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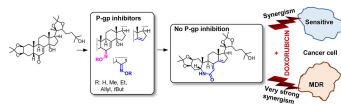
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ACCEPTED MANUSCRIPT

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

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20
21

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 ^1H 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.

Keywords: Ecdysterone; semi-synthesis; Beckmann-rearrangement; chemotherapy; adjuvant; p-glycoprotein; efflux pump inhibitor

43 Introduction

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

49 Currently, the antitumor activity of steroid oximes is by far the most deeply investigated and has
50 recently attracted great scientific attention. For example, oximes and lactams of cholest-4-en-6-
51 one were tested on two human cancer cell lines and were shown to have very high, tumor
52 selective anticancer activity on HeLa cells.^[4] Another study on the structure-activity relationships
53 (SAR) of hydroxyiminosteroids bearing the oxime group on the steroid A and/or B ring showed
54 that a C-6 oxime function is preferential over a 6-keto group concerning *in vitro* cytotoxic
55 activity of these type of compounds.^[5] In a follow-up study on the same compounds, the
56 importance of 3- and 6-hydroxy functions was highlighted.^[6] Furthermore, a set of *in vitro*
57 experiments on 63 novel estrone 16-oximes and oxime ethers revealed two oximes as promising
58 antiproliferative agents with selectivity towards HeLa cells; the compounds modulated cell cycle
59 and induced apoptosis through caspase-3.^[7] In a most recent study, a series of steroidal oximes
60 and lactams were described to possess significant *in vitro* antiproliferative activity, and a 6,23-
61 dioxime derivative, obtained from diosgenin acetate, was identified to be the most effective.^[8]
62 Several further recent reports can be found in the literature where well-defined mechanistic
63 changes could also be connected to the increase in the antiproliferative activity observed after
64 introducing an oxime moiety into an oxo-compound. For example, a number of α,β -unsaturated,
65 cyclohexanone-based oximes showed greatly increased activity as compared to their parental
66 oxo-compounds against BRAF^{V600E} (the most common mutation in the v-raf murine sarcoma viral
67 oncogenes homolog B1, involved in carcinogenesis and cancer aggressiveness) and/or epidermal
68 growth factor receptor TK kinases (involved in cell proliferation, evasion of apoptosis and
69 invasive capacity),^[9] or focal adhesion kinase (FAK; involved in stimulating metastasis and
70 tumor progression)^[10]. These reports suggest that the preparation of oxime derivatives from
71 ketosteroids, and particularly from those with an α,β -enone moiety, should be a reasonable
72 strategy to extend the chemical space towards new, potentially antitumor compounds.

73 Ecdysteroids are α,β -unsaturated 6-ketosteroids that occur in a wide range of plant species; as
74 analogs of the insect molting hormone ecdysone, these compounds possess several biological
75 functions in the flora and the fauna.^{[11][12]} Since the isolation of the most abundant ecdysteroid
76 20-hydroxyecdysone (20E), these compounds were reported to also exert various, beneficial
77 bioactivities in mammals.^{[13][14][15][16]} Additionally, our group revealed that relatively apolar
78 ecdysteroids can strongly sensitize cancer cells to chemotherapeutics (i.e. “chemo-sensitizing”
79 activity), and suggested 20-hydroxyecdysone 2,3:20,22-diacetonide (**1**) as a promising anticancer
80 lead compound.^[17] Interestingly, this sensitization towards various chemotherapeutics could be
81 observed both on multi-drug resistant (MDR) and drug susceptible cancer cell lines.^[18] After
82 several further studies, exploring this particular anticancer activity of ecdysteroids, we now know
83 that 1) apolar substituents on the 2,3-diol moiety are more important than those at positions 20
84 and 22,^[19] and 2) an oxidative side-chain cleavage knocks out the inhibitory activity on the efflux
85 function of P-glycoprotein (P-gp) while maintaining MDR selective sensitizing activity towards
86 doxorubicin.^[20] Regarding semi-synthetic modifications accompanied by the inclusion of
87 heteroatoms, a difluorinated derivative of 20E 2,3:20,22-diacetonide was found to be a stronger
88 P-gp inhibitor than its parental molecule (compound **1**), while, surprisingly, MDR selectivity of
89 the difluorinated compound was lower: it sensitized a P-gp expressing MDR cell line to
90 doxorubicin similarly to its parental compound **1**, and a stronger effect than that of **1** was
91 observed on a non-MDR cell line.^[21] The chemical structures of 20E and compound **1** are shown
92 in Figure 1.

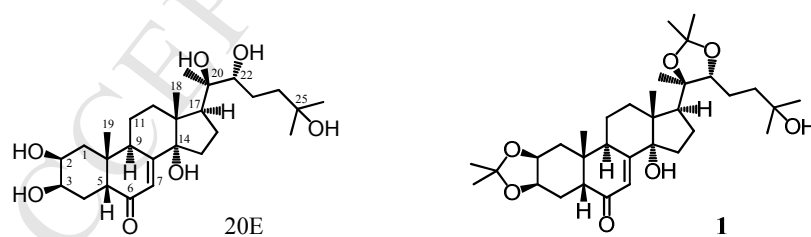


Figure 1. Chemical structures of 20-hydroxyecdysone (20E) and 20-hydroxyecdysone 2,3:20,22-diacetonide (**1**).

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

101 ecdysteroid oximes or lactams, the aim of the present work was to prepare a series of such
 102 compounds, and study their *in vitro* antitumor potential with a focus on their chemo-sensitizing
 103 activity.

