Jatrophane Diterpenes as P-Glycoprotein Inhibitors. First Insights of Structure−Activity Relationships and Discovery of a New, Powerful Lead

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Received January 23, 2003

The Mediterranean spurge *Euphorbia dendroides* L. afforded a series of 10 closely related jatrophane polyesters, nine of which are new, which served as a base for the establishment of structure−activity relationships within this class of P-glycoprotein inhibitors. The results, while pointing to the general role of lipophilicity for activity, also highlighted the relevance of the substitution pattern at the positions 2, 3, and 5, suggesting the involvement of this fragment in binding. The most powerful compound of the series, euphodendroidin D (**4**), outperformed cyclosporin by a factor of 2 to inhibit Pgp-mediated daunomycin transport.

Introduction

The development of resistance to anticancer drugs is a major limitation of cancer chemotherapy. One of the most effective mechanisms of multidrug resistance (MDR) involves the transporter P-glycoprotein (Pgp), a 170 KDa phosphorylated and glycosylated membrane protein belonging to the ATP-binding cassette (ABC) superfamily of transporter proteins.[1,2] Pgp consists of two homologous halves, each containing six hydrophobic domains and a hydrophilic nucleotide binding fold.[2,3] Pgp makes cells capable to resist lethal doses of certain cytotoxic drugs by pumping them out of the cells, substantially attenuating their cytotoxic effect. Furthermore, Pgp-mediated resistance is not restricted to a specific inducer but it is generally extended to chemically unrelated antitumor drugs (cross-resistance).

The spectrum of drugs interacting with Pgp encompasses various compounds of fungal and plant origin (e.g., anthracyclines, vinca alkaloids, taxanes), peptide antibiotics (e.g., gramicidin D, valinomycin), steroid hormones (e.g., cortisol, aldosterone, dexamethasone), and immunosuppressive agents (e.g., cyclosporin A, FK 506) as well as synthetic calcium channel blockers (e.g., verapamil, azidopine).[4−6] Since the substrates for Pgp are largely hydrophobic, access to its drugs binding site takes place seemingly from the lipid bilayer rather than from the aqueous phase. Accordingly, both transport and binding studies suggest that Pgp intercepts ligands from the inner leaflet of the membrane, extruding them into the extracellular medium with a "hydrophobic vacuum cleaner" process,[7] or alternatively with a "flippase" mechanism.[8] This model reconducts the unusual broad specificity of Pgp to the capacity of its substrates to intercalate into the lipid bilayer, making hydrophobicity or amphipathy crucial for Pgp interaction.

Recent studies by Hohmann et al. identified jatrophane diterpenoids from *Euphorbia* spp. as a new class of powerful inhibitors of Pgp. Owing to the structural diversity of the set of ligands investigated, structure−activity relationships could not be established, though lipophilicity was recognized as an important factor for the activity.[9] As part of an investigation on Mediterranean spurges, we have isolated a jatrophane fraction from the latex of *Euphorbia dendroides* L. endowed with a powerful inhibitory activity on Pgp. Characterization of this fraction yielded 10 closely related compounds, nine of which are new, which were individually investigated for their Pgp binding properties. The results showed the existence of definite structure−activity relationships, going beyond simple lipophilicity considerations, and suggesting the involvement of the "southwestern" fragment of the molecule in binding.

Results and Discussion

Latex obtained from different collections of *Euphorbia dendroides* L. was exhaustively extracted with EtOAc, and the obtained extract was filtered on silica gel in order to eliminate gummy compounds. The soluble material was separated by MPLC on silica gel column using a gradient system from hexane to EtOAc. The fractions containing diterpenes were repeatedly chromatographed by HPLC on silica gel to yield the new euphodendroidins A (**1**, 11 mg), B (**2**, 21 mg), C (**3**, 5 mg), D (**4**, 3 mg), E (**5**, 25 mg), F (**6**, 25 mg) G (**8**, 2 mg), H (**9**, 13 mg), and I (**10**, 4.5 mg). The known jatrophane derivative **7** (28 mg) was identified by comparison of its spectral data with those reported in the literature.[10]

Euphodendroidin A (**1**) was obtained as a colorless amorphous solid. The molecular formula $C_{37}H_{48}O_{13}$ was

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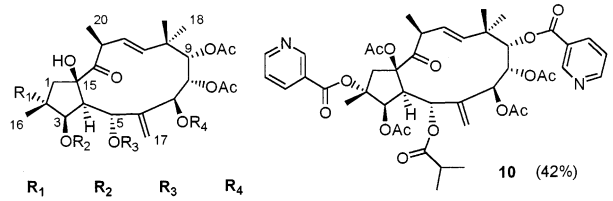
10.1021/jm030787e CCC: $25.00
Published on Web 06/17/2003

Chart 1[a]



10 (42%)

	R_1	R_2	R_3	R_4	
1	OAc	H	iBu	Bz	(97%)
2	OAc	H	MeBu	Bz	(105%)
3	OAc	H	Nic	Bz	(56%)
4	H	H	iBu	Bz	(183%)
5	H	Ac	iBu	Bz	(88%)
6	OH	Ac	iBu	Bz	(38%)
7	OAc	PhOAc	Ac	iBu	(75%)
8	OAc	Nic	Ac	iBu	(99%)
9	H	Bz	Ac	iBu	(74%)

iBu = isobutyryl
MeBu = 2-methylbutyryl
PhOAc = phenoxyacetyl
Bz = benzoyl
Nic = nicotinyl

[a] Values in parentheses represent the percentage of inhibition of cellular P-glycoprotein-mediated daunomycin efflux, relative to cyclosporin A. Complete data are reported in Table 3.

assigned to **1** on the basis of HR-FABMS. Structural assignment of euphodendroidin A was accomplished with the extensive use of 1D and 2D NMR spectroscopy. Inspection of ^{1}H NMR spectrum of **1** (Table 1) initially suggested the presence of a diterpene skeleton pentaesterified with three acetyls, an isobutyryl, and a benzoyl group. The nature of the diterpenoid core was more evident by combined inspection of the ^{13}C NMR (Table 2) and the 2D HSQC spectra of **1**, the latter allowing correlation of directly linked proton and carbon atoms. This analysis showed that, in addition to the signals attributable to the above ester groups, euphodendroidin A (**1**) contained a ketone carbonyl group (δ_C 212.5), two double bonds, one exocyclic and one trans disubstituted (δ_H 6.01 and 5.53, J = 16.0 Hz), and seven (five methines and two unprotonated) oxygenated sp^3 carbon atoms. Signals of four additional methyls, one methylene, and two methines were also present in the ^{1}H and ^{13}C NMR spectra of **1** (Tables 1 and 2). Application of ^{1}H−^{1}H correlation spectroscopy (COSY) allowed us to sequence the multiplets of the core diterpene structure into three

