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Research paper

Nitrogen-containing ecdysteroid derivatives vs. multi-drug resistance in cancer: Preparation and antitumor activity of oximes, oxime ethers and a lactam

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ABSTRACT

Multidrug resistance is a widespread problem among various diseases and cancer is no exception. We had previously described the chemo-sensitizing activity of ecdysteroid derivatives with low polarity on drug susceptible and multi-drug resistant (MDR) cancer cells. We have also shown that these molecules have a marked selectivity towards the MDR cells. Recent studies on the oximation of various steroid derivatives indicated remarkable increase in their antitumor activity, but there is no related bioactivity data on ecdysteroid oximes. In our present study, 13 novel ecdysteroid derivatives (oximes, oxime ethers and a lactam) and one known compound were synthesized from 20-hydroxyecdysone 2,3;20,22-diacetonide and fully characterized by comprehensive NMR techniques revealing their complete ^{1}H and ^{13}C signal assignments. The compounds exerted moderate to strong *in vitro* antiproliferative activity on HeLa, SiHa, MCF-7 and MDA-MB-231 cell lines. Oxime and particularly oxime ether formation strongly increased their inhibitory activity on the efflux of rhodamine 123 by P-glycoprotein (P-gp), while the new ecdysteroid lactam did not interfere with the efflux function. All compounds exerted potent chemo-sensitizing activity towards doxorubicin on a mouse lymphoma cell line and on its MDR counterpart, and, on the latter, the lactam was found the most active. Because of its MDR-selective chemo-sensitizing activity with no functional effect on P-gp, this lactam is of high potential interest as a new lead for further antitumor studies.

1. Introduction

Synthetic modification of steroidal compounds remains a promising strategy in the hunt for novel drug candidates since even minor changes in the substitution pattern of their chemical backbone may significantly modify specific bioactivities. Certain steroidal oximes and oxime ethers were shown to have antioxidant [1], antimicrobial [1], antineoplastic [2] or neuromuscular blocking [3] activities.

Currently, the antitumor activity of steroid oximes is by far the most deeply investigated and has recently attracted great scientific attention. For example, oximes and lactams of cholest-4-en-6-one were tested on two human cancer cell lines and were shown to have very high, tumor selective anticancer activity on HeLa cells [4]. Another study on the structure-activity relationships (SAR) of hydroxyiminosteroids bearing the oxime group on the steroid A and/or B ring showed that a C-6 oxime function is preferential over a 6-keto group concerning *in vitro* cytotoxic activity of these type of compounds [5]. In a follow-up study on the same compounds, the importance of 3- and 6-hydroxy functions was highlighted [6].

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Furthermore, a set of *in vitro* experiments on 63 novel estrone 16-oximes and oxime ethers revealed two oximes as promising antiproliferative agents with selectivity towards HeLa cells; the compounds modulated cell cycle and induced apoptosis through caspase-3 [7]. In a most recent study, a series of steroidal oximes and lactams were described to possess significant *in vitro* antiproliferative activity, and a 6,23-dioxime derivative, obtained from diosgenin acetate, was identified to be the most effective [8]. Several further recent reports can be found in the literature where well-defined mechanistic changes could also be connected to the increase in the antiproliferative activity observed after introducing an oxime moiety into an oxo-compound. For example, a number of α,β-unsaturated, cyclohexanone-based oximes showed greatly increased activity as compared to their parental oxo-compounds against $BRAF^{V600E}$ (the most common mutation in the v-raf murine sarcoma viral oncogenes homolog B1, involved in carcinogenesis and cancer agressiveness) and/or epidermal growth factor receptor TK kinases (involved in cell proliferation, evasion of apoptosis and invasive capacity) [9], or focal adhesion kinase (FAK; involved in stimulating metastasis and tumor progression) [10]. These reports suggest that the preparation of oxime derivatives from ketosteroids, and particularly from those with an α,β-enone moiety, should be a reasonable strategy to extend the chemical space towards new, potentially antitumor compounds.

Ecdysteroids are α,β-unsaturated 6-ketosteroids that occur in a wide range of plant species; as analogs of the insect molting hormone ecdysone, these compounds possess several biological functions in the flora and the fauna [11,12]. Since the isolation of the most abundant ecdysteroid 20-hydroxyecdysone (20E), these compounds were reported to also exert various, beneficial bioactivities in mammals [13,14,15,16]. Additionally, our group revealed that relatively apolar ecdysteroids can strongly sensitize cancer cells to chemotherapeutics (i.e. “chemo-sensitizing” activity), and suggested 20-hydroxyecdysone 2,3;20,22-diacetonide (**1**) as a promising anticancer lead compound [17]. Interestingly, this sensitization towards various chemotherapeutics could be observed both on multi-drug resistant (MDR) and drug susceptible cancer cell lines [18]. After several further studies, exploring this particular anticancer activity of ecdysteroids, we now know that 1) apolar substituents on the 2,3-diol moiety are more important than those at positions 20 and 22 [19], and 2) an oxidative side-chain cleavage knocks out the inhibitory activity on the efflux function of the ABCB1 transporter (P-glycoprotein; P-gp) while maintaining MDR selective sensitizing activity towards doxorubicin [20]. Regarding semi-synthetic modifications accompanied by the inclusion of heteroatoms, a difluorinated derivative of 20E 2,3;20,22-diacetonide was found to be a stronger P-gp inhibitor than its parental molecule (compound **1**), while, surprisingly, MDR selectivity of the difluorinated compound was lower: it sensitized a P-gp expressing MDR cell line to doxorubicin similarly to its parental compound **1**, and a stronger effect than that of **1** was observed on a non-MDR cell line [21]. The chemical structures of 20E and compound 1 are shown in Fig. 1.

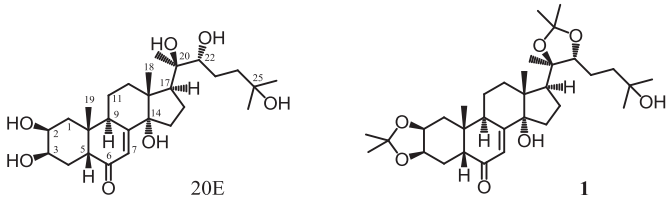


Fig. 1. Chemical structures of 20-hydroxyecdysone (20E) and 20-hydroxyecdysone 2,3;20,22-diacetonide (**1**).

Galyautdinov et al. have previously reported the successful preparation of several (*E*/*Z*)-isomeric ecdysteroid 6-oxime and some lactam derivatives [22]. Considering the above mentioned antitumor potential of steroidal oximes and the fact that no studies are available on the bioactivity of ecdysteroid oximes or lactams, the aim of the present work was to prepare a series of such compounds, and study their *in vitro* antitumor potential with a focus on their chemo-sensitizing activity.

2. Results and discussions

2.1. Chemistry

20-hydroxyecdysone 2,3; 20.22-diacetonide **1** and its 6-oxime and lactam derivatives were synthesized following previously published procedures [22,23]. Briefly, compound **1** was reacted with hydroxylamine or, aiming to prepare new oxime ethers, an alkoxylamine in pyridine at 70 °C. A total of 14 nitrogen-containing derivatives were prepared this way (Scheme 1).

Following each reaction, neutralization with KOH dissolved in anhydrous methanol was utilized with the aim of obtaining several different, structurally diverse and potentially bioactive products, including mixtures of 14,15-anhydro- and intact oxime derivatives: the oximes **2** and **3**, and oxime ethers with different 6-*O*-alkyl substituents **5**–**15**, respectively, were obtained through this method. Our results confirm previous observations that ecdysteroid 6-oximation can result in 3 different types of product mixtures depending on the neutralization procedure [22]: a mixture of 14,15-anhydro (*E*/*Z*)-isomeric oxime pairs form if the reaction does not include a neutralization step; a 2–4 components mixture of both intact and 14OH-eliminated derivatives is obtained if alkali dissolved in anhydrous methanol is added; and a mixture of intact (*E*/*Z*)-isomeric oxime pair with retained 14-OH groups is obtained if the neutralizing alkali is dissolved in anhydrous ethanol.

