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Isolation and Biological Evaluation of Jatrophane Diterpenoids from *Euphorbia dendroides*

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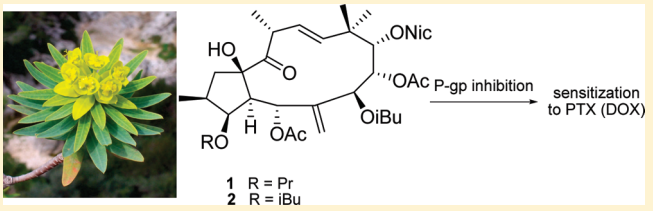
Supporting Information

ABSTRACT: From the Montenegrin spurge *Euphorbia dendroides*, seven new diterpenoids [jatrophanes (**1**−**6**) and a tigliane (**7**)] were isolated and their structures elucidated by spectroscopic techniques. The biological activity of the new compounds was studied against four human cancer cell lines. The most effective jatrophane-type compound (**2**) and its structurally closely related derivative (**1**) were evaluated for their interactions with paclitaxel and doxorubicin using a multidrug-resistant cancer cell line. Both compounds exerted a strong reversal potential resulting from inhibition of P-glycoprotein transport.



Euphorbia dendroides L. (Euphorbiaceae) is a small tree distributed in the Mediterranean region. Recent studies have shown macrocyclic jatrophane diterpenoids from *Euphorbia* species as a new structural class of inhibitors of P-gp.[1−3] As part of an investigation of spurges from the southeastern Balkan region, a jatrophane diterpenoid fraction was purified from *E. dendroides*. Isolation and structure determination work yielded six new jatrophanes (**1**−**6**) and a new tigliane (**7**).

Two of the jatrophanes (**1** and **2**) were shown to behave as potent multi-drug-resistant (MDR) modulators, each reversing resistance to paclitaxel in NCI-H460/R cells (a MDR cancer cell line).[4] The sensitivity of NCI-H460/R cells to another anticancer chemotherapeutic agent, doxorubicin, was also investigated in the presence of these jatrophanes. Described herein are the isolation, structure elucidation, and biological evaluation of compounds **1**−**7**.

RESULTS AND DISCUSION

The exhaustive extraction of a lyophilized aqueous ethanol extract of *E. dendroides* with hexane, followed by application of several preparative chromatographic techniques, afforded six new jatrophanes, euphodendrophanes A−F (**1**−**6**), and the tigliane euphodendriane A (**7**).

Euphodendrophane A (**1**) was obtained as a colorless, amorphous solid. The molecular formula, $C_{37}H_{49}NO_{12}$, was determined on the basis of an ion at m/z 700.3335 (calcd 700.3327) in the HRESIMS. The structural elucidation of **1** was achieved with the use of 1D and 2D NMR spectroscopy, as well as comparison of the NMR spectra obtained for jatrophane polyesters isolated previously from *E. dendroides*[5] and other *Euphorbia* species, possessing similar acylation patterns, namely, *E. turczaninowii*,[6] *E. obtusifolia*,[7] *E. terracina*,[8] *E. amygdaloides*,[9] and *E. altotibetic*.[10]

The overall NMR characteristics were suggestive of **1** being a diterpenoid bearing five ester functionalities. The NMR data (Tables 1 and 2) revealed the nature of the ester moieties as two acetates, a propionate, an isobutyrate, and a nicotinate. According to its ^{13}C and ^{1}H NMR spectra, compound **1** was found to contain six oxygenated sp^3 carbons (five secondary and one tertiary), with five of these bearing ester groups and the remaining one a hydroxy group (δ_H 2.81 s, exchangeable with D_2O). The NMR spectra also showed a signal for a keto carbonyl (δ_C 214.7), two double bonds (one exocyclic and one *trans*-disubstituted), an aliphatic methylene, four methyls (two ^{1}H NMR

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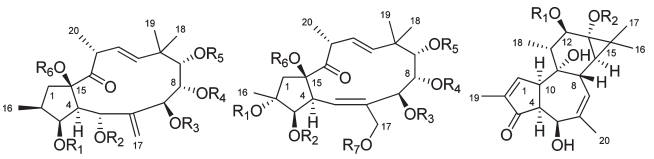
doublets and two singlets), and a sp^3 quaternary carbon (δ_C 40.6). This, in combination with the molecular formula, corresponding to 14 double-bond equivalents, indicated a bicyclic diterpene skeleton.

Chart 1



1-5 **6** **7**

	R_1	R_2	R_3	R_4	R_5	R_6	R_7
1	Pr	Ac	iBu	Ac	Nic	H	-
2	iBu	Ac	iBu	Ac	Nic	H	-
3	Pr	Ac	iBu	Ac	Nic	Ac	-
4	iBu	Ac	Ac	Bz	Ac	H	-
5	Pr	Ac	iBu	Bz	Ac	H	-
6	Ac	Ac	iBu	Ac	Nic	Ac	Ac
7	Bz	iBu	-	-	-	-	-

iBu – isobutanoyl; Ac – acetyl; Pr – propionyl; Nic – nicotinyl; Bz - benzoyl

The molecular framework of **1** was established by the application of 2D NMR techniques (COSY, HMBC, and NOESY). Thus, the observed COSY correlations (denoted in parentheses) enabled identification of three independent proton coupling networks: **A** (H_2-1/H-2, H-2/H_3-16, H-2/H-3, H-3/H-4, and H-4/H-5), **B** (H-7/H-8 and H-8/H-9), and **C** (H-11/H-12, H-12/H-13, and H-13/H_3-20) denoted with bold lines in Figure 1. The $^{2,3}J_{C-H}$ correlations inferred from the HMBC spectrum allowed the connections of **A**, **B**, and **C** to be established (Figure 1). Thus, the long-range correlations of H-5 and H-7 with C-6 and C-17 (exocyclic double bond) indicated the connection of **A** and **B** via the olefinic C-6. HMBC cross-peaks of protons attributed to the *gem*-dimethyl groups H_3-19 and H_3-18 (positioned at C-10) with C-9, C-10, and C-11 defined the connection between **B** and **C**, through C-10. Finally, a HMBC correlation of the ketone carbonyl (C-14) with H-13, H_3-20, and the H_2-1 methylene revealed the linkage of **C** and **A**. This evidence led to the diterpenoid carbon framework of **1** being proposed as a bicyclo[10.3.0]pentadecane with 2,10,10,13-tetramethyl-6-*exo*-methylene, commonly known as a jatrophane skeleton, with oxygenated carbons at positions 3, 5, 7, 8, 9, 14