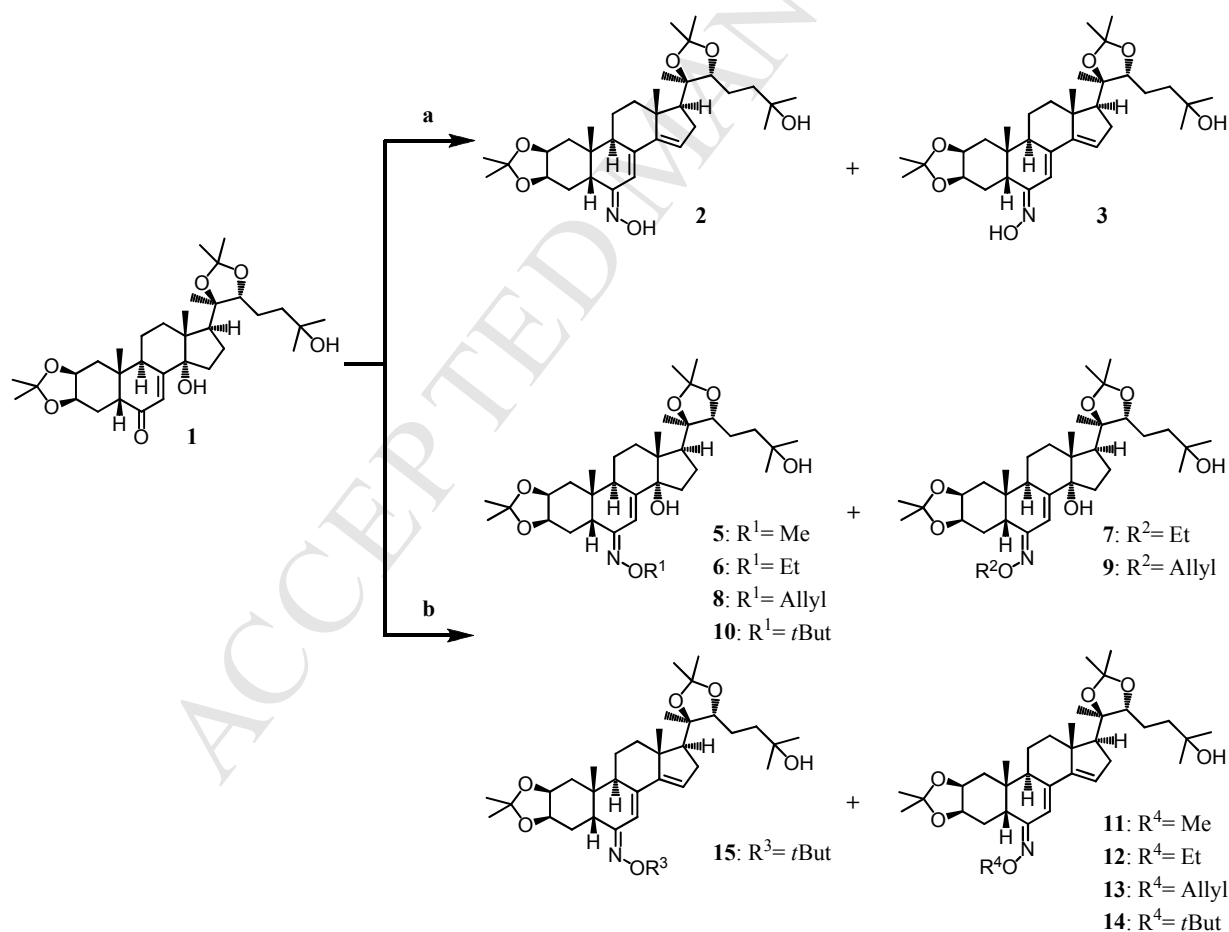
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105 **Results and Discussions**106 **Chemistry**

107 20-hydroxyecdysone 2,3;20.22-diacetonide **1** and its 6-oxime and lactam derivatives were
 108 synthesized following previously published procedures.^{[22][23]} Briefly, compound **1** was reacted
 109 with hydroxylamine or, aiming to prepare new oxime ethers, an alkoxyamine in pyridine at
 110 70°C. A total of 14 nitrogen-containing derivatives were prepared this way (Scheme 1).

111

112 **Scheme 1.** Synthesis of oxime and oxime ether derivatives of 20-hydroxyecdysone 2,3;20,22-
 113 diacetonide.



114

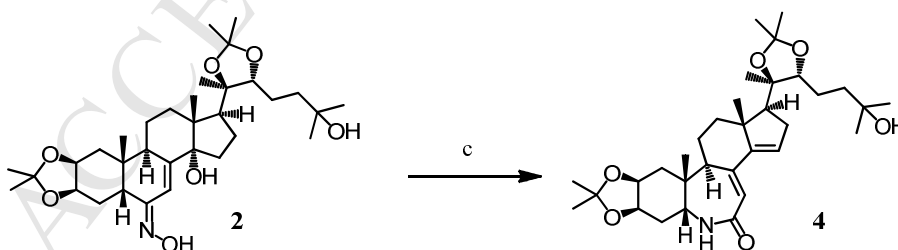
115 *Reagents and conditions:* a) pyridine, $\text{NH}_2\text{OH}\cdot\text{HCl}$, 70°C , 3 days; b) pyridine, $\text{NH}_2\text{OR}\cdot\text{HCl}$
 116 (mass equiv of **1**; R=Me, Et, Allyl, or *t*But), 70°C , 24 h; work-up with KOH in anhydrous
 117 MeOH.

118
 119 Following each reaction, neutralization with KOH dissolved in anhydrous methanol was utilized
 120 with the aim of obtaining several different, structurally diverse and potentially bioactive products,
 121 including mixtures of 14,15-anhydro- and intact oxime derivatives: the oximes **2** and **3**, and
 122 oxime ethers with different 6-*O*-alkyl substituents **5-15**, respectively, were obtained through this
 123 method. Our results confirm previous observations that ecdysteroid 6-oximation can result in 3
 124 different types of product mixtures depending on the neutralization procedure.^[22] a mixture of
 125 14,15-anhydro (*E/Z*)-isomeric oxime pairs form if the reaction does not include a neutralization
 126 step; a 2-4 components mixture of both intact and 14OH-eliminated derivatives is obtained if
 127 alkali dissolved in anhydrous methanol is added; and a mixture of intact (*E/Z*)-isomeric oxime
 128 pair with retained 14-OH groups is obtained if the neutralizing alkali is dissolved in anhydrous
 129 ethanol.