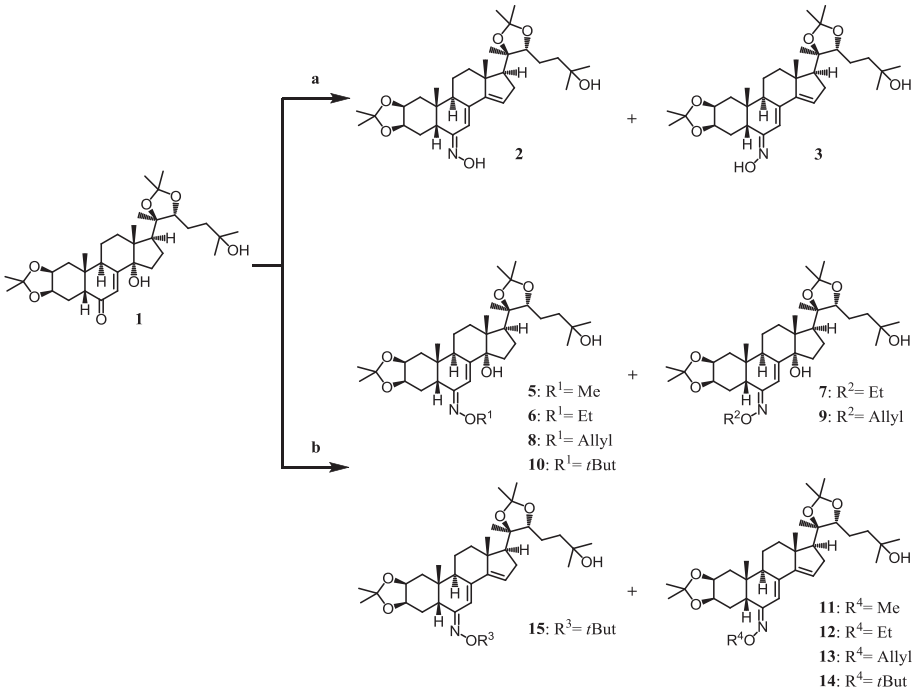
A second transformation involving the Beckmann-rearrangement of the (6*E*)-oxime compound **2** was performed utilizing *p*-toluenesulfonyl chloride (TsCl) in acetone in the presence of sodium carbonate to obtain a new ecdysteroid derivative, compound **4**, with a seven-membered lactam ring (Scheme 2). As expected, the (6*Z*)-oxime compound did not form the corresponding lactam but a tosylate was obtained (not presented, for more details see also reference [23]).

2.2. Structure elucidation

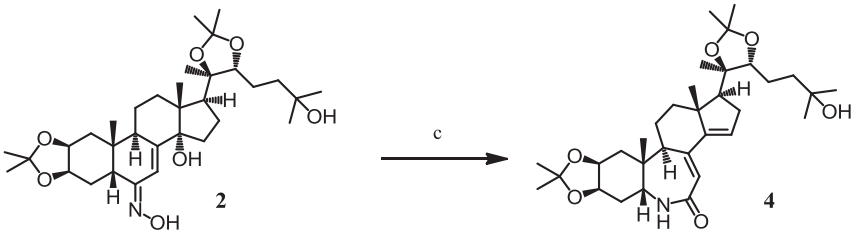
We have recently reported the structure elucidation and complete ^{1}H and ^{13}C signal assignment of a series of dioxolane derivatives of 20-hydroxyecdysone [19,20,21,24]. Here we discuss the complete ^{1}H and ^{13}C signal assignment of the corresponding 6-oxime and 6-oxime ether derivatives.

The structure and NMR signals of the products were assigned by comprehensive one- and two-dimensional NMR methods, such as ^{1}H, ^{13}C, DEPTQ, gradient-selected COSY, edited HSQC, HMBC, ROESY (**R**otating frame **O**verhauser **E**nchancement **S**pectroscopy) spectra and 1D-selective variants thereof. It is worth mentioning that due to the molecular mass (500–700 Da) the signal/noise value of the selective ROE experiments strongly exceeds that of the selective NOEs.

To facilitate the comparison of NMR signals of structurally analogous hydrogen and carbon atoms of the starting compound **1** with those of the 6-oxime **2**, and of its Beckmann rearranged product **4** and 6-oxime-ether derivatives **5**–**15**, we applied the usual steroid numbering, and for the central atoms of the 2,3;20,22-diacetonide moieties C-28 and C-29, respectively. The ^{13}C chemical shifts of compounds **1**, **2** and **4**–**15** in methanol-d_4 are compiled in



Scheme 1. Synthesis of oxime and oxime ether derivatives of 20-hydroxyecdysone 2,3;20,22-diacetonide.
Reagents and conditions: a) pyridine, $NH_2OH \cdot HCl$, 70 °C, 3 days; b) pyridine, $NH_2OR \cdot HCl$ (R = Me, Et, Allyl, or *t*But), 70 °C, 24 h; work-up with KOH in anhydrous MeOH.



Scheme 2. Beckmann rearrangement of ecdysteroid (6*E*)-oxime **2** into lactam **4**.
Reagents and conditions: c) acetone, *p*-toluenesulfonyl chloride (TsCl, 2 equiv of oxime **2**), Na_2CO_3 (1 equiv of oxime **2**), RT, 6 h.

Table 1. The characteristic ^{1}H data of compounds with a $\Delta^{14,15}$ C=CH ethylene moiety **2**, **4** and **11**–**15** are summarized in Table 2, whereas that of the HO-C(14) derivatives **5**–**10** are shown in Table 3.

It is well known that oximation of ketones is accompanied with characteristic changes of several ^{13}C and ^{1}H chemical shifts. Successful conversion of a C=O group to C=N-OH results of ca. 50 ppm diamagnetic shift of the corresponding carbon atom, whereas the chemical shift of α-CH carbon atom in the *syn* position with respect to the oxime hydroxyl group exhibits ~14 ppm, in the *anti* position ~9 ppm diamagnetic shift. The significant (Δδ *syn-anti*) parameters on C-5 and =C-7 signals successfully can be utilized for the assignment of (*Z*/*E*) isomers. Galyautdinov et al. reported some NMR data on 20-hydroxyecdysone oxime [22], including compound **3** (*Z* isomer), but they failed on isolating the isomeric compound **2** with *Z* configuration. In addition they have taken the NMR measurements in solvents with rather different anisotropic nature (e.g. pyridine-d_5, methanol-d_4) and so in some cases the solvation effect was comparable with the Δδ *syn-anti* parameters. To avoid this ambiguity, we have performed our NMR experiments exclusively in methanol-d_4.

On the basis of our data, all of the oxime derivatives in Table 1 with δC-5 ~ 38.6 and δC-7 ~ 117.5 ppm values, respectively, are *Z* isomers, while δC-5 ~ 43.8 and δC-7 ~ 111.0 ppm values assign the *E* isomers. It is worth noting that the less different δC-4 (~30/27 ppm) and δC-6 (~157/161 ppm) values also reflect on the *E* or *Z* isomers, respectively.

In case of compounds **2** and **4**, and the 6-oxime-ether derivatives **11**–**15** the DEPTQ and HSQC measurements revealed only seven methylene groups, one less than in the parent compound **1**, and simultaneously distinctive chemical shift changes appeared at δC-14: 85.4 → C= ~142 ppm and $\delta H_2\underline{C}$-15: 31.8 → H$\underline{C}$= ~124 ppm, respectively, indicating the emergence of an $\Delta^{14,15}$ C=CH ethylene moiety. All this means that in these compounds (**2**, **11**–**15**), simultaneously with the oximation, dehydration by the elimination of the 14-OH group also took place. The presence of the 14-OH substituent in compounds **5**–**10** appears straightforward, considering of the chemical shift of C-14 (δC-14–85 ppm) confirmed by the HMBC cross-peak H_3-18/C-14. Success of the Beckmann rearrangement of ecdysteroid (6*E*)-oxime **2** into lactam **4** could be expected from the E configuration of the parent oxime. Indeed, the significant (13.1 ppm) paramagnetic shift on δC-5 proves that in **4** the nitrogen atom coupled to C-5, the appearance of the signal at 170.6 ppm supports the formation of the lactam ring.

