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Jatrophane Diterpenes from *Euphorbia esula* as Antiproliferative Agents and Potent Chemosensitizers to Overcome Multidrug Resistance

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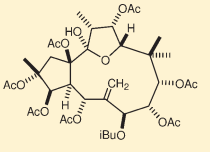
Supporting Information

ABSTRACT: Phytochemical study of whole, undried plants of *Euphorbia esula* led to the isolation of six new (**1**−**6**) jatrophane diterpene polyesters, named esulatins H−M, together with the known compounds 2α,3β,5α,7β,15β-pentaacetoxy-9α-nicotinoyloxyjatropha-6(17),11-dien-14-one (**7**), salicinolide (**8**), and euphosalicin (**9**). The structures and relative configuration of **1**−**6** were established on the basis of extensive spectroscopic analysis, including HRESIMS and one- and two-dimensional NMR techniques. All these compounds, together with diterpenes (**10**−**14**) isolated previously from this plant, were evaluated for their antiproliferative activity against HeLa, Ishikawa, and MCF7 cells. The multidrug-resistance-reversing activities were also investigated on L5178 mouse lymphoma cells transfected with the pHa MDR1/A retrovirus DNA. Preliminary structure−activity relationship data are discussed.



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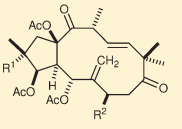
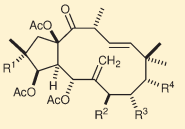


	R^1	R^2
2	OBz	OAc
3	H	O*i*Bu



	R^1	R^2	R^3	R^4
4	ONic	OAc	H	ONic
5	OAc	O*i*Bu	H	ONic
6	H	O*i*Bu	H	ONic

Plants of the genus *Euphorbia* are characterized by a unique profile of diterpenoid polyesters, often accumulated in relatively large amounts and generally occurring in complex mixtures, based on the acylation of several different skeletons (e.g., ingenane,[1] lathyrane,[2] tigliane,[3] jatrophane,[4] and daphnane[5]) with different acids. Certain of these compounds exhibit extremely strong skin-irritating and tumor-promoting effects, and some display therapeutically relevant biological properties, such as antiproliferative, cytotoxic, antiviral, antibacterial, platelet aggregation-inhibiting, and vasoconstrictor activities.[4−11]

The cytotoxic activities of jatrophane diterpenes have been studied previously in different test models. Compounds obtained from *E. esula* demonstrated in vitro cytotoxicity on cultured B16 cells,[12] and euphornin and helioscopinolide A from *E. helioscopia* proved active against HeLa and MDA-MB-231 cells.[13] Diterpenes of the jatrophane type isolated from *E. tuckeyana* were reported to exert moderate inhibitory activity on the growth of gastric and pancreatic tumor cell lines.[14] The microtubule-interacting activity of jatrophane polyesters of *E. semiperfoliata* was assessed, and they were found to stimulate purified tubulin assembly in vitro and to induce paclitaxel-like microtubules. These compounds inhibited the growth of some human cancer cells without inducing cell cycle arrest in the G2/M phase.[15]

The discovery of jatrophane diterpenes as a new class of potent inhibitors of P-glycoprotein (P-gp, ABCB1) has led to increasing interest in research on this type of compounds.[16] P-gp is one of the most important and best-studied ABC transporter proteins and acts as an energy-dependent pump of chemotherapeutic agents, thereby decreasing the intracellular concentration of drugs and resulting in multidrug resistance (MDR). Many powerful inhibitors have been identified among jatrophane and modified jatrophane diterpenes, which are promising compounds for drug development because of their manifold higher potencies than those of cyclosporin A or verapamil.[17,18]

Euphorbia esula L., or leafy spurge (Euphorbiaceae), produces a skin-irritant, toxic, milky latex and is a promising source of biologically active diterpenes. Ingenane,[19] lathyrane,[20] and jatrophane[12,21] polyesters were isolated previously from the root, seed, and herb of the plant. Some of them have antileukemic, cytotoxic, MDR-modifying, and anti-herpes simplex activities.[6,22−24]

In the course of our earlier work, six new jatrophane diterpenes, named esulatins A−F (**10**−**14**), were reported from *E. esula*.[25−27] We now report further six new (esulatins H−M, **1**−**6**) and three known (**7**−**9**) diterpenes from this plant. The antiproliferative activities of compounds **1**−**14** and their MDR-reversing activities have also been assessed.

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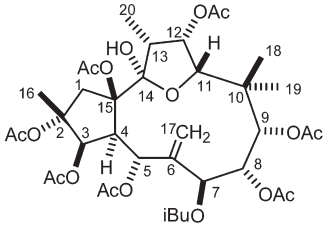
Chart 1



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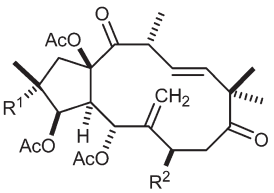
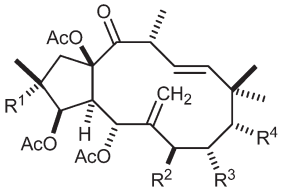
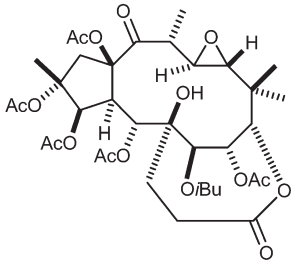


	R^1	R^2
2	OBz	OAc
3	H	O*i*Bu
10	OAc	OAc



	R^1	R^2	R^3	R^4
4	ONic	OAc	H	ONic
5	OAc	O*i*Bu	H	ONic
6	H	O*i*Bu	H	ONic
7	OAc	OAc	H	ONic
11	OAc	OAc	H	OAc
12	OAc	O*i*Bu	OAc	OAc



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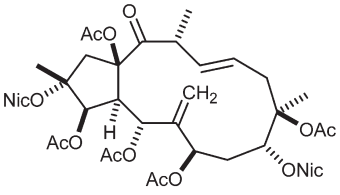

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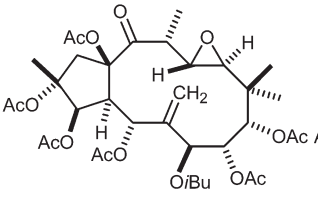

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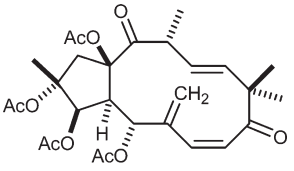

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RESULTS AND DISCUSSION

Whole plants of *E. esula* 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 procedures to yield pure compounds **1**−**9**.