Table 1. ^{1}H NMR Data of Euphodendroidins A−I (**1**−**6**, **8**−**10**) [$CDCl_3$, δ (ppm) and mult][a]

position		**1**	**2**	**3**	**4**	**5**	**6**	**8**	**9**	**10**
1α		2.80 d	2.80 d	2.82 d	2.49 dd	2.48 dd	2.41 d	2.78 d	2.45 dd	3.41 d
1β		2.09 d	2.09 d	2.05 d	1.60 m	1.66 dd	1.98 d	2.07 d	1.71 dd	2.93 d
2					2.35 m	2.48 m			2.45 m	
3		4.42 bd	4.42 bd	4.52 bd	4.26 dd	5.61 bs	5.40 bs	5.86 bs	5.70 bs	5.91 d
4		3.24 bs	3.22 bs	3.38 bs	2.88 bs	3.14 bs	3.78 bs	3.41 bs	2.91 bs	3.58 bd
5		5.38 bs	5.37 bs	5.68 bs	5.37 bs	5.52 bs	5.45 bs	5.38 bs	5.28 bs	5.49 bs
7		5.79 bs	5.76 bs	5.83 s	5.69 s	5.92 s	5.84 s	5.64 s	5.59 s	5.71 bs
8		5.17 s	5.19 s	5.26 s	5.14 s	5.19 s	5.05 s	4.96 s	4.99 s	5.15 s
9		5.01 s	5.00 s	5.03 s	4.98 s	4.97 s	4.98 s	4.93 s	4.92 s	5.16 s
11		6.01 d	6.01 d	6.05 d	5.97 d	5.97 d	5.97 d	5.93 d	5.94 d	5.72 d
12		5.53 dd	5.53 dd	5.58 dd	5.52 dd	5.51 dd	5.51 dd	5.50 dd	5.38 dd	5.60 dd
13		3.82 dq	3.82 dq	3.85 dq	3.77 dq	3.74 dq	4.21 dq	3.82 dq	3.73 dq	3.61 dq
16		1.52 s	1.52 s	1.53 s	1.20 d	1.03 d	1.40 s	1.60 s	1.02d	1.70 s
17		5.21 bs	5.18 bs	5.25 bs	5.18 bs	5.10 bs	5.08 bs		5.03 bs	5.03 bs
		5.29 bs	5.29 bs	5.41 bs	5.28 bs	5.16 bs	5.13 bs		5.19 bs	5.13 bs
18		0.92 s	0.92 s	0.93 s	0.90 s	0.90 s	0.91 s	0.93 s	0.90 s	0.85 s
19		1.33 s	1.33 s	1.38 s	1.33 s	1.30 s	1.32 s	1.25 s	1.22 s	1.30 s
20		1.30 d	1.30 d	1.31 d	1.13 d	1.34 d	1.33 d	1.30 d	1.32 d	1.21 d
15-OH		4.38 s	4.35 s	4.41 s	4.34 s	4.28 s	4.28 s	4.30 s	4.17 s	
3-OH		3.32 d	3.34 d	3.90 d	3.01 d					
2-OH							2.12 s			
Ac_s		1.73 s	1.73 s	1.72 s	1.55 s	1.49 s	1.49 s	2.00 s	1.51 s	2.00 s
		2.09 s	2.09 s	2.09 s	2.11 s	2.07 s	2.08 s	2.01 s	2.09 s	2.05 s
		2.28 s	2.28 s	2.30 s		2.12 s	2.11 s	2.05 s	2.09 s	2.10 s
								2.17 s		2.15 s
^{i}Bu	2	2.46 m			2.47 m	2.48 m	2.35 m	2.58 m	2.55 m	1.91 m
	3	1.13 d			1.11 d	0.95 d	1.06 d	1.15 d	1.12 d	0.89 d
	4	1.15 d			1.13 d	1.02 d	1.11 d	1.18 d	1.13 d	0.59 d
MeBu	2		2.30 tq							
	3		1.68 ddq							
			1.42 ddq							
	4		0.82 t							
	5		1.09 d							
Bz	2,6	8.05 d	8.05 d	8.07 d	8.10 d	8.10 d	8.11 d		8.11 d	
	3,5	7.49 t	7.49 t	7.48 t	7.49 t	7.49 t	7.49 t		7.48 t	
	4	7.60 d	7.60 d	7.60 d	7.60 d	7.60 d	7.62 d		7.60 d	
Nic	2			9.19 s				9.43 s		9.51 s
	4			8.22 bd				8.95 bd		8.59 bd
	5			7.32 dd				8.06 dd		7.56 dd
	6			8.72 bd				9.01 bd		8.87 bd
9-Nic	2									9.18 s
	4									8.27 bd
	5									7.46 dd
	6									8.83 bd

[a] J_{H-H} (values in Hz). **1**−**6**, **8**−**9**: 1α−1β = 14.0; 3−4 = 4−5 = 7−8 = 8−9 = 17a−17b = 1.2; 11−12 = 16.0; 12−13 = 9.5; 13−20 = 7.0. For **1**−**4**: 3-OH = 7.5. **4**, **5**, **9**: 1α−2 = 10.5; 1β−2 = 11.0; 2−16 = 6.5; 2−3 = 7.0. **10**: 1α−1β = 14.0; 3−4 = 4.0; 4−5 = 7−8 = 8−9 = 17a−17b = 1.2; 11−12 = 16.0; 12−13 = 9.5; 13−20 = 7.0. ^{i}Bu: 2−3 = 2−4 = 7.0. **MeBu**: 2−3a = 2−3b = 3a−4 = 3b-4 = 2−5 = 7.0; 3a−3b = 14.0. **Bz**: 2−3 = 3−4 = 7.4. **Nic**: 4−5 = 8.0; 5−6 = 5.0.