Thanks to the comprehensive one- and two-dimensional NMR techniques utilized in the structure elucidation process, a complete ^{1}H signal assignment could be achieved for all compounds. The

Table 1
^{13}C chemical shifts of compounds **2**, **4**–**15** as compared to that of their parental compound **1** (20-hydroxyecdysone 2,3; 20,22-diacetonide) [21]; in methanol-d_4.

No.	1	2	4[a]	5	6	7	8	9	10	11	12	13	14	15
1	39.0	39.5	43.2	39.7	39.7	39.4	39.7	39.4	39.8	39.1	39.1	39.1	39.3	39.5
2	73.7	73.4	73.2	73.6	73.6	73.6	73.6	73.6	73.7	73.4	73.5	73.5	73.6	73.6
3	73.3	74.0	75.5	74.0	74.0	73.9	74.0	73.8	74.2	73.7	73.8	73.8	74.0	74.1
4	27.9	30.3	30.9	30.0	30.0	27.0	29.9	27.0	30.0	27.3	27.2	27.2	27.3	30.4
5	52.7	43.5	56.6	43.8	43.8	38.6	43.8	38.7	44.0	38.4	38.5	38.6	38.2	43.7
6	205.8	157.0	170.6	157.2	156.9	160.3	157.4	160.7	155.7	160.8	160.6	161.0	159.4	155.8
7	122.0	110.0	119.9	110.7	110.9	117.5	110.8	117.3	111.3	117.0	117.2	117.0	118.3	110.8
8	167.1	151.5	151.6	154.1	153.8	150.7	154.1	151.0	152.3	151.0	151.1	151.1	151.3	151.6
9	35.9	40.2	45.9	35.5	35.5	34.4	35.5	34.4	35.7	39.1	39.2	39.2	39.2	40.2
10	38.9	38.0	40.7	37.8	37.7	37.0	37.7	37.0	37.6	37.1	37.1	37.1	37.0	37.9
11	21.8	21.9	25.4	21.5	21.5	21.5	21.5	21.5	21.5	21.8	21.8	21.9	21.9	21.9
12	32.5	41.3	42.4	32.6	32.6	32.5	32.6	32.5	32.6	41.1	41.1	41.1	41.2	41.3
13	48.7	49.0	50.2	49.0	48.6	48.3	48.6	48.3	48.6	48.6	48.6	48.7	48.6	48.7
14	85.4	144.3	154.4	85.9	85.9	85.7	85.9	85.7	86.0	142.4	142.1	142.4	140.6	143.8
15	31.8	125.3	125.6	32.0	32.0	32.1	32.0	32.1	32.0	124.4	124.3	124.4	123.6	125.0
16	22.6	32.4	32.6	22.6	22.6	22.7	22.6	22.6	22.6	32.3	32.3	32.4	32.3	32.4
17	50.6	59.0	59.3	50.6	50.6	50.7	50.6	50.7	50.6	58.9	58.9	59.0	59.0	59.1
18	17.8	19.7	19.6	18.0	18.0	18.0	18.0	18.0	18.1	19.6	19.6	19.6	19.6	19.7
19	24.2	23.9	18.1	24.3	24.3	24.3	24.3	24.3	24.3	24.1	24.0	24.1	24.1	24.9
20	86.0	84.9	84.7	86.0	86.0	86.1	86.0	86.1	86.0	84.9	84.9	85.0	85.0	84.9
21	22.8	22.0	21.9	22.7	22.7	22.7	22.7	22.7	22.7	22.0	22.0	22.0	22.0	22.0
22	83.5	83.1	83.1	83.4	83.4	83.4	83.4	83.4	83.4	83.2	83.2	83.2	83.2	83.2
23	24.9	24.8	24.8	24.8	24.8	24.8	24.8	24.8	24.8	24.8	24.8	24.9	24.9	24.9
24	42.4	42.1	42.1	42.3	42.3	42.4	42.3	42.4	42.3	42.1	42.1	42.1	42.1	42.7
25	71.3	71.2	71.2	71.2	71.2	71.2	71.2	71.2	71.2	71.1	71.2	71.2	71.2	71.2
26	29.1	29.0	29.1	29.1	29.1	29.1	29.1	29.1	29.1	29.1	29.0	29.0	29.0	29.0
27	29.0	29.7	29.6	29.6	29.6	29.6	29.6	29.5	29.6	29.7	29.7	29.7	29.7	29.7
28	109.6		109.5	109.4	109.3	109.3	109.3	109.3	109.3	109.3	109.3	109.3	109.2	109.4
28Meα	26.8		26.6	26.8	26.8	26.8	26.8	26.8	26.9	26.8	26.7	26.7	26.7	26.8
28Meβ	29.0		28.9	29.0	29.0	29.0	29.0	29.0	29.0	29.1	29.0	29.0	29.0	29.0
29	108.2		108.0	108.1	108.0	108.1	108.0	108.1	108.0	108.0	108.0	108.1	108.0	108.1
29Meα	29.5		29.3	29.5	29.5	29.5	29.5	29.4	29.5	29.4	29.4	29.4	29.4	29.4
29Meβ	27.3		27.3	27.3	27.3	27.3	27.4	27.3	27.3	27.3	27.3	27.2	28.0	27.3
1′				61.8	70.1	70.4	75.5	75.7	78.9	62.2	70.5	75.8	79.3	
2′					15.0	15.2	135.9	136.0	28.0		15.2	135.9	28.0	
3′							117.6	117.5				117.6		

[a] To facilitate the comparison of NMR data of the Beckman product **4** and the parental oxime ethers we applied the steroid atomic numbering also for compound **4**.

characteristic ^{1}H NMR data of the 14,15-anhydro derivatives **2**, **4** and **11**–**15** are summarized in Table 2, whereas that of the other compounds **5**–**10** in Table 3. The main difference between the two sets of data is that in Table 2, besides H-7, a second olefinic signal appears for H-15 (~δ5.80 dd) instead of the H_2-16 hydrogen signals.

The retained *cis* junction of the A/B rings in each compound was obvious by considering the strong H_3-19/Hβ-5 ROESY response, whereas the assignment of the α/β position of the diastereotopic methylene hydrogens of the skeleton were revealed by the one-dimensional selective ROESY measurements irradiating e.g. the H_3-18, H_3-19 and H-5 atoms in combination with the observed proton-proton coupling pattern.

Considering the data of Tables 2 and 3 it is clear that the values of δH-5 and δH-7 chemical shifts allow the easy and unequivocal differentiation between the *E* and *Z* isomers. In case of the 14,15-anhydro derivatives **2** and **11**–**15**, the H-5 signals resonate around 2.25 ppm in the *E* and at 3.15 ppm in the *Z* isomers, and the δH-7 chemical shifts appear at 6.76 ppm in the *E* and at 6.16 ppm in *Z* isomers. Similar trend was observed for the compounds in Table 3, the chemical shift of H-5 in the *anti* position with respect to the oxime hydroxyl group exhibits ~2.23 ppm, while in the *Z* isomer it is ~3.18 ppm. The corresponding values for H-7 are 6.45 and 5.88 ppm, respectively.

To facilitate the comparison between the NMR data of *Z* and *E* isomeric pairs, the stereo-structures with atomic numbering (in red) of compounds **7** (upper) and **6** (lower) are shown in Fig. 2. Blue numbers refer to ^{1}H chemical shifts; black numbers give the δ ^{13}C values.

2.3. Biology

Antiproliferative activity of compounds **4**–**15** was tested on a panel of gynecological cancer cell lines, including cervical (HeLa, SiHa) and breast cancer cell lines (MDA-MB-231, MCF7); the results are presented in Table 4.

Although most of the ecdysteroid analogs displayed moderate activities against the tested cell lines, the *t*-butyl substituted compound **10** was stronger than the positive control cisplatin on the HeLa and MDA-MB-231 cell lines. In our previous study, the antiproliferative IC_{50} values of compound **1** were 106.1 and 75.1 μM on the MDA-MB-231 and MCF7 cell lines, respectively [21], showing that the inclusion of certain oxime ether functions can increase this activity by nearly an order of magnitude. While the orientation of the oxime ether had no obvious effect on the activity, a larger alkyl group led to a stronger antiproliferative action. It appears to be clear that the retained 14-OH function is favorable over the $\Delta^{14,15}$ moiety in this regard on the MCF-7 cell line (compounds **7** vs. **12**, **9** vs. **13**, and **10** vs. **15**), while such a conclusion cannot be drawn on the other cell lines.