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

135

136 **Scheme 2.** Beckmann rearrangement of ecdysteroid (*6E*)-oxime **2** into lactam **4**.



137
 138 *Reagents and conditions:* c) acetone, *p*-toluenesulfonyl chloride (TsCl, 2 equiv of oxime **2**),
 139 Na_2CO_3 (1 equiv of oxime **2**), RT, 6 h.

140

141 *Structure elucidation*

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

146 The structure and NMR signals of the products were assigned by comprehensive one- and two-
147 dimensional NMR methods, such as ^1H , ^{13}C , DEPTQ, gradient-selected COSY, edited HSQC,
148 HMBC, ROESY (Rotating frame Overhauser Enhancement Spectroscopy) spectra and 1D-
149 selective variants thereof. It is worth mentioning that due to the molecular mass (500-700
150 Daltons) the signal/noise value of the selective ROE experiments strongly exceeds that of the
151 selective NOEs.

152 To facilitate the comparison of NMR signals of structurally analogous hydrogen and carbon
153 atoms of the starting compound **1** with those of the 6-oxime **2**, and of its Beckmann rearranged
154 product **4** and 6-oxime-ether derivatives **5 – 15**, we applied the usual steroid numbering, and for
155 the central atoms of the 2,3;20,22-diacetonide moieties C-28 and C-29, respectively. The ^{13}C
156 chemical shifts of compounds **1**, **2** and **4-15** in methanol- d_4 are compiled in Table 1. The
157 characteristic ^1H data of compounds with a $\Delta^{14,15}$ C=CH ethylene moiety **2**, **4** and **11-15** are
158 summarised in Table 2, whereas that of the HO-C(14) derivatives **5-10** are shown in Table 3.

159

160 **Table 1.** ^{13}C chemical shifts of compounds **2**, **4–15** as compared to that of their parental compound **1** (20-
 161 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
28Me α	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
28Me β	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
29Me α	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
29Me β	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		

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

164 **Table 2.** ¹H chemical shift, multiplicities and coupling constants of compounds **2**, **4**, **11-15** in methanol-*d*₄.

No.	2	<i>J</i> (Hz)	4 ^a	<i>J</i> (Hz)	11	<i>J</i> (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
28	Meα	1.30		1.30		1.31		1.31	1.31	1.32	1.32
	Meβ	1.47		1.46		1.49		1.49	1.49	1.50	1.49
29	Meα	1.40		1.40		1.40		1.40	1.40	1.40	1.40
	Meβ	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		

165
 166 ^a To facilitate the comparison of NMR data of the Beckman product **4** and the parental oximethers we
 167 applied also for **4** the steroidal atomic numbering.

168 ^b Because the stereostucture of the steroid frame is nearly identical within compounds **11-15** we described
 169 the *J* coupling constants only for **11**.

170

171

172 **Table 3.** ^1H 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
28Me α	1.31		1.31	1.32	1.31	1.32	1.32
28Me β	1.47		1.47	1.50	1.47	1.49	1.49
29Me α	1.39		1.39	1.39	1.39	1.39	1.39
29Me β	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	

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

175
 176 It is well known that oximation of ketones is accompanied with characteristic changes of several
 177 ^{13}C and ^1H chemical shifts. Successful conversion of a C=O group to C=N-OH results of ca. 50
 178 ppm diamagnetic shift of the corresponding carbon atom, whereas the chemical shift of α -CH
 179 carbon atom in the *syn* position with respect to the oxime hydroxyl group exhibits ~14 ppm, in

180 the *anti* position ~ 9 ppm diamagnetic shift. The significant ($\Delta\delta$ *syn-anti*) parameters on C-5 and
181 =C-7 signals successfully can be utilised for the assignment of (*Z/E*) isomers. Galyautdinov et al.
182 reported some NMR data on 20-hydroxyecdysone oxime,^[22] including compound **3** (*Z* isomer),
183 but they failed on isolating the isomeric compound **2** with *Z* configuration. In addition they have
184 taken the NMR measurements in solvents with rather different anisotropic nature (e.g. pyridine-
185 *d*₅, methanol-*d*₄) and so in some cases the solvation effect was comparable with the $\Delta\delta$ *syn-anti*
186 parameters. To avoid this ambiguity, we have performed our NMR experiments exclusively in
187 methanol-*d*₄.

188 On the basis of our data, all of the oxime derivatives in Table 1 with $\delta\text{C-5} \sim 38.6$ and $\delta\text{C-7} \sim$
189 117.5 ppm values, respectively, are *Z* isomers, while $\delta\text{C-5} \sim 43.8$ and $\delta\text{C-7} \sim 111.0$ ppm values
190 assign the *E* isomers. It is worth noting that the less different $\delta\text{C-4} (\sim 30/27 \text{ ppm})$ and $\delta\text{C-6}$
191 $(\sim 157/161 \text{ ppm})$ values also reflect on the *E* or *Z* isomers, respectively.