Table 2. ^{13}C NMR Data of Euphodendroidins A−I (**1**−**6**, **8**−**10**) [$CDCl_3$, δ (ppm) and mult]

position	**1**	**2**	**3**	**4**	**5**	**6**	**8**	**9**	**10**
1	51.26 t	51.26 t	51.20 t	45.95 t	45.95 tt]	52.24 t	51.40 t	45.43 t	47.02 t
2	89.90 s	89.90 s	89.70 s	37.71 d	37.71 d	86.33 s	88.32 s	38.31 d	87.90 s
3	78.39 d	78.10 d	78.25 d	77.10 d	76.73 d	80.50 d	78.12 d	75.69 d	77.34 d
4	47.53 d	47.53 d	47.50 d	47.00 d	50.26 d	47.11 d	47.60 d	51.50 d	47.05 d
5	68.46 d	68.46 d	68.70 d	70.36 d	70.36 d	68.75 d	67.80 d	68.94 d	68.26 d
6	144.61 s	144.61 s	144.40 s	145.37 s	144.60 s	144.60 s	143.60 s	144.61 s	144.00 s
7	70.39 d	70.39 d	69.95 d	70.39 d	68.01 d	68.74 d	67.11 d	68.86 d	67.09 d
8	68.46 d	68.46 d	68.40 d	68.46 d	70.35 d	70.35 d	70.50 d	70.27 d	70.06 d
9	80.34 d	80.34 d	80.42 d	80.34 d	80.64 d	80.50 d	80.10 d	80.28 d	81.47 d
10	40.67 s	40.67 s	40.66 s	40.67 s	40.36 s	40.49 s	40.59 s	40.87 s	40.41 s
11	137.39 d	137.68 d	137.30 d	137.39 d	136.95 d	136.87 d	137.85 d	137.39 d	135.43 d
12	129.80 d	129.55 d	129.75 d	129.80 d	129.75 d	130.06 d	129.26 d	129.75 d	130.69 d
13	43.99 d	43.99 d	43.90 d	43.99 d	44.21 d	43.37 d	43.76 d	44.68 d	43.89 d
14	212.51 s	212.51 s	212.50 s	212.50 s	212.51 s	212.51 s	211.56 s	212.58 s	211.70 s
15	88.10 s	88.10 s	88.10 s	88.10 s	86.46 s	79.31 s	87.01 s	88.85 s	87.09 s
16	20.30 q	20.30 q	20.60 q	14.50 q	14.55 q	23.21 q	20.28 q	14.16 q	18.20 q
17	111.75 t	111.75 t	112.50 t	111.75 t	111.72 t	111.74 t	112.87 t	112.52 t	111.90 t
18	26.34 q	26.34 q	26.30 q	26.34 q	25.73 q	26.00 q	26.00 q	25.94 q	26.50 q
19	23.20 q	23.20 q	23.30 q	23.20 q	23.39 q	23.40 q	23.30 q	22.83 q	23.30 q
20	20.30 q	20.30 q	20.40 q	20.30 q	20.21 q	20.28 q	20.23 q	19.58 q	19.22 q
Ac_s	169.01 s	169.01 s	169.00 s	169.89 s	169.89 s	169.89 s	169.34 s	169.75 s	169.24 s
	169.89 s	169.89 s	169.87 s	169.99 s	169.97 s	169.90 s	169.63 s	169.80 s	169.35 s
	170.09 s	170.09 s	170.09 s	20.15 q	170.28 s	170.05 s	169.91 s	170.09 s	169.61 s
	20.15 q	20.15 q	20.14 q	21.12 q	20.14 q	20.20 q	170.02 s	20.26 q	169.72 s
	20.30 q	20.30 q	20.30 q		20.73 q	20.68 q	20.58 q	20.60 q	20.62 q
	21.00 q	21.00 q	21.03 q		21.13 q	21.25 q	20.68 q	20.61 q	20.63 q
							21.12 q		20.69 q
							22.34 q		20.94 q
^{i}Bu	174.37 s			174.35 s	175.18 s	175.22 s	175.62 s	175.79 s	175.43 s
	33.90 d			33.89 d	33.86 d	34.00 d	33.87 d	34.33 d	33.45 d
	18.50 q			18.50 q	18.50 q	18.55 q	18.25 q	18.72 q	17.42 q
	18.69 q			18.69 q	18.69 q	18.79 q	19.21 q	19.03 q	18.29 q
MeBu		174.37 s							
		41.18 d							
		26.14 t							
		11.45 q							
		16.20 q							
Bz	165.37 s	165.35 s	165.37 s	165.37 s	165.37 s	165.43 s		165.14 s	
	130.90 s	129.55 s	130.85 s	129.55 s	129.49 s	129.79 s		129.27 s	
	130.56 d	129.39 d	130.57 d	130.21 d	130.01 d	130.06 d		129.29 d	
	128.74 d	128.74 d	128.79 d	128.70 d	128.66 d	128.73 d		128.66 d	
	133.58 d	133.53 d	133.51 d	133.55 d	133.53 d	133.74 d		133.52 d	
Nic			165.40 s				165.36 s		165.50 s
			151.03 d				151.15 d		151.02 d
			125.70 s				125.74 s		125.56 s
			137.47 d				137.52 d		137.46 d
			123.24 d				123.21 d		123.24 d
			153.40 d				153.37 d		153.43 d
9-Nic									165.60 s
									151.14 d
									125.21 s
									137.46 d
									123.51d
									153.52 d

spin systems: C-3 to C-5, C-7 to C-9, and C-11 to C-20. It should be noted that although the methine protons of the fragment C-7 to C-9 appeared as broad singlets, pointing to a preferred conformation with dihedral angles near 90°, correlation peaks of H-8 with both H-7 and H-9 were evident in the COSY spectrum of **1**.