Esulatin H (**1**) was isolated as an amorphous solid with $[\alpha]^{22}_D$ −4 (*c* 0.1, $CHCl_3$). Its HRESIMS showed the molecular formula, $C_{38}H_{54}O_{18}$, through the presence of a peak at *m*/*z* 821.3204 $[M + Na]^+$. The ^1H and ^{13}C NMR spectra of **1** revealed the occurrence of seven acetate groups [δ_H 2.27 s, 2.24 s, 2.20 s, 2.15 s, 2 × 2.13 s and 1.80 s; δ_C 170.7, 3 × 170.3, and 3 × 169.5 (*C*O) and 22.7, 22.5, 22.0, 21.5, 2 × 21.2, and 20.5 (*C*H_3)] and one isobutanoate group [δ_H 2.60 m, 1.25 d and 1.24 d; δ_C 176.1, 34.6, 19.5, and 18.5] (Table 1). Additionally, the ^1H NMR spectrum exhibited signals attributed to four methyls (1.67 s, 1.44 d, 1.40 s, and 0.95 s). The JMOD and HSQC spectra suggested a carbon skeleton consisting of 20 carbons: four methyls, two methylenes, nine methines, and five quaternary carbons. The $^1H-^1H$ COSY spectrum demonstrated three structural fragments with correlated protons, −CHR−CH−CHR− (A) (δ_H 5.80, 4.58, and 6.38), −CHR−CHR−CHR− (B) (δ_H 7.23, 6.30, and 5.41), and −CH−CHR−CH (CH_3)− (C) (δ_H 3.80, 5.80, 2.98, and 1.44), and one exomethylene group (δ_H 6.39 s and 6.06 s; δ_C 123.5). Their connectivities were determined from the long-range C−H correlations observed in the HMBC spectrum (Table 1). The long-range correlations of the quaternary carbons (C-2, C-6, C-10, C-14, and C-15) with protons of the structural fragments helped establish a jatrophane diterpene, with O-functionalities at C-2, 3, 5, 7, 8, 9, 11, 12, 14, and 15. The positions of the ester groups were established via an HMBC experiment. The correlation of the carbonyl signal at

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Table 1. NMR Data of Compound 1 [500 MHz (^{1}H), 125 MHz (^{13}C), pyridine-d_5, δ (ppm) (J = Hz)]

position	δ_H	δ_C	HMBC (H → C)	NOESY
1α	4.13 d (16.5)	40.9	16	20
1β	3.30 d (16.5)			16
2		86.2	1α, 16	
3	5.80 brs	80.4	1α, 16	4
4	4.58 dd (11.3, 4.1)	49.1	1α, 5	3, 7, OH-14
5	6.38 d (11.3)	77.7	17a, 17b	8, 17b
6		140.7	4, 7, 17a, 17b	
7	7.23 d (9.9)	69.2	5, 17a, 17b	4, 19
8	6.30 d (9.9)	74.0	7, 10	5, 9, 8-Ac
9	5.41 s	80.1	8, 11, 18, 19	8, 18, 11
10		41.5	11, 18, 19	
11	3.80 s	86.0	10, 18, 19	9, 18, 13, 12, 12/OAc-9
12	5.80 brs	78.0	20	11, 18, 13
13	2.98 q (6.9)	41.5	20	11, 12
14		108.4	1α, 1β, 4, 13, OH-14, 20	
15		94.9	1β, 3, OH-14	
16	1.67 s	19.7		1β, OAc-2
17a	6.39 s	123.5	5, 7	5
17b	6.06 s			
18	0.95 s	22.7	19	9, 11, 12
19	1.40 s	18.1		7, OAc-12
20	1.44 d (6.9)	10.3	13	1a, OH-14, OAc-15
OH-14	7.39 s			4, 20
Acetyls				
*C*O-2		169.5		
CO*Me*-2	2.27 s	21.2		16
*C*O-3		170.3	CO*Me*-3, 3	
CO*Me*-3	2.24 s	21.2		3′, 4′
*C*O-5		169.5	CO*Me*-5, 5	
CO*Me*-5	2.20 s	22.7		
*C*O-8		170.3	CO*Me*-8, 8	
CO*Me*-8	2.15 s	22.0		2′, 3′, 4′
*C*O-9		170.7	CO*Me*-9, 9	
CO*Me*-9	2.13 s	21.5		
*C*O-12		170.3	CO*Me*-12, 12	
CO*Me*-12	2.13 s	20.5		11, 19, 20
*C*O-15		169.5	CO*Me*-15	
CO*Me*-15	1.80 s	22.5		20
O*i*Bu-7				
1′		176.1	2′, 3′, 4′, 7	
2′	2.60 sept (7.0)	34.6	3′, 4′	3′, 4′, OAc-8
3′	1.25 d (7.0)	18.5	2′, 4′	2′, OAc-8, OAc-3
4′	1.24 d (7.0)	19.5	2′, 3′	2′, OAc-8, OAc-3

δ_C 176.1 (isobutanoyl *C*O) with the proton signals at δ_H 7.23 (H-7), 1.25 (H-3′), and 1.24 (H-4′) indicated the presence of the isobutanoyl group at C-7. Similarly, the long-range couplings of the carbonyl carbon signals at δ_C 170.7, 170.3, and 169.5 with the proton signals at δ_H 6.38 (H-5), 6.30 (H-8), 5.80 (H-3), 5.80 (H-12), and 5.41 (H-9) demonstrated the presence of acetyl groups at C-3, C-5, C-8, C-9, and C-12. The two remaining acetyl groups did not show any long-range correlations, and it was therefore supposed that they are attached to quaternary carbons. The ^{1}H NMR and JMOD data on **1** were compared with those on analogous compounds with 2,3,15-triacetyl substitution on the five-membered ring, and similar δ values were found.[25] The two acetyl groups (δ_H 2.27 and 1.80) were therefore assigned to positions C-2 and C-15. The singlet signal at δ_H 7.39, which did not show a correlation to any carbon in the HSQC spectrum, was assigned to an OH group. This OH group was located at C-14 with regard to its HMBC correlation with C-14. The chemical shifts of C-14 (δ_C 108.4) and C-11 (δ_C 86.0) indicated a hemiacetal structure for **1**, which was in agreement with its molecular composition.