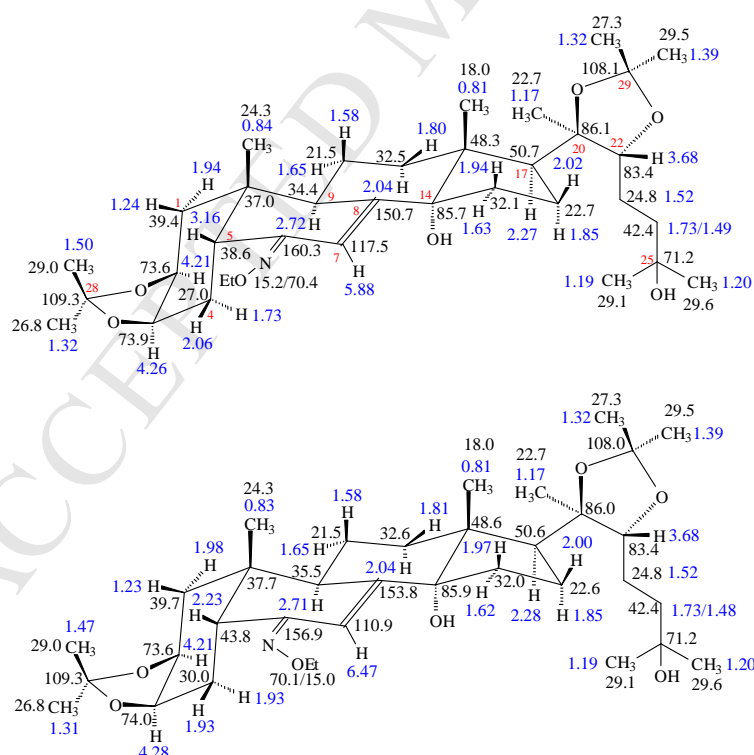
192 In case of compounds **2** and **4**, and the 6-oxime-ether derivatives **11–15** the DEPTQ and HSQC
193 measurements revealed only seven methylene groups, one less than in the parent compound **1**,
194 and simultaneously distinctive chemical shift changes appeared at $\delta\text{C-14}: 85.4 \rightarrow \text{C}=\sim 142 \text{ ppm}$
195 and $\delta\text{H}_2\text{C-15}: 31.8 \rightarrow \text{HC}=\sim 124 \text{ ppm}$, respectively, indicating the emergence of an $\Delta^{14,15} \text{C}=\text{CH}$
196 ethylene moiety. All this means that in these compounds (**2**, **11–15**), simultaneously with the
197 oximation, dehydration by the elimination of the 14-OH group also took place. The presence of
198 the 14-OH substituent in compounds **5–10** appears straightforward, considering of the chemical
199 shift of C-14 ($\delta\text{C-14} \sim 85 \text{ ppm}$) confirmed by the HMBC cross-peak H₃-18/C-14. Success of the
200 Beckmann rearrangement of ecdysteroid (6*E*)-oxime **2** into lactam **4** could be expected from the
201 *E* configuration of the parent oxime. Indeed, the significant (13.1 ppm) paramagnetic shift on $\delta\text{C-}$
202 **5** proves that in **4** the nitrogen atom coupled to C-5, the appearance of the signal at 170.6 ppm
203 supports the formation of the lactam ring.

204 Thanks to the comprehensive one- and two-dimensional NMR techniques utilized in the structure
205 elucidation process, a complete ¹H signal assignment could be achieved for all compounds. The
206 characteristic ¹H NMR data of the 14,15-anhydro derivatives **2**, **4** and **11–15** are summarized in
207 table 2, whereas that of the other compounds **5–10** in table 3. The main difference between the
208 two sets of data is that in Table 2, besides H-7, a second olefinic signal appears for H-15 (~ 85.80
209 dd) instead of the H₂-16 hydrogen signals.

210 The retained *cis* junction of the A/B rings in each compound was obvious by considering the
 211 strong H₃-19/Hβ-5 ROESY response, whereas the assignment of the α/β position of the
 212 diastereotopic methylene hydrogens of the skeleton were revealed by the one-dimensional
 213 selective ROESY measurements irradiating e.g. the H₃-18, H₃-19 and H-5 atoms in combination
 214 with the observed proton-proton coupling pattern.

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

222 To facilitate the comparison between the NMR data of *Z* and *E* isomeric pairs, the stereo-
 223 structures with atomic numbering (in red) of compounds **7** (upper) and **6** (lower) are shown in
 224 Figure 2. Blue numbers refer to ¹H chemical shifts; black numbers give the δ¹³C values.



225
 226 **Figure 2.** Characteristic NMR spectra on differentiation and NMR assignments of the isomeric **6**
 227 and **7** ecdysteroid 6-oxime ethers are given in the supporting information.

228 **Biology**

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

232
 233 **Table 4.** Antiproliferative properties of compounds **4-15** against four human gynecological
 234 cancer cell lines. Inhibition concentration at 50% growth (IC₅₀) values of each compound and the
 235 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]

236
 237 Although most of the ecdysteroid analogs displayed moderate activities against the tested cell
 238 lines, the *i*-butyl substituted compound **10** was stronger than the positive control cisplatin on the
 239 HeLa and MDA-MB-231 cell lines. In our previous study, the antiproliferative IC₅₀ values of
 240 compound **1** were 106.1 and 75.1 μM on the MDA-MB-231 and MCF7 cell lines, respectively,^[21]
 241 showing that the inclusion of certain oxime ether functions can increase this activity by nearly an
 242 order of magnitude. While the orientation of the oxime ether had no obvious effect on the
 243 activity, a larger alkyl group led to a stronger antiproliferative action. It appears to be clear that
 244 the retained 14-OH function is favorable over the Δ^{14,15} moiety in this regard on the MCF-7 cell
 245 line (compounds **7** vs. **12**, **9** vs. **13**, and **10** vs. **15**), while such a conclusion cannot be drawn on
 246 the other cell lines.

247 Compounds **2-15** were also tested for their cytotoxic activity on a murine lymphoma cell line
 248 pair, including L5178 and its multi-drug resistant counterpart transfected to express the human

249 ABCB1 transporter, L5178_{MDR}. Following this, the compounds were tested for their potential to
 250 inhibit the ABCB1 efflux transporter through measuring the intracellular accumulation of
 251 rhodamine 123 by flow cytometry. Degree of inhibition (%) values were calculated by means of
 252 the rhodamine 123 accumulation of the ABCB1 transfected L5178_{MDR} cells (i.e. 0 % inhibition)
 253 and that of the L5178 cells (i.e. 100% inhibition); results are presented in Table 5.

254
 255 **Table 5.** Cytotoxicity of compounds **1-15** on L5178 and L5178_{MDR} cells, and functional
 256 inhibition of the ABCB1 transporter. Dox=doxorubicin; for the ABCB1 inhibition, positive
 257 control: 100 nM of tariquidar (112.4% inhibition), negative control: 2% DMSO (-0.07%
 258 inhibition).