Table 1. ^{1}H NMR Data for Compounds 1−5 [500 MHz, $CDCl_3$, TMS, δ (ppm) (J = Hz)]

position	1	2	3	4	5
1α	2.51 dd (14; 11)	2.53 dd (14; 11)	3.06 dd (14; 6.5)	2.51 brs	2.40 m
1β	1.59 dd (14; 11)	1.60 dd (14; 11)	1.64 bt (14)	1.58 dd	1.60 t (12)
2	2.26 m	2.26 m	2.29 m	2.25 brs	2.29 bm
3	5.55 t (4.5)	5.55 t (4.5)	5.41 bs	5.57 brs	5.60 brs
4	3.13 brs	3.13 brs	2.79 d (3.0)	3.12 brs	3.07 brs
5	5.43 brs	5.41 brs	5.83 brs	5.47 brs	5.42 brs
7	5.08 brs	5.07 brs	5.08 brs	4.88 brs	4.82brs
8	5.63 brs	5.68 brs	5.53 brs	5.65 brs	5.70 brs
9	5.22 brs	5.22 brs	5.21 d (2)	5.06 brs	5.06 brs
11	5.92 d (15.5)	5.92 d (15.5)	5.94 d (16)	5.90 d (16.0)	5.86 d (16.0)
12	5.71 m	5.71 m	5.76 m	5.83 m	5.92 m
13	4.25	4.25	3.61 m	4.20 m	4.21 m
16	0.93 d (6.5)	0.93 d (6.5)	0.92 d (6.5)	0.93 d (6.5)	0.93 d (6.5)
17a	5.06 brs	5.05 brs	5.06 brs	5.01 brs	4.96 brs
17b	5.14 brs	5.14 brs	5.09 brs	5.08 brs	5.03 brs
18	1.08 brs	1.07 brs	1.06 brs	0.98 brs	0.96 brs
19	1.33 brs	1.34 brs	1.25 brs	1.39 brs	1.42 brs
20	1.22 d (7.02)	1.23 d (7.02)	1.20 d (6.0)	1.22 d (7.0)	1.16 d (7.0)
OR_1-3	2.41 dq (7.5; 4.5)	2.64 h (7.0)	2.40 dq (7.5; 4.5)	2.60 h (7.0)	2.40 q (7.5)
	1.19 t (7.5)	1.27 (7.0)	1.19 d (7.5)	1.26 d (7.0)	1.17 t (7.5)
		1.22 (7.0)		1.19 d (7.0)	
OR_2-5	2.07 s	2.08 s	2.03 s	2.09 s	2.09 s
OR_3-7	1.95 bm	1.98 bm	2.49 h (7.5)	2.07 s	2.06 h (7.0)
	0.86 brs	0.86 d (7.0)	0.93d (7.0)		1.20 d (7.0)
	0.65 brs	0.63 d (7.0)	0.73 brs		1.16 d (7.0)
OR_4-8	1.98 s	1.98	2.10 s	8.01 d (8.5)	8.02 d (8.5)
				7.54 t (7.5)	7.56 t (7.5)
				7.42 (8.0)	7.43 (8.0)
OR_5-9	9.25 brs	9.26 brs	9.21 brs	1.95 s	1.97 s
	8.80 brs	8.82 brs	8.81 brs		
	8.26 brd	8.26 brd	8.20 brd		
	7.45 brdd	7.47 brdd	7.45 brdd		
OR_6-15	2.81 s	2.81 s	2.12 s		

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Table 2. ^{13}C NMR Data for Compounds 1−5 [(125 MHz, $CDCl_3$, TMS, δ(ppm)]

position	1	2	3	4	5[a]
1	49.9	50.0	45.8	50.0	49.7
2	379	38.0	38.3	38.2	38.3
3	77.7	77.6	76.2	77.5	77.3
4	53.8	53.7	52.8	53.6	53.9
5	68.0	68.0	67.8	68.8	68.7
6	144.9	144.8	144.7	145.5	145.9
7	69.0	69.1	68.4	69.7	69.0
8	70.8	70.8	71.1	71.2	71.3
9	81.4	81.4	80.0	80.7	80.6
10	40.6	40.9	40.2	40.5	40.6
11	137.6	137.6	137.0	137.8	137.7
12	127.7	127.8	131.5	127.1	127.0
13	40.8	40.6	42.8	39.9	39.8
14	214.7	214.6	212.3	214.7	215.1
15	87.7	87.9	92.9	87.5	87.1
16	13.7	13.7	13.4	13.6	13.5
17	113.6	113.8	111.1	111.8	110.9
18	25.2	25.3	26.9	26.3	26.6
19	24.9	24.8	24.0	23.8	23.4
20	18.6	18.6	19.9	18.2	18.3
OR_1-3					
1′	173.1	175.6	173.0	175.5	173.2
2′	27.7	34.3	28.0	34.4	27.8
3′	9.1	19.8	9.2	18.6	9.3
4′		18.5		19.7	
5-R_2					
1″	170.0	169.1	169.5	169.2	169.2
2″	20.8	21.1	21.1	20.7	20.8
OR_3-7					
1‴	174.9	174.9	175.5	169.2	174.9
2‴	33.7	33.7	33.7	20.8	34.1
3‴	17.9	17.9	18.8		19.2
4‴	18.0	18.0	17.9		18.5
OR_4-8					
1⁗	169.1	170.0	170.5	165.2	165.2
2⁗	21.0	20.8	21.1	130.0	129.8
3⁗				129.9	129.9
4⁗				128.3	128.6
5⁗				132.98	133.15
OR_5-9					
1⁗′	164.1	164.1	164.0	169.9	170.0
2⁗′	151.4	151.5	151.3	21.2	21.0
3⁗′	125.3	125.4	125.3		
4⁗′	137.1	137.1	137.0		
5⁗′	123.5	123.5	123.6		
6⁗′	153.7	153.8	153.9		
OR_6-15					
1⁗″			169.0		
2⁗″			20.6		

[a] Data obtained from the HMBC and HSQC spectra.

(ketone), and 15, with the last bearing a free hydroxy group, according to a HMBC cross-peak correlation between OH/C-15.

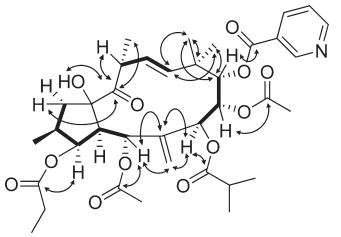

Figure 1. COSY (—) and selected HMBC correlations of **1**.

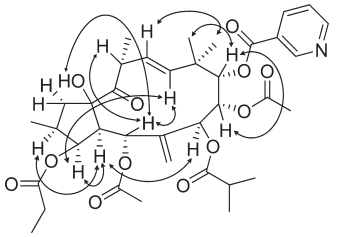

Figure 2. Key NOESY correlations of **1**.