The relative configuration of **1** was studied in a NOESY experiment (Table 1). It was deduced by starting from the

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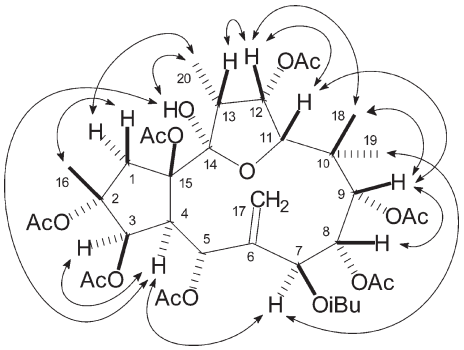

Figure 1. Key NOESY correlations for compound **1**.

α-orientation of H-4, characteristic of all jatrophane derivatives isolated to date (Figure 1). Cross-peaks between H-4/H-3 and H-4/H-7 proved the β-orientation of the acetyl group on C-3 and the isobutanoyl group at C-7. The NOE effect observed between OH-14 and H-4 indicated the presence of an α-hydroxy group on C-14. The nuclear Overhauser effect between OH-14 and the 13-methyl group indicated an α-oriented methyl group at C-20. The NOE interaction of H-7 and one of the geminal methyl groups on C-10 dictated the α-orientation of this methyl group (C-19). The NOE effects of the 18-methyl group with H-9 and the NOEs between H-9/H-8, H-9/H-11, H-11/H-12, H-12/H-18, and H-12/H-13 indicated the β-orientation of all these protons. Further, the NOESY cross-peaks between H-20/H-1α and between H-1β/H-16 indicated the β-position of the 16-methyl group. All of the above evidence confirmed the structure of **1** as 2α,3β,5α,8α,9α,12α,15β-heptaacetoxy-11,14-epoxy-14α-hydroxy-7β-isobutanoyloxyjatropha-6(17)-ene, which has been named esulatin H. This compound, containing eight ester groups, is the most highly esterified diterpenoid identified to date from a plant in the family Euphorbiaceae.

Esulatin I (**2**) was isolated as white crystals with $[\alpha]^{22}_{D}$ −152 (*c* 0.1, $CHCl_3$). It gave the molecular formula $C_{35}H_{42}O_{12}$, as determined from HRESIMS and NMR analysis. From the ^1H and ^{13}C NMR spectra, four acetyls [δ_H 2.21, 2.18, 2.15, and 2.07; δ_C 170.2, 169.9, 169.6, and 169.2 (*C*O), and 22.9, 21.4, 20.7, and 20.1 (*C*H$_3$)] and one benzoyl group (δ_H 8.11, 7.40, and 7.53; δ_C 165.6, 131.1, 130.0, 128.1, and 132.6) were identified. Additionally, the ^{13}C NMR and JMOD spectra exhibited resonances for four methyls, three methylenes, seven methines, and six quaternary carbons, including two keto groups (δ_C 209.3 and 209.5) (Table 3). The ^1H NMR and $^1H-^1H$ COSY spectra revealed the structural elements −CH$_2$−CR−CH−CH−CH−C(=CH$_2$)−CH−CH$_2$− (C-1−C-8) and −CH=CH−CH(CH$_3$)− (−C-11−C-13(C-20)−). For the identification of these elements, some weak $^4J_{H,H}$ correlations (H-1a/H-3, H-17/H-3, and H-17/H-7) were also taken into consideration. The connection of these partial structures was determined on the basis of two- and three-bond long-range correlations detected in the HMBC spectrum. The correlations of the quaternary carbons to proximate protons supported the structure of compound **2** as 3,5,7,15-tetraacetoxy-2-benzoyloxyjatropha-6(17),11-diene-9,14-dione.

The relative configuration of **2** was investigated by NOESY measurement. Starting from the α-orientation of H-4, the NOE effects between H-4/H-1a, H-4/H-2′,6′ and H-1b/H-16 indicated the α-orientation of the benzoyl group and the β-position of the 2-methyl group. The location of the 2β-OBz group was further evidenced by the NOEs observed between H-2′,6′ and H-4, H-3, and H-7. In connection with the 12-membered ring, nuclear Overhauser effects were detected between H-4/H-7, H-7/H-12, H-12/H-20, and H-12 and H-19, indicating that these protons and methyl groups are directed below the plane of the macrocycle. On the other hand, the NOE interactions between H-8a/H-11, H-11/H-13, H-13/H-5, H-5/H-11, H-8a/H-18, and H-11/H-18 dictated the positions of all these protons and the methyl group to be above the plane (β) of the 12-membered ring. All of these data are compatible with the structure proposed for esulatin I (**2**) as 3β,5α,7β,15β-tetraacetoxy-2α-benzoyloxyjatropha-6(17),11*E*-diene-9,14-dione.

Esulatin J (**3**) was isolated as a colorless, amorphous solid with $[\alpha]^{22}_{D}$ −113 (*c* 0.1, $CHCl_3$). Its HRESIMS displayed a quasi-molecular ion peak at *m*/*z* 585.2664 $[M + Na]^+$, indicating a molecular mass of 562 Da, corresponding to the formula $C_{30}H_{42}O_{10}$. The ^1H NMR and JMOD spectra of **3** revealed three acetates and one isobutanoate group. Additionally, the spectra exhibited resonances closely related to those of **2**, except for the appearance of a H-2 signal (δ_H 2.19 m) and a change in the chemical shift of C-2 (δ_C 38.3 in **3**; 87.2 in **2**). After the ^1H and ^{13}C NMR data on **3** had been assigned by analysis of its $^1H-^1H$ COSY, HSQC, and HMBC spectra, it was apparent that compounds **2** and **3** are based on the same parent system and differ only in the substituents at C-2 and C-7. The absence of acetate and benzoyl signals 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-7 and the carbon signal at δ_C 176.0 (isobutanoyl CO). A careful comparison of the NOESY spectra of **2** and **3** enabled the same relative configuration to be inferred for esulatin J (**3**) and esulatin I (**2**). The structure of esulatin J was elucidated therefore as 3β,5α,15β-triacetoxy-7β-isobutanoyloxyjatropha-6(17),11*E*-diene-9,14-dione (**3**).

