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Enhancing Macrocyclic Diterpenes as Multidrug-Resistance Reversers: Structure−Activity Studies on Jolkinol D Derivatives

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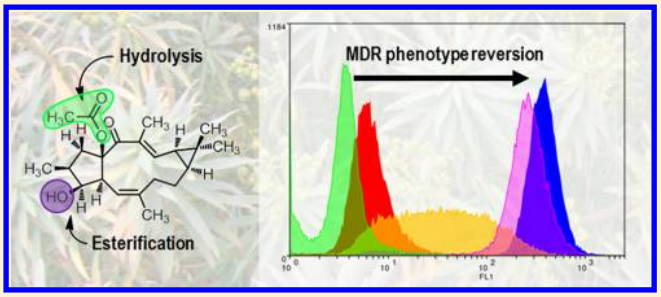
Supporting Information

ABSTRACT: The phytochemical study of *Euphorbia piscatoria* yielded jolkinol D (**1**) in a large amount, whose derivatization gave rise to 12 ester derivatives (**2**−**13**) and hydrolysis to compound **14**. The in vitro modulation of P-gp of compounds **1**−**14** was evaluated through a combination of transport and chemosensitivity assays, using the L5178 mouse T lymphoma cell line transfected with the human MDR1 gene. Apart from jolkinol D, all derivatives (**2**−**14**) showed potential as MDR reversal agents. In this small library of novel bioactive macrocyclic lathyrane diterpene derivatives, designed to evaluate structure−activity relationships essential in overcoming multidrug resistance (MDR), some correlations between MDR reversal and molecular weight, accessible solvent areas, and octanol/water partition coefficient were identified that can contribute to the development of new selective P-gp reversal agents.



1. INTRODUCTION

Multidrug resistance (MDR) designates a phenomenon where resistance to one drug is accompanied by resistance to drugs that are structurally and functionally unrelated. The development of MDR and subsequent failure of chemotherapy are widespread problems in many types of tumors. One of the most significant mechanisms of MDR results from overexpression of membrane efflux pumps belonging to the evolutionarily conserved family of ATP binding cassette (ABC) proteins, which transports anticancer drugs out of the cells, preventing them from reaching their cellular targets. A very important member of this superfamily is P-glycoprotein (P-gp).[1−3] This protein, with 170 kDa, is the product of the human multidrug resistance (*MDR1*) gene. It is classified as a pseudosymmetrical heterodimer, where each monomer is composed of six membrane spanning segments (transmembrane domain, TMD) and one nucleotide-binding domain (NBD). The TMDs mediate the recognition and transport of substrates, and the NBDs are responsible for the ATP-binding and hydrolysis and consequently for the generation of conformational changes on the protein.[4−7]

One of the most accepted strategies to overcome MDR mediated by P-gp is based on the development of reversal agents that when coadministered with an anticancer drug will circumvent its efflux and consequently will avoid chemotherapy failure.[8,9] In this manner, a considerable number of natural and synthetic compounds have been described in the literature as P-gp reversal agents. Nevertheless, despite encouraging results in in vitro assays, to date, there are no reversal agents clinically available.[10−12] For instance, first-generation drugs like verapamil[13] and cyclosporine A,[14] besides revealing low affinity for P-gp, had distinct pharmacological actions on the cardiovascular (calcium channel blocker) and immune (immunosuppressant) systems. As the high serum concentrations required for P-gp inhibition severely increased their adverse effects, analogues of these drugs (without intrinsic pharmacological activity) were designed as a way to reduce their side effects (second-generation modulators); however, they also showed increased toxicity[15] and alterations in the pharmacokinetics of cytotoxic drugs.[16] Third generation MDR modulators, like tariquidar (XR9576)[17] and zosuquidar (LY335979),[18] inhibit P-gp at the nanomolar level and were until recently under development but have not been approved for clinical use so far. The hampering of progress in this area can be attributable to the lack of knowledge concerning drug−transporter interactions to a certain extent because of the absence of detailed structural information of human P-gp. Meanwhile, in 2009 the structure of the first mammalian ABC transporter (murine, with 87% sequence identity to human P-gp) was published, showing for the first time P-gp complexed with a ligand.[19] Despite its low resolution (3.8 Å), it represents

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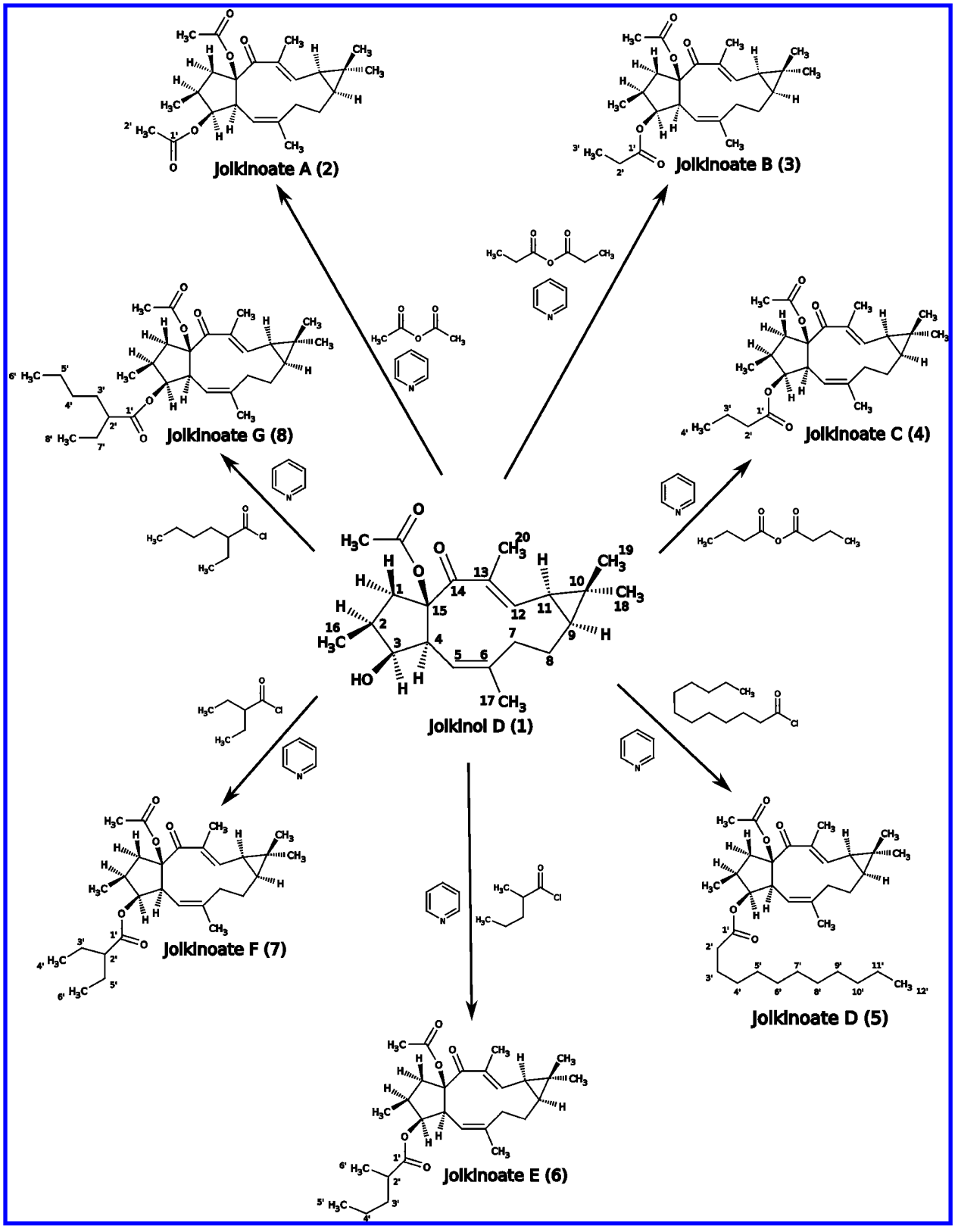

Figure 1. Scheme showing how jolkinol D aliphatic esters were prepared (**2**–**8**).

a huge step toward allowing structure-based drug design approaches. Aller et al. characterized a large hydrophobic chamber of approximately 6.000 Å^3 as the presumptive drug-binding pocket (DBP), comprising mostly of hydrophobic and aromatic residues near the periplasmatic lipid bilayer interface and, on the opposite side, more polar and possibly charged residues. These authors also identified three possible overlapping drug-binding sites in which residues Phe724 and Val978 seem to display a central role in the ligand-protein interactions, being common to the three binding sites.[19,20]

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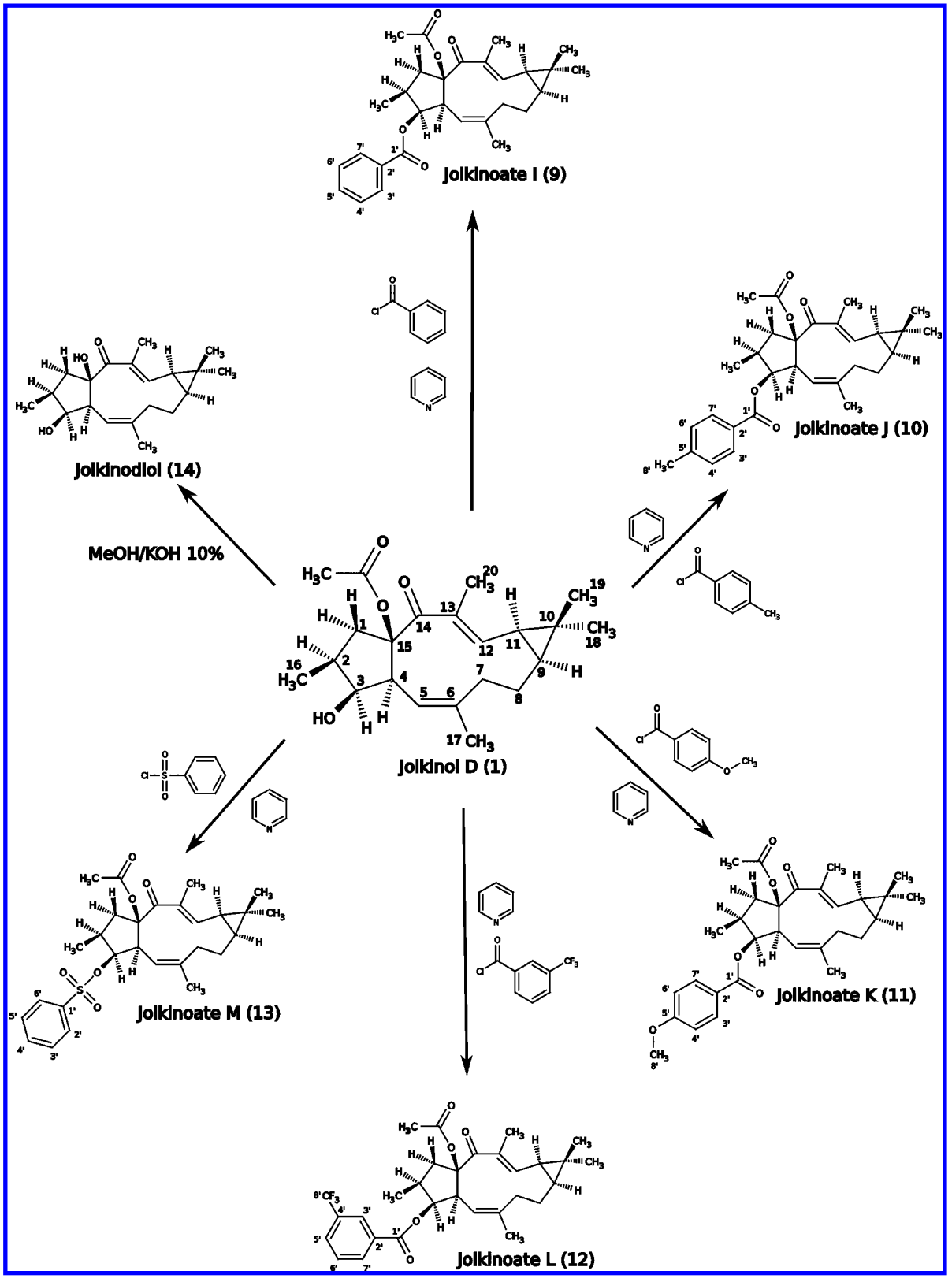