Compound	Change in the B-ring of 1 ^a	14-OH or $\Delta^{14,15}$	IC ₅₀ (μ M) [95% confidence intervals] ^b		ABCB1 inhibition (%)	
			L5178	L5178 _{MDR}	2 μ M	20 μ M
1	-	14-OH	110.3 [77.50-157.1]	97.69 [71.07-134.3]	2.54	20.91
2	(<i>E</i>)-oxime	$\Delta^{14,15}$	20.91 [17.68-24.74]	24.63 [19.82-30.63]	10.57	82.95
3	(<i>Z</i>)-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	(<i>E</i>); R=Me	14-OH	40.92 [35.66-46.97]	55.05 [41.53-72.98]	2.25	25.05
6	(<i>E</i>); R=Et	14-OH	35.02 [25.35-48.38]	47.00 [31.14-70.93]	17.54	78.79
7	(<i>Z</i>); R=Et	14-OH	37.26 [25.65-54.11]	42.16 [41.24-43.10]	18.96	75.03
8	(<i>E</i>); R=Allyl	14-OH	31.48 [23.71-41.80]	51.91 [42.69-63.13]	20.98	89.39
9	(<i>Z</i>); R=Allyl	14-OH	36.66 [28.32-47.44]	49.29 [43.07-56.40]	24.17	81.80
10	(<i>E</i>); R= <i>t</i> -But	14-OH	28.06 [21.30-36.98]	29.12 [25.12-33.76]	38.75	112.4
11	(<i>Z</i>); R=Me	$\Delta^{14,15}$	45.95 [36.97-57.11]	53.14 [43.54-64.86]	33.36	106.2
12	(<i>Z</i>); R=Et	$\Delta^{14,15}$	53.20 [38.64-73.26]	58.94 [45.86-75.74]	56.41	107.7
13	(<i>Z</i>); R=Allyl	$\Delta^{14,15}$	55.28 [46.21-66.13]	52.72 [39.97-65.53]	61.13	102.7
14	(<i>Z</i>); R= <i>t</i> -But	$\Delta^{14,15}$	63.23 [58.57-68.26]	51.22 [39.13-67.04]	58.99	78.76
15	(<i>E</i>); R= <i>t</i> -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]	-	-

259 ^a R groups refer to the alkyl substituents of the oxime ethers as in Scheme 1

260 ^b IC₅₀ values were calculated by the CompuSyn software as the median cytotoxic activities (Dm)
 261 from the control lanes on the checkerboard plates of the combination studies, n=2.

262
 263 While the compounds also exerted weak to moderate cytotoxic activities on the mouse lymphoma
 264 cell line pair, all of them were more potent than their parental compound **1**. No cross resistance
 265 was observed to any of them on the ABCB1 over-expressing MDR cells. The oximes **2** and **3**
 266 showed the strongest activity on either cell lines with IC₅₀ values ca. 4-5 times below that of
 267 compound **1**, and the *E*-oxime (**2**) was more cytotoxic than the *Z*-oxime (**3**). The oxime ethers
 268 typically exerted weaker cytotoxic activities than the non-substituted oximes, with the exception

269 of compound **10** where a bulky *t*-butyl substituent and a retained 14-OH group were present.
270 When comparing corresponding analogs with a retained 14-OH group or a $\Delta^{14,15}$ moiety, there
271 appeared to be a clear tendency for the former structural element to be associated with a stronger
272 cytotoxic activity on the mouse lymphoma cells, similarly to the case of MCF-7 cells (see above).
273 Evaluation of the results obtained from the rhodamine accumulation assay reveals that the lactam
274 derivative (**4**) is the only one among the compounds that was completely inactive in this regard at
275 as much as 20 μ M concentration. For the other compounds, several structure-activity
276 relationships could be observed. The oxime formation markedly increased the ABCB1 inhibitory
277 activity, and this was particularly true for oxime ethers. The orientation of the oxime group had
278 little if any influence on the ABCB1 inhibition (compound **2** vs. **3**, **6** vs. **7**, **8** vs. **9**, and **14** vs. **15**),
279 while the 14-OH elimination, forming a $\Delta^{14,15}$ double bond in the ecdysteroid D-ring, clearly
280 increased this activity (compound **7** vs. **12**, **9** vs. **13**, and **10** vs. **15**). When comparing the activity
281 of oximes and oxime ethers between analogs containing the same type of D-ring and orientation
282 of oxime but different substituents on the latter, the following order of bioactivity could be
283 concluded: H < Me < Et < Allyl \leq *t*-But.

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

291 **Table 6.** Chemo-sensitizing activity of compounds **1-15** on the L5178 and L5178_{MDR} cell lines
 292 towards doxorubicin at 50, 75 and 90% of growth inhibition (ED₅₀, ED₇₅ and ED₉₀, respectively).
 293 CI: combination index; CI_{avg}: weighted average CI value; CI_{avg} = (CI₅₀ + 2CI₇₅ + 3CI₉₀)/6. CI < 1,
 294 CI = 1, and CI > 1 represent synergism, additivity, and antagonism, respectively. Dm, m, and r
 295 represent antilog of the x-intercept, slope, and linear correlation coefficient of the median-effect
 296 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

297
 298 All tested derivatives showed strong synergism ($0.1 < CI_{avg} < 0.3$)^[25] with doxorubicin on the P-gp
 299 expressing L5178_{MDR} cells, similarly to their parental compound (**1**). As it was previously

300 reported by us, chemo-sensitizing activity of ecdysteroids has little if any correlation to their
301 (most typically weak) inhibitory effect on the efflux function of P-gp.^[20] This was clearly
302 confirmed in the present study as well: even though for example compounds **11-15** are much
303 stronger P-gp inhibitors than their parental compound **1**, no difference can be observed in the
304 strength of synergism with the P-gp substrate doxorubicin on the MDR cell line. Most
305 interestingly, among all derivatives obtained, the ecdysteroid lactam **4** was found to express the
306 strongest chemosensitization on the MDR cells, while being the only one to show no interference
307 with P-gp function. Accordingly, this compound has a further advantage over the diacetone of
308 20E, namely that it would likely be free from the potential adverse effects and unwanted drug-
309 drug interactions connected to P-gp inhibitors.^{[26][27]}

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

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

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

331

332 **Conclusions**

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

339 Evaluation of the antiproliferative and cytotoxic activity of the compounds on several cancer cell
340 lines revealed several structure-activity relationships (SAR). A new, *i*-butyl substituted
341 ecdysteroid oxime ether (**10**) was found to exert stronger antiproliferative effect on HeLa cells
342 than cisplatin. The $\Delta^{14,15}$ *E*-oxime derivative (**2**) exerted a substantially increased cytotoxic and P-
343 gp inhibitory activities in the L5178/L5178_{MDR} cell line pair, as compared to its parental
344 compound.