Esulatin K (**4**) was isolated as an amorphous solid with $[\alpha]^{22}_{D}$ −140 (*c* 0.1, $CHCl_3$). Its molecular formula, $C_{40}H_{46}O_{13}N_2$, was derived from the HRESIMS ($[M + H]^+$ *m*/*z* 763.3075, calcd 763.3073), combined with the NMR data. The ^1H NMR and JMOD spectra exhibited typical signals for two nicotinoyl (δ_H 9.31 s, 8.44 d, 7.37 dd, and 8.77 d; 9.18 s, 8.13 d, 7.30 dd, and 8.77 d; δ_C 164.1, 151.2, 125.8, 137.5, 123.3, and 152.9, and 164.3, 150.9, 127.4, 136.7, 123.5, and 153.6) and four acetyl groups [δ_H 2.19 s, 2.18 s, 2.16 and 1.52 s; δ_C 168.8, 169.5, 169.6, and 170.3 (CO) and 20.4, 21.1, and 2 × 21.3 (CH$_3$)]. After the ^1H and ^{13}C NMR data of **4** had been assigned by analysis of its $^1H-^1H$ COSY, HSQC, and HMBC spectra, it was concluded that esulatin K was based on the same parent system as **2** and **3**, having differences in the esterification pattern; as in the case of **4**, the 9-keto group was replaced by a nicotinoyl substituent.

The relative configuration of **4** was investigated by a NOESY measurement. For a more exact evaluation, the solvent $CDCl_3$ was replaced by benzene-d_6, which gave better-separated proton signals. NOE effects between H-4/H-1α, H-4/H-3, H-4/H-7, and H-7/H-18 indicated the α-orientation of these protons. The β-oriented H-5, H-9, H-13, and H-19 could be proposed from the NOESY cross-peaks between H-1β/H-16, H-5/H-13, H-13/H-11, H-11/H-9, and H-9/H-19. The NOE effects also provided evidence for the locations of the ester groups and especially those connected to quaternary carbons. The presence of the nicotinoyl group in the 2α-position was indicated by the NOEs between H-2‴/OAc-5, H-4/H-2‴, H-7/H-2‴, and H-3/H-2‴, while OAc-15β was suggested by the NOESY correlation between

Table 2. ^{1}H NMR Data of Compounds 2−6 [500 MHz (^{1}H), $CDCl_3$, δ (ppm) (J = Hz)]

position	2[a]	3	4[b]	4[c]	5	6
1a	4.06 d (15.8)	3.03 dd (13.6, 7.3)	3.90 d (16.1)	4.26 d (16.0)	3.80 d (16.1)	3.02 dd (14.0, 7.7)
1b	2.05 d (15.8)	1.60 m	2.12 m	2.14 d (16.0)	1.96 d (16.1)	1.63 t (14.0)
2		2.19 m				2.27 m
3	5.75 d (3.2)	5.49 (3.0)	5.80 d (2.7)	6.20 d (3.4)	5.41 d (3.0)	5.40 s
4	2.98 d (3.2)	2.48 d (3.0)	3.08 d (2.7)	3.45 d (3.4)	3.00 d (3.3)	2.66 s
5	5.91 s	5.76 s	5.91s	6.12 s	5.96 s	5.85 s
7	5.03 d (9.5)	4.92 d (9.8)	4.94 brs	5.15 t (5.6, 4.5)	5.01 t (5.8, 5.1)	4.91 t (2, 4)
8a	3.28 d (13.9)	3.23 d (13.9)	2.13 m	2.23 m	2.14 m	2.19 m (α)
8b	2.30 dd (13.9, 9.7)	2.32 dd (14.0, 10.0)	2.13 m	2.23 m	2.14 m	2.12 m (β)
9			5.09 m	5.20 m	5.08 dd (8.0, 3.4)	5.09 m
11	6.07 d (15.8)	6.07 d (15.8)	5.92 d (15.8)	6.00 d (16.0)	5.96 d (16.0)	5.94 d (16.0)
12	5.40 dd (15.8, 9.4)	5.53 dd (15.9, 9.5)	5.51 dd (15.9, 9.3)	5.62 dd (16.0, 9.2)	5.62 dd (16.0, 9.3)	5.62 dd (16.0, 9.2)
13	3.57 dq (9.4, 6.7)	3.59 dq (9.4, 6.8)	3.52 dq (9.3, 6.7)	3.62 dq (9.2, 6.7)	3.51 dq (9.2, 6.7)	3.54 dq (9.2, 7.0)
16	1.64 s	0.90 d (6.6)	1.63 s	1.61 s	1.50 s	0.90 d (7.1)
17a	5.17 s	5.07 s	5.10 s	5.34 s	5.18 s	5.09 s
17b	5.12 s	5.06 s	5.09 s	5.19 s	5.14 s	5.09 s
18	1.16 s	1.17 s	1.10 s	0.93 s	1.14 s	1.11 s
19	1.13 s	1.13 s	1.12 s	0.87 s	1.11 s	1.11 s
20	1.07 d (6.7)	1.15 d (6.7)	1.13 d (6.6)	1.27 d (6.7)	1.17 d (6.6)	1.19 d (6.6)
Acetyls						
OAc-2					2.18 s	
OAc-3	2.21 s	2.15 s	2.19 s	1.74 s	2.16 s	2.12 s
OAc-5	2.18 s	2.14 s	1.52 s	1.17 s	2.15 s	2.16 s
OAc-7	2.07 s		2.18 s	1.69 s		
OAc-15	2.15 s	2.13 s	2.16 s	1.62 s	2.13 s	2.14 s
O*i*Bu-7						
2′		2.54 sept (7.2)			2.10 m	2.07 m
3′		1.11 d (7.2)			0.95 d (7.1)	0.92 d (7.0)
4′		1.13 d (7.2)			0.74 d (7.0)	0.73 d (6.9)
ONic-9						
2‴			9.18 s	9.51 s	9.20 s	9.22 s
4‴			8.14 d (7.8)	8.04 d (7.8)	8.22 d (7.9)	8.22 d (7.8)
5‴			7.30 dd (7.8, 5.0)	6.79 dd (7.8, 4.8)	7.38 dd (7.9, 4.9)	7.44 dd (7.8, 4.9)
6‴			8.77 d (5.0)	8.52 m	8.78 d (4.8)	8.79 d (4.8)

[a] 2-*O*-Benzoyl group: δ_H 8.11 (2H, d, J = 7.5 Hz, H-2′,6′). 7.40 (2H, t, J = 7.7 Hz, H-3′,5′), 7.53 (1H, t, J = 7.4 Hz, H-4′). [b] 2-*O*-Nicotinoyl: 9.31 (1H, s, H-2″), 8.44 (1H, d, J = 7.8 Hz, H-4″), 7.37 (1H, t, J = 7.8, 5.0 Hz, H-5″), 8.77 (1H, d, J = 5.0 Hz, H-6″). [c] Measuered in benzene-d_6 at 600 MHz; 2-*O*-nicotinoyl group: δ_H 9.85 (1H, s, H-2″), 8.52 (1H, m, H-4″), 6.88 (1H, dd, J = 7.8, 4.8 Hz, H-5″), 8.57 (1H, d, J = 4.8 Hz, H-6″).