Compounds **2**–**15** were also tested for their cytotoxic activity on a murine lymphoma cell line pair, including L5178 and its multidrug resistant counterpart transfected to express the human ABCB1 transporter, $L5178_{MDR}$. Following this, the compounds were tested for their potential to inhibit the ABCB1 efflux transporter through measuring the intracellular accumulation of rhodamine 123 by flow cytometry. Degree of inhibition (%) values were calculated by means of the rhodamine 123 accumulation of the ABCB1 transfected $L5178_{MDR}$ cells (i.e. 0% inhibition) and that of the

Table 2
^{1}H chemical shift, multiplicities and coupling constants of compounds **2**, **4**, **11**–**15** in methanol-d_4.

No.		2	*J* (Hz)	4[a]	*J* (Hz)	11	*J* (Hz)[b]	12	13	14	15
1	α	1.98	dd; 14.0, 6.5	2.19	dd; 14.0, 6.8	1.95	dd; 13.9, 6.3	1.94	1.95	1.92	1.98
	β	1.25		1.30		1.26		1.28	1.28	1.29	1.25
2		4.19	ddd; 11.0, 6.5, 4.5	4.25	ddd; 12.0, 6.8, 5.0	4.18	ddd; 10.8, 6.3, 4.5	4.19	4.19	4.19	4.19
3		4.26	td; 4.5, 1.7	4.39	dt; 5.0, 3.0	4.24	td; 4.5, 1.2	4.25	4.25	4.24	4.27
4	α	1.77		1.29		1.60		1.60	1.61	1.57	1.77
	β	1.97		2.06		2.10		2.11	2.14	2.11	1.95
5		2.25	dd; 12.1, 4.2	3.30	dd; 10.2, 6.5	3.14	dd; 12.8, 4.6	3.15	3.19	3.15	2.26
7		6.81	d; 2.7	5.94	d; 2.6	6.14	d; 2.6	6.16	6.16	6.20	6.70
9		2.27		2.37	ddd; 11.5, 3.6, 2.6	2.31		2.31	2.31	2.29	2.24
11	α	1.65		1.88		1.63		1.63	1.62	1.61	1.64
	β	1.72		1.74		1.68		1.68	1.67	1.67	1.71
12	α	1.53		1.60		1.50		1.50	1.50	1.50	1.52
	β	2.23		2.21		2.22	dt; 12.7, 3.0	2.22	2.22	2.22	2.22
15		5.86	dd; 3.5, 2.0	5.74	dd; 3.5, 1.9	5.81	dd; 3.3, 2.1	5.81	5.81	5.79	5.82
16	α	2.33		2.33		2.32		2.32	2.31	2.31	2.32
	β	2.60		2.58		2.58		2.58	2.58	2.58	2.59
17		2.04	dd; 10.7, 7.7	2.11	dd; 10.7, 7.8	2.02	dd; 10.8, 7.7	2.02	2.02	2.01	2.03
18		1.06		1.06		1.05		1.05	1.05	1.05	1.05
19		0.83		0.96		0.84		0.84	0.85	0.84	0.81
21		1.22		1.21		1.22		1.22	1.22	1.22	1.22
22		3.76		3.75		3.76		3.76	3.76	3.77	3.76
23	a	1.53		1.53		1.53		1.53	1.53	1.53	1.54
	b	1.53		1.53		1.53		1.53	1.53	1.53	1.54
24	a	1.48		1.48		1.48		1.48	1.48	1.48	1.48
	b	1.72		1.72		1.72		1.72	1.72	1.72	1.72
26		1.20		1.20		1.19		1.19	1.19	1.19	1.19
27		1.21		1.21		1.21		1.20	1.20	1.21	1.21
28Meα		1.30		1.30		1.31		1.31	1.31	1.32	1.32
28Meβ		1.47		1.46		1.49		1.49	1.49	1.50	1.49
29Meα		1.40		1.40		1.40		1.40	1.40	1.40	1.40
29Meβ		1.30		1.30		1.30		1.30	1.30	1.30	1.31
1′						3.86		4.11	4.56	–	–
2′								1.27	6.00	1.29	1.29
3′	*Z*								5.19		
	E								5.29		

[a] To facilitate the comparison of NMR data of the Beckman product **4** and the parental oximethers, we applied the steroid atomic numbering also for **4**.
[b] Because the stereostucture of the steroid frame is nearly identical within compounds **11**–**15,** we described the *J* coupling contents only for **11**.

L5178 cells (i.e. 100% inhibition); results are presented in Table 5.

While the compounds also exerted weak to moderate cytotoxic activities on the mouse lymphoma cell line pair, all of them were more potent than their parental compound **1**. No cross resistance was observed to any of them on the ABCB1 over-expressing MDR cells. The oximes **2** and **3** showed the strongest activity on either cell lines with IC_{50} values ca. 4–5 times below that of compound **1**, and the *E*-oxime (**2**) was more cytotoxic than the *Z*-oxime (**3**). The oxime ethers typically exerted weaker cytotoxic activities than the non-substituted oximes, with the exception of compound **10** where a bulky *t*-butyl substituent and a retained 14-OH group were present. When comparing corresponding analogs with a retained 14-OH group or a $\Delta^{14,15}$ moiety, there appeared to be a clear tendency for the former structural element to be associated with a stronger cytotoxic activity on the mouse lymphoma cells, similarly to the case of MCF-7 cells (see above).

Evaluation of the results obtained from the rhodamine accumulation assay reveals that the lactam derivative (**4**) is the only one among the compounds that was completely inactive in this regard at as much as 20 μM concentration. For the other compounds, several structure-activity relationships could be observed. The oxime formation markedly increased the ABCB1 inhibitory activity, and this was particularly true for oxime ethers. The orientation of the oxime group had little if any influence on the ABCB1 inhibition (compound **2** vs. **3**, **6** vs. **7**, **8** vs. **9**, and **14** vs. **15**), while the 14-OH elimination, forming a $\Delta^{14,15}$ double bond in the ecdysteroid D-ring, clearly increased this activity (compound **7** vs. **12**, **9** vs. **13**, and **10** vs. **15**). When comparing the activity of oximes and oxime ethers between analogs containing the same type of D-ring and orientation of oxime but different substituents on the latter, the following order of bioactivity could be concluded: H < Me < Et < Allyl ≤ *t*-But.

The compounds were also tested for their ability to sensitize the susceptible/resistant mouse lymphoma cell line pair towards the cytotoxic activity of doxorubicin. Since each compound showed a measurable cytotoxic activity on both cell lines when applied alone, combination indices could be determined through the checkerboard microplate method similarly to our previous related studies [17,19]. Table 6 shows the strongest activity observed for each compound on the L5178 and $L5178_{MDR}$ cell lines; further details and results at other compound:doxorubicin ratios are available in supporting information Table S1.

All tested derivatives showed strong synergism ($0.1 < CI_{avg} < 0.3$) [25] with doxorubicin on the P-gp expressing $L5178_{MDR}$ cells, similarly to their parental compound (**1**). As it was previously reported by us, chemo-sensitizing activity of ecdysteroids has little if any correlation to their (most typically weak) inhibitory effect on the efflux function of P-gp [20]. This was clearly confirmed in the present study as well: even though for example compounds **11**–**15** are much stronger P-gp inhibitors than their parental compound **1**, no difference can be observed in the strength of synergism with the P-gp substrate doxorubicin on the MDR cell line. Most interestingly, among all derivatives obtained, the ecdysteroid lactam **4** was found to express the strongest chemosensitization on the MDR cells, while being the only one to show no interference with P-gp function. Accordingly, this compound has a further advantage over the diacetonide of 20E, namely that it would likely be free from the potential adverse effects and unwanted drug-drug interactions

Table 3
^{1}H chemical shifts, multiplicities and coupling constants of compounds **5**–**10** in methanol-d_4.

