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Ph.D. Thesis

Preparation of novel bioactive semi-synthetic ecdysteroid derivatives

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LIST OF PUBLICATIONS RELATED TO THE THESIS

I. Martins, A.; **Csábi, J.**; Balázs, A.; Kitka, D.; Amaral, L.; Molnár, J.; Simon, A.; Tóth, G.; Hunyadi, A.; Synthesis and Structure-Activity Relationships of Novel Ecdysteroid Dioxolanes as MDR Modulators in Cancer.
Molecules, **2013**, *18*(12), 15255-15275. **IF: 2.095**
(Shared first authorship) *Medicine (miscellaneous): Q1*

II. Balázs, A.; Hunyadi, A.; **Csábi, J.**; Jedlinszki, N.; Martins, A.; Simon, A.; Tóth, G.; ^{1}H and ^{13}C NMR investigation of 20-hydroxyecdysone dioxolane derivatives, a novel group of MDR modulator agents.
Magnetic Resonance in Chemistry, **2013**, *51*(12), 830-836. **IF: 1.559**
Chemistry (miscellaneous): Q2

III. Martins, A.; Sipos, P.; Dér, K.; **Csábi, J.**; Miklós, W.; Berger, W.; Zalatnai, A.; Amaral, L.; Molnár, J.; Szabó-Révész, P.; Hunyadi, A.; Ecdysteroids Sensitize MDR and Non-MDR Cancer Cell Lines to Doxorubicin, Paclitaxel, and Vincristine but Tend to Protect Them from Cisplatin.
BioMed Research International, **2015**, Article ID: 895360. **IF: 2.134**
Biochemistry, Genetics and Molecular Biology (miscellaneous): Q2

IV. **Csábi, J.**; Hsieh, T-J.; Hasanpour, F.; Martins, A.; Kele, Z.; Gáti, T.; Simon, A.; Tóth, G.; Hunyadi, A.; Oxidized Metabolites of 20-Hydroxyecdysone and Their Activity on Skeletal Muscle Cells: Preparation of a Pair of Desmotropes with Opposite Bioactivities.
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Complementary and Alternative Medicine: D1

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Magnetic Resonance in Chemistry, **2016**, *54*, 391-395. **IF: 1.226**
Chemistry (miscellaneous): Q2

VI. **Csábi, J.**; Martins, A.; Sinka, I.; Csorba, A.; Molnár, J.; Zupkó, I.; Tóth, G.; Tillekeratne, L.M.V.; Hunyadi, A.; Synthesis and *in vitro* evaluation of the antitumor potential and chemo-sensitizing activity of fluorinated ecdysteroid derivatives.
Medicinal Chemical Communications, **2016**, *7*, 2282-2289. **IF: 2.319**
(Front page article) *Pharmaceutical Science: Q1*

VII. Müller, J.; Martins, A.; **Csábi, J.**; Fenyvesi, F.; Könczöl, Á.; Hunyadi, A.; Balogh, T.Gy.; BBB penetration-targeting physicochemical lead selection: Ecdysteroids as chemo-sensitizers against CNS tumors.
European Journal of Pharmaceutical Sciences, **2017**, *96*, 571-577. **IF: 3.773**
Pharmaceutical Science: Q1

VIII. Hunyadi, A.; **Csábi, J.**; Martins, A.; Molnár, J.; Balázs, A.; Tóth, G.; Backstabbing P-gp: side-chain cleaved ecdysteroid 2,3-dioxolanes hyper-sensitize MDR cancer cells to doxorubicin without efflux inhibition.
Molecules, **2017**, *22*, 199. **IF: 2.465**
Organic chemistry: Q2

LIST OF ABBREVIATIONS

1D	one dimensional
2D	two dimensional
^{1}H NMR	proton nuclear magnetic resonance spectroscopy
^{13}C NMR	carbon nuclear magnetic resonance spectroscopy
20E	20-hydroxyecdysone
ANOVA	analysis of variance
APCI	atmospheric-pressure chemical ionization
aq.	aqueous
AUC	area under curve
BBB	blood-brain barrier
CC	column chromatography
CI	combination index
COSY	correlation spectroscopy
CPC	centrifugal partition chromatography
DAD	diode array detector
DAST	(diethylamino)sulfur trifluoride
δ	chemical shift
DEPT	distorsionless enhancement by polarization transfer
DEPTQ	distorsionless enhancement by polarization transfer with retention of quaternaries
DMEM	Dulbecco's modified Eagle's medium
DTT	dithiothreitol
EMEM	Eagle's minimum essential medium
ESI	electrospray ionization
equiv.	equivalent
FAR	fluorescence activity ratio
FBS	fetal bovine serum
FID	free induction decay
FL	mean of fluorescence
gs	gradient-selected
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
HMBC	heteronuclear multiple-bond correlation
HMQC	heteronuclear multiple-quantum correlation
HSQC	heteronuclear single quantum correlation
IC_{50}	fifty percent inhibitory concentration
λ_{max}	wavelength of maximum absorbance
LD_{50}	fifty percent lethal dose
MDR	multi-drug resistance
MS	mass spectroscopy
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NOE	nuclear Overhauser-enhancement
NOESY	nuclear Overhauser-enhancement spectroscopy
P-gp	P-glycoprotein
PIFA	[bis(trifluoroacetoxy)iodo]benzene
PVDF	polyvinylidene fluoride
ROE	rotated frame nuclear Overhauser-enhancement
ROESY	rotated frame nuclear Overhauser-enhancement spectroscopy
RPC	rotational planar chromatography
RP-HPLC	reversed-phase high performance liquid chromatography
SAR	structure-activity relationship
SDS	sodium dodecyl sulfate
sel	selective
SEM	standard error of the mean
TLC	thin layer chromatography
TOCSY	total correlation spectroscopy
UV	ultraviolet

