings detected in the ^{1}H–^{1}H COSY spectrum clearly revealed three sequences of correlated protons: –CH_2–C–CH(OR)–CH–CH(OR)–C(=CH_2)– (*A*), CH(OR)–CH_2– (*B*) and *trans*-CH_2–CH=CH–CH(CH_3)– (*C*). The overall structure was assembled by analysis of the long-range ^{1}H–^{13}C connectivities (Table 1) gleaned from an HMBC experiment. The long-range couplings of the quaternary carbon signals at δ_C 88.7 (C-2) and 94.1 (C-15) (listed in Table 1) clearly showed that partial structure *A* involves the C-1–C-6(C-17) part of the molecule, including the methyl-substituted

Table 2. NMR spectral data on diterpenes **3** and **4** [$CDCl_3$, TMS, δ (ppm), (J=Hz)]

Atom	3		4	
	^1H	^{13}C	^1H	^{13}C
1a	3.75 d (16.2)	46.9	3.73 d (15.4)	46.3
1b	2.04 d (16.2)		2.06 d (15.4)	
2	–	87.5	–	86.7
3	5.58 brd (3.4)	78.8	5.58 brs	78.2
4	2.96 dd (3.4, 1.9)	50.6	2.99 brs	49.5
5	6.09 brs	68.4	6.04 s	67.8
6	–	142.5	–	140.6
7	5.42 brs	69.4	5.36 s	68.4
8	5.51 d (4.5)	69.4	5.50 d (3.9)	69.2
9	4.94 d (4.5)	77.4	4.93 d (3.9)	77.7
10	–	40.2	–	39.3
11	3.00 d (2.1)	59.2	3.02 d (1.8)	58.2
12	3.33 dd (2.1, 4.7)	57.8	3.32 m	57.0
13	3.67 dq (6.8, 4.7)	37.8	3.66 m	37.0
14	–	210.9	–	210.4
15	–	93.3	–	93.0
16	1.52 s	18.8	1.52 s	18.1
17a	5.07 brs	116.0	5.12 s	111.7
17b	5.01 brs		5.03 s	
18	0.98 s	24.3	0.99 s	23.3
19	0.69 s	18.3	0.71 s	17.5
20	1.18 d (6.8)	15.9	1.18 d (6.8)	15.0
Acetyls				
2-CO	–	170.4	–	169.8
2-COMe	2.11 s	22.9	2.09 s	20.4
3-CO	–	169.2	–	168.4
3-COMe	2.10 s	21.9	2.11 s	21.0
5-CO	–	168.8	–	168.0
5-COMe	2.16 s	21.9	2.14 s	21.0
7-CO	–	–	–	168.6
7-COMe	–	–	2.10 s	21.0
9-CO	–	169.8	–	169.0
9-COMe	2.05 s	21.5	2.05 s	20.4
15-CO	–	170.6	–	169.3
15-COMe	2.12 s	21.8	2.10 s	21.9
7-OiBu				
1′	–	175.5	–	–
2′	2.60 sept (7.0)	34.6	–	–
3′	1.22 d (7.0)	19.8	–	–
4′	1.19 d (7.0)	19.4	–	–
8-OiBu				
1″	–	176.0	–	175.3
2″	2.54 sept (7.0)	34.7	2.55 sept (7.2)	33.7
3″	1.13 d (7.0)	19.7	1.12 d (7.2)	19.0
4″	1.12 d (7.0)	19.1	1.11 d (7.2)	18.7