Figure 2. Scheme showing how jolkinol D aromatic (**9**–**13**) and hydrolysis products (**14**) were prepared.

In our previous studies, many macrocyclic diterpenes and some policyclic derivatives have been isolated from several species of *Euphorbia* genus. Most of them were found to be very strong P-gp modulators, exhibiting as well synergistic interactions with cytotoxic drugs.[21−25] In addition, the acylation pattern of these compounds also seems to play a significant role in the reversal of MDR.

To identify more potent P-gp modulators, a new phytochemical study of *Euphorbia piscatoria* Ait. has been carried out. Since a large amount of the lathyrane diterpene

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jolkinol D[26] was isolated, 13 derivatives could be prepared by esterification of the hydroxyl group at C-3 with different groups and by hydrolysis of the ester function at C-15. Therefore, a new set of homologous bioactive macrocyclic lathyrane diterpenes was created for which the MDR reversal potency could be assessed. These chemical modifications were evaluated in vitro through the rhodamine-123 exclusion assay (for the modulatory effect) and a chemosensitivity assay over a checkerboard microplate method (drug interactions between compounds and doxorubicin) in human *MDR1* gene transfected mouse lymphoma cells.

The combination of the functional biological results and some physicochemical descriptors allowed a better characterization of some structure−activity relationships correlating chemical derivatization and anti-MDR activity.

2. RESULTS AND DISCUSSION

2.1. Chemistry. Fractionation of the ethyl acetate soluble fraction of the methanol extract of the aerial parts of *E. piscatoria* by chromatographic methods and crystallization provided the known macrocyclic diterpene jolkinol D (**1**) (Figure 1). This compound, previously isolated from *Euphorbia jolkini* Boiss, was identified by comparison of its ^{1}H NMR spectrum with that reported in the literature.[26] The combination of ^{13}C NMR, DEPT, and 2D-NMR spectra (COSY, HMQC, and HMBC) allowed the assignment of the ^{13}C resonances, which are reported here for the first time. The chemical derivatization of the free hydroxyl group at C-3 with alkanoyl anhydrides and alkanoyl/aroyl/sulfonyl chlorides yielded 12 derivatives in 50−97% yields (**2**−**13**, Figures 1 and 2). Hydrolysis of the ester function at C-15 yielded compound **14** in 81% yield (Figure 2). The new compounds were characterized by comparison of their spectroscopic data with those of jolkinol D (**1**). The main differences between the NMR data of compound **1** and the data of the ester derivatives (**2**−**13**) were observed for the carbon and proton chemical shifts of the pentacyclic ring. In the ^{1}H NMR spectra of the compounds **2**−**13**, a significant paramagnetic effect was observed for the signal of H-3, which appeared at approximately 1.0−1.6 ppm downfield. As expected, in the ^{13}C NMR spectra, similar paramagnetic effects at α-carbons were also observed, displaying the highest shifts of C-3 of compounds **12** and **13** ($\Delta\delta_C$ = +2.9 and +11.3 ppm, respectively). The β-carbons C-2 and C-4 of the esters also showed predictable diamagnetic effects ($\Delta\delta_C \cong$ 0.9−1.3 and 1.1−1.9 ppm, respectively).

On the contrary, for the hydrolyzed derivative of **1**, jolkinodiol (**14**), the α-carbon C-15 appeared upfield by 2.7 from its position in **1** and C-1, C-4, and C-14 (β-carbons) were shifted downfield ($\Delta\delta_C$ = +2.0, + 0.5, + 2.6 ppm, respectively). The marked effect at C-14 (β-carbon) could also be explained by an intramolecular hydrogen bond between the corresponding ketone function and the free hydroxyl group at C-15. This effect could also be observed at both H-12 and C-12 of the enone system because of the mesomeric effect ($\Delta\delta_H$ = +0.8 ppm and $\Delta\delta_C$ = +5.1 ppm, respectively).

2.2. Biological Activities. *In Vitro Antiproliferative Assay.* The antiproliferative activity of jolkinol D (**1**) and derivatives (**2**−**14**) was evaluated using the MTT assay in L5178Y mouse lymphoma cells (PAR cells) and in human MDR1-gene transfected L5178Y mouse lymphoma cells (MDR cells). The IC_{50} values are presented in Table 1. All the derivatives showed a higher antiproliferative activity when compared with jolkinol D (**1**). The determination of the selectivity index (Table 1) did not show a significant discrimination between cell lines, instead indicating that the compounds are equally active against both PAR cells (chemo-sensitive) and MDR cells (chemoresistant by overexpression of P-gp).

Table 1. Antiproliferative Activity of Jolkinol D (1) and Derivatives (2−14) on L5178Y Mouse Lymphoma Cells (PAR Cells) and in Human MDR1-Gene Transfected Mouse Lymphoma Cells (MDR Cells)

	IC_{50} [a] (μM)		
compd	PAR cell	MDR cell	selectivity index[b]
jolkinol D (**1**)	89.87 ± 0.80	99.67 ± 4.53	0.9
jolkinoate A (**2**)	38.21 ± 5.52	60.35 ± 1.25	0.6
jolkinoate B (**3**)	18.33 ± 4.00	22.16 ± 3.94	0.8
jolkinoate C (**4**)	23.99 ± 0.45	26.84 ± 5.32	0.9
jolkinoate D (**5**)	38.66 ± 6.30	60.27 ± 6.43	0.6
jolkinoate E (**6**)	17.20 ± 2.55	18.76 ± 3.11	0.9
jolkinoate F (**7**)	14.34 ± 2.64	14.74 ± 0.73	1
jolkinoate G (**8**)	20.58 ± 7.11	48.92 ± 4.16	0.4
jolkinoate I (**9**)	34.70 ± 6.93	33.08 ± 5.44	1
jolkinoate J (**10**)	41.16 ± 3.03	45.07 ± 3.33	0.9
jolkinoate K (**11**)	21.67 ± 6.60	32.71 ± 5.17	0.7
jolkinoate L (**12**)	43.99 ± 3.67	52.60 ± 3.84	0.8
jolkinoate M (**13**)	12.13 ± 0.46	14.95 ± 4.33	0.8
jolkinodiol (**14**)	36.42 ± 5.77	48.92 ± 4.16	0.7
DMSO (2%)	1.21 ± 0.51	1.46 ± 0.11	

[a]Values of IC_{50} are the mean ± standard error of the mean of three independent experiments. [b]Selectivity index = IC_{50}(PAR cells)/IC_{50}(MDR cells).

Inhibition of Rhodamine-123 Efflux P-gp-Mediated. The rhodamine-123 exclusion assay was used to access the potential P-glycoprotein-mediated MDR reversing activity of this set of compounds (**1**−**14**) (Table 2). In this assay, the fluorescence activity ratio (FAR) was evaluated through the accumulation ratio of rhodamine-123 (substrate of P-gp) between MDR and PAR cells and considering that when the FAR values are higher than 1, MDR reversal has occurred. As such, compounds that exhibit FAR values higher than 10 can be classified as strong MDR modulators.[27] Verapamil, a well-known modulator,[13] was used as positive control. To study whether MDR reversal activity had dose−effect dependence, compounds **1**−**14** were tested in several concentrations (2, 20, 40, and 80 μM). However, under the assay conditions, some of the compounds (**3**, **4**, **6**, **7**, and **8**) revealed toxicity above 40 μM (observed in the variations of the forward scatter and side scatter light parameters, data not shown). For this reason, only the MDR-reversal activities at 2 and 20 μM were chosen to be summarized in Table 1. At 20 μM, except for jolkinol D (**1**) and jolkinodiol (**14**), all the compounds showed a strong effect on P-gp modulation, with FAR values ranging from 12.13 to 109.17, suggesting that a modification at position C-3 greatly contributed to increase P-gp inhibitory activity. At 2 μM, compounds **8**, **9**, **10**, **11** and **13** showed FAR values higher than 10 (10.55 − 28.09), leading us to classify them as strong MDR modulators (Figure 3). These results are of particular interest, since the basal MDR reversal activity of verapamil at 22 μM is approximately 20.