Extensive study of the $^{2,3}J_{C-H}$ correlations, inferred from the heteronuclear multiple-bond correlation (HMBC) spectrum (Figure 1), allowed the connection of all the above deduced moieties. In particular, the long-range correlations of both H-5 and H-7 with the unprotonated sp^2 carbon at δ 144.6 and with the sp^2 methylene carbon at δ 111.7 allowed us to connect the first two fragments. Analogously, the correlations exhibited by the methyl singlets H_3-18 and H_3-19 with C-9, C-10, and C-11 indicated the linkage of both the second and the third fragment to the unprotonated dimethyl substituted

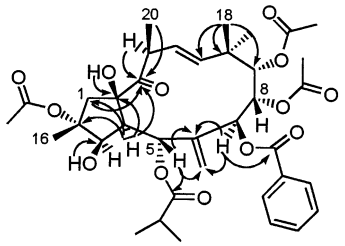


Figure 1. Selected HMBC correlations exhibited by euphodendroidin A (**1**).

C-10. On the "western" side of the molecule, $^{2,3}J_{C-H}$ correlations of the ketone carbon were a key point for the final assembling of the diterpenoid core. In particular, this carbon must be adjacent to C-13 (HMBC coupling with H-13 and H_3-20) and to the oxygenated carbon C-15 (^3J HMBC coupling with the isolated

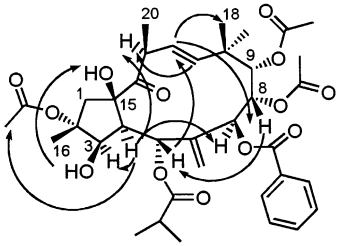


Figure 2. Selected ROESY correlations exhibited by euphodendroidin A (**1**).

methylene H_2-1 and with H-4, in turn further coupled with the two unprotonated carbons C-2 and C-15 and with C-1). This evidence allowed the final definition of the diterpenoid carbon framework of euphodendroidin A (**1**) as a bicyclo[10.3.0]pentadecane with 2,10,10,13-tetramethyl-6-*exo*-methylene branching, commonly known as jatrophane. The above analysis allowed us also to locate the oxygenated carbons at positions 2, 3, 5, 7, 8, 9, 14 (ketone), and 15. Only two of these oxygen atoms were present as free hydroxyl groups, and these functionalities were located at C-3 and C-15, respectively, by interpretation of the HMBC cross-peaks OH-3/C-3 and OH-15/C-15. With these data in our hands, the final task to define the planar structure of euphodendroidin A (**1**) was the correct location of the five acyl groups. The HMBC cross-peak of H-7 with the relatively high-field shifted ester carbon at δ 165.4 allowed the unambiguous location of the benzoyl group at C-7. Analogously, HMBC coupling of both the isobutyryl methine and H-5 to the same ester carbon at δ 174.4 indicated the linkage of the isobutyryl group at C-5. As a consequence, the other esterified carbons (C-2, C-8, and C-9) must be all acetylated, thus fully defining the planar structure of euphodendroidin A. It is noteworthy that the methyl singlet of the acetyl group linked at C-8 is considerably upfield shifted (δ_H 1.73) because of the deshielding effect of the adjacent benzoyl group.

The relative configuration of the stereogenic centers of euphodendroidin A (**1**) was deduced from coupling constant values, spatial proximities evidenced through a ROESY experiment (Figure 2) and comparison with literature data. In particular, the ROESY cross-peak of OH-3 with OH-15 and those of H-4 with both H-3 and Ac-2 were instrumental to define both the relative stereochemistry of the five-membered ring and the trans geometry of the ring junction. On the other hand, as already reported in the literature,[10] the very small value of the coupling constants $J_{H-7/H-8}$ and $J_{H-8/H-9}$ is consistent with the cis-orientation of C-8 and C-9 acyl groups and the trans-orientation of C-7 and C-8 acyl groups. The key ROESY cross-peak of H-4 with H-7 connected the relative stereochemistry of this fragment with that described above for the five-membered ring. Finally, the spatial proximities H-5/H-8 and H-4/H-13, revealed through the ROESY experiment, allowed the assignment of the relative orientation to H-5 and to H-13, respectively, thus completely defining the relative stereochemistry pattern of the jatrophane core of **1** (Figure 2). Assuming the *S* configuration at C-4, typical of all the members of the jatropahane family isolated to date (deduced by X-ray analysis of some derivatives[11]), the absolute stereochemistry reported in structure **1** can be confidently assigned to euphodendroidin A. Moreover, it should be noted that the small value of $J_{H-4/H-5}$ (1.2 Hz) has been correlated with a conformation of the 12-member ring with the *exo*-methylene group pointing outward and H-5 pointing inward.[12] Accordingly, H-5 showed ROESY cross-peak with both OH-15 and with H-12.

Euphodendroidins B−I (**2**−**6**, **8**−**10**) are closely related to compound **1**. In particular, with the only exception of **10**, they differ from euphodendroidin A (**1**) only for the acylation pattern at C-2, C-3, C-5, or C-7. Consequently, stereostructure elucidation of these molecules was greatly aided by comparison of their spectroscopic data with those obtained for **1**. Anyway, it should be noted that the complete set of 2D NMR spectra (COSY, HSQC, HMBC) was acquired for each metabolite in order to gain the complete and unambiguous assignment of ^{1}H and ^{13}C NMR resonances as listed in Tables 1 and 2, respectively. The same stereochemistry previously assigned to euphodendroidin A (**1**) was also confirmed for euphodendroidins B−I (**2**−**6**, **8**−**10**) by 2D NMR ROESY spectroscopy.

Euphodendroidin B (**2**), a colorless amorphous solid with molecular formula $C_{38}H_{50}O_{13}$, showed ^{1}H and ^{13}C NMR data nearly identical to those of **1**. In agreement with the molecular formula, the only difference was confined to the presence of an additional methylene group. In particular, in the case of **2**, the isobutyrate ester was replaced by a 2-methylbutyrate group, whose location at C-5 was secured by HMBC cross-peaks.

Similar considerations allowed us to easily infer the structure of euphodendroidin C (**3**), a colorless amorphous solid with molecular formula $C_{39}H_{45}NO_{13}$. In addition to the benzoate protons, the "aromatic" region of the ^{1}H NMR spectrum of **3** (Table 1) contained a singlet at δ 9.19, two doublets at δ 8.22 and 8.72, and a double doublet at δ 7.32. These proton signals, and the corresponding carbon resonances, associated with the use of the 2D HSQC experiment, suggested the presence of a nicotinate group. On the other hand, in high-field regions of ^{1}H and ^{13}C NMR spectra, the signals of **1** attributed to the isobutyrate group were lacking. Detailed analysis of 2D NMR data confirmed that euphodendroidin C (**3**) is an analogue of euphodendroidin A (**1**) where the isobutyrate group at C-5 has been replaced by a nicotinate group.

