Jatrophane Diterpenes as Modulators of Multidrug Resistance. Advances of Structure−Activity Relationships and Discovery of the Potent Lead Pepluanin A

Gabriella Corea,[†] Ernesto Fattorusso,[†] Virginia Lanzotti,*[,‡] Riccardo Motti,[§] Pierre-Noël Simon,[∥] Charles Dumontet,[∥] and Attilio Di Pietro[⊥]

Dipartimento di Chimica delle Sostanze Naturali, Università di Napoli "Federico II", Via D. Montesano 49, I-80131 Napoli, Italy, DISTAAM, Università degli Studi del Molise, Via F. De Sanctis, I-86100 Campobasso, Italy, Dipartimento di Arboricoltura, Botanica e Patologia Vegetale, Università di Napoli "Federico II", Via Università 100, I-80055 Portici (NA), INSERM U590, 8 Avenue Rockefeller, 69880 Lyon, France, and Institut de Biologie et Chimie des Proteines, UMR 5086 CNRS/Université Claude Bernard-Lyon I et IFR 128, Passage du Vercors 7, 69367 Lyon Cedex 07, France

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From the whole plant of *Euphorbia peplus* L., five new diterpenes based on a jatrophane skeleton (pepluanins A−E, **1**−**5**) were isolated, together with two known analogues (**6** and **7**), which served to divulge in detail the structure−activity relationships within this class of P-glycoprotein inhibitors. The results revealed the importance of substitutions on the medium-sized ring (carbons 8, 9, 14, and 15). In particular, the activity is collapsed by the presence of a free hydroxyl at C-8, while it increases with a carbonyl at C-14, an acetoxyl at C-9, and a free hydroxyl at C-15. The most potent compound of the series, pepluanin A, showed a very high activity for a jatrophane diterpene, outperforming cyclosporin A by a factor of at least 2 in the inhibition of Pgp-mediated daunomycin transport.

Introduction

Plants of the genus *Euphorbia* have a rich ethnopharmacology already documented in Greek and Roman medical literature. Thus, the plants were used to reduce toothache, to remove warts, as purgatives, and in asthma and bronchial catarrh.[1] Anyway, the plants are so inimical to human flesh that they border on toxic and have generally been avoided, if not feared, and used very rarely and cautiously. Recently, the discovery of jatrophane diterpenes as a new class of powerful inhibitors of P-glycoprotein has open new frontiers for research studies on this genus.[2]

P-glycoprotein (Pgp) belongs to a family of plasma membrane proteins encoded by the MDR (multidrug resistance) gene.[3,4] Pgp functions as a membrane-localized drug transport mechanism that has the ability to actively pump out all currently prescribed HIV-protease inhibitors from the intracellular cytoplasm, substantially attenuating their cytotoxic effect.[5,6] Furthermore, Pgp-mediated resistance is not restricted to a specific inducer but it is generally extended to chemically unrelated antitumor drugs (cross-resistance).[7−9]

In a search for natural revertant agents, we have recently reported the isolation of nine new jatrophane diterpenoids from *Euphorbia dendroides* L. These compounds were based on a structurally homogeneous skeleton and this gave us the possibility to propose a first insight into the structure−activity relationships within this class of P-glycoprotein inhibitors.[10] The results showed the existence of a definite structure−activity relationship that suggested the involvement of the southwestern fragment of the molecule (C-2/C-5) in binding, highlighting the relevance of a free OH group at C-3.[10]

We now describe from *Euphorbia peplus* L. the structure elucidation of five new jatrophane diterpenes, together with two known compounds, which were individually investigated for their Pgp-binding properties. The results were used to extend the structure−activity relationships to the other oxygenated carbon atoms of the medium-sized ring (C-7/C-15). In particular, one of the isolated metabolites, pepluanin A (**1**), showed an especially potent activity detected by now for a jatrophane diterpene, outperforming cyclosporin A by a factor of at least 2 in the inhibition of Pgp-mediated daunomycin transport.

Results and Discussion

The EtOAc extract of *E. peplus*, collected in the volcanic area of Vesuvio (Campania, Italy) in March 2002 was subjected to successive MPLC and HPLC chromatographic separations on a silica gel column (hexane−EtOAc, gradient) to afford compounds **1**−**7**.