No.		5	*J* (Hz)[a]	6	7	8	9	10
1	α	1.98		1.98	1.94	1.98	1.95	1.98
	β	1.22		1.23	1.24	1.23	1.24	1.23
2		4.21	ddd; 10.5, 6.7, 5.1	4.21	4.21	4.22	4.21	4.22
3		4.28		4.28	4.26	4.28	4.27	4.28
4	α	1.93		1.93	1.73	1.93	1.74	1.92
	β	1.93		1.93	2.06	1.93	2.08	1.92
5		2.22	dd; 12.2, 5.5	2.23	3.16	2.24	3.19	2.26
7		6.44	d; 2.7	6.47	5.88	6.49	5.88	6.47
9		2.72	ddd; 11.8, 6.9, 2.7	2.71	2.72	2.72	2.73	2.70
11	α	1.65		1.65	1.65	1.64	1.63	1.64
	β	1.59		1.58	1.58	1.59	1.58	1.59
12	α	2.03	td; 12.0, 5.5	2.04	2.04	2.04	2.04	2.03
	β	1.80	dm; 12.0	1.81	1.80	1.81	1.80	1.80
15	α	1.61		1.62	1.63	1.62	1.63	1.62
	β	1.96		1.97	1.94	1.97	1.94	1.96
16	α	1.85		1.85	1.85	1.86	1.85	1.85
	β	2.00		2.00	2.02	2.01	2.02	2.02
17		2.28	dd; 9.1, 7.8	2.28	2.27	2.29	2.27	2.28
18		0.80		0.81	0.81	0.81	0.81	0.81
19		0.83		0.83	0.84	0.83	0.85	0.82
21		1.17		1.17	1.17	1.17	1.17	1.17
22		3.68		3.68	3.68	3.68	3.68	3.68
23	a	1.52		1.52	1.52	1.52	1.52	1.52
	b	1.52		1.52	1.52	1.52	1.52	1.52
24	a	1.48		1.48	1.49	1.48	1.49	1.49
	b	1.73		1.73	1.73	1.73	1.73	1.74
26		1.19		1.19	1.19	1.19	1.19	1.19
27		1.20		1.20	1.20	1.20	1.20	1.20
28Meα		1.31		1.31	1.32	1.31	1.32	1.32
28Meβ		1.47		1.47	1.50	1.47	1.49	1.49
29Meα		1.39		1.39	1.39	1.39	1.39	1.39
29Meβ		1.32		1.32	1.32	1.32	1.32	1.32
1′		3.82		4.07	4.10	4.53	4.55	–
2′				1.25	1.26	5.98	5.99	1.28
3′	*Z*					5.18	5.19	
	E					5.26	5.28	

[a] Because the stereo-structure of the steroid frame is nearly identical within this set of compounds, the *J* coupling constants are given only once.

connected to P-gp inhibitors [26,27].

Considering structure-activity relationships, the several highly active compounds obtained in this work led us to follow our previously applied "best ratio" principle [17]. This means that we aimed to compare the compounds' chemo-sensitizing activities at their strongest, regardless of the compound vs. doxorubicin ratio where this activity was observed.

The length or nature of the alkyl function had no apparent effect on the compounds potency in sensitizing the MDR cells to doxorubicin, all compounds showed similarly high activity in this regard. A slight tendency may be observed for the $\Delta^{14,15}$ compounds (**2**–**4**, **11**–**15**) acting stronger in this regard than their corresponding analogs where the 14-OH group was retained (**5**–**10**), but the differences are so small that it is hard to make a sound judgment on the relevance of this phenomenon.

On the other hand, larger differences were observed between the compounds' activities on the non-MDR L5178 cells. On this cell line, the strongest synergism with doxorubicin was observed for the lactam (**4**) and compound **11**, a methyl substituted $\Delta^{14,15}$ *(Z)*-oxime ether. The oxime formation together with the elimination of the 14-OH group (**2** and **3**) decreased the strength of synergism with doxorubicin as compared to the case of compound **1**. In case of the oxime ethers, the 14,15-anhydro derivatives typically exerted stronger sensitizing activity to doxorubicin than their analogs with intact 14-OH groups, except for compounds **10** vs. **15**. Since oxime ethers substituted with bulky *t*-buthyl groups seem to show a tendency for decreased activity as compared to the corresponding analogs with ethyl groups (**6** vs **10** and **12** vs. **14**), one could hypothesize that the effect of the *t*-butyl group in the oxime ether function may overwrite that of the $\Delta^{14,15}$ moiety in compound **15**.

3. Conclusions

The present study reports the preparation and *in vitro* pharmacological investigation of 14 ecdysteroid diacetonide oximes, oxime ethers and a lactam, with 13 novel derivatives obtained in pure form for the first time. The synthetic procedure was utilized in a way to obtain product mixtures in order to increase chemical diversity, and subsequent use of high-performance separation techniques allowed us to obtain the compounds in high purity. All compounds are reported with a complete NMR signal assignment.

Evaluation of the antiproliferative and cytotoxic activity of the compounds on several cancer cell lines revealed several structure-activity relationships (SAR). A new, *t*-butyl substituted ecdysteroid oxime ether (**10**) was found to exert stronger antiproliferative effect on HeLa and MDA-MB-231 cells than cisplatin. The $\Delta^{14,15}$ *E*-oxime derivative (**2**) exerted a substantially increased cytotoxic and P-gp inhibitory activities in the L5178/L5178$_{MDR}$ cell line pair, as compared to its parental compound.

Clear SAR was observed for the compounds' activity as functional P-gp inhibitors, and many of them were identified as highly potent MDR-selective chemo-sensitizers. In particularly, a novel $\Delta^{14,15}$ δ-lactam ecdysteroid derivative (**4**) was revealed as a most promising new lead compound with low intrinsic cytotoxicity, and strong ability to sensitize MDR and also non-MDR cancer cells towards doxorubicin without interfering with the efflux function of P-gp. Accordingly, it can be expected that a combined treatment of cancer with this compound as a chemo-sensitizer and a chemotherapeutic agent would 1) be effective on the initial, susceptible state of the tumor, and 2) have a strong chance to prevent the acquisition of P-gp mediated resistance through an increased killing effect on the cell population becoming adapted to the chemotherapy.

4. Experimental section

4.1. Chemistry

All applied reagents were purchased from Sigma (Sigma-Aldrich Co., USA). Solvents were obtained from Macron Fine Chemicals (Avantor Performance Materials, USA).

^{1}H (500.1 MHz) and ^{13}C (125.6 MHz) NMR spectra were recorded at room temperature on a Bruker Avance-II spectrometer and on Avance-III spectrometer equipped with a cryo probehead. Regarding the compounds, amounts of approximately 1–10 mg were dissolved in 0.1 ml of methanol-d_4 and transferred to 2.5 mm Bruker MATCH NMR sample tube. Chemical shifts are given on the δ-scale and are referenced to the solvent (MeOH-d_4: $\delta_C = 49.1$ and $\delta_H = 3.31$ ppm). Pulse programs of all experiments (^{1}H, ^{13}C, DEPTQ, DEPT-135, one-dimensional sel-ROE (mixing time: 300 ms), edited gs-HSQC and gs-HMBC) were taken from the Bruker software library. The NMR signals of the product were assigned by comprehensive one- and two-dimensional NMR methods using widely accepted strategies [28,29,30]. Most ^{1}H assignments were accomplished using general knowledge of chemical shift dispersion with the aid of the proton-proton coupling pattern (^{1}H NMR spectra). Mass spectra were obtained on a Waters Acquity iClass UPLC coupled with Thermo Q Exactive Plus with HESI source (Waters Co., USA).

Reaction progress was monitored by thin layer chromatography (TLC) on Kieselgel 60F$_{254}$ silica plates obtained from Merck (Merck, Germany), and examined under UV illumination at 254 nm.