345 Clear SAR was observed for the compounds' activity as functional P-gp inhibitors, and many of
346 them were identified as highly potent MDR-selective chemo-sensitizers. In particular, a novel
347 $\Delta^{14,15}$ δ -lactam ecdysteroid derivative (**4**) was revealed as a most promising new lead compound
348 with low intrinsic cytotoxicity, and strong ability to sensitize MDR and also non-MDR cancer
349 cells towards doxorubicin without interfering with the efflux function of P-gp. Accordingly, it
350 can be expected that a combined treatment of cancer with this compound as a chemo-sensitizer
351 and a chemotherapeutic agent would 1) be effective on the initial, susceptible state of the tumor,
352 and 2) have a strong chance to prevent the acquisition of P-gp mediated resistance through an
353 increased killing effect on the cell population becoming adapted to the chemotherapy.

354

355 **Experimental section**

356 **Chemistry**

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

359 ¹H (500.1 MHz) and ¹³C (125.6 MHz) NMR spectra were recorded at room temperature on a
360 Bruker Avance-II spectrometer and on Avance-III spectrometer equipped with a cryo probehead.
361 Regarding the compounds, amounts of approximately 1 - 10 mg were dissolved in 0.1 mL of
362 methanol-*d*₄ and transferred to 2.5 mm Bruker MATCH NMR sample tube. Chemical shifts are

363 given on the δ -scale and are referenced to the solvent (MeOH- d_4 : $\delta_C = 49.1$ and $\delta_H = 3.31$ ppm).
364 Pulse programs of all experiments (^1H , ^{13}C , DEPTQ, DEPT-135, one-dimensional sel-ROE
365 (mixing time: 300 ms), edited gs-HSQC and gs-HMBC) were taken from the Bruker software
366 library. The NMR signals of the product were assigned by comprehensive one- and two-
367 dimensional NMR methods using widely accepted strategies.^{[28][29][30]} Most ^1H assignments were
368 accomplished using general knowledge of chemical shift dispersion with the aid of the proton-
369 proton coupling pattern (^1H NMR spectra). Mass spectra were obtained on a Waters Acquity
370 iClass UPLC coupled with Thermo Q Exactive Plus with HESI source (Waters Co., USA).
371 Reaction progress was monitored by thin layer chromatography (TLC) on Kieselgel 60F₂₅₄ silica
372 plates obtained from Merck (Merck, Germany), and examined under UV illumination at 254 nm.
373 Compounds were purified by flash chromatography with adequately chosen eluents of *n*-hexane –
374 dichloromethane – methanol on 12 g RediSep NP-silica flash columns (TELEDYNE Isco, USA).
375 For the RP-HPLC separation of isomeric oxime derivatives a Kinetex XB-C18 250 x 21.4 mm 5
376 μm preparative (Phenomenex Inc., USA) or an Agilent Eclipse XDB-C8 250 x 9.4 mm 5 μm
377 semi-preparative column (Agilent Technologies Inc., USA) was applied with the use of isocratic
378 grade eluents of acetonitrile and water. Purity of obtained compounds was determined by RP-
379 HPLC with the use of a Kinetex XB-C18 250 x 4.6 mm 5 μm analytical column (Phenomenex
380 Inc., USA). For data collection a Jasco HPLC instrument equipped with an MD-2010 Plus PDA
381 detector (Jasco Analytical Instruments, Japan) was applied in a detection range of 210-400 nm.
382 Ecdysteroid substrate **1** was synthesized from 20-hydroxyecdysone (20E) obtained from Shaanxi
383 KingsSci Biotechnology Co., Ltd. (Shanghai, People's Republic of China) at 90% purity and was
384 recrystallized (EtOAc:MeOH – 2:1) to a RP-HPLC purity of 97.8%. During the synthetic
385 procedure, 20E (10g) is dissolved in acetone in the concentration of g/100 cm³ and
386 phosphomolybdic acid is added (10g) under stirring. After 5 minutes of stirring at RT the reaction
387 is complete and the mixture is neutralized with 10% aqueous NaHCO₃. Acetone is evaporated
388 under reduced pressure and the mixture is extracted with EtOAc (3x50 ml) followed by drying
389 with Na₂SO₄. After filtration, solvent is evaporated under reduced pressure and the crude mixture
390 is purified by flash chromatography with isocratic grade eluents of dichloromethane:methanol –
391 99:1. (Yield: 51%)

392 **Synthesis of ecdysteroid 6-oximes (2-3).** 1g **1** (1,78 mmol) was dissolved in pyridine (10 ml)
393 and 1 g hydroxylamine hydrochloride (14.39 mmol) was added to the solution under stirring.

394 After 3 days of stirring at 70°C the reaction was complete and the solvent was evaporated under
395 reduced pressure. Following water addition (50 ml), the mixture was extracted with EtOAc (3x50
396 ml) and the combined organic phase was dried with Na₂SO₄. A filtration was made to remove
397 drying agent and the solvent was evaporated under reduced pressure. Purification of the crude
398 mixture was carried out by preparative RP-HPLC to obtain (*E/Z*)-isomeric oximes **2-3**,
399 respectively.

400 **Synthesis of ecdysteroid lactam derivative (4).** 0.138 g oxime **2** (0,25 mmol) was dissolved in
401 anhydrous acetone (10 ml), then 0.027 g Na₂CO₃ (0.25 mmol) and 0.096 g *p*-toluenesulfonyl
402 chloride (0,5 mmol) was added to the solution under stirring. After 6 hours of stirring at RT, the
403 reaction was stopped and the mixture was cooled to 0°C. Under stirring, water (10 ml) was added
404 and the mixture was extracted into ethyl acetate (3x50 ml). After evaporation under reduced
405 pressure, the mixture was purified with semi-preparative RP-HPLC to obtain lactam derivative **4**.