Euphodendroidin D (**4**), $C_{35}H_{46}O_{11}$ by HRFABMS, was disclosed to be the 2-desacetoxy analogue of euphodendroidin A (**1**). Indeed, in addition to unchanged C-7 to C-9 and C-11 to C-20 spin systems, COSY spectrum of **4** showed that H-3 is coupled not only with H-4 but also with a methine multiplet at δ 2.35 (H-2). In turn, this signal showed coupling with H_3-16 (δ 1.20) and with the methylene protons H_2-1 (δ 2.49 and 1.60). Of course, while signals of all the other protons of the molecule appeared nearly superimposable to those of euphodendroidin A (**1**), the replacement of an acetate group with a proton at C-2 caused dramatic effects on the ^{1}H NMR resonances of all the protons belonging to the five-membered ring. In particular, a consistent high-field shift was observed for H_2-1, H-3, H-4, and H_3-16 (see Table 1). The β-orientation of the methyl group at C-2 was deduced by the ROESY cross-peak of H-2 with H-4, implying the cis relationship of these two protons.

Euphodendroidin E (**5**), $C_{37}H_{48}O_{12}$ by HRFABMS, was isolated as a colorless amorphous solid. Both ^1H and ^{13}C NMR spectra of **5** (Tables 1 and 2, respectively), fully assigned through inspection of 2D NMR experiments, closely resembled those obtained for euphodendroidin D (**4**). In particular, the differences in the ^1H NMR spectrum of **5** were confined to the presence of an additional acetyl signal, to the substantial downfield shift of H-3 (δ 5.61 instead of δ 4.26), and to a small downfield shift of the signals attributed to the neighboring protons. These evidences, in agreement with the molecular formula and with ^{13}C NMR data, pointed for euphodendroidin B (**5**) to be the 3-acetylated analogue of euphodendroidin D (**4**). Standard acetylation (pyr/Ac_2O, rt) of **4** afforded **5** in high yield (in these conditions the tertiary alcohol at C-15 is not reactive), thus confirming the relationship between the two molecules and indicating that **4** and **5** share also the stereochemistry of the chiral centers.

Euphodendroidin F (**6**), $C_{37}H_{48}O_{13}$ by HRFABMS, is a close analogue of compound **5**. Indeed, inspection of 1D and 2D NMR data revealed that these two molecules differ almost uniquely for chemical shifts and multiplicities of C/H atoms belonging to the five-membered ring. In particular, in the case of compound **6**, C-2 revealed to be an oxygenated and unprotonated carbon atom (δ_C 86.3, s) and, accordingly, H_2-1 resonated as an uncoupled diastereotopic methylene (δ_H 2.41 and 1.98, mutually coupled doublets), while H-3 showed vicinal coupling only with H-4. In agreement with its molecular formula, showing only an oxygen atom more than that of compound **5**, the above observations pointed for euphodendroidin F (**6**) to be the 2-hydroxy derivative of the previously described euphodendroidin E (**5**). ROESY correlations indicated that compound **6** shows the same stereochemistry at C-2 as the other euphodendroidins oxygenated at that carbon (**1**−**3**).

Compound **7**, one of the major constituents of the jatrophane pool of *E. dendroides*, resulted to be a known compound already isolated from the latex of *E. terracina*.[13] Anyway, NMR spectra obtained for this molecule revealed to be a particularly useful tool for the structural elucidation of two further new euphodendroidins, namely G (**8**) and H (**9**).

Euphodendroidin G (**8**), a white amorphous solid with the molecular formula $C_{38}H_{49}NO_{14}$ deduced via HR-FABMS, differs from compound **7** only for the presence of a nicotinate group (δ_H 9.43, s; 8.95, d; 8.06, dd; 9.01, d) in place of the phenoxyacetate as acylating group at C-3. Analogously, euphodendroidin H (**9**) shows the linkage of a benzoate group at C-3. However, this molecule differs from compound **7** also for the replacement of the acetate group at C-2 with an hydrogen atom. As described above for euphodendroidin D (**4**), this change was particularly evident in the $^1H-^1H$ COSY spectrum of **9** where a single spin system connects C-1 to C-5, encompassing the methyl substitution at C-2.

The last novel jatrophane, euphodendroidin I (**10**), possesses some unique structural features when compared to the other metabolites of the same class isolated from *E. dendroides*. In particular, in this case all the oxygenated sp^3 carbons of the jatrophane core (positions 2, 3, 5, 7, 8, 9, and 15) are acylated, and, consequently, euphodendroidin I (**10**) is the only jatrophane diterpene of *E. dendroides* lacking free hydroxyls. The seven acyl groups were easily recognized as four acetate, one isobutyrate, and two nicotinate groups. The 2D HMBC spectrum allowed the correct location of the ester moieties on the diterpenoid polyol core. In particular, three of the four acetyl groups were located at C-3, C-7, and C-8, respectively, on the basis of the HMBC cross-peaks of the corresponding diterpenoid oxymethine protons with the relevant acetyl carbonyls. Analogously, the isobutyrate group was located at C-5 and one of the nicotinate groups was unambiguously located at C-9 taking advantage of the HMBC cross-peaks of the ester carbonyl at δ_H 165.6 with both H-9 and the broad singlet at δ_H 9.18 attributed to H-2 of the pyridine nucleus. Unfortunately, HMBC correlations could not help to infer the location of the two remaining esters (one acetate and one nicotinate), since these groups must be linked at unprotonated carbon atoms (C-2 and C-15, respectively). However, careful examination of proton resonances reported for all the series of jatrophane derivatives till now isolated suggested that the presence of an aromatic (benzoate or nicotinate) ester at C-2 results in a discrete downfield shift of H_3-16 when compared with the presence of an aliphatic ester at the same position.[14,15] Since H_3-16 of euphodendroidin I (**10**) resonates as a singlet at δ_H 1.70 (instead of the normal value of δ_H 1.52−1.53 for the acetylated C-2) the nicotinate group was confidently linked at C-2, and, consequently, the acetate must be positioned at C-15. This conclusion was further supported by the ROESY cross-peak between OAc-15 and CH_3-20, thus unambiguously defining the structure of euphodendroidin I (**10**).