Pepluanin A, isolated in relatively high yield (10.1 mg), gave in the HRFABMS spectrum a molecular ion at *m*/*z* 822.3362, corresponding to the formula $C_{43}H_{51}O_{15}N$. These data, together with the NMR profiles, were indicative of a diterpenoid polyol bearing several ester groups. The nature of the ester groups has been identified by ^1H and ^{13}C NMR spectra (Tables 1 and 2) that showed the presence of five acetates, one benzoate, and one nicotinate.

* To whom correspondence should be addressed. Telephone: +39-0874-404649. Fax: +39-0874-404652. E-mail: lanzotti@unimol.it.
[†] Dipartimento di Chimica delle Sostanze Naturali, Università di Napoli "Federico II".
[‡] DISTAAM, Università degli Studi del Molise.
[§] Dipartimento di Arboricoltura, Botanica e Patologia Vegetale, Università di Napoli "Federico II".
[∥] INSERM U590.
[⊥] UMR 5086 CNRS/Université Claude Bernard-Lyon I et IFR 128.

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Table 1. ^{1}H NMR Data ($CDCl_3$) of Pepluanins A−E (1−5) (500 MHz, $CDCl_3$)[a]

	δ (ppm) and multiplicity				
H	**1**	**2**	**3**	**4**	**5**
1α	2.82 d	2.87 d	2.61 d	3.00 dd	2.98 d
1β	2.15 d	2.06 d	2.23 d	1.60 dd	2.11 d
2				2.21 m	
3	5.98 brd	5.94 brd	5.80 brd[b]	5.43 brs	5.95 brd
4	3.63 brs[b]	3.76 brs	3.27 brs	2.64 brs	3.60 brs
5	5.83 brs	5.80 brs	5.79 brs[b]	5.80 brs	5.30 brs
7	5.58 s	5.35 s	5.59 s	4.76 brdd[b]	5.43 s
8	5.38 s	4.15 d	5.25 s	2.05 m	3.90 d
9	5.14 s	5.08 s	4.96 s	4.77 brdd[b]	5.02 s
11	6.10 d	6.14 d	5.92 d	5.84 d	6.14 d
12	5.75 dd	5.66 dd	5.66 dd	5.55 dd	5.55 dd
13	2.83 dq[b]	2.88 dq[b]	2.68 dq	3.48 dq	4.01 dq
14	5.23 s	5.14 s	5.10 s		
16	1.46 s	1.46 s	1.54 s	0.89 d	1.56 s
17	4.45 brs	4.47 brs	4.45 brs	5.05 brs	4.87 brs
	4.82 brs	4.80 brs	4.91 brs	5.09 brs	5.04 brs
18	1.02 s	1.08 s	0.93 s	1.00 s	1.09 s
19	1.26 s	1.36 s	1.38 s	1.04 s	1.29 s
20	1.19 d	1.12 d	1.15 d	1.15 d	1.33 d
4-OH					
5- OH					
8-OH		2.99 d			3.15 d
15-OH	3.62 s	3.65	3.59 s		4.18 s
OAc	1.97 s	2.02 s	1.93 s	1.99 s	1.80 s
	2.03 s	2.11 s	1.99 s	2.00 s	2.21 s
	2.03 s	2.21 s	2.03 s	2.15 s	
	2.11 s		2.10 s	2.15 s	
	2.25 s		2.17 s	2.17 s	
OiBu 2			2.62 m[b]		1.90 m
OiBu 3			1.18 d		0.50 d
OiBu 4			1.22 d		0.94 d
OMeBu 2		1.98 tq			
OMeBu 3		1.52 ddq			
OMeBu 3′		1.29 ddq			
OMeBu 4		0.60 t			
OMeBu 5		0.91 d			
OBz 2,6	8.06 d	8.05 d	8.09 d		8.19 d
OBz 3,5	7.43 t[b]	7.43 t[b]	7.45 t		7.43 t
OBz 4	7.57 t	7.58 t	7.57 d		7.56 t
ONic 2	9.30 brs	9.30 brs			9.26 brs
ONic 4	8.35 d	8.38 d			8.34 d
ONic 5	7.42 brdd[b]	7.42 brdd[b]			7.44 brdd[b]
ONic 6	8.82 brd	8.71 brd			8.84 brd