1. INTRODUCTION

1.1. Occurrence and structural diversity of ecdysteroids

Ecdysteroids represent a large and diverse family of steroid hormones in nature. The first ecdysteroid compound ever discovered, ecdysone, was isolated from silkworm pupae by *Butenandt and Karlson* in 1954 [1]. The name of these steroid compounds derives from the Latin word *ecdysis* (molting), referring to their role in the regulation of molting and reproduction in arthropods [2]. These compounds are not only present in insects, but can be found in much larger quantities in several terrestrial plant genera like *Leuzea* and *Serratula* [2, 3]. Though ecdysteroids are also present in certain fungi and algae, their most important source is considered to be plants, where their concentration can reach as much as 1,000-fold higher than that typically found in arthropods [4, 5, 6]. Phytoecdysteroids can be found in all parts of plants: they are located in roots, rhizomes, seeds, bark, flowers and fruits [2]. Since ecdysteroids possess insect molting hormone activity, most assuredly in plants they play a defensive role against non-adapted phytophagous invertebrates [6]. Nonetheless, these compounds have very limited application as natural pesticides, since – due to their relatively high polarity – they do not possess the required properties to be successful in respect of pest control [7]. The most abundant phytoecdysteroid in plants is 20-hydroxyecdysone (20E, **1, Fig. 1.**), which is also recognized as the major biologically active ecdysteroid in arthropods [2]. 20E is usually accompanied by a large number of minor phytoecdysteroids – present in typically much lower amounts –, and over the past five decades, more than 480 structural analogues have been isolated from plant sources [2, 8]. Approximately one quarter of the known natural ecdysteroids have been discovered by the research group of Prof. Báthori at the University of Szeged [8].

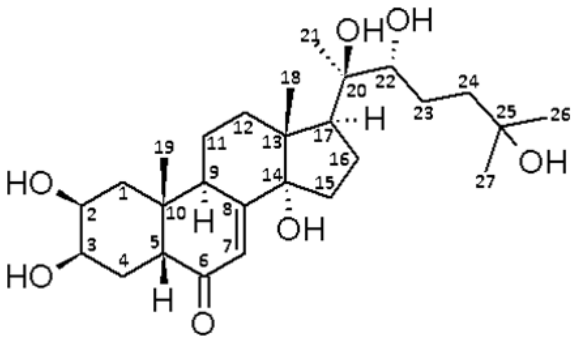


Figure 1 – Structure of 20-hydroxyecdysone (20E, **1**)

Concerning their structure, ecdysteroids can be characterized with a steroid skeleton (C_{27}) derived biosynthetically from cholesterol [2]. The A/B ring junction is normally *cis* (5β-H), while the C/D ring junction is almost always *trans* with the exceptions of 14-epi-20-hydroxyecdysone and 14-epi-ponasterone A 22-glucoside [2]. On ring B a 7-en-6-one chromophore group can be found, giving a

characteristic ultraviolet (UV) absorption – with λ_{max} at *ca.* 242 nm in methanol – to most ecdysteroids. Usually, a β-side-chain is present at position C-17, similar to that of cholesterol, though partial or complete metabolic removal of this side-chain may result in ecdysteroids possessing a C_{24} (e.g. sidisterone), C_{21} (e.g. poststerone) or C_{19} (e.g. rubrosterone) parent skeleton [2]. The side-chain may contain unsaturated bonds or can form a cyclic ether or a 5- or 6-membered lactone ring [9]. Usually several hydroxyl groups are attached to the carbon skeleton of ecdysteroids, typically at the position C-2, C-3, C-14, C-20, C-22 and C-25, which, together with occasionally appearing further carbonyl groups other than that at C-6, result in a relatively high polarity of these compounds. The occasional glycosidic, ester or ether bond formation of the hydroxyl groups further contribute to the structural variability of ecdysteroids [2].