The efficiency of euphodendroidins to inhibit P-glycoprotein-mediated daunomycin efflux was investigated by monitoring the intracellular accumulation of this drug. Euphodendroidin D (**4**) was found to be a highly potent inhibitor since it was almost 2-fold more efficient at 5 μM (183 ± 17%) than cyclosporin A (Cs A), the "golden standard" of Pgp modulators (Figure 3). The concentration dependency of inhibition is shown in Figure 4, in the case of Euphodendroidin B (**2**): a maximal inhibition was observed in the 5−10 μM range, with a half-maximal effect around 1−2 μM. Structure−activity relationships within the set of compounds showed that the substitution pattern at C-2, C-3, and C-5 is important for activity (Table 3). Thus, the relevance of a free hydroxyl at C-3 is highlighted by comparison of **4**, **5**, and **9**, which showed a marked decrease of activity upon acylation of the 3-hydroxyl. A second important feature is the lack of oxygenation at C-2, since hydroxylation or acyloxylation at this carbon was also detrimental for activity. Comparison of **4** with **1**−**3** cogently demonstrates this point. An additional decrease of efficiency was observed by acetylation of the 3-hydroxyl and oxygenation of C-2 as in **6**, **7**, and **10**. Within compounds acetoxylated at C-2, acylation of the 3-hydroxyl was less detrimental with nicotinic than phenoxyacetic acid (cf. **6** and **7**). Finally, the acyloxyl at C-5 could also modulate the inhibitory efficiency, since the nicotinyl derivative euphodendroidin C (**3**) was 2-fold less active (56 ± 4.8%) compared to its 2-methylbutyryl (euphodendroidin A, **2**) and isobutyryl (in euphodendroidin A, **1**) analogues.

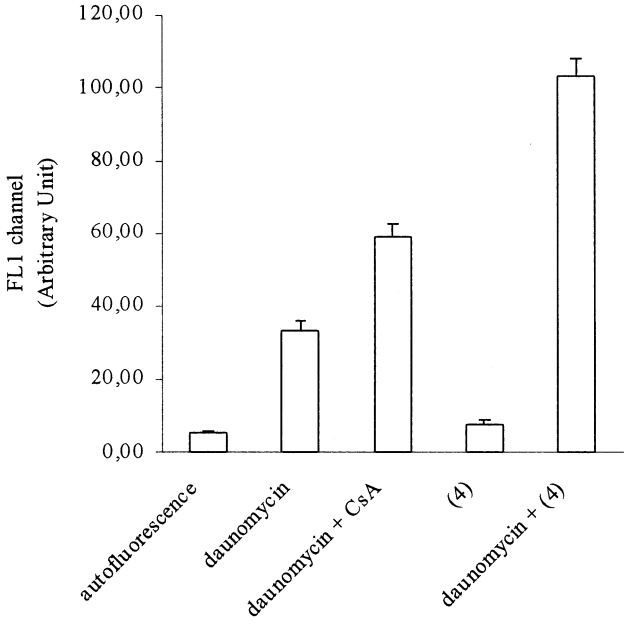


Figure 3. Accumulation of daunomycin in P-glycoprotein-overexpressing human K562/R7 leukemic cells, as monitored by flow cytometry. The cells were exposed to the drug for 1 h in the absence or presence of either cyclosporin A (CsA) or euphodendroidin D (**4**) and quickly washed in ice-cold PBS; intracellular drug concentrations were compared by the shift in fluorescence (FL channel). The results represent the mean ± SD of at least three independent experiments.

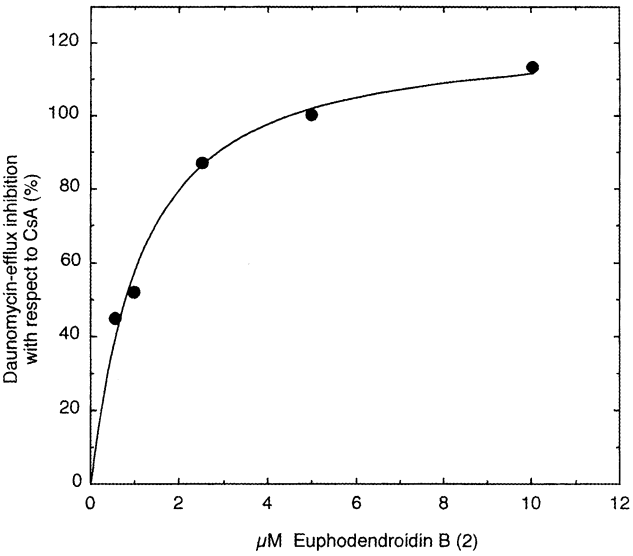


Figure 4. Concentration dependence of euphodendroidin B inhibition against daunomycin efflux. The human leukemic cells were incubated with daunomycin under conditions of Figure 3, in the presence of the increasing concentrations of euphodendroidin B (**2**), in the 0.5−10 μM range as indicated. The inhibition levels of daunomycin efflux were compared to that produced by a fixed concentration of CsA.

Previous studies on a structurally heterogeneous set of jatrophane polyesters pointed to the positive effect of overall lipophilicity on Pgp binding and suggested the importance of the oxygen substituent at C-9.[9] On the other hand, the structural diversity of the set of compounds investigated prevented the analysis of point mutations. In contrast, the present series of jatrophanes with closely related structures has allowed us to demonstrate the critical role of hydroxyl group at position 3, the negative effect of any substitution at adjacent position 2 and the differential effect of substituent at position 5. Within the set of compounds investigated, the powerful Pgp inhibiting activity of euphodendroidin D (**4**) qualifies this compound as a very promising lead for chemosensitization and validates the biological potential of jatrophane polyesters.

Table 3. Inhibition by Jatrophanes, Relatively to Cyclosporin A, of Cellular P-Glycoprotein-Mediated Daunomycin Efflux Monitored by Intracellular Drug Accumulation[a]

compound name	daunomycin-efflux inhibition with respect to CsA, %
euphodendroidin A (**1**)	97 ± 13.5
euphodendroidin B (**2**)	105 ± 12.7
euphodendroidin C (**3**)	56 ± 4.8
euphodendroidin D (**4**)	183 ± 17
euphodendroidin E (**5**)	88 ± 6.7
euphodendroidin F (**6**)	38 ± 4.1
jatrophane diterpene (**7**)	75 ± 12.2
euphodendroidin G (**8**)	99 ± 6.5
euphodendroidin H (**9**)	74 ± 9.5
euphodendroidin I (**10**)	42 ± 7.4

[a] The increase in daunomycin accumulation produced by each jatrophane derivative was determined under the same conditions as in Figure 3. The effect produced by cyclosporin A (CsA) was taken as 100%.