OAc-15/H-13. The 9α-nicotinoyl group was proved by the cross-peaks between H-18/H-2″, OAc-5/H-2″, OAc-5/H-4″, H-7/H-2″, H-7/H-4″, and H-12/H-4″. All these data led to the identification of esulatin K as depicted in structural formula **4**.

Esulatin L (**5**) was isolated as an amorphous solid with $[\alpha]^{22}_D$ −82 (*c* 0.1, $CHCl_3$). Its molecular formula, $C_{38}H_{49}O_{13}N$, was determined by means of HRESIMS, which displayed a protonated molecular ion peak at *m/z* 728.3277 $[M + H]^+$. The ^{1}H NMR and JMOD spectra exhibited typical signals for one nicotinoyl group (δ_H 9.20 s, 8.22 d, 7.38 dd, and 8.78 d; δ_C 164.0, 151.1, 125.7, 137.0, 123.3, and 153.6) and four acetyl groups (δ_H 2.18 s, 2.16 s, 2.15 s, and 2.13 s; δ_C 170.5, 170.2, 169.5, and 168.9 (CO) and 21.3, 21.2, 21.1, and 21.0 (CH_3)). Evaluation of the ^{1}H−^{1}H COSY, HSQC, and HMBC spectra of **5** led to the conclusion that esulatin L was based on the same diterpene polyol as that of esulatin K (**4**), but in compound **5** the 2-*O*-nicotinoyl group was replaced by an acetyl group and the 7-*O*-acetyl group by an isobutanoyl group. A careful comparison of the NOESY spectra of **4** and **5** enabled the same relative configuration for esulatin L (**5**) and esulatin K (**4**) to be assumed. The structure of esulatin L was therefore determined as 2α,3β,5α,15β-tetraacetoxy-7β-isobutanoyloxy-9α-nicotinoyloxyjatropha-6(17),11*E*-dien-14-one (**5**).

Esulatin M (**6**) was isolated as an amorphous solid with $[\alpha]^{22}_D$ −42 (*c* 0.1, $CHCl_3$). It gave the molecular formula $C_{36}H_{47}O_{11}N$ from the HRESIMS (*m/z* 670.3222 $[M + H]^+$) and from NMR analysis. Interpretation of the ^{1}H, ^{13}C, ^{1}H−^{1}H COSY, NOESY, HSQC, and HMBC NMR spectra led to the structure 3β,5α,15β-triacetoxy-7β-isobutanoyloxy-9α-nicotinoyloxyjatropha-6(17),11-dien-14-one for this compound, and the NMR assignments are presented in Tables 2 and 3. The only difference between esulatin L (**5**) and esulatin M (**6**) is the lack of an acetyl group at C-2 in **6**.

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Table 3. ^{13}C NMR Data of Compounds 2−6 [125 MHz, $CDCl_3$]

position	2[a]	3	4[b]	5	6
1	46.6	45.3	48.3	47.0	46.0
2	87.2	38.3	88.1	86.4	38.3
3	78.4	76.6	77.6	78.1	76.5
4	49.7	52.8	49.8	49.3	52.8
5	68.0	68.1	68.5	68.8	68.6
6	146.6	146.9	146.3	146.4	146.8
7	69.3	69.1	68.9	67.7	68.4
8	45.7	46.0	34.4	35.1	34.6
9	209.3	209.6	75.7	75.6	75.8
10	49.3	49.3	40.2	40.6	40.8
11	137.4	137.2	138.4	138.8	138.3
12	132.7	132.7	130.6	130.7	130.5
13	44.6	44.7	43.8	43.9	43.6
14	209.5	211.8	210.9	211.2	212.4
15	92.1	92.0	92.6	92.6	92.5
16	17.8	13.2	18.2	17.9	13.3
17	110.4	109.6	110.8	111.3	110.5
18	26.8	26.8	22.2	22.2	26.8
19	22.9	23.0	27.5	27.0	22.5
20	20.1	20.0	19.6	19.5	19.3
Acetyls					
COMe-2				170.5	
COMe-2				21.3	
COMe-3	169.0	169.8	168.8	168.9	169.7
COMe-3	21.4	21.3	21.3	21.0	21.2
COMe-5	169.7	169.8	169.5	169.5	169.6
COMe-5	21.3	21.3	21.1	21.1	21.2
COMe-7	169.4		169.6		
COMe-7	20.1		20.4		
COMe-15	170.1	170.3	170.3	170.2	170.4
COMe-15	20.4	21.0	21.3	21.2	21.2
O-*i*Bu-7					
1′		176.0		175.2	175.7
2′		33.8		33.5	33.7
3′		18.8		18.8	18.8
4′		18.7		17.9	18.0
O-nicotinoyl-9					
CO			164.1	164.0	164.1
2″			150.9	151.1	151.1
3″			125.8	125.7	125.8
4″			136.7	137.0	136.9
5″			123.5	123.3	123.5
6″			153.6	153.6	153.6

[a] 2-*O*-Benzoyl group: δ_C 165.6 (CO), 131.1 (C-1′), 130.0 (C-2′,6′), 128.1 (C-3′,5′), 132.6 (C-4′). [b] 2-*O*-Nicotinoyl group: δ_C 164.3 (CO), 151.2 (C-2″), 127.4 (C-3″), 137.5 (C-4″), 123.2 (C-5″), 152.9 (C-6″).