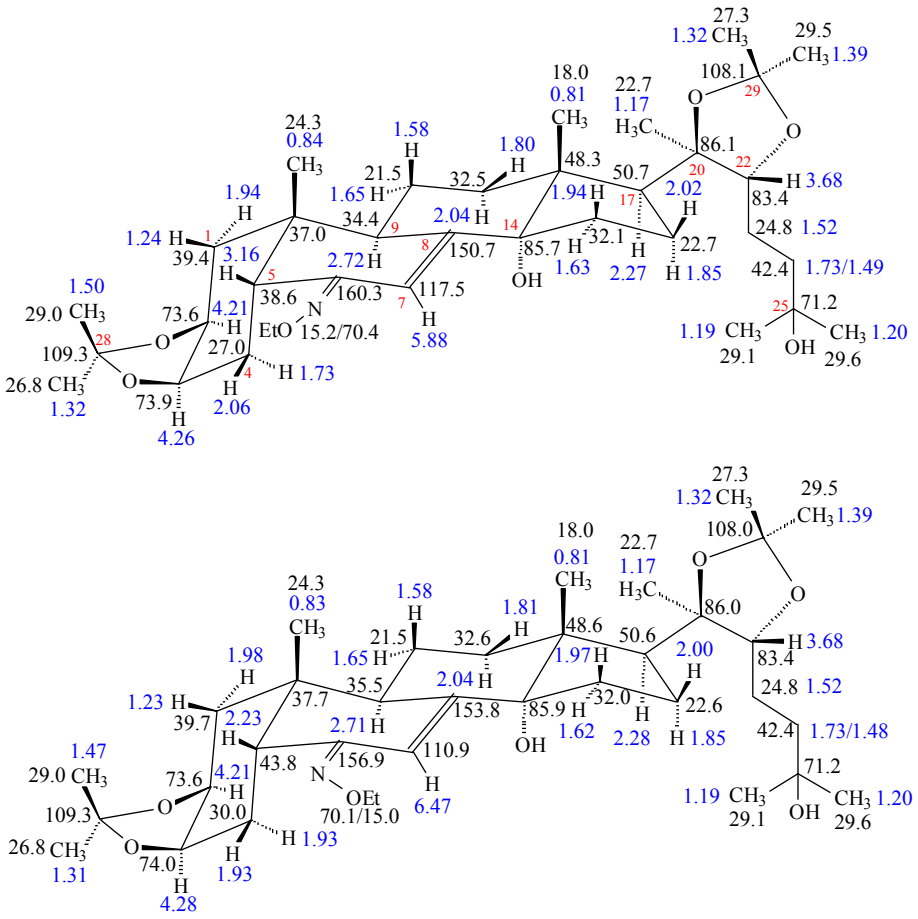


Fig. 2. Characteristic NMR spectra on differentiation and NMR assignments of the isomeric **6** and **7** ecdysteroid 6-oxime ethers are given in the supporting information.

Table 4
Antiproliferative properties of compounds **4**–**15** against four human gynecological cancer cell lines. Inhibition concentration at 50% growth (IC_{50}) values of each compound and the 95% confidence intervals are given for each cell line.

Compound	IC_{50} (μM)			
	HeLa	SiHa	MDA-MB-231	MCF7
4	>30	>30	>30	>30
5	>30	>30	>30	>30
6	>30	>30	>30	22.55 [17.24–29.50]
7	29.12 [24.00–32.94]	>30	25.12 [17.74–35.57]	13.10 [10.89–15.77]
8	15.55 [13.69–17.66]	25.52 [21.95–29.68]	21.36 [18.86–24.19]	13.63 [11.91–15.60]
9	17.55 [14.77–20.84]	>30	26.90 [23.34–31.00]	17.22 [15.21–19.50]
10	8.43 [4.66–9.29]	16.13 [13.02–19.99]	12.36 [11.00–13.89]	11.06 [9.96–12.29]
11	15.43 [12.87–18.50]	>30	25.99 [21.67–29.50]	18.03 [15.86–20.50]
12	29.96 [27.03–33.20]	>30	26.00 [23.44–28.85]	19.59 [17.09–22.46]
13	>30	>30	29.37 [26.11–33.03]	24.16 [20.36–28.68]
14	20.71 [18.63–23.02]	8.14 [5.62–11.79]	15.70 [13.50–18.25]	17.29 [15.33–19.52]
15	26.06 [22.45–30.25]	14.17 [10.60–18.94]	16.93 [14.71–19.49]	19.34 [16.51–22.66]
Cisplatin	14.02 [12.65–15.56]	7.87 [5.83–10.63]	18.65 [16.67–20.85]	6.01 [5.33–6.79]

Compounds were purified by flash chromatography with adequately chosen eluents of *n*-hexane – dichloromethane – methanol on 12 g RediSep NP-silica flash columns (TELEDYNE Isco, USA).

For the RP-HPLC separation of isomeric oxime derivatives a Kinetex XB-C18 250 × 21.4 mm 5 μm preparative (Phenomenex Inc., USA) or an Agilent Eclipse XDB-C8 250 × 9.4 mm 5 μm semi-preparative column (Agilent Technologies Inc., USA) was applied with the use of isocratic grade eluents of acetonitrile and water. Purity of obtained compounds was determined by RP-HPLC with the use of a Kinetex XB-C18 250 × 4.6 mm 5 μm analytical column (Phenomenex Inc., USA). For data collection a Jasco HPLC instrument equipped with an MD-2010 Plus PDA detector (Jasco Analytical Instruments, Japan) was applied in a detection range of 210–400 nm.

Ecdysteroid substrate **1** was synthesized from 20-hydroxyecdysone (20E) obtained from Shaanxi KingsSci Biotechnology Co., Ltd. (Shanghai, People's Republic of China) at 90% purity and recrystallized (EtOAc:MeOH – 2:1) to a RP-HPLC purity of 97.8%. During the synthetic procedure, 20E (10 g) was dissolved in

Table 5
Cytotoxicity of compounds **1**–**15** on L5178 and L5178$_{MDR}$ cells, and functional inhibition of the ABCB1 transporter. Dox = doxorubicin; for the ABCB1 inhibition, positive control: 100 nM of tariquidar (112.4% inhibition), negative control: 2% DMSO (−0.07% inhibition).

Compound	Change in the	14-OH or	IC_{50} (μM) [95% confidence intervals][b]		ABCB1 inhibition (%)	
	B-ring of 1 [a]	$\Delta^{14,15}$	L5178	L5178$_{MDR}$	2 μM	20 μM
1	–	14-OH	110.3 [77.50–157.1]	97.69 [71.07–134.3]	2.54	20.91
2	(*E*)-oxime	$\Delta^{14,15}$	20.91 [17.68–24.74]	24.63 [19.82–30.63]	10.57	82.95
3	(*Z*)-oxime	$\Delta^{14,15}$	34.22 [28.21–41.51]	28.35 [21.97–36.58]	7.15	81.09
4	δ-lactam	$\Delta^{14,15}$	63.42 [47.51–84.65]	72.35 [64.39–81.29]	1.16	4.27
5	(*E*); R = Me	14-OH	40.92 [35.66–46.97]	55.05 [41.53–72.98]	2.25	25.05
6	(*E*); R = Et	14-OH	35.02 [25.35–48.38]	47.00 [31.14–70.93]	17.54	78.79
7	(*Z*); R = Et	14-OH	37.26 [25.65–54.11]	42.16 [41.24–43.10]	18.96	75.03
8	(*E*); R = Allyl	14-OH	31.48 [23.71–41.80]	51.91 [42.69–63.13]	20.98	89.39
9	(*Z*); R = Allyl	14-OH	36.66 [28.32–47.44]	49.29 [43.07–56.40]	24.17	81.80
10	(*E*); R = *t*-But	14-OH	28.06 [21.30–36.98]	29.12 [25.12–33.76]	38.75	112.4
11	(*Z*); R = Me	$\Delta^{14,15}$	45.95 [36.97–57.11]	53.14 [43.54–64.86]	33.36	106.2
12	(*Z*); R = Et	$\Delta^{14,15}$	53.20 [38.64–73.26]	58.94 [45.86–75.74]	56.41	107.7
13	(*Z*); R = Allyl	$\Delta^{14,15}$	55.28 [46.21–66.13]	52.72 [39.97–65.53]	61.13	102.7
14	(*Z*); R = *t*-But	$\Delta^{14,15}$	63.23 [58.57–68.26]	51.22 [39.13–67.04]	58.99	78.76
15	(*E*); R = *t*-But	$\Delta^{14,15}$	63.84 [45.70–89.19]	65.44 [55.66–76.94]	67.46	93.95
Dox	–	–	0.080 [0.053–0.12]	4.49 [3.43–5.89]	–	–

[a] R groups refer to the alkyl substituents of the oxime ethers as in Scheme 1.
[b] IC_{50} values were calculated by the CompuSyn software as the median cytotoxic activities (Dm) from the control lanes on the checkerboard plates of the combination studies, n = 2.