Experimental Section

General Experimental Procedures. Low- and high-resolution FAB mass spectra (glycerol matrix) were measured on a Prospec Fisons mass spectrometer. Optical rotations were determined on a Perkin-Elmer 192 polarimeter equipped with a sodium lamp (589 nm) and 10-cm microcell. ^{1}H and ^{13}C NMR spectra were recorded at 500 and 125 MHz, respectively, on a Bruker AMX-500 spectrometer. Chemical shifts were referred to the residual solvent signal ($CDCl_3$: δ_H 7.26, δ_C 77.0). The multiplicities of ^{13}C NMR resonances were determined by DEPT experiments. ^{1}H connectivities were determined by using COSY experiments; one-bond heteronuclear ^{1}H−^{13}C connectivities were determined with 2D HSQC pulse sequence with an interpulse delay set for $^1J_{CH}$ of 130 Hz. Two and three bond heteronuclear ^{1}H−^{13}C connectivities were determined with 2D NMR HMBC experiments, optimized for $^{2-3}J_{CH}$ of 8 Hz. Measurement of spatial coupling was obtained through 2D ROESY experiments. Medium-pressure liquid chromatography (MPLC) was performed on a Büchi 861 apparatus using silica gel (230−400 mesh) as stationary phase. HPLC in isocratic mode was performed on a Varian apparatus equipped with an RI-3 refractive index detector.

Plant Material, Extraction, and Isolation. The latexes of *Euphorbia dendroides* were collected around Arzana (Nuoro, Italy) in March 1999, February 2000, and March 2000. The spurge has been identified by M. B. and a voucher specimen deposited at the Dipartimento di Scienze Botaniche, Cagliari (Italy). Each sample of latex (ca. 12, 7, and 6 mL, respectively) was extracted with three aliquots of EtOAc, and the obtained extract was filtered on silica gel in order to eliminate the gummous fraction. The EtOAc soluble material obtained from the three different collections were concentrated in vacuo to afford three crude organic extract. (collection March 1999: 3 g; collection February 2000: 2.1 g; collection March 2000: 0.9 g). The organic extract of the first collection was chromatographed by MPLC on silica gel column (230−400 mesh) using a gradient system from hexane to EtOAc. The following fractions contained diterpenes and were further purified: fr. 1 (731.7 mg) eluted in hexane/EtOAc 1:1, fr. 2 (497.9 mg) eluted in hexane/EtOAc 45:55, fr. 3 (266.3 mg) eluted in hexane/EtOAc 4:6.

Fraction 1 was first separated by MPLC (hexane/EtOAc 6:4) and afforded a mixture of compounds (100.0 mg) further purified by HPLC on silica gel (hexane/EtOAc 7:3) to give euphodedroidin B (**2**, 21 mg), D (**4**, 3 mg), F (**6**, 25 mg), and H

(**9**, 13 mg). Fraction 2 was first separated by MPLC (from hexane/EtOAc 6:4 to hexane/EtOAc 4:6) and afforded two fractions: 2A (157.7 mg) and 2B (264.5). Fraction 2A was purified by silica gel HPLC (hexane/EtOAc 65:35) to give euphodedroidin A (**1**, 11 mg) and E (**5**, 25 mg). Fraction 2B was purified by HPLC (hexane/EtOAc 55:45) to give compound **7** (28 mg). Fraction 3 was first separated by MPLC (from hexane/EtOAc 2:8 to EtOAc) and afforded two fractions: 3A (23.8 mg) and 3B (40.4 mg). Fraction 3A was purified by HPLC (hexane/EtOAc 2:8) to give euphodedroidin C (**3**, 5 mg) and G (**8**, 2 mg). Fraction 3B was purified by HPLC on silica gel (hexane/EtOAc 25:75) to give euphodedroidin I (**10**, 4.5 mg). The EtOAc extracts obtained from the collections of February and March 2000, subjected to the same purification procedure, showed a nearly identical jatrophane diterpene composition.

Euphodendroidin A (1). Colorless amorphous solid. $[\alpha]^{25}_D$ +43.1 (c = 0.1 $CHCl_3$); IR (KBr): ν_{max} 3450, 1748, 1709, 1480 cm^{-1}. HRFABMS (positive ion): found m/z 701.3165 $[M + H]^+$; calculated for $C_{37}H_{49}O_{13}$ m/z 701.3173; ^1H NMR data: Table 1. ^{13}C NMR data: Table 2.

Euphodendroidin B (2). Colorless amorphous solid. $[\alpha]^{25}_D$ +2.9 (c = 0.1 $CHCl_3$); IR (KBr): ν_{max} 3450, 1749, 1709, 1480 cm^{-1}.HRFABMS (positive ion): found m/z 715.3327 $[M + H]^+$; calculated for $C_{38}H_{51}O_{13}$ m/z 715.3330; ^1H NMR data: Table 1. ^{13}C NMR data: Table 2.

Euphodendroidin C (3). Colorless amorphous solid. $[\alpha]_D^{25}$ −5.0 (c = 0.1 $CHCl_3$); IR (KBr): ν_{max} 3453, 1751, 1711, 1482 cm^{-1}. HRFABMS (positive ion): found m/z 736.2975 $[M + H]^+$; calculated for $C_{39}H_{46}NO_{13}$ m/z 736.2969; ^1H NMR data: Table 1. ^{13}C NMR data: Table 2.

Euphodendroidin D (4). Colorless amorphous solid. $[\alpha]^{25}_D$ +9.3 (c = 0.1 $CHCl_3$); IR (KBr): ν_{max} 3444, 1744, 1711, 1466 cm^{-1}. HRFABMS (positive ion): found m/z 643.3113 $[M + H]^+$; calculated for $C_{35}H_{47}O_{11}$ m/z 643.3118; ^1H NMR data: Table 1. ^{13}C NMR data: Table 2.