five-membered ring, present in many types of Euphorbiaceae diterpenes.[8] The two and three-bond correlations of the carbon signal at δ_C 212.9 (C-14) located a keto group at C-14 and indicated that structural moiety *C* comprises the C-18–C-11–C-13–C-20 part of the molecule. Further building of the structure with the aid of HMBC cross-peaks between C-18/H-19, C-10/H-19, C-9/H-19 and C-7/H-5 led to the conclusion that the parent diterpene involved a new carbon skeleton,[9] which is the first representative of an unusual type of cembrene cation-derived Euphorbiaceae diterpene. The new skeletal system is similar to that of jatrophane, but one of the geminal dimethyl groups is incorporated in the carbocycle, resulting in a 13-membered macrocycle. This rearrangement may be the explanation of the observed flexibility of the molecule. The positions of the ester groups were determined via two HMBC experiments using 60 and 100 ms evolution times. On the basis of the $^3J_{C-H}$ couplings between the oxymethine protons and carbonyl carbons, the presence of acetyl groups at C-3, C-5 and C-7 was evident. The locations of acyl groups situated on quaternary carbons were confirmed through the $^4J_{C-H}$ couplings observed in the spectrum recorded at 100 ms between C-10, C-15 and the acetyl methyl protons, and between the nicotinoyl carbonyl carbon and H-16. The remaining nicotinoyl group, which did not exhibit any long-range correlations because of the flexibility of the molecule, was of necessity situated on C-9.

The stereochemistry of all stereogenic centres and the preferred conformations were deduced from the NOESY spectrum. A convenient point of reference was the proton at the ring junction (H-4), which was chosen to be α as is usual in the literature.[2,10–13] Diagnostic NMR features, the small $J_{4,5}$ value (0–4 Hz) and the detection of H-4/H-5 and H-4/H-7 NOE interactions suggested that the 6,17-exomethylene group is parallel to the plane of the macrocycle, in the ‘exo conformation’, and H-4 and H-5 are almost orthogonal, as described for jatrophanes earlier.[10,14] This conformation involves a β-oriented H-5, which points inwards into the macrocycle. The nuclear Overhauser effects between H-5 and H-13 and between H-13 and the 15-OAc demonstrated the β position of H-13 and the 15-OAc group. NOE interactions between H-4 and H-3 and between H-4 and H-7 revealed β-oriented acyl groups at C-7 and C-3. The NOESY correlations from H-4 to nicotinoyl protons (H-6′, H-6″) and between the two nicotinoyl groups (H-2′/H-2″) suggested the α configuration of the nicotinoyl groups at C-2 and C-9. Further important NOE effects were observed between H-17a and the 3-OAc and 5-OAc groups, and between H-17b and the 7-OAc and 10-OAc groups, from which the β-orientation of the acetyl at C-10 was concluded. All of the above data led to the formulation of euphosalicin as **1**. Its relative configurations at C-2-C-9, C-13 and C-15 are the same as found in compounds **2–4**, discussed below. The relative configuration of **1** elucidated here is in accordance with biogenetic aspects: all jatrophane derivatives, including compounds **2–4** substituted at C-7 and C-9 have the same stereochemistry.[2,10–17]

2.2. Structures of compounds 2–4

Mass spectrometry and ^1H and ^{13}C NMR investigations indicated that compound **2** was identical with esulatin A, isolated previously from *E. esula*.[15] Compound **3** gave a quasimolecular ion in the FABMS at *m/z* 767.3539 $[M+H]^+$, Δ=4.9 ppm) appropriate for a molecular formula of $C_{38}H_{54}O_{16}$. It displayed IR absorption band at 1746 cm^{-1}, characteristic of ester groups. The ^1H and ^{13}C NMR spectra of **3** revealed five acetate and two isobutanoate groups. Additionally, the spectra exhibited resonances closely related to those of **2**. After the ^1H and ^{13}C NMR data on **3** had been assigned by analysis of its ^1H–^1H COSY, HMQC and HMBC spectra, it was obvious that compounds **2** and **3** were based on the same parent system and differed only in the esterification. The absence of an acetate signal and the appearance of signals of a further isobutanoate indicated the replacement of one of the acetyl residues with an isobutanoyl group. The position of this substituent was concluded from the HMBC cross-peak between H-8 and the carbon signal at δ 176.0 (*i*-butanoyl CO). A careful comparison of the NOESY spectra of **2** and **3** enabled us to assume