The cross-peaks of the ester carbonyls with oxymethine protons showed the positions of the ester groups. Thus, HMBC correlations of H-9 with the nicotinate carbonyl (δ_C 164.1), H-7 with the isobutyrte carbonyl (δ_C 174.9), and H-3 with the propionate carbonyl (δ_C 173.0) supported the locations of the nicotinate at C-9, the isobutyrate at C-7, and the propionate at C-3, respectively. In addition, HMBC cross-peaks of H-5 and H-8 with the carbonyl of both acetates (δ_C170.0 and 169.1, respectively) were used to define C-5 and C-8 as the positions of the acetoxy groups. The parallel orientation of the ester groups at C-7 and C-9 was reflected in the upfield shift of the methyls from the isobutyrate moiety (δ_H 0.86 and 0.65), caused by the aromatic ring current effect of the nicotinate at C-9.[11]

The relative configuration of **1** was assigned from its scalar and dipolar couplings (Figure 2) as well as comparison of the NMR spectroscopic data with those of closely related compounds.[12−14] Assuming a H-4α-configuration on a biogenetic basis,[15] the NOE cross-peaks of this proton (H-4/H-3 and H-4/H-2) were consistent with the β-orientation of both the 3-propionate and 2-methyl moieties. The 5β-H-configuration (and consequently OAc-5α) was deduced on the basis of vicinal coupling between H-4 and H-5 ($J_{4,5}$ = 0 Hz), suggesting an orthogonal (*trans*) relationship of these protons.

The NOESY correlation between H-4 and H-7 could be rationalized in terms of the 7β-position of the isobutyrate group in compound **1**. The absence of dipolar coupling between H-7 and H-8 was in accordance with an OAc-8α substituent. The occurrence of a NOE interaction between H-5 (δ_H 5.43) and H-11 (δ_H 5.92) indicated their spatial proximity and their disposition on the same (β) side of the macrocyclic ring. The cross-peaks observed between the pairs H-11/H-3 and H-5/H-13 defined the 13α-orientation of the methyl group. A large coupling constant between the H-11 olefinic protons ($J_{11,12}$ = 15.5 Hz) indicated the *E*-geometry of the endocyclic double bond, with H-11 located at the α-side of the diterpenoid core. The occurrence of a NOE between H-12 and one of the geminal methyl groups at C-10 (H_3-18, δ_H 1.08) was used to fix this methyl in the α-position and the remaining geminal methyl (H_3-19) in the β-position. NOE correlations between H_3-19 and H-9,

as well as between H-8 and H-9, indicated an α-orientation of the C-9-nicotinyloxy group. A NOE interaction of OH-15 with H-5 was used to define the relative configuration at C-15, with an OH-15β substituent occurring. The absence of a NOESY cross-peak between H-4 and OH-15 supported the *trans*-fusion of the cyclopentane ring, which is usual in jatrophane derivatives.[16,17]

The co-occurring euphodendrophanes B−F (**2**−**6**) were determined as being closely related to **1** structurally. With the exception of **6**, they were found to differ from euphodendrophane A (**1**) only in their acylation patterns. The elucidation of the structure and relative configuration of **2**−**6** was based on their NMR spectroscopic data (Tables 1 and 2) and by comparison with those of **1** and known jatrophane diterpenoids.[5−10]

Euphodendrophane B (**2**), an amorphous solid, gave a $[M + H]^+$ ion at m/z 714.3489 (calcd 714.3484) in the HRESIMS, corresponding to a molecular formula of $C_{38}H_{51}NO_{12}$, differing from that of **1** by an additional 14 amu (CH_2). Its ^1H and ^{13}C NMR spectra were almost identical with those of **1**. The only difference was the occurrence of signals typical for an additional isobutyrate ester group (instead of those for a propionate ester bonded to C-3 in **1**), including resonances at δ_H 2.64 (H-2′), 1.27 (H_3-3′), and 1.22 (H_3-4′) and δ_C 175.6 (C-1′), 34.28 (C-2′), 19.8 (C-3′), and 18.5 (C-4′). A three-bond HMBC correlation of the isobutyrate carbonyl (C-1′) with H-3 was also observed. The above evidence was in accordance with the structure of **2** being very similar to that of **1**, differing from the latter in the presence of a 3-isobutyrate group instead of a 3-propionate group. Additional proof for the relative configuration of **2** being the same as that in **1** was obtained by comparison of their NOESY spectra.

Compound **3** (euphodendrophane C) was isolated as an amorphous solid with the molecular formula $C_{39}H_{51}NO_{13}$, as established by the $[M + H]^+$ ion at m/z 742.3438 (calcd 742.3433) in the HRESIMS. ^1H and ^{13}C NMR data of **3**, fully assigned through 2D NMR experiments, closely resembled those of **1**. The only difference was the appearance of signals of an additional acetoxy group (δ_H 2.12 and δ_C 20.6 and 169.0) and the absence of resonances for a hydroxy group. The molecular formula of **3** was in accordance with the proposed structural elements, as evidenced in their ^1H and ^{13}C NMR spectra. The locations of the ester groups were confirmed through HMBC cross-peaks between the ester carbonyl carbons and the neighboring methine protons. The carbonyls of the nicotinate, propionate, and isobutyrate groups exhibited cross-peaks with H-9, H-3, and H-7, respectively, whereas two of the acetoxy carbonyls gave correlations with H-5 and H-8, thus indicating the same locations of these ester groups as in **1**. The remaining acetyl group (δ_H 2.12), without any long-range correlations with the protons from the rings, was situated at the ring junction (C-15). The acetoxy group caused paramagnetic shifts of H-1α and H-5 ($\Delta\delta$ = 0.55 and 0.40 ppm, respectively), while H-4 and H-13 underwent diamagnetic shifts ($\Delta\delta$ = −0.34 and −0.64 ppm, respectively), compared to compound **1**. The relative configuration of **3**, determined on the basis of a NOESY experiment, proved to be the same as in **1**. Euphodendrophane C is the 15-acetylated analogue of euphodendrophane A.

Euphodendrophane D (**4**) was obtained as an amorphous solid, with a molecular formula of $C_{37}H_{48}O_{12}$, assigned by HRESIMS from the m/z 702.3483 $[M + NH_4]^+$ ion (calcd 702.3484). Comparison of its ^1H and ^{13}C NMR data (Tables 1 and 2) with those of compound **1** showed close similarites between these compounds, again differing only in their ester portions. The

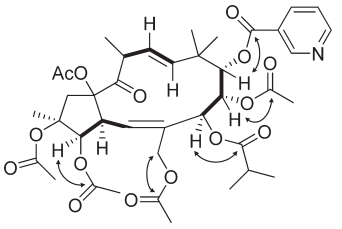

Figure 3. COSY (—) and selected HMBC correlations of **6**.