Compounds **7**−**9** were identified as known metabolites of the genus *Euphorbia*, but are described for the first time from *E. esula*. ^{1}H and ^{13}C NMR spectroscopic investigations proved that compound **7** was identical with 2α,3β,5α,7β,15β-pentaacetoxy-9α-nicotinoyloxyjatropha-6(17),11-dien-14-one, isolated previously from *E. peplus*.[28,29] Compound **8** proved to be identical with the 17-ethyl bis-homojatrophane-type lactone salicinolide, and compound **9** with the modified jatrophane euphosalicin. The latter two compounds were isolated previously from *E. salicifolia*.[30,31]

The isolated compounds (**1**−**9**), together with five jatrophane diterpenes (**10**−**14**) identified in our earlier experiments, were tested for their antiproliferative activity against a set of human adherent cell lines of gynecological origin [HeLa (cervix adenocarcinoma), Ishikawa (endometrial adenocarcinoma), and MCF7 (breast epithelial adenocarcinoma)] using the MTT test and cisplatin as positive control. The antiproliferative assay revealed that the isolated diterpenes possess different cell growth-inhibitory activities (Table 4); substantial effects were recorded only at the higher tested concentration: 30 μg/mL. Esulatins J (**3**), A (**13**), and E (**14**) were the most effective against all cell lines; especially esulatin J (**3**) exhibited high cell growth-inhibitory activity on Ishikawa (98.4% at 30 μg/mL) and MCF7 (81.4% at 30 μg/mL) cells. Esulatin I (**2**) and esulatin B (**10**) displayed marked inhibitory effects on MCF7 (60.1% and 43.3% at 30 μg/mL). The structure−activity relationships are not easy to evaluate, because the structural variations within the set of compounds are very complex, stemming from the variations in the substitution at C-2, C-7, and C-9, from the nature of the ester groups at C-2, C-3, C-5, C-7, C-8, and C-9, from the number and positions of the double bonds, and from the presence or absence of a keto or epoxy functionality. The most potent compounds found, esulatins I, J, B, and E (**2**, **3**, **10**, and **14**), are tetra- or pentaesters of jatrophane polyols, which contain a keto group at C-9. Moreover, esulatin A (**13**), containing an epoxy group at C-11−C-12, proved also be effective against all three cell lines.

Compounds **1**−**13** were tested for their MDR-reversing activity on L5178 mouse lymphoma cells, using a standard functional assay with Rhodamine 123 as a fluorescent substrate analogue of epirubicin. The results showed that the isolated diterpenes differ significantly in the inhibition of the efflux pump activity of P-gp in tumor cells (Table 5). Within the set of compounds investigated, esulatin J (**3**) [fluorescence activity ratio (R) = 52.5 at 40 μg/mL] and esulatin M (**6**) (R = 119.9 at 40 μg/mL) were found to be the most powerful inhibitors of efflux pump activity. Their efficacy was 2−5-fold higher than that of the standard modulator verapamil, taken as a positive control (R = 23.2 at 10 μg/mL); thus, both compounds **3** and **6** appear to be promising leads for the drug development to overcome the MDR of cancer cells. Compounds **2**, **4**, **5**, **8**, **9**, **11**, and **12** revealed lower anti-MDR potency, and **1**, **7**, **10**, and **13** were ineffective in the test model used.

In terms of preliminary structure−activity relationships, only a few conclusions can be made, because the compounds tested do not comprise a structurally uniform series. The present set of compounds suggested the involvement of ring A in P-gp binding, since the lack of oxygenation at C-2 dramatically increased the MDR-modulatory activity in the cases of **3** and **6**, relative to all other compounds evaluated. A similar observation was reported earlier for analogous jatrophane polyesters isolated from *E. dendroides*.[18] Furthermore, the presence of an aromatic ester group (benzoyl or nicotinoyl) instead of an acetyl group at C-2 has a positive role in the modulation of the drug accumulation of mouse lymphoma cells, as exemplified by the efficacy of the compared pairs **4** and **7** or **2** and **10**. On the other hand, substitution at C-7 also influenced the resistance-modifying ability, as demonstrated by the fluorescence activity ratios of compounds **7** (R = 1.7 at 4 μg/mL) and **5** (R = 23.8 at 4 μg/mL), which differed only in the 7-*O*-acetyl/7-*O*-isobutanoyl groups. In

Table 4. Inhibition (%) of Tumor Cell Proliferation of Diterpenes 1−14

	inhibition (%) ± SEM					
	HeLa		Ishikawa		MCF7	
compound	10 μg/mL	30 μg/mL	10 μg/mL	30 μg/mL	10 μg/mL	30 μg/mL
esulatin H (**1**)	10.6 ± 2.3	17.8 ± 1.1	12.0 ± 0.9	17.7 ± 1.5	9.8 ± 2.9	11.4 ± 2.4
esulatin I (**2**)	15.4 ± 1.0	23.8 ± 3.0	8.0 ± 2.9	29.4 ± 1.3	12.7 ± 2.1	60.1 ± 2.7
esulatin J (**3**)	19.1 ± 3.0	64.5 ±2.3	18.4 ± 2.0	98.4 ± 0.1	46.8 ± 2.1	81.4 ± 2.6
esulatin K (**4**)	0.5 ± 2.5	0.6 ± 2.2	7.5 ± 0.5	0.1 ± 2.2	1.3 ± 2.8	21.3 ± 1.2
esulatin L (**5**)	6.4 ± 2.1	1.1 ± 1.0	17.5 ± 2.9	26.6 ± 2.7	3.6 ± 1.6	16.7 ± 2.8
esulatin M (**6**)	18.3 ± 1.4	4.5 ± 1.9	5.9 ± 2.4	16.4 ± 2.1	18.5 ± 2.1	14.4 ± 2.1
7	13.9 ± 0.6	17.9 ± 0.3	8.1 ± 2.7	13.9 ± 0.1	20.3 ± 1.8	36.3 ± 0.8
8	5.3 ± 1.2	20.8 ± 1.7	31.5 ± 0.1	33.0 ± 2.5	24.8 ± 1.3	29.2 ± 2.5
9	14.5 ± 1.3	18.1 ± 2.2	0.9 ± 3.3	7.6 ± 2.0	6.3 ± 1.1	20.0 ± 2.8
esulatin B (**10**)	17.3 ± 3.0	36.3 ± 2.3	11.6 ± 1.8	35.0 ± 2.1	29.9 ± 1.8	43.3 ± 1.6
esulatin D (**11**)	11.4 ± 1.1	26.7 ± 2.7	29.4 ± 2.5	29.9 ± 1.2	9.5 ± 1.3	13.5 ± 1.9
esulatin F (**12**)	2.9 ± 2.4	10.9 ± 2.3	21.4 ± 0.7	17.1 ± 2.4	8.7 ± 2.3	23.3 ± 2.4
esulatin A (**13**)	16.3 ± 0.9	62.6 ± 2.4	20.1 ± 2.8	53.8 ± 2.0	21.4 ± 2.8	47.9 ± 2.0
esulatin E (**14**)	19.5 ± 2.8	58.1 ± 2.5	35.6 ± 2.8	54.1 ± 2.9	30.4 ± 2.4	61.4 ± 2.7
cisplatin	12.4 ± 1.1[a]		3.5 ± 0.5[a]		9.6 ± 0.8[a]	