The Overton cumulative histogram subtraction algorithm was calculated for compounds **9**, **10**, **11**, and **13**. This algorithm subtracts histograms on a channel-by-channel basis to provide a percentage of positive cells,[28] allowing calculation of the percentage of the population that reverted to a chemosensitive

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Table 2. Multidrug Resistance Reversal Activities of Jolkinol D (1) and Derivatives (2−14) on Human MDR1-Gene Transfected Mouse Lymphoma Cells (MDR Cells)

compd	concn (μM)	FSC[a]	SSC[b]	FL-1[c]	SD[d]	peak	FAR[e]
PAR cells		1592	680	48.44	17.91	45.09	
MDR cells		1718	751	0.67	0.64	0.53	
jolkinol D (**1**)	2	1680	673	0.44	0.60	0.37	0.79
	20	1686	673	0.47	0.57	0.35	0.92
jolkinoate A (**2**)	2	1738	718	0.65	0.54	0.53	1.16
	20	1791	877	18.40	16.30	11.50	33.76
jolkinoate B (**3**)	2	1746	717	1.65	2.86	0.63	2.95
	20	1667	762	16.70	11.30	16.00	30.64
jolkinoate C (**4**)	2	1766	731	2.88	4.24	1.00	5.15
	20	1798	819	58.80	26.60	47.00	107.89
jolkinoate D (**5**)	2	1756	711	0.97	1.09	0.58	1.74
	20	1751	831	14.00	10.20	15.40	25.69
jolkinoate E (**6**)	2	1772	725	3.99	4.98	1.07	7.14
	20	1573	533	6.14	8.06	3.79	12.13
jolkinoate F (**7**)	2	1210	104	2.79	3.18	2.29	4.99
	20	1834	963	42.10	21.10	42.20	77.25
jolkinoate G (**8**)	2	1708	721	5.90	6.99	3.40	10.55
	20	1825	857	28.70	11.50	26.40	52.66
jolkinoate I (**9**)	2	1765	702	13.50	10.40	13.80	24.15
	20	1698	776	45.90	21.40	39.20	84.22
jolkinoate J (**10**)	2	1785	716	11.80	8.85	9.31	21.11
	20	1844	886	31.00	11.40	31.60	56.88
jolkinoate K (**11**)	2	1736	708	15.70	10.90	16.00	28.09
	20	1776	839	59.50	21.50	56.20	109.17
jolkinoate L (**12**)	2	1790	719	5.21	5.08	3.28	9.32
	20	1756	777	14.50	10.20	15.40	28.66
jolkinoate M (**13**)	2	1758	706	8.98	9.29	7.77	16.06
	20	1756	773	26.70	14.80	29.40	52.77
jolkinodiol (**14**)	2	1753	725	0.47	0.34	0.42	0.84
	20	1742	798	1.71	1.94	0.65	3.38
verapamil	22	1689	754	5.70	5.08	4.00	20.00

[a]FSC: forward scatter count of cells in the samples. [b]SSC: side scatter count of cells in the samples. [c]FL-1: mean fluorescence intensity of the cells. [d]SD: standard deviation. [e]FAR (fluorescence activity ratio) values were calculated by using the equation given in the Experimental Section.

phenotype. The data are presented in Figure 4 and show that jolkinoate K (**11**) and jolkinoate I (**9**) reverted 95% and 70.2% of the population of MDR cells to a chemosensitive phenotype, respectively (at 20 μM). Moreover, the direct correlation between the increase of concentration and number of cells with a chemosensitive phenotype is an indicator of a dose-dependent inhibitory effect on the MDR reversal activity (Figure 4).

Drug Combination Assays. Since the studied compounds showed a good MDR reversal activity, the following step was the investigation of the combined effect of these compounds and an antineoplastic drug, such as doxorubicin. This chemotherapeutic agent not only is transported by P-gp but also induces its expression in cancer cells, and therefore, its cytostatic efficacy is limited by P-gp activity.[29] As can be observed in Table 3, all tested compounds but jolkinoate D (**5**) synergistically enhanced the cytotoxicity of doxorubicin (combination index, CI = 0.18−0.65). These results are in accordance with the assumption that a promising P-gp inhibitor would consequently be able to increase the action of the cytotoxic drug because of greater efflux impairment. Jolkinol D (**1**) did not display a good MDR reversal ability but showed a synergistic interaction with doxorubicin comparable to the most active inhibitors (**10**, **12**), suggesting that other distinct targets/pathways besides the P-gp inhibition can be involved and should be the subject of further investigation. Jolkinoate D (**5**) was a strong inhibitor at 20 μM but in the combination assay had an antagonistic effect.

2.3. Structure−Activity Relationships. *Physicochemical Properties: K-Means Clustering and Linear Regression Analysis.* The monoacylated derivatives at C-3 are a good set of homologous compounds to determine structure−activity relationships. Therefore, some physicochemical properties of compounds **1**−**14** were determined (Table 4) and subdivided by the K-means cluster analysis into five main clusters in which a high degree of similarity was found (Table 4).

Cluster I comprises compounds **1** and **14** (Table 4), molecules that have a molecular weight below 400 g·mol^{-1} and log *P* lower than 4.0 and that are often considered poor P-gp substrates.[30] Cluster II includes compounds **2**, **3**, and **4** (Table 4), only differing in the size of the ester side chain (one or two methylene units). In this group and despite the low number of molecules, it was possible to correlate the increase in P-gp inhibitory activity with the increase of the molecular weight (MW, r^2 = 0.96) and solvent accessible area (ASA, r^2 = 0.94 at 2 μM and at 20 μM, with ASA, r^2 = 0.77). Cluster III comprises compounds **6**, **7**, **9**, and **13** (Table 4). Within this group, a single correlation could be determined with ASA (r^2 = 0.84, 20 μM). However, compounds bearing an aromatic moiety (**9** and **13**) displayed higher MDR reversal activity at 2 μM when compared with all the alkanoyl derivatives. This

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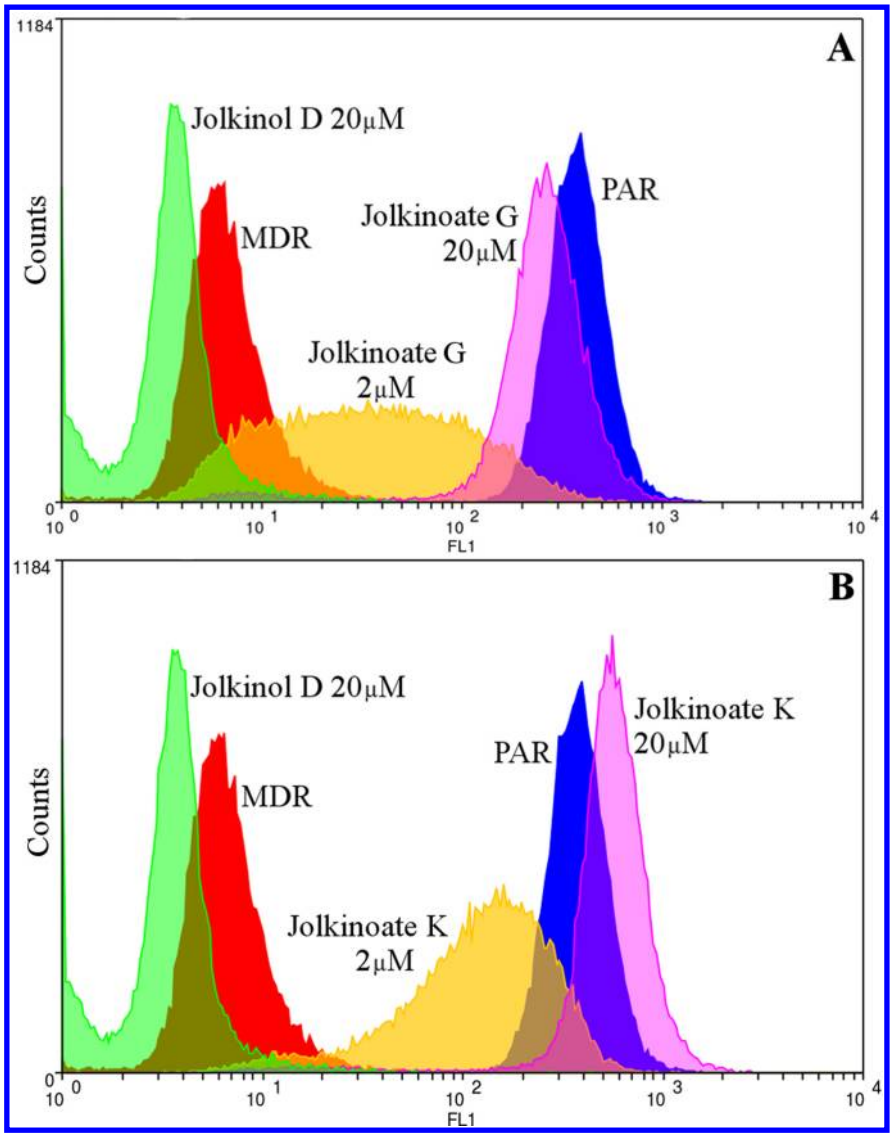

Figure 3. Reversal of the MDR phenotype by jolkinoates G (A) and K (B) at 2 and 20 μM, in comparison with jolkinol D (20 μM): MDR, chemoresistant cell by overexpression of P-gp; PAR, parental chemosensitive cell.

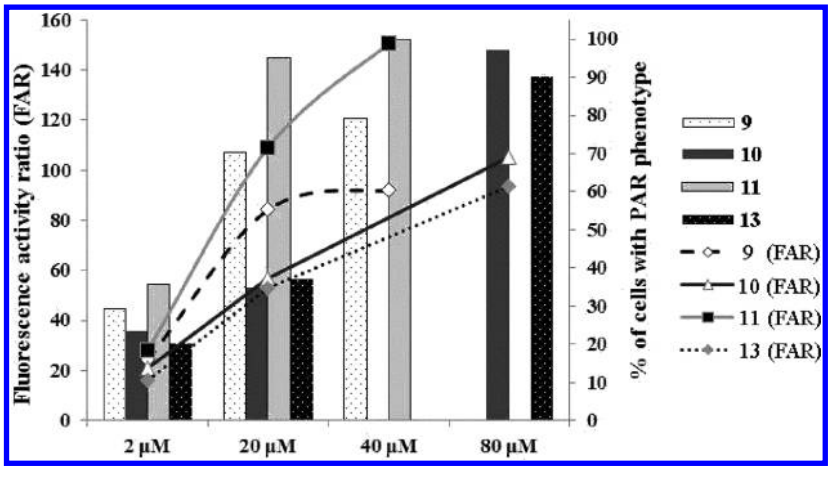

Figure 4. Relation between fluorescence activity ratio (FAR, lines) and percentage of MDR cells that reverted to a PAR phenotype (bars) for compounds **9**–**11** and **13**. This analysis clearly shows the dose-dependent inhibitory effect on P-gp modulation.

observation is in agreement with previous studies by our group[31−33] that identified aromatic moieties to be important for the establishment of additional hydrophobic interactions within the DBP. Cluster IV includes the most active molecules

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Table 3. Evaluation of the Interaction between Compounds 1−14 and Doxorubicin on Human MDR1-Gene Transfected Mouse Lymphoma Cells (MDR Cells)

drug combination with doxorubicin	ratio[a]	CI ± SE for IC_{50}[b]	interaction
jolkinol D (**1**)	40:1	0.26 ± 0.05	synergy
jolkinoate A (**2**)	38:1	0.18 ± 0.03	synergy
jolkinoate B (**3**)	8:1	0.34 ± 0.04	synergy
jolkinoate C (**4**)	20:1	0.39 ± 0.04	synergy
jolkinoate D (**5**)	5:1	2.02 ± 0.26	antagonism
jolkinoate E (**6**)	4:1	0.23 ± 0.07	synergy
jolkinoate F (**7**)	30:1	0.25 ± 0.04	synergy
jolkinoate G (**8**)	65:1	0.31 ± 0.05	synergy
jolkinoate I (**9**)	40:1	0.35 ± 0.06	synergy
jolknoate J (**10**)	33:1	0.26 ± 0.03	synergy
jolkinoate K (**11**)	10:1	0.30 ± 0.04	synergy
jolkinoate L (**12**)	18:1	0.27 ± 0.04	synergy
jolkinoate M (**13**)	10:1	0.21 ± 0.05	synergy
jolkinodiol (**14**)	23:1	0.65 ± 0.11	synergy

[a]Data are shown as the best combination ratio of the tested compounds and doxorubicin. [b]Combination index (CI) values listed in the table are the mean ± standard error (SE) for an inhibitory concentration of 50% (IC_{50}). CI < 1: synergy. CI = 1: additivity. CI > 1: antagonism. Values were determined experimentally and calculated using the Chou and Talalay method and CalcuSyn software.

identified in this particular study, namely, compounds **8** and **10**−**12** (Table 4). The only strong correlation found within this group was between octanol/water partition coefficient (log *P*, r^2 = 0.96 and r^2 = 0.86 at 2 and 20 μM, respectively) and the biological activity (these two properties are inversely proportional). Jolkinoate D (**5**) remained alone in cluster V because its physicochemical features are very dissimilar to those of the remaining compounds (Table 4).