the stereochemistry for **3** as that of **2**, whose configuration was previously established by X-ray crystallography. Compound **4**, a minor metabolite of *E. salicifolia*, was shown to have the same molecular formula $C_{36}H_{50}O_{16}$ as that of **2**. Interpretation of the ^{1}H NMR, ^{1}H–^{1}H COSY, NOESY, HMQC and HMBC spectra led to structure **4** for this compound and the NMR assignments in Table 2. The only difference between **2** and **4** was found in the position of the isobutanoyl group.

2.3. Assay for multidrug resistance (mdr) reversing effect

Euphosalicin (**1**), **2** and **3** were examined as concerns the reversal of mdr.[18] Compound **1** displayed considerable potency in inhibiting the efflux-pump activity of mdr *p*-glycoprotein in mouse lymphoma cells; a fluorescence activity ratio (*R*; see Experimental) of 22.46 was measured when 2 μl of the 1.0 mg/ml stock solution was applied in the test. At the same concentration, compound **2** and **3** was found to be inactive in the reversal of mdr. The positive control verapamil was less potent than **1**: it exhibited $R=8.49$ at the same final concentration of 2 μg/ml.

3. Experimental

3.1. General

Melting points are uncorrected. HRFABMS measurements were carried out on a VG ZAB2-SEQ high-resolution mass spectrometer. FAB ionization with glycerol as matrix was used for measurements; the resolution setting was 10 000. NMR spectra were recorded on a Bruker Avance DRX 500 spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C). The signals of the deuterated solvents were taken as the reference. Two-dimensional experiments were performed with standard Bruker software. IR spectra of KBr discs were run on a Perkin–Elmer Paragon 1000 PC FTIR instrument. Optical rotations were determined in MeOH by using a Perkin–Elmer 341 polarimeter. The UV spectra were recorded on a Shimadzu UV-2101 PC spectrometer. For column chromatography, polyamide (ICN) and RP-Si gel C18 (32–63 μm) 60A (ICN), and for flash chromatography, Si gel (Kieselgel GF_{254} 15 μm, Merck) were used. Preparative TLC was performed on Si gel plates (Merck 5715). HPLC was carried out on a Waters Millipore instrument, with RI detection on LiChrospher Si 100 and LiChrospher RP-18 (5 μm, 200×4 mm) columns, with cyclohexane–EtOAc–EtOH (20:10:1) and acetonitrile–H_2O (4:1), respectively, as mobile phase.

3.2. Plant material

E. salicifolia was collected in Budapest (Pesthidegkút), Hungary, in August 1998 and was identified by Tamás Rédei (Department of Taxonomy and Ecology, Eötvös Lóránd University, Budapest, Hungary). A voucher specimen (No. 511) has been deposited at the Department of Pharmacognosy, University of Szeged, Szeged, Hungary.

3.3. Extraction and isolation

The fresh plant material (2600 g) was percolated with MeOH (16 l) at room temperature. The crude extract was concentrated in vacuo to 600 ml and partitioned between CH_2Cl_2 and H_2O. Evaporation of the organic phase gave a residue (40 g), which was chromatographed on a polyamide column (180 g) with mixtures of MeOH–H_2O (2:3, 3:2 and 4:1) as eluents. The fractions obtained with MeOH–H_2O (2:3) were subjected to Si gel flash chromatography, with a gradient system of *n*-hexane–EtOAc–EtOH (60:20:1, 60:20:2, 60:30:3, 60:50:5 and 60:60:10). Upon standing, fractions 2–9 (each 50 ml), eluted with the first of these eluents, afforded a crystalline material, which was purified by preparative TLC on Si gel plates with cyclohexane–EtOAc–EtOH (20:10:1) as mobile phase, to yield compounds **2** (60 mg) and **3** (8 mg). Fractions 49–71 obtained with *n*-hexane–EtOAc–EtOH 60:50:5 and 60:60:10 were transferred to RP-Si gel CC eluted with MeOH–H_2O (4:1) and further fractionated by normal- and reverse-phase HPLC to afford compounds **1** (15 mg) and **4** (1.5 mg).