^1H and ^{13}C NMR spectra of **4** indicated the presence of a benzoate, an isobutyrate, and three acetates (Table 1). The presence of a benzoate ester was apparent from ^1H and ^{13}C NMR signals at δ_H 7.42, 7.54, and 8.01 and δ_C 165.2, 130.0, 129.9, 128.4, and 133.0, respectively. A HMBC correlation of H-8 and the carbonyl carbon of the benzoate indicated C-8 as the position of this group. The positions of the remaining ester groups, namely, 3-isobutyrate and 5,7,9-triacetate, were also determined on the basis of the long-range correlations between the ester carbonyl carbons and the neighboring oxymethine protons.

The HRESIMS of euphodendrophane E (**5**) showed a $[M + NH_4]^+$ ion at m/z 716.3652 (calcd 716.3641), consistent with the molecular formula $C_{38}H_{50}O_{12}$. Both the ^1H and ^{13}C NMR spectroscopic data of **5**, fully assigned through 2D NMR experiments (Tables 1 and 2, respectively), closely resembled those obtained for compound **4** and differed only in the signals for a propionate group at C-3 instead of isobutyrate, and an isobutyrate at C-7, instead of an acetate group. The assignment of the positions of these ester groups was based on the HMBC cross-peaks of the corresponding ester carbonyl carbons (δ_C 173.2 and 174.9) with H-3 and H-7, respectively. The remaining HMBC correlations for the rest of the molecule were in agreement with the proposed structure.

Euphodendrophane F (**6**) was obtained as an amorphous solid. The molecular formula was determined as $C_{40}H_{51}NO_{15}$ by HRESIMS, showing a $[M + H]^+$ ion at m/z 786.3346 (calcd 786.3332). The structure of **6** was deduced using 1D and 2D NMR spectroscopy, as well as comparison of the NMR spectra with those of the co-occurring jatrophanes **1**−**5** and related analogues.[11] These data indicated a jatrophane polyol structure with seven ester groups, which were identified as five acetates, an isobutyrate, and a nicotinate according to their NMR data (Tables 1 and 2). They could be associated with seven oxygenated sp^3 carbons: one primary, four secondary, and two tertiary. The NMR spectra also showed a keto carbonyl (δ_C 211.5, C-14), two endocyclic double bonds (Δ^5 and Δ^{11}) (*trans*-disubstituted Δ^{11} as in **1**−**5** and trisubstituted Δ^5: δ_H 5.14 brs, δ_C 111.7, 125.2), an aliphatic methylene (H_2-1), four methyls (one secondary: δ_H 1.17 d, H_3-20, and three tertiary (δ_H 1.07 s, 1.24 s, 1.50 s, H_3-18, -19, and -16, respectively), and one sp^3 quaternary carbon (δ_C 40.1, C-10). This, in combination with the molecular formula, corresponding to 14 double-bond equivalents, and COSY and HMBC correlations (Figure 3) indicated a bicyclic diterpene (jatrophane skeleton), differing from **1**−**5** by the endocyclic (Δ^5) double bond instead of an exocyclic ($\Delta^{6(17)}$) functionality. Moreover, the occurrence of a NOESY cross-peak between H-4 (δ_H 3.17) and H_2-17 (δ_H 5.05, 5.92) was in agreement with the *E*-configuration of this double bond, the same as that observed in the previously reported jatrophane-type diterpenoids from *Euphorbia helioscopia*.[18] Three-bond HMBC cross-peaks between H-3 (δ_H 5.47), H-8 (δ_H 5.38), and H_2-17

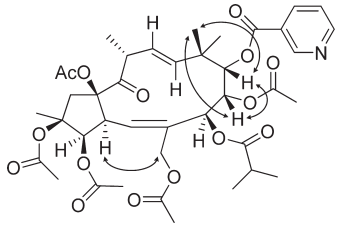

Figure 4. Key NOESY correlations of **6**.

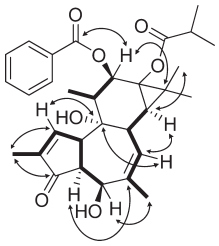

Figure 5. COSY (—) and selected HMBC correlations of **7**.

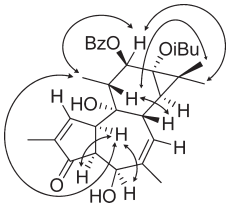

Figure 6. Key NOESY correlations of **7**.

with the acetate carbonyl carbons (δ_C 170.6, 168.9, and 170.3, respectively) indicated a 3,8,17-triacetoxy substitution pattern. The HMBC correlation between H-7 (δ_H 5.05) and the isobutyrate carbonyl (δ_C 174.8), as well as between H-9 (δ_H 5.18) and the nicotinate carbonyl (δ_C 163.9), were consistent with a 7-isobutyrate, 9-nicotinate structure.

The carbonyl signals of the remaining acetate groups (δ_C 168.9 and 169.5) in **6**, exhibiting no HMBC correlations, were positioned at C-2 and C-15 (δ_C 86.4, 92.8), as 2α- and 15β-substituents, according to the similarity of the chemical shifts of C(1)H_2, C-2, C(3)H, and C(16)H_3 (Experimental Section) with those of 2,3,5,7,15-pentaacetoxy-9-nicotinoyloxy-14-oxojatropha-6(1),11*E*-diene from *Euphorbia peplus*,[11] exhibiting a 3α,15β-diacetate moiety. The relative configurations of the remaining stereocenters were deduced from the coupling constants, a NOESY experiment (Figure 4), and comparison with literature data[5,11,14] of the related compounds as well as those of **1**−**5**. The NOEs between H-8, H-9, and H_3-19 (δ 1.24), with the latter assigned, according to the literature,[5,14,18] as 10β-methyl, indicated the usual 8α,9α-diacyloxy arrangement. The *cis*-arrangement of H-8 and H-9 was also supported by an observed NOE correlation between these protons. On the other hand, a very small value of the $J_{7,8}$ coupling constant was in agreement with the *trans*-orientation of the 7- and 8-acyloxy groups,[5,11] and a 7β-acyloxy substituent. The 13α-methyl configuration was also supported by the similarity of the NMR data of H-13 (δ_H 3.58), C-13 (δ_C 43.3), H_3-20 (δ_H 1.17), and C-20 (δ_C 20.0) to those of the co-occurring compound **3**, also bearing an OAc-15β group.