406 **General Procedure for the synthesis of ecdysteroid 6-oxime ethers (5-15).** 200 mg **1** (0,35
407 mmol) was dissolved in pyridine (8 ml) and depending on the oxime ether to be obtained, 200 mg
408 alkoxyamine-hydrochloride was added to the solution under stirring. After stirring at 70°C for 24
409 hours, the mixture was cooled down to 0°C, neutralized with KOH dissolved in anhydrous
410 methanol and evaporated under reduced pressure. Water (50 ml) was added and the mixture was
411 extracted with EtOAc (3x50 ml). The combined layers were dried with Na₂SO₄ and after
412 filtration, the organic solvent was evaporated under reduced pressure. Purification of crude
413 material was carried out by flash chromatography on silica gel to obtain compounds **5-15**,
414 respectively. In cases of oxime pairs **2-3**, **6-7**, **8-9**, **14-15** preparative RP-HPLC was applied to
415 separate the isomeric oxime and oxime ether derivatives.

416
417 **Compound 4:** White solid; yield: 8% (11.04mg); RP-HPLC purity: 98.1%; for ¹H- and ¹³C-
418 NMR data, see Tables 2 and 3, respectively; HR-HESI-MS: C₃₃H₅₂O₆N, calcd. 558.3789, found:
419 558.3737.

420 **Compound 5:** White solid; yield: 28.3% (59.53mg); RP-HPLC purity: 99.8%; for ¹H- and ¹³C-
421 NMR data, see Tables 1 and 3, respectively; HR-HESI-MS: C₃₄H₅₆O₇N, calcd. 590.4051, found:
422 590.4045.

423 **Compound 6:** White solid; yield: 15.2% (32.75 mg); RP-HPLC purity: 99.6%; for ¹H- and ¹³C-
424 NMR data, see Tables 1 and 3, respectively; HR-HESI-MS: C₃₅H₅₈O₇N, calcd. 604.4208, found:
425 604.4198.

426 **Compound 7:** White solid; yield: 2.8% (6.06 mg); RP-HPLC purity: 98.7%; for ^1H - and ^{13}C -
427 NMR data, see Tables 1 and 3, respectively; HR-HESI-MS: $\text{C}_{35}\text{H}_{58}\text{O}_7\text{N}$, calcd. 604.4208, found:
428 604.4199.

429 **Compound 8:** White solid; yield: 15.5% (34.05 mg); RP-HPLC purity: 98.3%; for ^1H - and ^{13}C -
430 NMR data, see Tables 1 and 3, respectively; HR-HESI-MS: $\text{C}_{36}\text{H}_{58}\text{O}_7\text{N}$, calcd. 616.4208, found:
431 616.4201.

432 **Compound 9:** White solid; yield: 1.6% (3.5 mg); RP-HPLC purity: 99.6%; for ^1H - and ^{13}C -NMR
433 data, see Tables 1 and 3, respectively; HR-HESI-MS: $\text{C}_{36}\text{H}_{58}\text{O}_7\text{N}$, calcd. 616.4208, found:
434 616.4200.

435 **Compound 10:** White solid; yield: 38.9% (87.67 mg); RP-HPLC purity: 98.5%; for ^1H - and ^{13}C -
436 NMR data, see Tables 1 and 3, respectively; HR-HESI-MS: $\text{C}_{37}\text{H}_{62}\text{O}_7\text{N}$, calcd. 632.4521, found:
437 632.4515.

438 **Compound 11:** White solid; yield: 43.3% (88.32 mg); RP-HPLC purity: 97.7%; for ^1H - and ^{13}C -
439 NMR data, see Tables 2 and 3, respectively; HR-HESI-MS: $\text{C}_{34}\text{H}_{54}\text{O}_6\text{N}$, calcd. 572.3946, found:
440 572.3937.

441 **Compound 12:** White solid; yield: 33.3% (69.59 mg); RP-HPLC purity: 97.5%; for ^1H - and ^{13}C -
442 NMR data, see Tables 2 and 3, respectively; HR-HESI-MS: $\text{C}_{35}\text{H}_{56}\text{O}_6\text{N}$, calcd. 586.4102, found:
443 586.4099.

444 **Compound 13:** White solid; yield: 2% (4.25 mg); RP-HPLC purity: 98.3%; for ^1H - and ^{13}C -
445 NMR data, see Tables 2 and 3, respectively; HR-HESI-MS: $\text{C}_{36}\text{H}_{56}\text{O}_6\text{N}$, calcd. 598.4102, found:
446 598.4094.

447 **Compound 14:** White solid; yield: 8.3% (18.17 mg); RP-HPLC purity: 98.7%; for ^1H - and ^{13}C -
448 NMR data, see Tables 2 and 3, respectively; HR-HESI-MS: $\text{C}_{37}\text{H}_{60}\text{O}_6\text{N}$, calcd. 614.4415, found:
449 614.4411.

450 **Compound 15:** White solid; yield: 2.5% (5.48 mg); RP-HPLC purity: 95.8%; for ^1H - and ^{13}C -
451 NMR data, see Tables 2 and 3, respectively; HR-HESI-MS: $\text{C}_{37}\text{H}_{60}\text{O}_6\text{N}$, calcd. 614.4415, found:
452 614.4407.