[a] J_{H-H}. For **1**−**3**, **5**: 1α∠1β = 14.0; 3−4 = 4−5 = 7−8 = 8−9 =17a−17b =1.2 Hz; 11−12 = 16.0 Hz; 12−13 = 9.5 Hz; 13−20 = 7.0 Hz. For **2**, **4**: 8-OH = 11.5 Hz. **4**: 1α∠1β =14.0 Hz; 1α∠2 = 7.7 Hz; 1β∠2 = 11.0 Hz; 2−16 = 6.5 Hz; 2−3 = 7.0 Hz. ^{i}Bu: 2−3 = 2−4 = 7.0 Hz. MeBu: 2−3a = 2−3b = 3a−4 = 3b−4 = 2−5 = 7.0 Hz; 3a−3b = 14.0 Hz. Bz: 2−3 = 3−4 = 7.4 Hz. Nic: 4−5 = 8.0 Hz; 5−6 = 5.0 Hz. [b] Overlapped by other signals.

Table 2. ^{13}C NMR Data ($CDCl_3$) of Pepluanins A−E (1−5) (125 MHz, $CDCl_3$)

	δ (ppm) and multiplicity				
C	**1**	**2**	**3**	**4**	**5**
1	49.91 t	49.68 t	50.77 t	46.15 t	51.03 t
2	88.42 s	88.53 s	88.22 s	38.23 d	88.86 s
3	80.47 d	80.55 d	80.01 d	76.52 d	77.64 d
4	44.76 d	44.78 d	44.58 d	52.70 d	47.83 d
5	71.63 d	71.87 d	71.50 d	68.46 d	68.04 d
6	143.07 s	144.03 s	144.14 s	145.96 s	143.45 s
7	68.04 d	68.01 d	66.79 d	69.02 d	68.54 d
8	70.63 d	70.33 d	70.93 d	34.19 t	69.72 d
9	79.42 d	86.75 d	80.43 d	74.67 d	86.90 d
10	41.00 s	40.44 s	40.68 s	40.38 s	40.67 s
11	133.87 d	133.82 d	134.27 d	138.30 d	136.83 d
12	131.87 d	131.48 d	130.98 d	129.73 d	130.06 d
13	37.34 d	37.54 d	37.37 d	43.23 d	43.79 d
14	81.79 d	79.38 d	79.34 d	211.81 s	211.70 s
15	84.17 s	84.43 s	83.73 s	92.76 s	87.79 s
16	23.76 q	23.58 q	21.98 q	13.33 q	20.88 q
17	109.69 t	109.00 t	110.03 t	110.14 t	112.09 t
18	26.72 q	27.48 q	26.55 q	26.27 q	26.77 q
19	22.92 q	23.05 q	23.25 q	23.05 q	22.90 q
20	23.55 q	23.53 q	23.48 q	19.28 q	19.53 q
OAc	170.81 s	171.15 s	170.45 s	170.41 s	170.27 s
	170.80 s	170.25 s	170.44 s	169.94 s	169.30 s
	169.75 s	168.59 s	169.65 s	169.84 s	
	169.74 s		169.64 s	169.75 s	
	168.00 s		168.06 s	169.47 s	
	22.47 q	22.48 q	22.29 q	21.23 q	22.23 q
	20.87 q	21.16 q	20.82 q	21.17 q	20.72 q
	20.62 q	20.51 q	20.64 q	20.88 q	
	20.48 q		20.55 q	20.82 q	
	20.28 q		20.44 q	20.70 q	
OiBu			175.01 s		174.55 s
			33.82 d		33.34 d
			19.30 q		18.86 q
			18.41 q		17.17 q
OMeBu		174.37 s			
		41.18 d			
		26.14 t			
		11.45 q			
		16.20 q			
OBz	163.87 s	164.75 s	164.68 s		164.80 s
	129.72 s	130.03 s	130.08 s		129.97 s
	129.67 d	129.62 d	129.64 d		129.96 d
	128.33 d	128.39 d	128.37 d		128.27 d
	133.13 d	133.09 d	133.10 d		133.03 d
ONic	164.50 s	165.67 s			165.45 s
	151.27 d	151.55 d			150.68 d
	125.98 s	125.05 s			125.71 s
	137.27 d	137.62 d			137.99 d
	123.29 d	123.33 d			123.72 d
	153.80 d	153.98 d			153.54 d