Table 6
Chemo-sensitizing activity of compounds **1**–**15** on the L5178 and L5178$_{MDR}$ cell lines towards doxorubicin at 50, 75 and 90% of growth inhibition (ED_{50}, ED_{75} and ED_{90}, respectively). CI: combination index; CI_{avg}: weighted average CI value; $CI_{avg} = (CI_{50} + 2CI_{75} + 3CI_{90})/6$. CI < 1, CI = 1, and CI > 1 represent synergism, additivity, and antagonism, respectively. Dm, m, and r represent antilog of the x-intercept, slope, and linear correlation coefficient of the median-effect plot, respectively.

Compound	Cell line	Drug ratio	CI at			Dm	m	r	CI_{avg}
			ED_{50}	ED_{75}	ED_{90}				
1 [21]	L5178$_{MDR}$	20.4: 1	0.27	0.14	0.07	11.678	3.246	0.964	0.13
	L5178	163: 1	0.67	0.55	0.46	11.236	2.103	0.942	0.53
2	L5178$_{MDR}$	15: 1	0.26	0.16	0.12	4.454	6.638	1.000	0.16
	L5178	150: 1	0.80	0.79	0.78	10.748	2.572	0.997	0.78
3	L5178$_{MDR}$	30: 1	0.32	0.25	0.20	7.595	3.981	0.994	0.24
	L5178	150: 1	0.98	0.76	0.61	16.049	3.239	0.986	0.72
4	L5178$_{MDR}$	15: 1	0.20	0.12	0.09	6.419	4.953	0.970	0.12
	L5178	150: 1	0.40	0.42	0.46	10.477	2.033	0.966	0.44
5	L5178$_{MDR}$	15: 1	0.17	0.16	0.16	6.605	3.721	0.978	0.16
	L5178	150: 1	1.06	0.79	0.62	14.306	2.947	0.971	0.75
6	L5178$_{MDR}$	7.5: 1	0.18	0.14	0.12	5.001	5.858	1.000	0.14
	L5178	37.5: 1	0.55	0.58	0.60	8.598	2.495	0.972	0.59
7	L5178$_{MDR}$	3.75: 1	0.27	0.16	0.13	3.030	3.329	0.993	0.16
	L5178	37.5: 1	0.63	0.52	0.45	8.078	3.858	0.952	0.50
8	L5178$_{MDR}$	15: 1	0.17	0.13	0.13	4.939	3.193	0.955	0.14
	L5178	150:1	1.03	0.81	0.69	8.970	2.178	0.991	0.79
9	L5178$_{MDR}$	15: 1	0.17	0.16	0.17	7.338	3.771	0.947	0.17
	L5178	75: 1	0.70	0.83	1.03	8.202	1.722	0.956	0.91
10	L5178$_{MDR}$	7.5: 1	0.30	0.20	0.17	3.928	4.610	1.000	0.20
	L5178	37.5: 1	0.58	0.63	0.70	7.606	2.502	0.966	0.66
11	L5178$_{MDR}$	7.5: 1	0.17	0.16	0.15	5.224	3.722	0.971	0.16
	L5178	37.5: 1	0.77	0.47	0.31	8.165	3.044	0.982	0.44
12	L5178$_{MDR}$	7.5: 1	0.21	0.14	0.11	6.133	4.890	0.992	0.14
	L5178	75: 1	0.49	0.50	0.52	7.864	2.094	0.961	0.51
13	L5178$_{MDR}$	3.75: 1	0.25	0.15	0.11	5.614	5.805	1.000	0.15
	L5178	37.5: 1	0.46	0.47	0.47	8.295	2.882	0.981	0.47
14	L5178$_{MDR}$	7.5: 1	0.34	0.26	0.23	8.365	3.378	0.939	0.26
	L5178	37.5: 1	0.53	0.59	0.66	9.652	2.400	0.961	0.62
15	L5178$_{MDR}$	7.5: 1	0.27	0.24	0.23	8.739	3.813	0.960	0.24
	L5178	37.5: 1	1.16	0.85	0.64	7.199	3.273	0.977	0.80

acetone in the concentration of g/100 cm^3 and phosphomolybdic acid was added (10 g) under stirring. After 5 min of stirring at RT, the reaction mixture was neutralized with 10% aqueous $NaHCO_3$. Acetone was evaporated under reduced pressure and the mixture was extracted with EtOAc (3 × 50 ml) followed by drying with Na_2SO_4. After filtration, the solvent was evaporated under reduced pressure and the crude mixture was purified by flash chromatography with isocratic grade eluents of dichloromethane:methanol – 99:1. (Yield: 51%).

Synthesis of ecdysteroid 6-oximes (2–3). 1 g of compound **1** (1,78 mmol) was dissolved in pyridine (10 ml) and 1 g of hydroxylamine hydrochloride (14.39 mmol) was added to the solution under stirring. After 3 days of stirring at 70 °C the reaction was complete and the solvent was evaporated under reduced pressure. Following water addition (50 ml), the mixture was extracted with EtOAc (3 × 50 ml) and the combined organic phase was dried with

Na_2SO_4. A filtration was made to remove drying agent and the solvent was evaporated under reduced pressure. Purification of the crude mixture was carried out by preparative RP-HPLC to obtain (*E*/*Z*)-isomeric oximes **2**–**3**, respectively.

Synthesis of ecdysteroid lactam derivative (4). 0.138 g of oxime **2** (0,25 mmol) was dissolved in anhydrous acetone (10 ml), then 0.027 g of Na_2CO_3 (0.25 mmol) and 0.096 g of *p*-toluenesulfonyl chloride (0,5 mmol) was added to the solution under stirring. After 6 h of stirring at RT, the reaction was stopped and the mixture was cooled to 0 °C. Under stirring, water (10 ml) was added and the mixture was extracted into ethyl acetate (3 × 50 ml). After evaporation under reduced pressure, the mixture was purified with semi-preparative RP-HPLC to obtain lactam derivative **4**.

General Procedure for the synthesis of ecdysteroid 6-oxime ethers (5–15). 200 mg of **1** (0,35 mmol) was dissolved in pyridine (8 ml), and, depending on the oxime ether to be obtained, 200 mg of the appropriate alkoxyamine-hydrochloride was added to the solution under stirring. After stirring at 70 °C for 24 h, the mixture was cooled down to 0 °C, neutralized with KOH dissolved in anhydrous methanol, and evaporated under reduced pressure. Water (50 ml) was then added, and the mixture was extracted with EtOAc (3 × 50 ml). The combined organic layers were dried with Na_2SO_4, and, after filtration, the solvent was evaporated under reduced pressure. Purification of the crude material was carried out by flash chromatography on silica gel to obtain compounds **5**–**15**, respectively. In cases of oxime pairs **2**–**3, 6**–**7, 8**–**9, 14**–**15** preparative RP-HPLC was applied to separate the isomeric oxime and oxime ether derivatives.

Compound 4: White solid; yield: 8% (11.04 mg); RP-HPLC purity: 98.1%; for ^1H and ^{13}C NMR data, see Tables 2 and 3, respectively; HR-HESI-MS: $C_{33}H_{52}O_6N$, calcd. 558.3789, found: 558.3737.

Compound 5: White solid; yield: 28.3% (59.53 mg); RP-HPLC purity: 99.8%; for ^1H and ^{13}C NMR data, see Tables 1 and 3, respectively; HR-HESI-MS: $C_{34}H_{56}O_7N$, calcd. 590.4051, found: 590.4045.

Compound 6: White solid; yield: 15.2% (32.75 mg); RP-HPLC purity: 99.6%; for ^1H and ^{13}C NMR data, see Tables 1 and 3, respectively; HR-HESI-MS: $C_{35}H_{58}O_7N$, calcd. 604.4208, found: 604.4198.