Euphodendriane A (**7**) was obtained as an amorphous solid, and its molecular formula, $C_{31}H_{38}O_7$, was found to be the same as that of a phorbol ester (Euphorbia factor Pr_2) isolated from *Euphorbia prolifera*.[19] This was deduced from the $[M + Na]^+$ peak at *m*/*z* 545.2515 (calcd for $C_{31}H_{38}O_7Na$, 545.2510) in the HRESIMS. The ^1H and ^{13}C NMR spectra (Experimental Section) showed signals typical for phorbol esters with a tigliane-type diterpene skeleton.[7] The ^1H NMR spectra displayed signals for five methyl groups (δ_H 1.20 s, H-16; 1.34 s, H-17; 1.15 d, *J* = 6.5 Hz, H-18; 1.82 brs, H-19; 1.91 brs, H-20) and two olefinic protons (δ_H 7.07 brs, H-1 and 4.89 brs, H-7). Two ^1H NMR doublets (δ_H 5.70, *J* = 10 Hz; 0.82, *J* = 4.5 Hz) were attributed to H-12 and H-14, respectively. Three methine protons (δ_H 3.14, dd, *J* = 6.5 and 4.5 Hz, H-4; 4.46, dd, *J* = 12.5 and 4.5 Hz, H-5; 3.65, m, H-10) were evident from the ^1H NMR spectrum of **7**. The COSY correlations revealed three independent spin systems (Figure 5), namely, at H_3-19/H-1/H-10/H-4/H-5, H_3-20/H-7/H-8/H-14, and H_3-18/H-11/H-12. A downfield singlet at δ_H 6.14 and a doublet at δ_H 6.05, both without any correlations in the HSQC spectrum, were assigned to hydroxy groups at C-9 and C-5, respectively. The ^1H and ^{13}C NMR spectra also exhibited signals typical for a benzoate group (δ_H 8.06 dd, 7.61 dt and 7.49 t; δ_C 166.1, 129.7, 129.6, 128.5, and 133.1) and an isobutyrate group (δ_H 2.58 sept, 1.19 and 1.16 d; δ_C 179.6, 34.2, 18.5, and 18.5). The position of the benzoate ester at C-12 was established by a three-bond HMBC correlation between H-12 (δ_H 5.70) and the benzoate carbonyl carbon (δ_C 166.1). The absence of any HMBC correlations of the carbonyl (δ_C 179.6) of the remaining ester function, assigned as an isobutyrate, indicated its attachment to a carbon bearing no hydrogen (C-13). The 13-isobutyrate ester unit was also supported by the almost identical chemical shift of C-3 (δ_C 64.7) with that of the related 4-deoxyphorbol derivatives.[8] The proposed skeletal structure of **7** was further supported by the correlations observed in the HMBC spectrum (Figure 5).

The relative configuration of **7** was deduced from the NOESY spectrum (Figure 6). All tigliane diterpenoids discovered to date possess H-8β, OH-9α, and H-10α configurations.[20] The observed NOE correlations between H-11/H-8 and H-11/H-17 indicated that these protons are on the same (β) side of the molecule. The coupling constant, $J_{11,12}$ = 10 Hz, suggested the opposite configuration of H-11 and H-12, and the NOE effect between H-12/H_3-18 indicated the α-orientation of H-12. The absence of any NOE effect between H-8β and H-14 suggested that H-14 is α-oriented. The NOE between H_3-18 and H-10 confirmed the α-orientation of H-10. Since H-4 exhibited a NOE with H-10, both protons were assigned as α-oriented. The α-arrangement of H-4 was also supported by the absence of a NOE with H-8β. Compound **7** exhibited the same gross structure as Euphorbia factor Pr_2 from *E. prolifera*.[19] According to the differences in ^1H NMR data, most particularly those concerning H-1, H-5, H-7, and H-10, tigliane **7** was assigned as the 4-*epi* derivative of Euphorbia factor Pr_2.

Compounds **1**−**7** were evaluated for their capacity to inhibit the in vitro growth of four human cancer cell lines (NCI-H460, NCI-H460/R, DLD-1, and U-87 MG) using a sulforhodamine B (SRB) assay. The most effective were **2** and **7** (Table 3).

The MDR phenotype in a NCI-H460/R cell line that was highly resistant to paclitaxel and doxorubicin did not significantly change the inhibitory pattern of the compounds tested compared to the corresponding sensitive cell line, NCI-H460. Only the sensitivity to **6** was decreased considerably using the NCI-H460/R cell line. The degree of growth inhibition of **1** and **2** did not

Table 3. Growth Inhibitory Effects of Compounds Tested against Four Different Cancer Cell Lines

	NCI-H460		NCI-H460/R[b]		DLD-1		U-87 MG	
compound	IC_{30} (μM)[a]	IC_{50} (μM)[a]	IC_{30} (μM)	IC_{50} (μM)	IC_{30} (μM)	IC_{50} (μM)	IC_{30} (μM)	IC_{50} (μM)
1	31.0	46.6	26.6	44.8	7.3	59.3	74.7	155.3
2	6.1	16.2	3.9	17.2	4.8	22.1	6.8	107.7
4	11.7	21.3	14.6	30.1	14.2	42.7	10.4	52.6
5	8.3	21.9	15.2	49.8	13.7	37.9	14.1	73.1
6	4.8	14.8	51.9	90.8	38.8	75.3	27.3	65.5
7	7.0	14.3	15.7	26.0	13.3	27.4	33.2	60.1
paclitaxel[c]	3.6	9.4	440	2078	23	39	49	90

[a] Values represent the average from five independent experiments. IC_{30} and IC_{50} were calculated by linear regression analysis using Excel software. [b] NCI-H460/R is a multi-drug-resistant cancer cell line obtained by drug selection from its sensitive counterpart NCI-H460.[4] [c] Data for the positive control (paclitaxel) are expressed in nM.