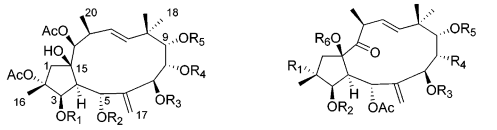
Besides these signals, the ^{1}H and ^{13}C NMR spectra also indicated the presence of four methyls (three singlets and one doublet), two double bonds (one exocyclic and one trans disubstituted), and eight oxygenated sp^3 carbons (six were secondary and two tertiary), seven of which being esterified. The remaining oxygenated function has been ascribed to an hydroxyl group by the observation of a singlet at δ 3.62 in the ^{1}H NMR spectrum that exchanges with D_2O.

A 2D HSQC spectrum of **1** allowed correlations of protons with directly linked carbon atoms. By COSY and HOHAHA techniques the following sequences could be extracted: C-3 to C-5, C-7 to C-9, and C-11 to C-14, including C-20.

Extensive study of the $^{2,3}J_{C-H}$ correlations, obtained from the 2D HMBC spectrum, allowed the connection of all the above-deduced moieties. In particular, HMBC cross-peaks of H-1α and H-1β with C-2 and C-15; H_3-16 with C-1, C-2, and C-3; H-3 with C-2, C-4, and C-15; and 15-OH with C-1 and C-4 built ring A. Further cross-peaks of H-5 with C-4 and C-6; H_2-17 with C-5 and C-7; and H-8 with C-7, C-9, and C-10 extended the structure up to this point. Similarly, correlations of H_3-18 and H_3-19 with C-9, C-10, and C-11; H_3-20 with C-12, C-13, and C-14; and H-14 with C-12, C-4, and C-15 closed a 12-membered jatrophane ring. The cross-peaks of H-3 with the benzoate carbonyl and of H-9 with the nicotinate carbonyl ensured the position of benzoate and nicotinate at C-3 and C-9, respectively. As a consequence, the other esterified carbons (C-2, C-5, C-7, C-8, and C-14) must be all acetylated, thus fully defining the planar structure of pepluanin A.

The relative configuration of **1** was determined by analyzing the *J* pattern and the results of NOE correlations. NOE effects of 15-OH with H-1β, H_3-16, and the ortho benzoyl protons and of H-4 with H-3 defined the relative configuration of ring A, as depicted in Chart 1. Concerning the macrocyclic ring protons, we observed

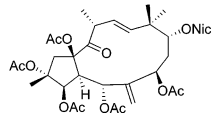
Chart 1



	R_1	R_2	R_3	R_4	R_5
1	Bz	Ac	Ac	Ac	Nic
2	Bz	Ac	MeBu	H	Nic
3	Ac	iBu	Bz	Ac	Ac
6	Bz	Ac	Ac	H	Ac

iBu = isobuthyryl
MeBu =2-methyl-buthyryl
Bz = benzoyl
Nic = nicotinyl

	R_1	R_2	R_3	R_4	R_5	R_6
4	H	Ac	Ac	H	Ac	Ac
5	OAc	Bz	iBu	OH	Nic	H



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a rather peculiar coupling pattern of compound **1**, with all coupling constants on the order of 0−2 Hz, thus suggesting an orthogonal relationship between the protons of the macrolide. This feature has already been found for all $\Delta^{6(17)}$,Δ^{11}-jatrophanes isolated so far from other *Euphorbia* species.[11] Among the NOE effects, those of the methyls with the macrocyclic protons (H_3-19/H-8, H_3-18/H-11, H_3-18/H-7, H_3-20/H_2-17) and among the protons (H-13/H-4, H-13/H-11, H-13/H-1α, H-11/H-7, H-5/H-8) were diagnostic, allowing us to completely define the relative stereochemistry pattern of the jatrophane core of **1** (Chart 1). Moreover, it should be noted that the small value $J_{H-4/H-5}$ (1.2 Hz) has been correlated with a predominant conformer of the 12-membered 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.