3. CONCLUSIONS

The common scaffold of this particular set of compounds and the limited variation achieved through the derivatization at C-3 provide an ideal model for structure−activity relationship studies. Biological studies clearly demonstrated the effect of C-3 pattern modification in the MDR reversal activity. As the combination index between these compounds and doxorubicin revealed a synergistic effect, the combination of cytotoxic and MDR reversal effects would hold promise in the treatment of MDR.

K-means analysis obtained five main clusters for which strong correlations between activity and log *P*, MW, or ASA could be specifically identified . However, as each group had its own behavior regarding physicochemical descriptors (no correlation or correlation in different descriptors), the hypothesis of a multifactorial inhibition mechanism is reinforced, thus contributing to a better clarification of the polispecificity in the P-gp efflux reversion.

It has become clear in several studies the importance of aromatic moieties for P-gp efflux modulation. This new SAR analysis, in a different perspective, corroborated the importance of the aromatic ring for binding inside the DBP, namely, through its electronic and steric effects. Hydrophobicity remains a critical factor, but the new data acquired in this study provided a different insight on how the acylation of a single hydroxyl group, with different moieties, can affect the modulation ability of P-gp efflux.

4. EXPERIMENTAL SECTION

4.1. Chemistry. *General Procedures.* Pyridine of HPLC grade was dried with solid KOH followed by distillation and stored with Linde 4A molecular sieves. All the other reagents and solvents were obtained from commercial suppliers and were used without further purification. The infrared spectra were collected on an Affinity-1 (Shimadzu) FTIR spectrophotometer. High resolution mass spectra were recorded on a FTICR-MS Apex Ultra (Brüker Daltonics) 7 T instrument. Column chromatography was performed on silica gel (Merck 9385). Merck silica gel 60 F254 plates were used in analytical TLC, with visualization under UV light and by spraying with sulfuric acid/methanol (1:1), followed by heating. NMR spectra were recorded on a Bruker 400 Ultra-Shield instrument (^{1}H 400 MHz, ^{13}C 100.61 MHz). ^{1}H and ^{13}C chemical shifts are expressed in δ (ppm) referenced to the solvent used and the proton coupling constants *J* in hertz (Hz). Spectra were assigned using appropriate COSY, DEPT, HMQC, and HMBC sequences. HPLC was performed on a Merck-Hitachi HPLC system, with UV detection. All tested compounds were purified to ≥95% purity as determined by HPLC (Merck LiChrospher 100 RP-18; 5 μm, 125 mm × 4 mm column; MeOH/H_2O as the mobile phase).

Plant Material. Euphorbia piscatoria Ait. was collected in the Garcia de Orta garden, Lisbon, Portugal (April 2011), and was identified by Dr. Teresa Vasconcelos (plant taxonomist) of Instituto Superior de Agronomia, Technical University of Lisbon, Portugal. A voucher specimen (no. 276) has been deposited at the herbarium of Instituto Superior de Agronomia.

Extraction and Isolation. The air-dried powdered plant (8.4 kg) was exhaustively extracted with methanol (9 × 6 L) at room temperature. Evaporation of the solvent (under vacuum, 40 °C) from the crude extract yielded a residue of 1.3 kg. This residue was resuspended in MeOH/H_2O solution (1:2, 4 L) and extracted with EtOAc (8 × 3 L). The ethyl acetate soluble fraction was dried (Na_2SO_4) and evaporated under vacuum, yielding a residue (462.4 g) that was chromatographed over SiO_2 (2 kg), using mixtures of *n*-hexane−EtOAc (1:0 to 0:1) and EtOAc−MeOH (9:1 to 3:7) as eluents. According to differences in composition as indicated by TLC, 14 crude fractions were obtained (fractions A−O). Jolkinol D (**1**) was obtained (2.9 g) through crystallization (EtOAc/*n*-hexane) from the crude fraction G (17.58 g).

*Jolkinol D, 15β-Acetoxy-3β-hydroxylathyra-5E,12E-dien-14-one (**1**).* ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.65 (1H, d, *J* = 11.4 Hz, H-12), 5.67 (1H, d, *J* = 10.9 Hz, H-5), 3.91 (1H, dd, *J* = 8.2, 3.6 Hz, H-3), 3.49 (1H, dd, *J* = 14.0, 8.1 Hz, H-1α), 2.57 (1H, d, *J* = 13.3 Hz, H-7α), 2.38 (1H, dd, *J* = 10.9, 3.6 Hz, H-4), 2.21 (1H, ddd, *J* = 14.5, 6.8, 3.3 Hz, H-8α), 2.04 (1H, m, H-2), 2.01 (3H, s, Me-22), 1.83 (3H, s, Me-20), 1.77 (1H, td, *J* = 13.1, 6.5 Hz, H-7β), 1.57−1.48 (2H, m, H-1β, H-8 β), 1.46 (3H, s, Me-17), 1.40 (1H, dd, *J* = 11.4, 8.2 Hz, H-11), 1.17 (3H, s, Me-18), 1.08 (3H, d, *J* = 6.7 Hz, Me-16), 1.06 (1H, m, H-9), 1.04 (3H, s, Me-19) ppm; ^{13}C NMR (101 MHz, $CDCl_3$) δ 195.5 (C-14), 170.0 (C-21), 146.7 (C-12), 143.2 (C-6), 132.3 (C-13), 119.6 (C-5), 95.4 (C-15), 80.1 (C-3), 52.8 (C-4), 44.1 (C-1), 39.4 (C-2), 36.9 (C-7), 34.4 (C-9), 29.8 (C-11), 29.4 (C-18), 28.5 (C-8), 24.6 (C-10), 21.8 (C-22), 21.1 (C-17), 16.6 (C-19), 13.9 (C-16), 12.5 (C-20) ppm.

*General Preparation of Jolkinol D Derivatives **2**−**13**.* A solution of jolkinol D (1 equiv) in dry pyridine (2 mL for 0.039 mol of jolkinol D) was stirred for 5 min at room temperature before addition of the suitable anhydride or chloride (2 equiv). The mixture was stirred for 48 h at room temperature. The reaction mixture was concentrated under vacuum at 40 °C, and the obtained residue was purified by flash column chromatography.

*Jolkinoate A, 3β,15β-Diacetoxylathyra-5E,12E-dien-14-one (**2**).* was obtained from reaction with acetic anhydride (Merck KGaA, Darmstradt, Germany; 10.2 mg, 0.1 mol). The residue was purified by flash column chromatography (silica gel, CH_2Cl_2/MeOH 100:0 to 99:1) to afford 13 mg (0.031 mol, 56% yield) of an amorphous white powder. IR (NaCl) ν_{max} 1730 (C═O), 1270 (C−O) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.66 (1H, d, *J* = 11.4, H-12), 5.34 (1H, dt, *J* = 10.7, 1.8 Hz, H-5), 5.23 (1H, *t*, *J* = 3.6 Hz, H-3), 3.50 (1H, dd, *J* =

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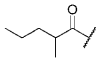
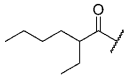
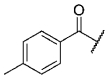
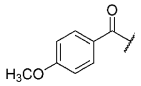
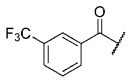
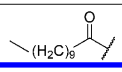
Table 4. Cluster Analysis (K-Means Algorithm) of Compounds 1−14[b]

Compound	Substituent		FAR	Physico-chemical properties [a]					
	C-3	C-15	(2 μM)	MW	MV	log*P*	MR	TPSA	ASA
Cluster I									
Jolkinol D (**1**)	H		0.79	360	377.4	3.8	10.1	63.6	587.01
Jolkinodiol (**14**)	H	H	0.84	318	339.0	3.3	9.0	57.5	540.04
Cluster II									
Jolkinoate A (**2**)			1.16	402	414.5	4.4	11.1	69.7	629.51
Jolkinoate B (**3**)			2.95	430	433.3	4.8	11.6	69.7	661.45
Jolkinoate C (**4**)			5.15	416	449.6	5.2	12.1	69.7	642.79
Cluster III									
Jolkinoate E (**6**)			7.14	459	486.0	5.8	13.0	69.7	695.65
Jolkinoate F (**7**)			4.99	458	486.9	5.8	13.0	69.7	686.88
Jolkinoate I (**9**)			24.15	465	477.6	5.7	13.2	69.7	698.05
Jolkinoate M (**13**)			16.06	501	488.6	5.2	13.6	86.7	699.81
Cluster IV									
Jolkinoate G (**8**)			10.55	486	520.9	6.6	13.9	69.7	734.80
Jolkinoate J (**10**)			21.11	476	492.9	6.0	13.6	69.7	732.92
Jolkinoate K (**11**)			28.09	492	503.4	5.7	13.8	78.9	746.18
Jolkinoate L (**12**)			9.32	533	503.4	7.0	13.8	69.7	736.54
Cluster V									
Jolkinoate D (5)			1.74	543	592.4	8.3	15.8	69.7	916.70

[a]Physicochemical properties were calculated using MOE, version 2010.10.[34] [b]Grouping variables are physicochemical properties: molecular weight (MW), molecular volume (MV), logarithm of the octanol/water partition coefficient (log *P*), molar refractivity (MR), topological polar surface area (TPSA), accessible solvent area (ASA), and FAR (at 2 μM).