Compound 7: White solid; yield: 2.8% (6.06 mg); RP-HPLC purity: 98.7%; for ^1H and ^{13}C NMR data, see Tables 1 and 3, respectively; HR-HESI-MS: $C_{35}H_{58}O_7N$, calcd. 604.4208, found: 604.4199.

Compound 8: White solid; yield: 15.5% (34.05 mg); RP-HPLC purity: 98.3%; for ^1H and ^{13}C NMR data, see Tables 1 and 3, respectively; HR-HESI-MS: $C_{36}H_{58}O_7N$, calcd. 616.4208, found: 616.4201.

Compound 9: White solid; yield: 1.6% (3.5 mg); RP-HPLC purity: 99.6%; for ^1H and ^{13}C NMR data, see Tables 1 and 3, respectively; HR-HESI-MS: $C_{36}H_{58}O_7N$, calcd. 616.4208, found: 616.4200.

Compound 10: White solid; yield: 38.9% (87.67 mg); RP-HPLC purity: 98.5%; for ^1H and ^{13}C NMR data, see Tables 1 and 3, respectively; HR-HESI-MS: $C_{37}H_{62}O_7N$, calcd. 632.4521, found: 632.4515.

Compound 11: White solid; yield: 43.3% (88.32 mg); RP-HPLC purity: 97.7%; for ^1H and ^{13}C NMR data, see Tables 2 and 3, respectively; HR-HESI-MS: $C_{34}H_{54}O_6N$, calcd. 572.3946, found: 572.3937.

Compound 12: White solid; yield: 33.3% (69.59 mg); RP-HPLC purity: 97.5%; for ^1H and ^{13}C NMR data, see Tables 2 and 3, respectively; HR-HESI-MS: $C_{35}H_{56}O_6N$, calcd. 586.4102, found: 586.4099.

Compound 13: White solid; yield: 2% (4.25 mg); RP-HPLC purity: 98.3%; for ^1H and ^{13}C NMR data, see Tables 2 and 3, respectively; HR-HESI-MS: $C_{36}H_{56}O_6N$, calcd. 598.4102, found: 598.4094.

Compound 14: White solid; yield: 8.3% (18.17 mg); RP-HPLC purity: 98.7%; for ^1H and ^{13}C NMR data, see Tables 2 and 3, respectively; HR-HESI-MS: $C_{37}H_{60}O_6N$, calcd. 614.4415, found: 614.4411.

Compound 15: White solid; yield: 2.5% (5.48 mg); RP-HPLC purity: 95.8%; for ^1H and ^{13}C NMR data, see Tables 2 and 3, respectively; HR-HESI-MS: $C_{37}H_{60}O_6N$, calcd. 614.4415, found: 614.4407.

4.2. Biology

Cell cultures. The human gynecological cancer cell lines MDA-MB-231 and MCF7 (breast cancers), and HeLa (cervical adenocarcinoma) were purchased from ECACC (European Collection of Cell Cultures, Salisbury, UK), while SiHa (cervical carcinoma) was purchased from ATCC (American Tissue Culture Collection, Manassas, Virginia, USA). The cells were grown in Minimum Essential Medium (MEM) supplemented with 10% fetal calf serum (FCS), 1% non-essential aminoacids, and 1% penicillin-streptomycin. All media and supplements for these experiments were obtained from Lonza Group Ltd. (Basel, Switzerland). The cells were maintained at 37 °C in humidified atmosphere containing 5% CO_2. Two mouse lymphoma cell lines were also used: a drug susceptible cell line, L5178 mouse T-cell lymphoma (ECACC catalog number 87111908, U.S. FDA, Silver Spring,MD, U.S.), and its multidrug resistant counterpart ($L5178_{MDR}$) obtained by transfection with pHa MDR1/A retrovirus [31]. Cells were cultured in McCoy's 5A media supplemented with nystatin, L-glutamine, penicillin, streptomycin, and inactivated horse serum, at 37 ◦C and 5% CO_2. The MDR cell line was selected by culturing the infected cells with 60 g/L colchicine (Sigma). Media, fetal bovine serum, horse serum, and antibiotics were purchased from Sigma.

Antiproliferative assay on human gynecological cancer cell lines. The growth-inhibitory activities of the prepared ecdysteroid analogs were determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method on four human adherent cancer cell lines of gynecological origin [32]. Briefly, cells were seeded into 96 well plates (5000 cells/well) and incubated with increasing concentrations of the tested compounds (0.1–30.0 μM) under cell-culturing conditions. After incubation for 72 h, 5 mg/ml MTT solution was added and the samples were incubated for another 4 h. The precipitated formazan crystals than were dissolved in dimethyl sulfoxide and the absorbance was measured at 545 nm with a microplate reader. Cisplatin, a clinically used anticancer agent was used as a positive control. In order to calculate fifty percent inhibitory concentrations (IC_{50}), sigmoidal dose–response curves were fitted to the measured points by using the non-linear regression model log (inhibitor) vs. normalized response and variable slope with a least squares (ordinary) fit of GraphPad Prism 5.01 software (GraphPad Software Inc., San Diego, CA, USA).

Cytotoxicity assay on murine lymphoma cell lines. Cytotoxic activities on the L5178 and $L5178_{MDR}$ cell lines were performed as described before [18]. Briefly, 5×10^4 cells/well were incubated with serial dilutions of each compound (n = 3) in McCoy's 5 A medium (Sigma-Aldrich) for 48 h at 37 °C, 5% CO_2. Then, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added to each well at a final concentration of 0.5 mg/mL per well and after 4 h of incubation, 100 μL of sodium dodecyl sulfate (SDS) 10% (Sigma-Aldrich) in 0.01 M HCl was added to each well. Plates were further incubated overnight, the optical densities were read at 540 and 630 nm using an ELISA reader (Multiskan EX, Thermo Labsystem, Milford, MA, USA), and IC_{50} values were calculated as described above.

Rhodamine 123 accumulation assay. ABCB1 inhibitory activities of the compounds were studied through their effect on the

accumulation of rhodamine 123, a fluorescent dye that is an ABCB1 substrate. Flow cytometry was used as described before [15]. Briefly, 2×10^6 cells/mL were treated with 2 or 20 μM of each compound. After 10 min incubation, rhodamine 123 (Sigma-Aldrich) was added to a final concentration of 5.2 μM and the samples were incubated at 37 °C in a water bath for 20 min. Samples were centrifuged (Heraeus Labofuge 400, Thermo Fisher Scientific, Waltham, MA, USA) (2000 rpm, 2 min) and washed twice with phosphate buffer saline (PBS, Sigma). The final samples were re-suspended in 0.5 mL PBS and its fluorescence measured with a Partec CyFlow flow cytometer (Partec, Münster, Germany). 100 nM of tariquidar was used as positive control, which was kindly provided by Dr. Milica Pesic from the Institute for Biological Research Sinisa Stankovic, Belgrade, Serbia.

Cytotoxicity assay in combination with doxorubicin. The checkerboard microplate method was utilized to test the combined activity of doxorubicin (Teva, Budapest, Hungary) and the ecdysteroid derivatives on the L5178 and $L5178_{MDR}$ cell lines, as described before [17]. Briefly, 5×10^4 cells/well were incubated with doxorubicin and the compound to be tested in McCoy's 5 A medium (Sigma-Aldrich) for 48 h at 37 °C, 5% CO_2. Then, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added to each well at a final concentration of 0.5 mg/mL per well, and after 4 h of incubation, 100 μL of sodium dodecyl sulfate (SDS) 10% (Sigma-Aldrich) in 0.01 M HCl was added to each well. The plates were further incubated overnight, and the optical densities were read at 540 and 630 nm using an ELISA reader (Multiskan EX, Thermo Labsystem, Milford, MA, USA). The interaction was evaluated using the CompuSyn software (CompuSyn Inc., Paramus, NJ, USA) at each constant ratio of compound *vs.* doxorubicin (M/M), and combination index (CI) values were obtained for 50%, 75%, and 90% of growth inhibition. Single-drug data obtained from the duplicate control lanes of each plate were utilized to determine cytotoxic activities for each compound.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ejmech.2017.12.032.

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