[a] IC_{50} values expressed in μM.

our experiment, the positive effect played by a carbonyl versus an *O*-acyl group at position C-9 (reported earlier for jatrophane diterpenes in another test system) could not be observed.[18]

In conclusion, the most promising compound in the isolated set of *E. esula* metabolites was esulatin J (**3**), which exerted the highest antiproliferative effect against the tested cancerous cells and also inhibited the efflux pump activity of tumor cells. This compound can be regarded as promising for drug development and worthy of more detailed studies.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were determined in chloroform by using a Perkin-Elmer 341 polarimeter. NMR spectra were recorded on a Bruker Ultrashield Plus 600 spectrometer at 600 MHz (^{1}H) and 150 MHz (^{13}C) and on a Bruker Avance DRX 500 spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C), with TMS as internal standard. Two-dimensional data were acquired and processed with standard Bruker software. In the two-dimensional NMR experiments, gradient-enhanced versions were used. Low-resolution ESI mass spectra were recorded on an Applied Biosystems 3200QTrap instrument. Samples were acquired in flow injection mode. High-resolution MS data were recorded on a Shimadzu IT-TOF mass spectrometer equipped with an electrospray source. The resolution was over 10 000. Column chromatography was carried out on polyamide (ICN); vacuum liquid chromatography (VLC) on silica gel G (15 μm, Merck); rotation planar chromatography (RPC) on silica gel 60 GF_{254} with a Chromatotron instrument (Harrison Research); HPLC on a LiChrospher RP-18 (5 μm, 250 × 4 mm, Merck) column with a Waters instrument; preparative thin-layer chromatography (preparative TLC) on silica gel 60 F_{254} and RP-18 F_{254} plates (Merck); and HPLC on a Waters instrument with detection at 254 nm on LiChrospher RP-18 (5 μm, 250 × 4 mm, Merck) column using MeOH−H_2O (7:3) as mobile phase at 3.0 mL/min flow rate.

Plant Material. *Euphorbia esula* was collected in the flowering period from wild stock on the banks of the River Tisza, in Szeged, Hungary, in June 2004. 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. 773) has been deposited at the Herbarium of the Department of Pharmacognosy, University of Szeged, Szeged, Hungary.

Extraction and Isolation. The whole fresh plants of *E. esula* (8 kg) were extracted with MeOH (60 L) at room temperature. The crude extract was concentrated in vacuo and partitioned between CH_2Cl_2 (5 × 1.5 L) and H_2O. On evaporation, the organic phase residue (95 g) was obtained, which was chromatographed over a polyamide column (500 g) with mixtures of H_2O−MeOH (4:1, 3:2, 2:3, and 1:4) as eluents. The fractions obtained with H_2O−MeOH (4:1) were subjected to silica gel VLC using a gradient system of cyclohexane−EtOAc−EtOH. Fractions were combined into six fractions (I−VI) according to TLC monitoring. Compound **8** (105 mg, mp 262−268 °C) was crystallized from fractions II and III, which were eluted with cyclohexane−EtOAc−EtOH, 40:10:0.5 and 70:30:1, respectively. Fraction II was subjected twice to RPC and eluted with cyclohexane−EtOAc−EtOH (from 80:20:0.5 to 30:10:1) and benzene−CH_2Cl_2−Et_2O (from 2:1:0.5 to 1:1:2) of increasing polarity to yield compounds **1** (5.7 mg) and **5** (11.8 mg). Fraction IV was separated by reversed-phase VLC with a gradient system of MeOH−H_2O (3:2, 7:3, 4:1, 9:1, and 1:0). From the fractions obtained with MeOH−H_2O (7:3), compound **7** (10.0 mg) was crystallized. Fraction V was subjected to silica gel RPC, using a gradient system of cyclohexane−EtOAc−EtOH (from 5:5:0 to 1:1:1). Fractions obtained by elution with cyclohexane−EtOAc−EtOH (5:5:0) were further purified by reversed-phase HPLC with MeCN−H_2O (3:2) as eluent at a flow rate of 1.0 mL/min, to afford compound **4** (3.9 mg). The fractions eluted with cyclohexane−EtOAc−EtOH (5:5:0) were rechromatographed on silica gel by preparative TLC, using cyclohexane−EtOAc−EtOH (5:5:2) as developing system, and then by reversed-phase HPLC, using MeCN−H_2O (3:2) as eluent at a flow rate of 1.0 mL/min, yielding compound **9** (30 mg, mp 134−138 °C). The combined fractions obtained with H_2O−MeOH (3:2) from the polyamide column were subjected to silica gel VLC, using a gradient system of cyclohexane−EtOAc−EtOH (from 30:10:1 to 5:5:4). Fraction III of this chromatography, eluted with cyclohexane−EtOAc−EtOH (30:10:1), was subjected to silica gel RPC, using cyclohexane−EtOAc−EtOH mixtures of increasing polarity (from 40:10:0 to 30:10:1). Final

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Table 5. Reversal of the Multidrug Resistance of Mouse Lymphoma Cells by Diterpenoids Isolated from *Euphorbia esula*

compound	concentration μg/mL	fluorescence activity ratio[a]
esulatin H (**1**)	4	1.4
	40	1.0
esulatin I (**2**)	4	3.9
	40	19.9
esulatin J (**3**)	4	24.9
	40	52.5
esulatin K (**4**)	4	7.2
	40	19.8
esulatin L (**5**)	4	23.8
	40	36.4
esulatin M (**6**)	4	16.6
	40	119.9
7	4	1.7
	40	2.3
8	4	5.9
	40	17.5
9	4	1.9
	40	12.3
esulatin B (**10**)	4	2.6
	40	7.8
esulatin D (**11**)	4	4.6
	40	15.2
esulatin F (**12**)	4	5.5
	40	14.8
esulatin A (**13**)	4	2.0
	40	1.9
verapamil	10	23.2

[a] For determination of fluorescence activity ratio, see the Experimental Section.

purification of the fractions obtained with cyclohexane−EtOAc−EtOH (70:20:2 and 30:10:1) was carried out by reversed-phase HPLC with MeOH−H_2O (7:3) at a flow rate of 3.0 mL/min. The compounds detected at retention times of 23.7, 26.1 and 35.4 min were **2** (17.6 mg), **3** (5.2 mg), and **6** (25.0 mg), respectively.