Table 4. Relative Reversion of Resistance to Paclitaxel and Doxorubicin by Simultaneous Treatment with Compounds 1 and 2 and Verapamil

test compound(s)	FAR[a]	concentration (μM)	IC_{50} (μM)[b]	relative reversion[c]	combination index (CI)
paclitaxel		(0.005−5)	2.078 ± 0.385		
		1	0.692 ± 0.092	3	CI < 0.5 (SS)
1 + paclitaxel		2.5	0.109 ± 0.003	19	CI < 1 (S)[d]
	2.1	5	0.055 ± 0.001	38	CI < 0.5 (SS)[e]
	3.2	20			
		1	0.189 ± 0.019	11	CI < 1 (S)
2 + paclitaxel		2.5	0.083 ± 0.002	25	CI < 1 (S)
	1.9	5	0.035 ± 0.005	60	CI < 0.5 (SS)
	3.0	20			
		1	0.104 ± 0.002	20	CI < 1 (S)
verapamil + paclitaxel		2.5	0.094 ± 0.003	22	CI < 0.5 (SS)
	0.9	5	0.045 ± 0.017	46	CI < 0.5 (SS)
	1.8	20			
doxorubicin		(0.05−5)	4.307 ± 0.233		
1 + doxorubicin		5	0.227 ± 0.011	19	CI < 0.5 (SS)
2 + doxorubicin		5	0.154 ± 0.006	28	CI < 0.5 (SS)
verapamil + doxorubicin		5	0.215 ± 0.013	20	CI < 0.5 (SS)

[a] The fluorescence activity ratio was calculated on the basis of the measured fluorescence values (FL2-H) expressed via the following equation: FAR = $FL2\text{-}H_{MDR\ treated}/FL2\text{-}H_{MDR\ control}$. [b] Results were obtained with a SRB assay. Values represent the average ± standard deviation from five independent experiments. IC_{50} values were calculated by linear regression analysis using Excel software. [c] Relative reversion was calculated as IC_{50} for a cytostatic drug/IC_{50} for its combination with reversal agent. [d] S, synergism (CI < 1). [e] SS, strong synergism (CI < 0.5).

differ substantially between the sensitive NCI-H460 and the resistant NCI-H460/R cell lines. However, the effectiveness of **2** was significantly higher in comparison to **1** (Table 3).

Since macrocyclic diterpenes of the jatrophane type previously isolated from *E. dendroides* have been investigated as P-gp pump inhibitors,[5] it was considered that the new compounds obtained in this investigation may share such properties. Indeed, a marked increase in the accumulation of P-gp substrate Rho123 (assessed by flow cytometry) was observed in the MDR cancer cell line (NCI-H460/R) when treated with **1** and **2**. The overexpression of *mdr1* mRNA, which codes for P-gp, was reported in the NCI-H460/R cell line previously.[4] The effects of **1** and **2** on Rho123 accumulation in NCI-H460/R cells were compared with untreated resistant NCI-H460/R cells by the fluorescence activity ratio (FAR, Table 4). Rho123 accumulation was about 2-fold higher in untreated NCI-H460 cells compared to NCI-H460/R cells. A significantly higher accumulation of Rho123 in the NCI-H460/R cell line was obtained with **1** and **2**, compared to the effect of the standard P-gp inhibitor, verapamil (Table 4). Earlier findings have highlighted the positive role of certain pharmacophoric elements in the activities of jatrophane diterpenoids toward P-gp,[1] such as a free hydroxy group at C-5 and an acetate group at C-8, which are both present in jatrophanes **1** and **2**.

Next, the effects of **1** and **2**, each in simultaneous combination with two anticancer agents (paclitaxel and doxorubicin), were investigated in an MDR cancer cell line. Resistant NCI-H460/R cells were treated for 72 h with combinations of 1, 2.5, and 5 μM **1** or **2** and 0.005−5 μM paclitaxel (Table 4). The ability of **1** and **2** to reverse drug resistance was compared to that of verapamil. As shown in Table 4, NCI-H460/R cells were also exposed to combinations of 5 μM **1** or **2** and 0.05−5 μM doxorubicin for 72 h. The effects of **1**, **2**, and verapamil on paclitaxel or doxorubicin sensitivity were assessed using a SRB assay. The IC_{50} value for paclitaxel decreased in combination with **1**, demonstrating 3-,

19-, and 38-fold reversal. An even more pronounced effect was obtained for **2**, exibiting 11-, 25-, and 60-fold reversal. There were no significant differences in reversal activity at concentration levels of 2.5 and 5 μM between compounds **1** and **2** and verapamil. Both jatrophane esters at 5 μM decreased the IC_{50} value for doxorubicin significantly, showing a similar reversal potential to verapamil (Table 4). The results obtained on combined treatment were subjected to computerized synergism/antagonism CalcuSyn software analysis. All of the combinations used in the course of treatment using resistant NCI-H460/R cells induced synergistic (CI < 1) or strong synergistic effect (CI < 0.5). These results point to the potential of **1** and **2** to reverse paclitaxel and doxorubicin resistance in the MDR cancer cell line used.

As shown earlier, a resistant NCI-H460/R cell line has been developed that displays cross-resistance to paclitaxel, vinblasine, doxorubicin, epirubicin, and etoposide.[4] A synergistic interaction between jatrophane diterpenes and anthracyclines (epirubicin and doxorubicin) was found previously.[21,22] Compounds **1** and **2** sensitized NCI-H460/R cells to paclitaxel in a concentration-dependent manner (Table 4). This is the first report of a synergistic effect observed between jatrophanes and the taxane paclitaxel.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were determined on a Autopol IV (Rudolph Research Analytical) polarimeter equipped with a sodium lamp (589 nm) and 10 cm microcell. ^{1}H and ^{13}C NMR data were measured on a Bruker Avance III 500 NMR spectrometer (500 MHz for ^{1}H and 150 MHz for ^{13}C NMR, in $CDCl_3$, with TMS as internal reference). High-resolution LC/ESI TOF mass spectra were measured on a HPLC instrument (Agilent 1200 Series, Agilent Technologies) with a Zorbax Eclipse Plus C_{18} column (150 × 4.6 mm i.d.; 1.8 μm) and a diode-array detector (DAD) coupled with a 6210 time-of-flight LC/MS system (Agilent Technologies). HPLC was performed on an Agilent 1100 Series instrument in a gradient mode on Zorbax XDB-C_{18} column with a DAD. Detection of analytes was performed at 260 nm.

Dry flash chromatography was performed on silica gel (70−230 mesh). Silica gel 60 F_{254} precoated aluminum sheets (0.25 mm, Merck) for TLC control and preparative TLC plates (2 and 0.5 mm Merck) for preparative purification were used.

Plant Material. The aerial parts of *E. dendroides* were collected around Petrovac (Montenegro) in April 2009. The spurge was identified by Petar Marin, and a voucher specimen (No. 030409) was deposited at the Herbarium of Botanical Garden "Jevremovac", University of Belgrade, Belgrade (Serbia).