3.3.1. Euphosalicin (1). Amorphous solid; $[\alpha]_D^{25}=-101$ (*c* 0.12, $CHCl_3$); IR (KBr) ν_{max} 2994, 2944, 1736, 1591, 1373, 1285, 1233, 1111, 1023, 742 cm^{-1}; UV λ_{max} (log ϵ) (MeOH) 219 (3.94), 263.5 (3.38); ^{1}H and ^{13}C NMR, see Table 1; HRFABMS *m/z* 821.3121 $[M+H]^+$, $\Delta=1.2$ ppm (calcd for 821.3133, $C_{42}H_{49}O_{15}N_2$).

3.3.2. Compound 2. Colourless crystals from MeOH; mp 218–219°C; spectral data are identical with published data.[15]

3.3.3. Compound 3. White needles; mp 228–229°C (from MeOH); $[\alpha]_D^{25}=-37$ (*c* 0.085, $CHCl_3$); IR (KBr) ν_{max} 2924, 1746, 1378, 1218, 1144, 1020 cm^{-1}; UV λ_{max} (log ϵ) (MeOH) 202.5 (4.01), 228 sh (3.45); ^{1}H and ^{13}C NMR, see Table 2; HRFABMS *m/z* 767.3539 $[M+H]^+$, $\Delta=4.9$ ppm (calcd for 767.3490, $C_{38}H_{55}O_{16}$).

3.3.4. Compound 4. Amorphous solid; $[\alpha]_D^{25}=-97$ (*c* 0.02, $CHCl_3$); IR (KBr) ν_{max} 2924, 1742, 1374, 1228, 1144, 1026 cm^{-1}; UV λ_{max} (log ϵ) (MeOH) 202.5 (4.07), 221.5 sh (3.82); ^{1}H and ^{13}C NMR, see Table 2; HRFABMS *m/z* 871.2152 $[M+Cs]^+$, $\Delta=0.1$ ppm (calcd for 871.2153, $C_{36}H_{50}O_{16}Cs$).

3.4. Cell and fluorescence uptake, mdr reversal effect

The L5178 mouse T cell lymphoma cell line was infected with the pHa MDR1/A retrovirus as previously described.[19] MDR1 expressing cell lines were selected by culturing the infected cells with 60 ng/ml colchicine to maintain expression of the MDR phenotype. The L5178 MDR cell line and the L5178Y parent cell line were grown in McCoy's 5AA medium with 10% heat-inactivated horse serum, L-glutamine and antibiotics. The cells were adjusted to a concentration of 2×10 ml^{-1}, resuspended in serum-free McCoy's 5AA medium and distributed in 0.5 ml aliquots into Eppendorf centrifuge tubes. From 2.0 to 20.0 μl of the 1.0 mg/ml stock solutions of the tested compounds in DMSO were then added and the samples were incubated for 10 min at room

temperature. 10 μl (5.2 μM final concentration) of the indicator Rhodamine 123 was next added to the samples and the cells were incubated for a further 20 min at 37°C, washed twice and resuspended in 0.5 ml phosphate-buffered saline for analysis. The fluorescence of the cell population was measured by flow cytometry with a Beckton Dickinson FACScan instrument. Verapamil was used as a positive control in the Rhodamine 123 exclusion experiments. The mean fluorescence intensity was calculated as a percentage of the control for the parental and mdr cell lines as compared to untreated cells. An activity ratio (*R*) was calculated on the basis of the measured fluorescence values via the following equation:

$$\mathrm{R} = \frac{\text{mdr treated/mdr control}}{\text{parental treated/parental control}}$$

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