13.9, 8.0 Hz, H-1α), 2.50 (2H, m, H-4, H-7α), 2.21−2.17 (2H, m, H-8α, H-2), 2.01 (3H, s, Me-22), 1.83 (3H, s, Me-20), 1.75 (1H, td, *J* = 13.1, 2.6 Hz, H-7β), 1.52, (1H, dd, *J* = 12.5, 2.8 Hz, H-8β), 1.47 (1H, dd, *J* = 8.4, 4.9 Hz, H-1β), 1.44 (3H, d, *J* = 1.3 Hz, Me-17), 1.39 (1H, dd, J = 7.5, 3.9 Hz, H-11), 1.17 (3H, s, Me-18), 1.06 (1H, m, H-9), 1.05 (3H, s, Me-19), 0.95 (3H, d, *J* = 6.7 Hz, Me-16), 2.13 (1H, s, Me-2′) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 195.4 (C-14), 170.8 (C-1′), 169.9 (C-21), 146.80 (C-12), 143.2 (C-6), 132.2 (C-13), 118.7 (C-5), 94.6 (C-15), 81.1 (C-3), 51.2 (C-4), 44.8 (C-1), 38.4 (C-2), 36.9 (C-7), 34.2 (C-9), 29.8 (C-11), 29.3 (C-18), 28.5 (C-8), 24.6 (C-10), 21.6 (C-22), 21.1 (C-2′), 21.0 (C-17), 16.4 (C-19), 13.9 (C-16), 12.4 (C-20) ppm.

*Jolkinoate B, 15β-Acetoxy-3β-propionatelathyra-5E,12E-dien-14-one (**3**).* **3** was obtained from reaction with propionic anhydride (Sigma-Aldrich Chemie GmbH, Riedstrasse D-89555, Steinhelm, Germany; 10.8 mg, 0.08 mol). The residue was purified by flash column chromatography (silica gel, CH_2Cl_2/MeOH = 100:0 to 99:1) to afford 10 mg (0.023 mol, 56% yield) of a yellow oil. IR (NaCl) ν_{max} 1737 (C═O), 1271 (C−O) cm^{-1}; MS *m*/*z* (rel intens) 439 [M + Na]$^+$ (100), 417 [M]$^+$ (38); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.65 (1H, d, *J* = 11.4 Hz, H-12), 5.33 (1H, dt, *J* = 10.7, 1.3 Hz, H-5), 5.24 (1H, *t*, *J* = 3.6 Hz, H-3), 3.52 (1H, dd, *J* = 13.9, 8.0 Hz, H-1α), 2.50 (1H, dd, *J* = 10.7, 3.8 Hz, H-4), 2.44 (1H, m, H-7α), 2.20−2.16 (2H, m, H-8α, H-2), 2.01 (3H, s, Me-22), 1.85 (3H, d, *J* = 1.0 Hz, Me-20), 1.75 (1H, td, *J* = 13.1, 2.6 Hz, H-7β), 1.50 (1H, dd, *J* = 14.4, 2.5 Hz, H-8β), 1.45 (3H, d, *J* = 1.4 Hz, Me-17), 1.41 (1H, dd, *J* = 7.9, 3.5 Hz, H-1β), 1.38−1.32 (1H, m, H-11), 1.17 (3H, s, Me-18), 1.06 (1H, m, H-9), 1.04 (3H, s, Me-19), 0.96 (3H, d, *J* = 6.7 Hz, Me-16), 2.44 (2H, *q*, *J* =

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15.1, 7.6, H-2′), 1.21 (3H, *t*, *J* = 7.6, Me-3′) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 195.0 (C-14), 173.7 (C-1′), 169.5 (C-21), 146.5 (C-12), 142.8 (C-6), 131.9 (C-13), 118.6 (C-5), 94.3 (C-15), 80.6 (C-3), 50.9 (C-4), 44.4 (C-1), 38.1 (C-2), 36.7 (C-7), 33.5 (C-9), 29.0 (C-11), 28.9 (C-18), 28.3 (C-8), 27.6 (C-2′), 24.3 (C-10), 21.2 (C-22), 20.6 (C-17), 16.0 (C-19), 13.6 (C-16), 12.0 (C-20), 9.2 (C-3′) ppm; HRMS-ESI-TOF *m*/*z* calcd $C_{25}H_{36}O_5Na$ (M^+ + Na) 439.2455, found 439.2458.

*Jolkinoate C, 15β-Acetoxy-3β-butanoatelathyra-5E,12E-dien-14-one (**4**).* **4** was obtained from reaction with butanoic anhydride (Sigma-Aldrich Chemie GmbH, Riedstrasse D-89555, Steinhelm, Germany; 12.6 mg, 0.08 mmol). The residue was purified by flash column chromatography (silica gel, *n*-hexane/EtOAc = 100:0 to 9:1) to afford 10 mg (0.024 mol, yield 61%) of a yellow oil. IR (NaCl) ν_{max} 1743 (C═O), 1259 (C−O) cm^{-1}; MS *m*/*z* (rel intens) 453 [M + Na]$^+$ (100), 431 [M]$^+$ (11), 381 [M + Na − $CH_3CH_2CH_2CO$]$^+$ (35); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.65 (1H, d, *J* = 11.4 Hz, H-12), 5.33 (1H, d, *J* = 10.6 Hz, H-5), 5.24 (1H, *t*, *J* = 3.6 Hz, H-3), 3.52 (1H, dd, *J* = 13.9, 8.0 Hz, H-1α), 2.49 (1H, dd, *J* = 10.7, 3.8 Hz, H-4), 2.37 (1H, td, *J* = 7.3, 1.9 Hz, H-7α), 2.22−2.12 (2H, m, H-8α, H-2), 2.00 (3H, s, Me-22), 1.82 (3H, s, Me-20), 1.74 (1H, td, *J* = 13.1, 2.9 Hz, H-7β), 1.48 (1H, dd, *J* = 13.5, 2.5 Hz, H-11), 1.48−1.46 (1H, m, H-1β, H-8β), 1.45 (3H, s, Me-17), 1.39 (1H, dd, *J* = 7.9, 3.5 Hz, H-1β), 1.16 (3H, s, Me-18), 1.08−1.04 (1H, m, H-9), 1.04 (3H, s, Me-19), 0.96 (3H, d, *J* = 7.4 Hz, Me-16), 2.30 (2H, t, *J* = 7.4 Hz, H-2′), 1.65 (2H, q, *J* = 7.4 Hz, H-3′), 0.94 (3H, t, *J* = 3.4 Hz, Me-4′) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 195.4 (C-14), 173.2 (C-1′), 169.8 (C-21), 146.8 (C-12), 143.0 (C-6), 132.2 (C-13), 118.8 (C-5), 94.6 (C-15), 80.4 (C-3), 50.9 (C-4), 44.5 (C-1), 38.2 (C-2), 35.6 (C-2′), 36.1 (C-7), 34.2 (C-9), 29.7 (C-11), 29.3 (C-18), 28.4 (C-8), 24.6 (C-10), 21.6 (C-22), 21.0 (C-17), 18.0 (C-3′), 16.0 (C-19), 13.8 (C-16), 13.5 (C-4′), 12.0 (C-20) ppm; HRMS-ESI-TOF *m*/*z* calcd $C_{26}H_{38}O_5Na$ (M^+ + Na) 453.2617, found 453.2606.

*Jolkinoate D, 15β-Acetoxy-3β-lauroyloxylathyra-5E,12E-dien-14-one (**5**).* **5** was obtained from reaction with lauroyl chloride (Sigma-Aldrich Chemie GmbH, Riedstrasse D-89555, Steinhelm, Germany; 17.4 mg, 0.08 mmol). The residue was purified by flash column chromatography (silica gel, *n*-hexane/EtOAc = 100:0 to 9:1) to afford 20 mg (0.036 mol, yield 91%) of an amorphous dark yellow powder. IR (NaCl) ν_{max} 1740 (C═O), 1280 (C−O) cm^{-1}; MS *m*/*z* (rel intens) 565 [M + Na]$^+$ (54), 543 [M]$^+$ (3), 381 [M + Na − $CH_3(CH_2)_{10}CO$]$^+$ (100); ^{1}H NMR (400 MHz, $CDCl_3$), δ 6.66 (1H, d, *J* = 11.3 Hz, H-12), 5.33 (1H, d, *J* = 10.7 Hz, H-5), 5.25 (1H, t, *J* = 3.5 Hz, H-3), 3.51 (1H, dd, *J* = 13.8, 8.0 Hz, H-1α), 2.50 (1H, dd, *J* = 10.6, 3.7 Hz, H-4), 2.39 (1H, td, *J* = 12.0, 2.4 Hz, H-7α), 2.21−2.17 (2H, m, H-8α, H-2), 2.01 (3H, s, Me-22), 1.83 (3H, s, Me-20), 1.75 (1H, td, *J* = 12.5, 3.0 Hz, H-7β), 1.47 (1H, dd, *J* = 7.4, 4.3 Hz, H-11), 1.46 (1H, dd, *J* = 12.4, 2.9 Hz, H-1β), 1.44 (3H, s, Me-17), 1.41 (1H, dd, *J* = 7.3, 4.1 Hz, H-8β), 1.17 (3H, s, Me-18), 1.08 (1H, m, H-9), 1.05 (3H, s, Me-19), 0.94 (3H, d, *J* = 6.7 Hz, Me-16), 2.34 (2H, t, *J* = 7.5 Hz, H-2′), 1.67−1.58 (18H, m, H-3′−H-11′), 0.87 (3H, t, *J* = 6.8 Hz, Me-12′) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 195.3 (C-14), 173.3 (C-1′), 169.7 (C-21), 146.7 (C-12), 142.9 (C-6), 132.1 (C-13), 118.7 (C-5), 94.5 (C-15), 80.7 (C-3), 51.2 (C-4), 44.8 (C-1), 38.3 (C-2), 36.8 (C-7), 34.6 (C-2′), 34.1 (C-9), 34.1−29.3 (C-3′−C-11′), 29.2 (C-11), 29.1 (C-18), 28.3 (C-8), 24.7 (C-10), 22.7 (C-22), 20.8 (C-17), 16.3 (C-19), 14.1 (C-16), 13.9 (C-12′), 12.3 (C-20) ppm; HRMS-ESI-TOF *m*/*z* calcd $C_{34}H_{54}O_5Na$ (M^+ + Na) 565.3863, found 565.3875.