Pepluanins B−E (**2**−**5**) are closely related to pepluanin A. In particular, apart from C-14, they differ from one another by their acylation pattern. Consequently, stereostructure elucidation of these molecules was greatly aided by comparison of their spectroscopic data with those obtained for pepluanin A and for the other known jatrophanes. Anyway, it should be noted that a 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 relative stereochemistry previously assigned to pepluanin A was also confirmed for pepluanins B−E by 2D NMR ROESY spectroscopy.

Pepluanin B (**2**, 2.5 mg) gave in the HRFABMS spectrum a molecular ion peak at *m*/*z* 822.3725, corresponding to the formula $C_{44}H_{55}O_{14}N$. The NMR spectra of **2** (Tables 1 and 2) revealed close similarities with those of **1** and showed the lack of an ester group, thus pointing to a hexasubstituted jatrophane skeleton for this compound. The nature of the ester groups has been determined by ^{1}H and ^{13}C NMR spectra as three acetates, one 2-methyl butanoate, one benzoate, and one nicotinate. The ^{1}H NMR spectra showed the presence of an additional hydroxyl group, resonating as an exchangeable doublet at δ 2.99, mutually coupled with a signal at δ 4.15 (d, J = 11.5 Hz). HMBC correlations allowed us to assign the last signal to H-8, thus locating the free hydroxyl group at C-8. HMBC correlations also placed the 2-methyl butanoate at C-7 and confirmed the same position of all the other ester groups as that of pepluanin A, as already suggested from the similarities of the NMR resonances for pepluanins A and B.

Pepluanin C (**3**, 7.1 mg), $C_{41}H_{54}O_{15}$ by HRFABMS, was isolated as a colorless amorphous solid. Analysis of its ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) indicated the same core lipid structure of pepluanins A and B and in addition revealed the presence of five acetyls, an isobuthyryl, and a benzoyl group. An exchangeable singlet at δ 3.59 was attributed to 15-OH by the same evidences as those obtained for **1** and **2**. HMBC cross-peaks of H-5 (δ 5.79 brs) with the carbonyl of the isobutyrate (δ 175.01) and of H-7 (δ 5.59 s) with the carbonyl of the benzoate (δ 164.68) indicated the linkage of isobuthyryl and benzoyl at C-5 and C-7, respectively. As a consequence, the other esterified carbons (C-2, C-8, C-9, and C-14) must be acetylated.

Pepluanin D (**4**, 6.3 mg), $C_{30}H_{42}O_{11}$ by HRFABMS, showed some remarkable differences when compared to pepluanins A−C: (i) in the ^{1}H NMR of **4** (Table 1), the signal due to H_3-16 appeared as a doublet shifted upfield (δ 0.89, d, in **4** vs δ 1.46, s, in **1**), coupled with a proton signal at δ 2.21 (H-2); (ii) the absence in the ^{1}H NMR of **4** of the characteristic singlet due to H-14 (see Table 1) and the presence in the ^{13}C NMR spectrum (Table 2) of a ketone carbonyl at δ 211.81, coupled in the HMBC spectrum with H-13, H-11, H-1α, and H-1β; (iii) a dramatic change in the ^{1}H NMR resonances of H-7/H-8 and H-9 in compound **4**. A combined analysis of the 2D COSY, HSQC, and HMBC spectra allowed us to identify at position 8 a methylene carbon (δ 2.05, m), to define the core structure of pepluanin D as depicted in Chart 1, and to locate the five acetyls at C-3, C-5, C-7, C-9, and C-15. The ^{1}H and ^{13}C NMR assignments of **4** are reported in Tables 1 and 2.