*Esulatin H (**1**):* amorphous solid; $[\alpha]^{22}_D$ −4 (*c* 0.1, $CHCl_3$); ^{1}H and ^{13}C NMR data see Table 1; HRESIMS *m/z* 821.3204 $[M + Na]^+$ (calcd for $C_{38}H_{54}O_{18}Na$, 821.3208).

*Esulatin I (**2**):* white crystals; mp 212−215 °C; $[\alpha]^{22}_D$ −152 (*c* 0.1, $CHCl_3$); ^{1}H and ^{13}C NMR data see Tables 2 and 3; HRESIMS *m/z* 677.2554 $[M + Na]^+$ (calcd for $C_{35}H_{42}O_{12}Na$, 677.2568), 672 $[M + NH_4]^+$, 473 $[533 - AcOH]^+$, 413 $[473 - AcOH]^+$, 353 $[413 - AcOH]^+$, 293 $[353 - AcOH]^+$.

*Esulatin J (**3**):* amorphous solid; $[\alpha]^{22}_D$ −113 (*c* 0.1, $CHCl_3$); ^{1}H and ^{13}C NMR data see Tables 2 and 3; HRESIMS *m/z* 585.2664 $[M + Na]^+$ (calcd for $C_{30}H_{42}O_{10}Na$, 585.2675).

*Esulatin K (**4**):* amorphous solid; $[\alpha]^{22}_D$ −140 (*c* 0.1, $CHCl_3$); ^{1}H and ^{13}C NMR data see Tables 2 and 3; HRESIMS *m/z* 763.3075 $[M + H]^+$ (calcd for $C_{40}H_{47}O_{13}N_2$, 763.3073), 640 $[(M + H) - C_6H_5NO_2]^+$, 580 $[640 - AcOH]^+$, 598 $[640 - CH_2CO]^+$, 538 $[598 - CH_3COOH]^+$, 478 $[538 - CH_3COOH]^+$, 418 $[478 - CH_3COOH]^+$, 312 $[(M + H) - C_6H_5NO_2 - 4 \times CH_3COOH - (CH_3)_2CHCOOH]^+$, 295 $[312 - H_2O]^+$.

*Esulatin L (**5**):* amorphous solid; $[\alpha]^{22}_D$ −82 (*c* 0.1, $CHCl_3$); ^{1}H and ^{13}C NMR data see Tables 2 and 3; HRESIMS *m/z* 728.3264 $[M + H]^+$ (calcd for $C_{38}H_{50}O_{13}N$, 728.3277), 626 $[686 - AcOH]^+$, 566 $[626 - AcOH]^+$, 506 $[566 - AcOH]^+$, 418 $[566 - i$BuOH$]^+$, 295 $[418 - NicOH]^+$.

*Esulatin M (**6**):* amorphous solid; $[\alpha]^{22}_D$ −42 (*c* 0.1, $CHCl_3$); ^{1}H and ^{13}C NMR data see Tables 2 and 3; HRESIMS *m/z* 670.3204 $[M + H]^+$ (calcd for $C_{36}H_{48}O_{11}N$, 670.3222).

Assay for Antiproliferative Effect. Antiproliferative effects were measured in vitro on three human cell lines (HeLa, Ishikawa, and MCF-7) by means of the MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) assay.[32] Briefly, a limited number of human cancer cells (5000/well) were seeded onto a 96-well microplate and became attached to the bottom of the well overnight. On the second day of the procedure, the original medium was removed and 200 μL of new medium containing the test substances was added. After an incubation period of 72 h, the living cells were assayed by the addition of 20 μL of 5 mg/mL MTT solution. MTT was converted by intact mitochondrial reductase and precipitated as blue formazan crystals during a 4 h contact period. The medium was then removed, and the precipitated formazan was dissolved in 100 μL of DMSO during a 60 min period of shaking. Finally, the reduced MTT was assayed at 545 nm using a microplate reader, with untreated cells being taken as the negative control. All in vitro experiments were carried out on two microplates with at least five parallel wells. Cisplatin were used as positive control substance. Stock solutions of 10 mg/mL of the tested compounds and extracts were prepared with DMSO. The highest DMSO concentration (0.3%) of the medium did not have any significant effect on the cell proliferation. Cisplatin was obtained from Ebewe Pharma GmbH, Unterach, Austria, as concentrated solution for intravenous use in human clinical practice.

Assay for MDR-Reversing Activity. The L5178 mouse T-cell lymphoma cell line was transfected with the pHa MDR1/A retrovirus, as previously described.[33] 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 2×10^6/mL, resuspended in serum-free McCoy's 5AA medium, and distributed in 0.5 mL aliquots into Eppendorf centrifuge tubes. The compounds to be tested were added (4.0 and 40.0 μg/mL in DMSO), and the samples were then incubated for 10 min at room temperature. Then, 10 μL (5.2 μM final concentration) of the indicator Rhodamine 123 (Sigma) was 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 of phosphate-buffered saline for analysis. The fluorescence of the cell population was measured by flow cytometry with a Becton Dickinson FACScan instrument. Verapamil (Sanofi-Synthelabo) was used as a positive control in the Rhodamine 123 exclusion experiments. The fluorescence activity ratio (R) was calculated from the drug accumulation of treated MDR and untreated MDR cells related to parental treated per untreated drug-sensitive cells, on the basis of the measured fluorescence values, via the following equation:

$$\text{Fluorescence activity ratio } (R) = \frac{\text{MDR treated/MDR control}}{\text{parental treated/parental control}}$$

■ ASSOCIATED CONTENT

Supporting Information. Copies of the 1D and 2D NMR spectra of compounds **1**−**6** are available free of charge via the Internet at http://pubs.acs.org.

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