*Jolkinoate E, 15β-Acetoxy-3β-(2-methylvaleroate)lathyra-5E,12E-dien-14-one (**6**).* **6** was obtained from reaction with 2-methylvaleryl chloride (Sigma-Aldrich Chemie GmbH, Riedstrasse D-89555, Steinhelm, Germany; 10.9 mg, 0.08 mol). The residue was purified by column chromatography (silica gel, CH_2Cl_2) followed by a preparative TLC with *n*-hexane/EtOAc (6:1) to afford 20 mg (0.044 mol, yield 93.5%) of a yellow oil. IR (NaCl) ν_{max} 1792 (C═O), 1271 (C−O) cm^{-1}; MS *m*/*z* (rel intens) 481 [M + Na]$^+$ (100), 430 [M − CH_2═CH_2]$^+$ (18); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.66 (1H, d, *J* = 11.4 Hz, H-12), 5.34 (1H, d, *J* = 10.7 Hz, H-5), 5.24 (1H, t, *J* = 4.1 Hz, H-3), 3.53 (1H, dd, *J* = 13.9, 8.0 Hz, H-1α), 2.55 (1H, dd, *J* = 13.7, 6.8 Hz, H-4), 2.50 (1H, ddd, *J* = 10.6, 3.7, 1.7 Hz, H-7α), 2.21−2.18 (2H, m, H-8α, H-2), 2.01 (3H, d, *J* = 2.5, Me-22), 1.83 (3H, s, Me-20), 1.75 (1H, td, *J* = 13.1, 2.5 Hz, H-7β), 1.48 (1H, dd, *J* = 5.7, 2.8 Hz, H-8β), 1.46 (1H, dd, *J* = 8.6, 2.3 Hz, H-1β), 1.44 (3H, d, *J* = 1.3 Hz, Me-17), 1.38 (1H, dd, *J* = 7.6, 3.6 Hz, H-11), 1.17 (3H, s, Me-18), 1.09 (1H, m, H-9), 1.04 (3H, d, *J* = 1.2 Hz, Me-19), 0.95 (3H, d, *J* = 6.6 Hz, Me-16), 2.54 (1H, m, H-2′), 1.21 (2H, dd, *J* = 7.0, 2.9 Hz, H-3′), 1.12 (2H, m, H-4′), 0.95 (3H, t, *J* = 7.1 Hz, Me-5′), 1.17 (3H, d, *J* = 6.7 Hz, Me-6′) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 195.2 (C-14), 175.9 (C-1′), 169.6 (C-21), 146.7 (C-12), 142.8 (C-6), 132.1 (C-13), 118.8 (C-5), 94.6 (C-15), 80.5 (C-3), 51.3 (C-4), 44.8 (C-1), 39.8 (C-2′), 38.3 (C-2), 36.07 (C-7), 34.12 (C-9), 29.59 (C-11), 29.39 (C-3′), 29.15 (C-18), 28.88 (C-4′), 28.22 (C-8), 24.49 (C-10), 21.39 (C-22), 20.8 (C-17), 20.31 (C-5′), 16.6 (C-6′), 16.3 (C-19), 13.8 (C-16), 12.3 (C-20) ppm; HRMS-ESI-TOF *m*/*z* calcd $C_{28}H_{42}O_5Na$ (M^+ + Na) 481.2924, found 481.2933.

*Jolkinoate F, 15β-Acetoxy-3β-(2-ethylbutyrate)lathyra-5E,12E-dien-14-one (**7**).* **7** was obtained from reaction with 2-ethylbutyryl chloride (Sigma-Aldrich Chemie GmbH, Riedstrasse D-89555, Steinhelm, Germany; 11 mg, 0.08 mol). The residue was purified by column chromatography (silica gel, CH_2Cl_2) followed by preparative TLC with *n*-hexane/EtOAc (9:1) to afford 19 mg (0.042 mol, yield 84%) of a yellow oil. IR (NaCl) ν_{max} 1734 (C═O), 1269 (C−O) cm^{-1}; MS *m*/*z* (rel intens) 481 [M + Na]$^+$ (100), 399 [M − CH_3CO_2]$^+$ (12); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.65 (1H, dd, *J* = 11.4, 0.9 Hz, H-12), 5.36 (1H, d, *J* = 10.7 Hz, H-5), 5.26 (1H, t, *J* = 3.5 Hz, H-3), 3.53 (1H, dd, *J* = 13.9, 8.0 Hz, H-1α), 2.50 (1H, dd, *J* = 10.7, 3.7 Hz, H-4), 2.44 (1H, dd, *J* = 13.2, 3.9 Hz, H-7α), 2.20−2.16 (2H, m, H-8α, H-2), 2.00 (3H, s, Me-22), 1.83 (3H, d, *J* = 0.9 Hz, Me-20), 1.74 (1H, td, *J* = 12.0, 2.4 Hz, H-7β), 1.49 (1H, dd, *J* = 7.2, 1.5 Hz, H-8β), 1.44 (1H, dd, *J* = 12.8, 7.3 Hz, H-1β), 1.44 (3H, d, *J* = 1.2 Hz, Me-17), 1.39 (1H, dd, *J* = 11.5, 8.1 Hz, H-11), 1.16 (3H, s, Me-18), 1.06 (1H, m, H-9), 1.04 (3H, s, Me-19), 0.89 (3H, d, *J* = 6.9 Hz, Me-16), 2.28 (1H, m, H-2′), 1.68−1.58 (4H, m, H-3′, H-5′), 0.93 (6H, t, *J* = 7.5 Hz, Me-4′, Me-6′) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 195.6 (C-14), 175.7 (C-1′), 169.8 (C-21), 146.9 (C-12), 142.8 (C-6), 132.2 (C-13), 119.1 (C-5), 94.7 (C-15), 80.8 (C-3), 51.4 (C-4), 48.4 (C-2′), 44.9 (C-1), 38.4 (C-2), 36.8 (C-7), 34.2 (C-9), 29.6 (C-18), 29.3 (C-11), 28.3 (C-8), 24.6 (C-10), 22.7 (C-3′/C-5′), 21.5 (C-22), 21.0 (C-17), 16.4 (C-19), 14.0 (C-16), 12.4 (C-4′/C-6′), 12.1 (C-20) ppm; HRMS-ESI-TOF *m*/*z* calcd $C_{28}H_{42}O_5Na$ (M^+ + Na) 481.2924, found 481.2934.

*Jolkinoate G, 15β-Acetoxy-3β-(2-ethylhexanoate)lathyra-5E,12E-dien-14-one (**8**).* **8** was obtained from reaction with 2-ethylhexanoyl chloride (Sigma-Aldrich Chemie GmbH, Riedstrasse D-89555, Steinhelm, Germany; 13.5 mg, 0.08 mol). The residue was purified by flash column chromatography (silica gel CH_2Cl_2/MeOH = 99:1) to afford 33.5 mg (0.069 mol, yield 97%) of a yellow oil. IR (NaCl) ν_{max} 1743 (C═O), 1269 (C−O) cm^{-1}; MS *m*/*z* (rel intens) 509 [M + Na]$^+$ (100), 427 [M − CH_3CO_2]$^+$ (12); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.67 (1H, d, *J* = 11.4 Hz, H-12), 5.39 (1H, d, *J* = 10.7 Hz, H-5), 5.28 (1H, t, *J* = 3.5 Hz, H-3), 3.55 (1H, dd, *J* = 13.9, 8.0 Hz, H-1α), 2.55 (1H, dd, *J* = 10.7, 3.7 Hz, H-4), 2.44 (1H, d, *J* = 13.4, H-7α), 2.30−2.20 (2H, m, H-8α, H-2), 2.00 (3H, s, Me-22), 1.83 (3H, s, Me-20), 1.76 (1H, td, *J* = 12,8, 2.7 Hz, H-7β), 1.66−1.56 (8H, m, H-1β, H-8β), 1.44 (3H, d, *J* = 1.0 Hz, Me-17), 1.39 (1H, dd, *J* = 11.5, 8.3 Hz, H-11), 1.16 (3H, s, Me-18), 1.06 (1H, m, H-9), 1.04 (3H, s, Me-19), 0.92 (3H, d, *J* = 7.4 Hz, Me-16), 2.26 (1H, m, H-2′), 1.66−1.56 (8H, m, H-3′, H-4′, H-5′, H-7′), 0.96 (3H, t, *J* = 7.3 Hz, Me-8′), 0.95 (3H, t, *J* = 8.8 Hz, Me-6′) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 195.3 (C-14), 175.5 (C-1′), 169.8 (C-21), 146.9 (C-12), 142.8 (C-6), 132.2 (C-13), 119.1 (C-5), 94.6 (C-15), 80.74 (C-3), 51.4 (C-4), 49.8 (C-2′), 44.9 (C-1), 38.5 (C-2), 36.9 (C-7), 34.26 (C-9), 29.7 (C-11), 29.3 (C-18), 28.3 (C-8), 25.3−25.0 (C-3′/C-4′/C-5′/C-7′), 24.6 (C-10), 21.5 (C-22), 20.9 (C-17), 16.4 (C-19), 14.1 (C-16), 12.4−12.2 (C-6′/C-8′), 12.1 (C-20) ppm; HRMS-ESI-TOF *m*/*z* calcd $C_{30}H_{46}O_5Na$ (M^+ + Na) 509.3237, found 509.3256.

*Jolkinoate I, 15β-Acetoxy-3β-benzoyloxylathyra-5E,12E-dien-14-one (**9**).* **9** was obtained from reaction with benzoyl chloride (Merck KGaA, Darmstradt, Germany; 14 mg, 0.10 mol). The residue was

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purified by two sequential flash column chromatography (silica gel, CH_2Cl_2 and silica gel, CH_2Cl_2/MeOH = 99:1) to afford 33 mg (0.07 mol, yield 96%) of an amorphous white powder. IR (NaCl) ν_{max} 3030 (C−H_{sp2}), 1740 (C═O), 1273 (C−O) cm^{-1}; MS *m*/*z* (rel intens) 487 $[M + Na]^+$ (100), 381 $[M + Na - C_6H_5CO]^+$ (9), 405 $[M - CH_3CO_2]^+$ (7); ^1H NMR (400 MHz, $CDCl_3$) δ 6.69 (1H, d, *J* = 11.4 Hz, H-12), 5.51 (1H, t, *J* = 3.4 Hz, H-3), 5.39 (1H, d, *J* = 10.6 Hz, H-5), 3.64 (1H, dd, *J* = 14.0, 8.1 Hz, H-1α), 2.63 (1H, dd, *J* = 10.6, 3.6 Hz, H-4), 2.42 (1H, d, *J* = 13.4 Hz, H-7α), 2.29 (1H, m, H-2), 2.14 (1H, m, H-8α), 2.08 (3H, s, Me-22), 1.86 (3H, s, Me-20), 1.71 (1H, td, *J* = 13.2, 2.5 Hz, H-7β), 1.64 (1H, dd, *J* = 13.9, 12.4, H-11), 1.47 (3H, d, *J* = 1.2 Hz, Me-17), 1.45−1.41 (2H, m, H-1β, H-8β), 1.17 (3H, s, Me-18), 1.06 (1H, m, H-9), 1.04 (3H, s, Me-19), 1.00 (3H, d, *J* = 6.7 Hz, Me-16), δ 8.11 (2H, t, *J* = 7.1 Hz, H-3′), 7.62 (1H, t, *J* = 7.4 Hz, H-5′), 7.48 (2H, t, *J* = 7.7 Hz, H-4′) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 195.3 (C-14), 171.2 (C-1′), 169.8 (C-21), 147.0 (C-12), 143.4 (C-6), 133.8 (C-13), 133.2 (C-5′), 130.3 (C-2′), 129.8 (C-3′/C-7′), 128.6 (C-4′/C-6′), 118.7 (C-5), 94.9 (C-15), 81.9 (C-3), 51.7 (C-4), 45.2 (C-1), 38.9 (C-2), 36.7 (C-7), 34.3 (C-9), 29.8 (C-11), 29.3 (C-18), 28.5 (C-8), 24.7 (C-10), 21.6 (C-22), 21.1 (C-17), 16.5 (C-19), 14.2 (C-16), 12.4 (C-20) ppm; HRMS-ESI-TOF *m*/*z* calcd $C_{29}H_{36}O_5Na$ (M^+ + Na) 487.2455, found 487.2453.