Pepluanin E (**5**, 5.1 mg) had the formula $C_{41}H_{49}O_{13}N$ by HRFABMS. A preliminary analysis of the MS and NMR data indicated a jatrophane core structure esterified with two acetyls, one benzoyl, one isobuthyryl, and one nicotinyl and bearing two hydroxyls (one on a methine carbon and the other on a quaternary carbon). A carbonyl function at δ 211.70 appeared in the ^{13}C NMR spectrum, thus indicating, as found for compound **4**, a ketone functionality at C-14. However, the substitution pattern of pepluanin E appeared more related to that of pepluanin B. A detailed analysis (2D COSY, HSQC, and HMBC) of **5** in comparison to **2** confirmed the same substitutions for both compounds, the differences being confined to the ester group at C-7 (iBu in **5** instead of MeBu in **2**) and to the group on C-14 (a ketone for **5** instead of an acetate for **2**). Thus, the structure of **5** has been assigned as indicated in Chart 1.

The EtOAc extract of *E. peplus* also afforded the known compounds **6** and **7**, which were characterized by comparing their NMR data with those reported in the literature.[11]

The biological activity of the isolated jatrophane diterpenes (**1**−**7**) was assayed through their ability to inhibit P-glycoprotein-mediated drug efflux from cancer cells leading to intracellular accumulation. The obtained percentages of inhibition for this series of compounds at 5 μM is reported in Table 3, related to cyclosporin A, the "golden standard" of Pgp modulators, taken here as reference (100%). The 5 μM jatrophane concentration

Table 3. Inhibition by Pepluanins A−E (1-5), Relatively to CsA, of Cellular P-glycoprotein-mediated Daunomycin Efflux Monitored by Its Intracellular Accumulation[a]

compd	substitution C-8	C-9	C-14	C-15	% inhibn of daunomycin efflux with respect to CsA
1	OAc	ONic	OAc	OH	207 ± 17
2	OH	ONic	OAc	OH	60 ± 4
3	OAc	OAc	OAc	OH	54 ± 5
4	H	OAc	O	OAc	76 ± 7
5	OH	ONic	O	OH	94 ± 5
6	OH	OAc	OAc	OH	80 ± 6
7	H	ONic	O	OAc	47 ± 7

[a] The increase in daunomycin accumulation produced by each compound was determined under the same conditions as in Figure 1, the effect produced by CsA being taken as 100% (±9.7%).

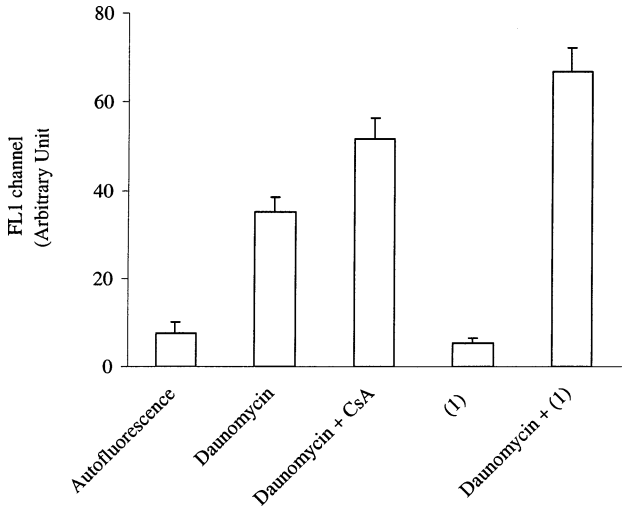

Figure 1. Accumulation of daunomycin in P-glycoprotein overexpressing human K562/R7 leukemic cells, as monitored by flow cytometry. The cells were exposed to daunomycin for 1 h in the absence or presence of either cyclosporin A (CsA) or pepluanin A (**1**) and quickly washed; intracellular drug concentrations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

was chosen because previously for analogues a maximal inhibition in the 5−10 μM range has been shown[10] with a half-maximal effect around 1−2 μM.

Our previous studies on a first series of jatrophane diterpenes, euphodendroidins A−I,[10] isolated from *E. dendroides*, evidenced the involvement of ring A in Pgp binding. In particular, the critical role of the hydroxyl group at position 3, together with the negative effect of this group at C-2 have been observed. In addition, substitution at the proximal C-5 with a large group also decreased the activity. We have now gone beyond these data and underlined the importance of the substitution on other carbons of the medium-sized ring in modulating the activity.