Acetylation of Euphodendroidin D (4) to E (5). Euphodendroidin D (**4**) (1.0 mg) was dissolved in dry pyridine (50 μL) and treated with Ac_2O (50 μL). After standing overnight at room temperature, the reaction was worked up by addition of a few drops methanol to destroy the excess Ac_2O, water (ca. 1 mL) and EtOAc (ca. 3 mL). The organic phase was washed sequentially with 2 N H_2SO_4, sat. $NaHCO_3$, and brine. After drying (Na_2SO_4) and removal of the solvent, the residue afforded 0.9 mg of **5**.

Euphodendroidin E (5). Colorless amorphous solid. $[\alpha]^{25}_D$ +34.4 (c = 0.1 $CHCl_3$); IR (KBr): ν_{max} 3448, 1748, 1712, 1480 cm^{-1}. HRFABMS (positive ion): found m/z 685.3226 $[M + H]^+$; calculated for $C_{37}H_{49}O_{12}$ m/z 685.3224; ^1H NMR data: Table 1. ^{13}C NMR data: Table 2.

Euphodendroidin F (6). Colorless amorphous solid. $[\alpha]^{25}_D$ +20.8 (c = 0.1 $CHCl_3$); IR (KBr): ν_{max} 3449, 1749, 1711, 1480 cm^{-1}. HRFABMS (positive ion): found m/z 701.3169 $[M + H]^+$; calculated for $C_{37}H_{49}O_{13}$ m/z 701.3173; ^1H NMR data: Table 1. ^{13}C NMR data: Table 2.

Euphodendroidin G (8). White amorphous solid. $[\alpha]^{25}_D$ +3.5 (c = 0.1 $CHCl_3$); IR (KBr): ν_{max} 3448, 1748, 1712, 1468 cm^{-1}. HRFABMS (positive ion): found m/z 744.3234 $[M + H]^+$; calculated for $C_{38}H_{50}NO_{14}$ m/z 744.3231; ^1H NMR data: Table 1. ^{13}C NMR data: Table 2.

Euphodendroidin H (9). Colorless amorphous solid. $[\alpha]^{25}_D$ +22.1 (c = 0.1 $CHCl_3$); IR (KBr): ν_{max} 3450, 1749, 1711, 1476 cm^{-1}. HRFABMS (positive ion): found m/z 685.3227 $[M + H]^+$; calculated for $C_{37}H_{49}O_{12}$ m/z 685.3224; ^1H NMR data: Table 1. ^{13}C NMR data: Table 2.

Euphodendroidin I (10). Colorless amorphous solid. $[\alpha]^{25}_D$ −1.3 (c = 0.1 $CHCl_3$); IR (KBr): ν_{max} 1755, 1718, 1456 cm^{-1}. HRFABMS (positive ion): found m/z 849.3441 $[M + H]^+$; calculated for $C_{44}H_{53}N_2O_{15}$ m/z 849.3446; ^1H NMR data: Table 1. ^{13}C NMR data: Table 2.

Biological Assay. For inhibition of P-glycoprotein-mediated drug efflux, 1 millon K562/R7 human leukemic cells, expressing high levels of P-glycoprotein, were incubated for 1 h at 37 °C in 1 mL of phosphate buffer saline containing a final concentration of 10 μM daunomycin, in the presence or absence of inhibitor. The cells were then washed two times with ice-cold phosphate buffer saline and kept on ice until analysis by flow cytometry on a FACS-II (Becton-Dickinson Corp., Mountain View, CA), as described previously.[16,17] Assays were performed in duplicate, in a least three separate experiments. Cyclosporin A, a potent inhibitor of P-glycoprotein was used as a positive control, at a final 2 μM concentration. The ability of 5 μM jatrophanes [or 0.5−10 μM euphodendroidin B (**2**)] to inhibit P-glycoprotein-mediated drug efflux was quantified by comparing the induced shift in fluorescence to that obtained with cyclosporin A.

Purity Criteria for Target Compounds. The degree of purity of all tested compounds (**1**−**10**) was over 95% as indicated by the appearance of a single peak using two different HPLC eluent systems. Retention times (t_R) are expressed in minutes.

Euphodendroidin A (**1**): Hexane/EtOAc 65:35 with t_R 28.5. CH_2Cl_2/EtOAc 7:3 with t_R 16.2.

Euphodendroidin B (**2**): Hexane/EtOAc 7:3 with t_R 22.5. CH_2Cl_2/EtOAc 75:25 with t_R 10.5.

Euphodendroidin C (**3**): Hexane/EtOAc 2:8 with t_R 12.6. CH_2Cl_2/EtOAc 3:7 with t_R 7.2.

Euphodendroidin D (**4**): Hexane/EtOAc 7:3 with t_R 20.4. CH_2Cl_2/EtOAc 75:25 with t_R 7.8.

Euphodendroidin E (**5**): Hexane/EtOAc 65:35 with t_R 32.1. CH_2Cl_2/EtOAc 7:3 with t_R 19.5.

Euphodendroidin F (**6**): Hexane/EtOAc 7:3 with t_R 31.5. CH_2Cl_2/EtOAc 75:25 with t_R 19.2.

Compound **7**: Hexane/EtOAc 55:45 with t_R 39.3. CH_2Cl_2/EtOAc 6:4 with t_R 27.0.

Euphodendroidin G (**8**): Hexane/EtOAc 2:8 with t_R 17.1. CH_2Cl_2/EtOAc 3:7 with t_R 12.

Euphodendroidin H (**9**): Hexane/EtOAc 7:3 with t_R 21.6. CH_2Cl_2/EtOAc 75:25 with t_R 9.3.

Euphodendroidin I (**10**): Hexane/EtOAc 25:75 with t_R 31.5. CH_2Cl_2/EtOAc 3:7 with t_R 18.9.

Acknowledgment. This work was supported by Italian MIUR (Progetto Sostanze Naturali ed Analoghi Sintetici ad Attività Antitumorale) and French grants from the Ligue Nationale contre le Cancer (commité du Rhône) and Association pour le Recherche sur le Cancer (ARC 4631). Mass and NMR spectra were recorded at the "Centro Interdipartimentale di Analisi Strumentale" of the University of Naples "Federico II". The assistance of the staff is gratefully acknowledged.

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JM030787E