*Jolkinoate J, 15β-Acetoxy-3β-(4-methylbenzoyloxy)lathyra-5E,12E-dien-14-one (**10**).* **10** was obtained from reaction with 4-methylbenzoyl chloride (Sigma-Aldrich Chemie GmbH, Riedstrasse D-89555, Steinhelm, Germany; 16.5 mg, 0.11 mol). The residue was purified by two sequential flash column chromatography (silica gel, *n*-hexane/CH_2Cl_2 = 2:1 and silica gel, CH_2Cl_2/MeOH = 99:1) to afford 15 mg (0.030 mol, yield 55%) of an amorphous white powder. IR (NaCl) ν_{max} 2960 (C−H_{sp2}), 1735 (C═O), 1269 (C−O) cm^{-1}; MS *m*/*z* (rel intens) 501 $[M + Na]^+$ (100), 419 $[M - CH_3CO_2]^+$ (7), 318 $[M - CH_3CO_2 - CH_3C_6H_4CO]^+$ (6); ^1H NMR (400 MHz, $CDCl_3$) δ 6.69 (1H, dd, *J* = 11.4, 0.9, H-12), 5.49 (1H, t, *J* = 3.3, H-3), 5.38 (1H, d, *J* = 10.6 Hz, H-5), 3.63 (1H, dd, *J* = 14.0, 8.1 Hz, H-1α), 2.62 (1H, dd, *J* = 10.6, 3.5 Hz, H-4), 2.42 (1H, m, H-7α), 2.27 (1H, m, H-2), 2.13 (1H, m, H-8α), 2.08 (3H, s, Me-22), 1.86 (3H, d, *J* = 0.9 Hz, Me-20), 1.70 (1H, td, *J* = 13.1, 2.5 Hz, H-7β), 1.60 (1H, dd, *J* = 13.9, 12.4 Hz, H-11), 1.46 (3H, d, *J* = 1.4 Hz, Me-17), 1.45 (1H, dd, *J* = 11.3, 8.0 Hz, H-8β), 1.26 (1H, dd, *J* = 7.6, 6.7 Hz, H-1β), 1.16 (3H, s, Me-18), 1.06 (1H, m, H-9), 1.03 (3H, s, Me-19), 1.00 (3H, d, *J* = 6.7 Hz, Me-16), 8.01 (2H, dd, *J* = 18.1, 8.2 Hz, H-3′/H-7′), 7.30 (2H, dd, *J* = 12.1, 8.0 Hz, H-4′/H-6′), 2.45 (3H, s, Me-8′) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 195.3 (C-14), 169.8 (C-21), 166.1 (C-1′), 147.0 (C-12), 143.9 (C-6), 132.3 (C-13), 130.8 (C-5′), 129.8 (C-4′/C-6′), 129.3 (C-3′/C-7′), 127.7 (C-2′), 118.82 (C-5), 94.9 (C-15), 81.6 (C-3), 51.7 (C-4), 45.2 (C-1), 38.9 (C-2), 36.7 (C-7), 34.3 (C-9), 29.8 (C-11), 29.3 (C-18), 28.5 (C-8), 24.7 (C-10), 22.0 (C-8′), 21.6 (C-22), 21.1 (C-17), 16.4 (C-19), 14.1 (C-16), 12.4 (C-20) ppm; HRMS-ESI-TOF *m*/*z* calcd $C_{30}H_{38}O_5Na$ (M^+ + Na) 501.2611, found 501.2610.

*Jolkinoate K, 15β-Acetoxy-3β-(4-methoxybenzoyloxy)lathyra-5E,12E-dien-14-one (**11**).* **11** was obtained from reaction with 4-methoxybenzoyl chloride (Sigma-Aldrich Chemie GmbH, Riedstrasse D-89555, Steinhelm, Germany; 7.5 mg, 0.039 mol). The residue was purified by two sequential flash column chromatography (silica gel, CH_2Cl_2 and silica gel, CH_2Cl_2/MeOH = 99:1) to afford 14 mg (0.028 mol, yield 73%) of an amorphous white powder. IR (NaCl) ν_{max} 3040 (C−H_{sp2}), 1740 (C═O), 1273 (C−O) cm^{-1}; MS *m*/*z* (rel intens) 517 $[M + Na]^+$ (100), 435 $[M - CH_3CO_2]^+$ (9); ^1H NMR (400 MHz, $CDCl_3$) δ 6.69 (1H, d, *J* = 11.4 Hz, H-12), 5.47 (1H, t, *J* = 3.3 Hz, H-3), 5.38 (1H, d, *J* = 10.6 Hz, H-5), 3.63 (1H, dd, *J* = 14.0, 8.1 Hz, H-1α), 2.61 (1H, dd, *J* = 10.7, 3.6 Hz, H-4), 2.42 (1H, d, *J* = 13.6 Hz, H-7α), 2.27 (1H, m, H-2), 2.14 (1H, m, H-8α), 2.08 (3H, s, Me-22), 1.86 (3H, d, *J* = 0.9 Hz, Me-20), 1.70 (1H, td, *J* = 13.0, 2.4 Hz, H-7β), 1.59 (1H, dd, *J* = 14.9, 11.2 Hz, H-11), 1.46 (3H, d, *J* = 1.4 Hz, Me-17), 1.40 (1H, m, H-8β), 1.25 (1H, m, H-1β), 1.17 (3H, s, Me-18), 1.06 (1H, m, H-9), 1.04 (3H, s, Me-19), 0.99 (3H, d, *J* = 6.7 Hz, Me-16), 8.05 (2H, d, *J* = 8.9 Hz, H-3′/H-7′), 6.96 (2H, d, *J* = 8.9 Hz, H-4′/H-6′), 3.89 (3H, s, H-6′) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 195.3 (C-14), 169.8 (C-21), 163.6 (C-1′), 146.9 (C-12), 143.3 (C-6), 133.3 (C-13), 132.3 (C-5′), 131.8 (C-3′/C-7′) 122.8 (C-2′), 118.9 (C-5), 113.8 (C-4′/C-6′), 94.9 (C-15), 81.7 (C-3), 55.7 (C-8′), 51.7 (C-4), 45.3 (C-1), 38.9 (C-2), 36.7 (C-7), 34.3 (C-9), 29.8 (C-11), 29.3 (C-18), 28.5 (C-8), 24.7 (C-10), 21.6 (C-22), 21.1 (C-17), 16.5 (C-19), 14.1 (C-16), 12.4 (C-20) ppm; HRMS-ESI-TOF *m*/*z* calcd $C_{30}H_{38}O_6Na$ (M^+ + Na) 517.2560, found 517.2565.

*Jolkinoate L, 15β-Acetoxy-3β-(3-trifluormethylbenzoyloxy)-lathyra-5E,12E-dien-14-one (**12**).* **12** was obtained from reaction with 3-trifluoromethylbenzoyl chloride (Sigma-Aldrich Chemie GmbH, Riedstrasse D-89555, Steinhelm, Germany). The residue was purified by column chromatography (silica gel, CH_2Cl_2/acetone = 19:1) and preparative TLC (two runs) with *n*-hexane/acetone (9:1) to afford 12.6 mg (0.024 mol, yield 50%) of an amorphous white powder. IR (NaCl) ν_{max} 3020 (C−H_{sp2}), 1743 (C═O), 1257 (C−O) cm^{-1}; MS *m*/*z* (rel intens) 555 $[M + Na]^+$ (100), 318 $[M - CH_3CO_2^- - F_3CC_6H_4CO]^+$ (13); ^1H NMR (400 MHz, $CDCl_3$) δ 6.70 (1H, d, *J* = 12.4, H-12), 5.53 (1H, t, *J* = 3.4, H-3), 5.37 (1H, d, *J* = 10.6, H-5), 3.66 (1H, dd, *J* = 14.1, 8.1, H-1α), 2.65 (1H, dd, *J* = 10.6, 3.6, H-4), 2.42 (1H, d, *J* = 13.3, H-7α), 2.31 (1H, m, H-2), 2.14 (1H, m, H-8α), 2.08 (3H, s, Me-22), 1.86 (3H, d, *J* = 1.0, Me-20), 1.72 (1H, td, *J* = 13.1, 2.5, H-7β), 1.59 (1H, dd, *J* = 14.0, 12.3, H-11), 1.47 (3H, d, *J* = 1.4, Me-17), 1.45−1.41 (2H, m, H-1β, H-8β), 1.17 (3H, s, Me-18), 1.06 (1H, m, H-9), 1.04 (3H, s, Me-19), 1.00 (3H, d, *J* = 6.7, Me-16), 8.34 (1H, s, H-3′), 8.29 (1H, d, *J* = 7.8, H-5′), 7.87 (1H, d, *J* = 7.8, H-7′), 7.65 (1H, t, *J* = 7.8, H-6′) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 195.5 (C-14), 170.2 (C-1′), 164.9 (C-21), 147.3 (C-12), 144.2 (C-6), 133.5 (C-13), 132.6 (C-7′), 131.7 (C-4′), 131.6 (C-2′), 130.1 (C-5′), 129.7 (C-6′), 126.7 (C-3′), 125.5 (C-8′), 118.7 (C-5), 95.1 (C-15), 83.0 (C-3), 51.9 (C-4), 45.6 (C-1), 39.2 (C-2), 37.0 (C-7), 34.7 (C-9), 30.1 (C-11), 29.6 (C-18), 28.8 (C-8), 25.1 (C-10), 21.6 (C-22), 21.4 (C-17), 16.74 (C-19), 14.5 (C-16), 12.7 (C-20) ppm; HRMS-ESI-TOF *m*/*z* calcd $C_{30}H_{35}F_3O_5Na$ (M^+ + Na) 555.2334, found 555.2331.