453

454 *Biology*

455 **Cell cultures.** The human gynecological cancer cell lines MDA-MB-231 and MCF7 (breast
456 cancers), and HeLa (cervical adenocarcinoma) were purchased from ECACC (European

457 Collection of Cell Cultures, Salisbury, UK), while SiHa (cervical carcinoma) was purchased from
458 ATCC (American Tissue Culture Collection, Manassas, Virginia, USA). The cells were grown in
459 Minimum Essential Medium (MEM) supplemented with 10% fetal calf serum (FCS), 1% non-
460 essential aminoacids, and 1% penicillin-streptomycin. All media and supplements for these
461 experiments were obtained from Lonza Group Ltd. (Basel, Switzerland). The cells were
462 maintained at 37 °C in humidified atmosphere containing 5% CO₂. Two mouse lymphoma cell
463 lines were also used: a drug susceptible cell line, L5178 mouse T-cell lymphoma (ECACC
464 catalog number 87111908, U.S. FDA, Silver Spring, MD, U.S.), and its multidrug resistant
465 counterpart (L5178_{MDR}) obtained by transfection with pHa MDR1/A retrovirus.^[31] Cells were
466 cultured in McCoy's 5A media supplemented with nystatin, L-glutamine, penicillin,
467 streptomycin, and inactivated horse serum, at 37°C and 5% CO₂. The MDR cell line was selected
468 by culturing the infected cells with 60 µg/L colchicine (Sigma). Media, fetal bovine serum, horse
469 serum, and antibiotics were purchased from Sigma.

470
471 **Antiproliferative assay on human gynecological cancer cell lines.** The growth-inhibitory
472 activities of the prepared ecdysteroid analogs were determined by the MTT (3-(4,5-
473 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method on four human adherent cancer
474 cell lines of gynecological origin.^[32] Briefly, cells were seeded into 96 well plates (5000
475 cells/well) and incubated with increasing concentrations of the tested compounds (0.1–30.0 µM)
476 under cell-culturing conditions. After incubation for 72 h, 5 mg/ml MTT solution was added and
477 the samples were incubated for another 4 h. The precipitated formazan crystals than were
478 dissolved in dimethyl sulfoxide and the absorbance was measured at 545 nm with a microplate
479 reader. Cisplatin, a clinically used anticancer agent was used as a positive control. In order to
480 calculate fifty percent inhibitory concentrations (IC₅₀), sigmoidal dose–response curves were
481 fitted to the measured points by using the non-linear regression model log (inhibitor) vs.
482 normalized response and variable slope with a least squares (ordinary) fit of GraphPad Prism 5.01
483 software (GraphPad Software Inc., San Diego, CA, USA).

484
485 **Cytotoxicity assay on murine lymphoma cell lines.** Cytotoxic activities on the L5178 and
486 L5178_{MDR} cell lines were performed as described before.^[18] Briefly, 5 x 10⁴ cells/well were
487 incubated with serial dilutions of each compound (n = 3) in McCoy's 5 A medium (Sigma-

488 Aldrich) for 48 h at 37°C, 5% CO₂. Then, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
489 bromide (MTT, Sigma) was added to each well at a final concentration of 0.5 mg/mL per well
490 and after 4 h of incubation, 100 µL of sodium dodecyl sulfate (SDS) 10% (Sigma-Aldrich) in
491 0.01 M HCl was added to each well. Plates were further incubated overnight, the optical densities
492 were read at 540 and 630 nm using an ELISA reader (Multiskan EX, Thermo Labsystem,
493 Milford, MA, USA), and IC₅₀ values were calculated as described above.

494
495 **Rhodamine 123 accumulation assay.** ABCB1 inhibitory activities of the compounds were
496 studied through their effect on the accumulation of rhodamine 123, a fluorescent dye that is an
497 ABCB1 substrate. Flow cytometry was used as described before.^[17] Briefly, 2 x 10⁶ cells/mL
498 were treated with 2 or 20 µM of each compound. After 10 min incubation, rhodamine 123
499 (Sigma-Aldrich) was added to a final concentration of 5.2 µM and the samples were incubated at
500 37 °C in a water bath for 20 min. Samples were centrifuged (Heraeus Labofuge 400, Thermo
501 Fisher Scientific, Waltham, MA, USA) (2000 rpm, 2 min) and washed twice with phosphate
502 buffer saline (PBS, Sigma). The final samples were re-suspended in 0.5 mL PBS and its
503 fluorescence measured with a Partec CyFlow flow cytometer (Partec, Münster, Germany). 100
504 nM of tariquidar was used as positive control, which was kindly provided by Dr. Milica Pesic
505 from the Institute for Biological Research Sinisa Stankovic, Belgrade, Serbia.

506
507 **Cytotoxicity assay in combination with doxorubicin.** The checkerboard microplate method
508 was utilized to test the combined activity of doxorubicin (Teva, Budapest, Hungary) and the
509 ecdysteroid derivatives on the L5178 and L5178_{MDR} cell lines, as described before.^[17] Briefly, 5 ×
510 10⁴ cells/well were incubated with doxorubicin and the compound to be tested in McCoy's 5 A
511 medium (Sigma-Aldrich) for 48 h at 37°C, 5% CO₂. Then, 3-(4,5-dimethylthiazol-2-yl)-2,5-
512 diphenyltetrazolium bromide (MTT, Sigma) was added to each well at a final concentration of
513 0.5 mg/mL per well, and after 4 h of incubation, 100 µL of sodium dodecyl sulfate (SDS) 10%
514 (Sigma-Aldrich) in 0.01 M HCl was added to each well. The plates were further incubated
515 overnight, and the optical densities were read at 540 and 630 nm using an ELISA reader
516 (Multiskan EX, Thermo Labsystem, Milford, MA, USA). The interaction was evaluated using the
517 CompuSyn software (CompuSyn Inc., Paramus, NJ, USA) at each constant ratio of compound vs.
518 doxorubicin (M/M), and combination index (CI) values were obtained for 50%, 75%, and 90% of

519 growth inhibition. Single-drug data obtained from the duplicate control lanes of each plate were
520 utilized to determine cytotoxic activities for each compound.

521

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528

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Fourteen nitrogen-containing ecdysteroid diacetoneides were prepared

The new compounds were characterized with complete NMR signal assignment

Ecdysteroid oxime ethers are potent P-gp inhibitors on MDR cancer cells

Oimes and oxime ethers sensitize MDR and non-MDR cancer cells to doxorubicin

A new ecdysteroid lactam strongly sensitizes cancer cells without P-gp inhibition

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