Extraction and Isolation. To the dried plant material (1 kg) was added 60% aqueous ethanol (3 L), and the mixture was left for 10 days at room temperature with stirring. The extract obtained after filtration was lyophilized at 40 °C to give a dried residue (105 g). This extract was mixed with hexane (350 mL) and placed in an ultrasound bath for 45 min, with the soluble portion decanted. The extraction was repeated under the same conditions, and the combined extracts were concentrated under reduced pressure. The hexane fraction (4.65 g) was subjected to dry flash chromatography on silica gel using toluene−ethyl acetate for elution. Fractions B−F were eluted with a higher percent of ethyl acetate in toluene. These fractions were purified by preparative TLC on silica gel plates, 20 cm × 20 cm. Final purification was performed by HPLC to obtain diterpene compounds **1**−**7**.

Fraction B (0.26 g, eluted with 30% ethyl acetate in toluene) was subjected to preparative TLC on silica gel, using as developing system hexane−acetone (7:3) (the plates were developed three times), to give subfraction B-1 (77.7 mg). Final purification by HPLC afforded pure compounds **4** (4.5 mg), **5** (4.8 mg), and **7** (5 mg). Fraction C (0.25 g, eluted with a second portion of 30% ethyl acetate in toluene) was subjected to preparative TLC on silica gel, eluted with hexane−acetone (7:3) (the plates were developed five times), to give two subfractions.

Subfraction C-1 (81 mg) was purified by HPLC to give **1** (8 mg) and **2** (16 mg). Subfraction C-2 (63 mg), after purification by HPLC, yielded an additional amount of **2** (8.6 mg). Preparative TLC of fraction D (0.16 g, eluted with 40% ethyl acetate in toluene) on silica gel, using hexane−acetone (7:3) (the plates were developed three times), afforded two subfractions. Subfraction D-1 (50 mg), after HPLC purification, afforded **1** (17 mg) and **3** (2 mg). Subfraction D-2 (51 mg) on HPLC yielded additional amounts of **1** (16 mg) and **2** (8 mg). Fraction E (0.09 g, eluted with 50%−100% ethyl acetate in toluene) was subjected to preparative TLC on silica gel, in hexane−acetone (65:35) (the plate was developed four times), to afford three subfractions. Subfractions E-1 (12.4 mg) and E-2 (23 mg), after purification by HPLC, both yielded compound **6** (2 and 4.4 mg, respectively). Purification of subfraction E-3 (21 mg) by HPLC afforded an additional amount of **1** (2.2 mg). Preparative TLC of fraction F (0.12 g, eluated with 10% methanol in ethyl acetate) on silica gel in hexane−acetone (65:35) (the plate was developed four times) afforded subfraction F-1 (30 mg). This was purified by HPLC to give an additional amount of compound **6** (2.5 mg).

*Euphodendrophane A (**1**):* colorless, amorphous solid; $[\alpha]^{20}_D$ +31.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 227 (3.48), 264 (3.28) nm; IR (film) ν_{max} 3446, 2926, 1738, 1278, 1240 cm^{-1}; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HRESIMS *m*/*z* 700.3335 [M + H]$^+$, calcd for $(C_{37}H_{49}NO_{12} + H)^+$ *m*/*z* 700.3328.

*Euphodendrophane B (**2**):* colorless, amorphous solid; $[\alpha]^{20}_D$ +41.6 (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ε) 217 (3.80), 264 (3.17) nm; IR (film) ν_{max} 3446, 2972, 1737, 1278,1240 cm^{-1}; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HRESIMS *m*/*z* 714.3489 [M + H]$^+$, calcd for $(C_{38}H_{51}NO_{12} + H)$ *m*/*z* 714.3484.

*Euphodendrophane C (**3**):* colorless, amorphous solid; $[\alpha]^{20}_D$ +10.1 (*c* 0.06, MeOH); UV (MeOH) λ_{max} (log ε) 213 (4.13), 264 (3.60) nm; IR (film) ν_{max} 2975, 1736, 1373, 1253, 1190 cm^{-1}; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HRESIMS *m*/*z* 742.3438 [M + H]$^+$, calcd for $(C_{39}H_{51}NO_{13}+H)^+$ *m*/*z* 742.3433.

*Euphodendrophane D (**4**):* colorless, amorphous solid; $[\alpha]^{20}_D$ +26.0 (*c* 0.08, MeOH); UV (MeOH) λ_{max} (log ε) 228.3 (3.98), 273.4 (2.97) nm; IR (film) ν_{max} 3478, 2929, 1740, 1273,1235 cm^{-1}; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HRESIMS *m*/*z* 702.3483 [M + NH_4]$^+$, calcd for $(C_{37}H_{48}O_{12} + NH_4)^+$ *m*/*z* 702.3484.

*Euphodendrophane E (**5**):* colorless, amorphous solid; $[\alpha]^{20}_D$ +28.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 229 (4.03) 273 (3.00) nm; IR (film) ν_{max} 3446, 2924, 1738, 1271, 1234 cm^{-1}; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HRESIMS *m*/*z* 716.3652 [M + NH_4]$^+$, calcd for $(C_{38}H_{50}O_{12}+NH_4)^+$ *m*/*z* 716.3640.

*Euphodendrophane F (**6**):* colorless, amorphous solid; $[\alpha]^{20}_D$ −8.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 217 (4.00), 264 (3.41) nm; IR (film) ν_{max} 3447, 2931, 1737,1231 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.99 (1H, d, *J* = 16.5 Hz, H-11), 5.92 (1H, brs, H-17b), 5.75 (1H, m, H-12), 5.47 (1H, brs, H-3), 5.38 (1H, brs, H-8), 5.18 (1H, d, *J* = 1.5 Hz, H-9), 5.14 (1H, brs, H-5), 5.05 (2H, brs, H-7, H-17a), 3.78 (1H, d, *J* = 16.5 Hz, H-1α), 3.58 (1H, m, H-13), 3.17 (1H, brs, H-4), 2.00 (1H, d, *J* = 16.5 Hz, H-1β), 1.50 (3H, s, H-16), 1.24 (3H, brs, H-19), 1.17 (3H, d, *J* = 6.5 Hz, H-20), 1.07 (3H, brs, H-18); five OAc: 2.20 (2 × 3H, s, OCOCH_3-3, -15) 2.13 (2 × 3H, s, OCOCH_3-8, -17), 2.04 (3H, s, OCOCH_3-2); OiBu-7: 2.10 (1H, bm, H-2′), 0.94, 0.68 (2 × 3H, two brd, *J* = 7 Hz, H-3′, 3H-4′); ONic-9: 9.20, (1H, s, H-2″), 8.80 (1H, brd, *J* = 5 Hz, H-6″), 8.20 (1H, brd, *J* = 8 Hz, H-4″), 7.38 (1H, dd, *J* = 8, 5 Hz, H-5″); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 211.5 (C, C-14), 137.1 (CH, C-12), 131.9 (CH, C-11), 125.2 (C, C-6), 111.7 (CH, C-5), 92.8