*Jolkinoate M, 15β-Acetoxy-3β-benzenesulfoxylathyra-5E,12E-dien-14-one (**13**).* **13** was obtained from reaction with benzenesulfonyl chloride (Sigma-Aldrich Chemie GmbH, Riedstrasse D-89555, Steinhelm, Germany). The residue was purified by column chromatography (silica gel, CH_2Cl_2/acetone (19:1)) and preparative TLC with *n*-hexane/acetone (9:1) to afford 13 mg (0.024 mol, yield 60%) of an amorphous white powder. IR (NaCl) ν_{max} 3015 (C−H_{sp2}), 1741 (C═O), 1378 (S═O) 1271 (C−O) cm^{-1}; MS *m*/*z* (rel intens) 523 $[M + Na]^+$ (100), 381 $[M + Na - C_6H_5O_3S]^+$; ^1H NMR (400 MHz, $CDCl_3$) δ 6.57 (1H, d, *J* = 11.8 Hz, H-12), 5.14 (1H, d, *J* = 10.5 Hz, H-5), 4.90 (1H, t, *J* = 3.4 Hz, H-3), 3.47 (1H, dd, *J* = 14.0, 8.0 Hz, H-1α), 2.45 (1H, dd, *J* = 10.5, 3.6 Hz, H-4), 2.19−2.06 (3H, m, H-7α, H-8α, H-2), 1.98 (3H, s, Me-22), 1.79 (3H, d, *J* = 0.5 Hz, Me-20), 1.60 (1H, td, *J* = 12.9, 2.2 Hz, H-7β), 1.45 (1H, dd, *J* = 13.5, 13.0 Hz, H-11), 1.36 (3H, d, *J* = 1.2 Hz, Me-17), 1.35−1.25 (2H, m, H-1β, H-8β), 1.15 (3H, s, Me-18), 1.05 (1H, m, H-9), 1.00 (3H, s, Me-19), 0.91 (3H, d, *J* = 6.7 Hz, Me-16), 7.91 (2H, d, *J* = 8.6 Hz, H-2′/H-6′), 7.63 (1H, t, *J* = 7.4 Hz, H-4′), 7.54 (2H, t, *J* = 7.6 Hz, H-3′/H-5′) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 194.91 (C-14), 169.7 (C-21), 147.0 (C-12), 143.2 (C-6), 137.6 (C-1′), 133.5 (C-13), 131.9 (C-2′/C-6′), 129.0 (C-4′), 127.8 (C-3′/C-5′), 118.6 (C-5), 93.7 (C-15), 91.4 (C-3), 51.5 (C-4), 44.1 (C-1), 38.7 (C-2), 36.5 (C-7), 34.2 (C-9), 29.6 (C-11), 29.1 (C-18), 28.1 (C-8), 24.6 (C-10), 21.4 (C-22), 20.7 (C-17), 16.3 (C-19), 14.0 (C-16), 12.2 (C-20) ppm; HRMS-ESI-TOF *m*/*z* calcd $C_{28}H_{36}O_6SNa$ (M^+ + Na) 523.2125, found 523.2127.

*Preparation of Jolkinodiol 3β,15β-Dihydroxylathyra-5E,12E-dien-14-one (**14**).* A mixture of Jolkinol D (20 mg, 0.055 mol) in MeOH/KOH at 10% was stirred for 72 h at room temperature. The residue was purified by column chromatography (silica gel, CH_2Cl_2/MeOH = 99:1 to 3:1) to afford 14 mg (0.044 mol, yield 81%) of an amorphous white powder. IR (NaCl) ν_{max} 3442, 3425, 1650, 1640 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.44 (1H, dd, *J* = 11.9, 0.8 Hz, H-12), 5.66 (1H, d, *J* = 10.7 Hz, H-5), 3.96 (1H, t, *J* = 3.0 Hz, H-3), 3.43 (1H, dd, *J* = 14.0, 9.3 Hz, H-1α), 2.54 (1H, d, *J* = 13.2 Hz, H-7α), 2.28 (1H, dd, *J* = 10.7, 3.1 Hz, H-4), 2.17 (1H, br d, *J* = 14.1 Hz, H-8α), 2.02 (1H, m, H-2), 1.82 (3H, d, *J* = 0.9 Hz, Me-20), 1.71 (1H, td, *J* = 13.1, 2.0 Hz, H-7β), 1.56 (1H, td, *J* = 14.3, 2.3 Hz, H-11), 1.47−1.41 (2H, m, H-1β, H-8β), 1.41 (3H, d, *J* = 1.2 Hz, Me-17), 1.18 (3H, s, Me-18), 1.11

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(3H, d, J = 6.9 Hz, Me-16), 1.08 (3H, s, Me-19), 1.05 (1H, m, H-9) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 198.1 (C-14), 151.8 (C-12), 141.9 (C-6), 132.4 (C-13), 120.2 (C-5), 92.7 (C-15), 81.3 (C-3), 53.3 (C-4), 46.1 (C-1), 38.9 (C-2), 36.7 (C-7), 35.1 (C-9), 29.9 (C-11), 29.3 (C-18), 28.3 (C-8), 24.9 (C-10), 21.0 (C-17), 16.4 (C-19), 14.4 (C-16), 12.5 (C-20) ppm; HRMS-ESI-TOF m/z calcd $C_{20}H_{30}O_3Na$ (M^+ + Na) 341.2087, found 341.2095.

4.2. Biological Studies. *Cell Lines and Cultures.* The L5178Y mouse T-lymphoma cells (ECACC catalog no. 87111908, U.S. FDA, Silver Spring, MD, U.S.) were transfected with the pHa MDR1/A retrovirus as described in the literature.[35] The MDR1-expressing cell line was selected by culturing the infected cells with colchicine (60 ng/mL), thus maintaining the MDR phenotype expression. L5178Y (parental, PAR) mouse T-cell lymphoma cells and the human MDR1-transfected subline (MDR) were cultured in McCoy's 5A supplemented with 10% heat-inactivated horse serum, 100 U/L L-glutamine, and 100 mg/L penicillin–streptomycin mixture, all obtained from Sigma-Aldrich (Sigma-Aldrich Kft, Budapest, Hungary). The cell lines were then incubated in a humidified atmosphere (5% CO2, 95% air) at 37 °C.

Antiproliferative Assay. The antiproliferative effects of the compounds were tested in a range of decreasing concentrations (2-fold dilutions) using PAR and MDR mouse lymphoma cell lines as experimental model. The cells were distributed into 96-well flat bottom microtiter plates at 1×10^5/mL for a final volume of 100 μL of medium per well. The different concentrations of each compound were added into duplicate wells. The plates were initially incubated at 37 °C for 72 h, and at the end of the incubation period, 15 μL of MTT solution (thiazolyl blue tetrazolium bromide dissolved in PBS to a final concentration of 5 mg/mL) was added to each well and incubated for another 4 h. Then 100 μL of 10% SDS solution (sodium dodecyl sulfate) was added into each well and the plates were further incubated overnight at 37 °C. Cell growth was determined by measuring the optical density (OD) at 550 nm (ref, 630 nm) with a Multiscan EX ELISA reader (Thermo Labsystems, Cheshire, WA, U.S.). The percentage of inhibition of cell growth was determined according to eq 1.

$$100 - \left[\frac{OD_{sample} - OD_{medium\ control}}{OD_{cell\ control} - OD_{medium\ control}} \right] \times 100 \quad (1)$$

All the experiments were performed in triplicate. The evaluation of the stability of the esters, under these experimental conditions, is reported in the Supporting Information.

Assay for Rhodamine 123 Accumulation. The cells were adjusted to a density of 2×10^6 cells/mL, resuspended in serum-free McCoy's 5A medium, and distributed in 500 μL aliquots. The test compounds were added at 2 and 20 μM. Verapamil (positive control, EGIS Pharmaceuticals PLC, Budapest, Hungary) was added at 22 μM and DMSO at 2% as solvent control. The samples were incubated for 10 min at room temperature, after which 10 μL (5.2 μM final concentration) of rhodamine-123 was added to the samples. After a 20 min incubation at 37 °C, the samples were washed twice, resuspended in 500 μL of phosphate buffered saline (PBS), and analyzed by flow cytometry (Partec CyFlow Space instrument, Partec GmbH, Münster, Germany). The resulting histograms were evaluated regarding mean fluorescence intensity (FL-1), standard deviation, and peak channel of 20 000 individual cells belonging to the total and gated populations. The fluorescence activity ratio (FAR) was calculated on the basis of the quotient between FL-1 of treated/untreated resistant cell line (MDR mouse lymphoma cells) over treated/untreated sensitive cell line (PAR mouse lymphoma cells), according to eq 2.

$$FAR = \frac{\left(\frac{FL-1MDR_{treated}}{FL-1MDR_{untreated}} \right)}{\left(\frac{FL-1PAR_{treated}}{FL-1PAR_{untreated}} \right)} \quad (2)$$

The evaluation of the stability of the esters, under these experimental conditions, is reported in the Supporting Information.

Drug Combination Assay. The combination studies were designed as suggested in the CalcuSyn[36] software manual, using a fixed ratio of the drugs across a concentration gradient. The dilutions of doxorubicin (14.7–0.1 μM) were made in a horizontal direction and the dilutions of resistance modifiers (at 2-fold of their IC_{50} values) vertically in a microtiter plate to a final volume of 200 μL of medium per well. The cells were distributed into plates at 2×10^5 cells/mL per well and were incubated for 48 h under the standard conditions. The cell growth rate was determined after MTT staining, as previously described. Drug interactions were evaluated according to Chou using the software CalcuSyn, version 2.[36,37] Each dose–response curve (individual agents as well as combinations) was fit to a linear model using the median effect equation in order to obtain the median effect value (corresponding to the IC_{50}) and slope (m). Goodness-of-fit was assessed using the linear correlation coefficient r, and only data from analyses with $r > 0.90$ were presented. The extent of interaction between drugs was expressed using the combination index (CI) for mutually exclusive drugs. A CI close to 1 indicates additivity. CI < 1 is defined as synergy and CI > 1 as antagonism.

Curve Fitting and Data Analysis. All results were expressed as the mean ± SD unless otherwise noted. IC_{50} values were obtained by best fitting the dose-dependent inhibition curves in GraphPad Prism 5 program.[38,39] K-means clustering was performed with Tanagra, version 1.4.41,[40,41] which subdivides the compounds (those with the nearest mean) into groups that exhibit a high degree of similarity. FAR values were submitted to statistical analysis, evaluating the coefficient determination (r^2) between each evaluated property and the logarithm of 1/FAR.[41]

■ ASSOCIATED CONTENT

Supporting Information

Evaluation of the stability of the esters under in vitro experimental conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

MDR, multidrug resistance; MDR1, multidrug resistance gene 1; TMD, transmembrane domain; NBD, nucleotide-binding domain; P-gp, P-glycoprotein; DBP, drug-binding pocket; FAR, fluorescence activity ratio; PAR, parental cell; log P, logarithm of the octanol/water partition coefficient; MW, molecular weight; ASA, accessible surface area; TPSA, topological polar surface area

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