Pepluanin A (**1**) was found here to be an even more powerful inhibitor, with an efficiency at least 2-fold higher (207 ± 17%, Table 3) than the conventional modulator cyclosporin A (Figure 1). This appears quite interesting also in the light of some structure−activity considerations that can be drawn. First of all, comparison of the activity of **1** and **2**, the only stuctural differences of which being confined to C-8 substitution, evidenced a collapse of the inhibitory potency (207 ± 17% in **1** vs 60 ± 4% in **2**). This clearly indicates that also the substitution pattern at the carbons of the medium-sized ring is of great importance for the activity. Another point to clarify is that pepluanin A, unlike the majority of the other tested jatrophanes, euphodendroidins A−I[10] and compounds **4**, **5**, **7** (Chart 1), has an acetoxyl function at C-14 instead of a carbonyl. It is rather difficult at this point to draw a definitive conclusion on this structural detail. Anyway, by comparing the activity of **5** (94 ± 5%) with that of **2** (60 ± 4%), essentially differing for this structural detail, we could hypothesize that the observed inhibitory potency of **1** might be further increased by a carbonyl at position 14. Similarly, a positive effect might be expected by substituting nicotinyl by acetoxyl at C-9, since **6** (80 ± 6%) is more efficient than **2** (60 ± 4%). An additional key point is the presence of a free hydroxyl group at C-15, as found in the most powerful compounds tested. In fact, compounds **4** (76 ± 7%) and **7** (47 ± 7%), presenting an ester function at this position, exhibited the lowest efficiency.

In conclusion, the present series of seven jatrophane diterpenes has allowed us to extend their structure−activity relationships, highlighting the importance of an acetoxyl at C-8, by comparison to a free hydroxyl, and of a free hydroxyl at C-15. Moreover, a carbonyl at C-14 and acetoxyl at C-9 are also favorable substitutions. Within the set of diterpenes tested by now, pepluanin A appears a powerful Pgp inhibitor, very promising to improve drug therapy in multidrug-resistant cancer. Further studies on structure−activity relationships, regarding the 3-D structure modeling on the jatrophanes and the interaction with the receptorial site of Pgp, are in progress.

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 the stationary phase. HPLC in isocratic mode was performed on a Varian apparatus equipped with a RI-3 refractive index detector using Merck columns [semipreparative LiChrocart Si60 column (10μ) with a flow rate of 2.5 mL/min and analytical LiChrospher Si60 (5 μm) with a flow rate of 1 mL/min].

Plant Material. Samples of *Euphorbia peplus* L. were collected in waste places in Parco Gussone, Portici (NA) in March 2002. The plant material was identified by R.M., and a voucher specimen is kept at the Dipartimento di Arboricoltura, Botanica e Patologia Vegetale.

Extraction and Isolation. Fresh, whole plants (2.097 kg, fresh plant), including latex and roots, were extracted five times with 7 L of EtOAc at room temperature. The obtained EtOAc extract (81.9 g) was partitioned between H_2O and EtOAc, to remove hydrophilic and gummy compounds, and then the sole apolar fraction (35.5 g) was chromatographed

using a gradient system from hexane 100% to EtOAc 100%. We have further purified fractions eluted in hexane:EtOAc 1:1, hexane:EtOAc 3:7, and hexane:EtOAc 2:8.

The first fraction (hexane:EtOAc 1:1) was successively purified on an HPLC direct phase column, using hexane:EtOAc 65:35 as the mobile phase, and afforded two new compounds, pepluanin C (7.1 mg) and pepluanin D (6.3 mg).

The second fraction (hexane:EtOAc 3:7) was further purified first on semipreparative columns and then on analytic HPLC columns (both eluted with hexane:EtOAc 6:4), affording the known compound **6** (8.2 mg) and the new compound pepluanin E (5.1 mg).