(C, C-15), 86.4 (C, C-2), 80.0 (CH, C-9), 77.9 (CH, C-3), 71.2 (CH, C-8), 68.0 (CH, C-7), 67.76 (CH_2, C-17), 49.9 (CH, C-4), 47.5 (CH_2, C-1), 43.3 (CH, C-13), 40.1 (C, C-10), 26.9 (CH_3, C-18), 24.1 (CH_3, C-19), 20.0 (CH_3, C-20), 18.8 (CH_3, C-16); five OAc: 170.6, 170.3, 169.5 (3 × C, CO-3, -15, -17), 168.9 (2 × C, CO-2, -8), 22.2, 21.3, 21.2, 21.1, 20.6 (5 × CH_3, $COCH_3$-15, -17, -8, -3, -2); OiBu-7: 174.8 (C, CO-7), 33.5 (CH, C-2′), 18.0, 17.7 (2 × CH_3, C-3′, -4′); ONic-9: 163.9 (C, CO-9), 153.8 (CH, C-6″), 151.3 (CH, C-2″), 137.1 (CH, C-4″), 125.2 (C, C-3″), 123.3 (CH, C-5″); HRESIMS m/z 786.3346 $[M + H]^+$, calcd for ($C_{40}H_{51}NO_{15}$ + H) m/z 786.3332.

*Euphodendriane A (**7**):* colorless, amorphous solid; $[\alpha]^{20}_D$ −2.7 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 230 (3.58), 271 (2.72) nm; IR (film) ν_{max} 3334, 2925, 1715, 1457, 1266 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 7.07 (1H, brs, H-1), 6.14 (1H, s, *H*O-9), 6.05 (1H, d, J = 12 Hz, *H*O-5), 5.70 (1H, d, J = 10 Hz, H-12), 4.89 (1H, brs, H-7), 4.46 (1H, dd, J = 12, 4.5 Hz, H-5), 3.65 (1H, m, H-10), 3.14 (1H, dd, J = 6.5, 4.5 Hz, H-4), 2.06 (1H, m, H-8), 1.91 (3H, brs, H-20), 1.85 (1H, m, H-11), 1.82 (3H, brs, H-19), 1.34 (3H, s, H-17), 1.20 (3H, s, H-16), 1.15 (3H, d, J = 6.5 Hz, H-18), 0.82 (1H, d, J = 4.5 Hz, H-14); OBz-12: 8.06, (2H, dd, J = 8.5, 1.5 Hz, H-2′, H-6′), 7.61 (1H, t, J = 7.5 Hz, H-4′). 7.49 (1H, t, J = 7.5 Hz, H-3′, H-5′); OiBu-13: 2.58 (1H, h, J = 7 Hz, H-2″), 1.19, 1.16 (2 × 3H, two brd, J = 7 Hz, 3H-3″, 3H-4″); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 207.6 (C, C-3), 154.4 (C, C-1), 143.8 (C, C-2), 136.9 (C, C-6), 125.5 (C, C-7), 78.2 (C, C-9), 75.5 (CH, C-12), 70.7 (CH, C-5), 64.7 (C, C-13), 56.0 (CH, C-4), 47.8 (CH, C-10), 43.5 (CH, C-11), 40.1 (CH, C-8), 38.6 (CH, C-14), 27.2 (CH_3, C-20), 23.8 (CH_3, C-16), 25.73 (C, C-15), 16.6 (CH_3, C-17), 11.8 (CH_3, C-18), 10.5 (CH_3, C-19); OBz-12: 166.1 (C, CO-12), 133.1 (CH, C-4′), 129.7 (C, C-1′), 129.6 (2 × CH, C-2′, C-6′,), 128.5 (2 × CH, C-3′, C-5′); OiBu-13: 179.6 (C, CO-13), 34.2 (CH, C-2″), 18.5 (2 × CH_3, C-3″, C-4″); HRESIMS m/z 545.2515 $[M + Na]^+$, calcd for ($C_{31}H_{38}O_7$ +Na), m/z 545.2510.

Cells and Cell Culture. The NCI-H460, DLD-1, and U-87 MG cell lines were purchased from the American Type Culture Collection, Rockville, MD. NCI-H460/R cells were selected originally from NCI-H460 cells and cultured in a medium containing 100 nM doxorubicin.[4] All cell lines were subcultured at 72 h intervals using 0.25% trypsin/EDTA and seeded into a fresh medium at the following densities: 8000 cells/cm^2 for NCI-H460 and DLD-1 and 16 000 cells/cm^2 for U-87 MG and NCI-H460/R.

Chemosensitivity Determination with a Sulforhodamine B Assay. Cells grown in 25 cm^2 tissue flasks were trypsinized, seeded into flat-bottomed 96-well tissue culture plates, and incubated overnight. NCI-H460 and DLD-1 cells were seeded at 2000 cells/well, while U-87 MG and NCI-H460/R cells were seeded at 4000 cells/well. Treatment with compounds **1**, **2**, and **4**−**7** (0.5−150 μM) lasted 72 h. The effects of **1** and **2** in simultaneous treatment with classic chemotherapeutic drugs (paclitaxel and doxorubicin) were studied in the MDR cell line (NCI-H460/R). The chemosensitivity assay was performed after 72 h. The cellular proteins were stained with SRB, following a slightly modified protocol.[23]

Median Effect Analysis. The nature of the interaction between selected compounds and two classical chemotherapeutic drugs was analyzed using Calcusyn software, which uses the combination index method based on the multiple drug effect equation.[24]

Rho123 Accumulation Assay. Rho123 accumulation was analyzed by flow cytometry utilizing the ability of Rho123 to emit fluorescence. The intensity of the fluorescence was proportional to Rho123 accumulation. Studies were carried out with verapamil and **1** and **2**. NCI-H460 and NCI-H460/R cells were grown to 80% confluence in 75 cm^2 flasks, trypsinized, and resuspended in 10 mL centrifuge tubes in a Rho123-containing medium. The cells were treated with **1** and **2** and verapamil (5 and 20 μM) and incubated at 37 °C in 5% CO_2 for 30 min. The samples were analyzed using a FACScalibur flow-cytometer (Becton Dickinson, Oxford, U.K.). The fluorescence of Rho123 was assessed on fluorescence channel 2 (FL2-H) at 530 nm. At least of 10 000 events were assayed for each sample.

■ ASSOCIATED CONTENT

Supporting Information. NMR spectra of compounds **1**−**7** are available free of charge via the Internet at http://pubs.acs.org.

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