From the third fraction (hexane:EtOAc 2:8) analyzed first on semipreparative HPLC column, using hexane:EtOAc 4:6 as the mobile phase, and then with analytic HPLC column, with hexane:EtOAc 6:4 as eluent, we isolated known compound **7** (7.7 mg), and the two new compounds, pepluanin A (10.1 mg) and pepluanin B (2.5 mg).

Pepluanin A: yield 10.1 mg, colorless amorphous solid; $[\alpha]^{25}_D$ +34.4° (c = 0.1, $CHCl_3$); HRFABMS (positive ion) found m/z 822.3362 $[M + H]^+$, calculated for $C_{43}H_{51}O_{15}N$ m/z 821.32804; ^1H NMR ($CDCl_3$) data, Table 1; ^{13}C NMR ($CDCl_3$) data, Table 2.

Pepluanin B: yield 2.5 mg, colorless amorphous solid; $[\alpha]^{25}_D$ +25.7° (c = 0.1, $CHCl_3$; HRFABMS (positive ion) found m/z 822.3725 $[M + H]^+$, calculated for $C_{44}H_{55}O_{14}N$ m/z 821.36434; ^1H NMR ($CDCl_3$) data, Table 1; ^{13}C NMR ($CDCl_3$) data, Table 2.

Pepluanin C: yield 7.1 mg, colorless amorphous solid; $[\alpha]^{25}_D$ +3.0° (c = 0.1, $CHCl_3$); HRFABMS (positive ion) found m/z 787.3521 $[M + H]^+$, calculated for $C_{41}H_{54}O_{15}$ m/z 786.3447; ^1H NMR ($CDCl_3$) data, Table 1; ^{13}C NMR ($CDCl_3$) data, Table 2.

Pepluanin D: yield 6.3 mg, colorless amorphous solid; $[\alpha]^{25}_D$ −39.4° (c = 0.1, $CHCl_3$; HRFABMS (positive ion) found m/z 579.2789 $[M + H]^+$, calculated for $C_{30}H_{42}O_{11}$ m/z 578.2715; ^1H NMR ($CDCl_3$) data, Table 1; ^{13}C NMR ($CDCl_3$) data, Table 2.

Pepluanin E: yield 5.1 mg, colorless amorphous solid; $[\alpha]^{25}_D$ +28.8° (c = 0.1, $CHCl_3$); HRFABMS (positive ion) found m/z 764.3309 $[M + H]^+$, calculated for $C_{41}H_{49}O_{13}N$ m/z 763.3226; ^1H NMR ($CDCl_3$) data, Table 1; ^{13}C NMR ($CDCl_3$) data, Table 2.

Purity Criteria for Target C ompounds. The degree of purity of all tested compounds (**1**−**7**) 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.

Pepluanin A: hexane/EtOAc 4:6 with t_R 33.0; CH_2Cl_2/EtOAc 45:55 with t_R 20.5.

Pepluanin B: hexane/EtOAc 6:4 with t_R 37.6; CH_2Cl_2/EtOAc 65:35 with t_R 25.1.

Pepluanin C: hexane/EtOAc 6:4 with t_R 54.2; CH_2Cl_2/EtOAc 65:35 with t_R 42.3.

Pepluanin D: hexane/EtOAc 6:4 with t_R 60.0; CH_2Cl_2/EtOAc 65:35 with t_R 48.1.

Pepluanin E: hexane/EtOAc 6:4 with t_R 25.5; CH_2Cl_2/EtOAc 65:35 with t_R 13.3.

Compound **6**: hexane/EtOAc 62:38 with t_R 36.3; CH_2Cl_2/EtOAc 7:3 with t_R 27.1.

Compound **7**: hexane/EtOAc 6:4 with t_R 47.1; CH_2Cl_2/EtOAc 65:35 with t_R 35.3.

Biological Assay. P-Glycoprotein-overexpressing K562/R7 human leukemic cells were incubated with 10 μM daunomycin, as described previously,[13,14] in the presence or absence of inhibitor. The cells were then washed and analyzed by flow cytometry on a FACS-II apparatus. 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 pepluanin to inhibit P-glycoprotein-mediated drug efflux was quantified by comparing the induced shift in fluorescence to that obtained with cyclosporin A.

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