1.2. Biological activity and potential application of ecdysteroids

Based on their ability to disrupt hormonal processes in arthropods upon ingestion, ecdysteroids have been investigated to develop safe natural insecticides. But due to their high polarity, these compounds do not penetrate the exoskeleton of insects, therefore they cannot be used efficiently as a spray in the control of pests. Bisacylhydrazines, synthetic analogs of ecdysteroids (e.g. *tebufenozide, methoxyfenozide*), however, appear to be good candidates for pest control and have reached the market [10, 11]. Ecdysteroids exert their regulating effect in insects by targeting a heterodimeric complex comprising two members of the steroid hormone receptor superfamily, namely the ecdysteroid receptor (EcR) and the product of the *ultraspinacle* gene (USP) [10]. Since these proteins do not naturally occur in vertebrates, and because the structure of ecdysteroids significantly differs from that of mammalian steroid hormones, one could expect a low toxicity of ecdysteroids on mammals [6, 12]. Indeed, it is extremely low: for example, the LD_{50} value of 20E in mice is 6.4 g/kg for intraperitoneal injection and more than 9 g/kg after oral application [13]. Ecdysteroids do not appear to interact with the vertebrate steroid hormone system, still, they have been found to exert numerous beneficial effects on mammals as extensively reviewed from several aspects [14].

Probably the most important and by far the most investigated of these effects is their anabolic activity, which has been in the focus of scientific research since the late 1960s. The studies involved demonstrated increase in growth in a number of vertebrate animals, like rats [15], sheep [16], quails [17], pigs [18], and mice [19,20]. It was shown by *Otaka et al.*, that some ecdysteroids (20E, turkesterone and cyasterone) cause the stimulation of protein synthesis in mouse liver [21-24]. *Gorelick-Feldman et al.* demonstrated that application of some ecdysteroids (20E, turkesterone, polypodine B and ponasterone A) and ecdysteroid containing plant extracts can stimulate protein synthesis by up to 20% in mouse and human myotubules [25], while in a study carried out in our research group by *Tóth et al.*, 20E was shown to increase muscle fiber size in rats [26]. Though numerous results of *in vitro* and/or

animal studies are available concerning the anabolic activity of ecdysteroids, there have been only a few human trials executed so far, typically with low number of patients [27,28].

In spite of the large number of studies in the topic, the exact mechanism of action of ecdysteroids exerting anabolic activity is still not fully understood and needs further clarification. In a mouse skeletal muscle cell line (C_2C_{12}), 20E caused a rapid elevation in intracellular calcium that led to sustained Akt-activation and increased protein synthesis. This effect could be inhibited by a G-protein coupled receptor (GPCR) inhibitor, a phospholipase C (PLC) inhibitor and a phosphoinositide kinase-3 (PI3K) inhibitor, which suggest the involvement of these elements in the molecular pathway behind 20E's anabolic activity [25,29]. As already mentioned above, ecdysteroids do not show the typical adverse side-effects associated with vertebrate steroid hormones: no androgenic, estrogenic (or antiestrogenic) effects were reported [30,31]. As it was concluded by *Báthori et al.*, these compounds do not appear to interact with vertebrate nuclear steroid receptor systems [14], and it is tempting to hypothesize that some membrane-bound receptors are involved in their pathway [29].

It is a question whether ecdysteroids exert their anabolic activity directly or their metabolites are responsible for their biological effect. The metabolism of 20E in mice was studied by *Kumpun et al.* – in their experiment, they injected 20E intraperitoneally to mice and collected urine and faeces to identify metabolites in them. They found that the metabolism of 20E involves dehydroxylation at C-14 and side-chain cleavage between C-20 and C-22 as major steps, thus yielding poststerone (**51**), 14-deoxy-20-hydroxyecdysone and 14-deoxypoststerone as main metabolites, which then undergo several metabolic steps of reduction [32]. *Ramazanov et al.* gave 20E to rats (50 mg/kg) directly into their stomach with a special probe and collected urine over the following 10 days. Besides unchanged 20E, three new metabolites were isolated. According to their observation, metabolic conversion resulted in the removal of the 7-en-6-one chromophore group, dehydroxylation at C-22 and epimerization at C-5 [34].

It appears that 20E is rapidly eliminated in mammals. In mice, the half-life for 20E was measured to be 8.15 min [35], while in lambs 0.2, 0.4 and 2 hours after intravenous, oral and intramuscular administration, respectively [36]. *Simon and Koolman* investigated the pharmacokinetic behavior of 20E in a human volunteer, measuring an effective half-time of elimination of 9 hours after oral administration [36]. Both fecal and urinary routes appear to be used for the elimination of 20E [35].

Despite the absence of properly designed clinical studies, the anabolic activity of ecdysteroids – with special regards to 20E – has been utilized by athletes and body builders for decades by consuming food supplementary products containing mainly 20E. These ecdysteroid-containing products are marketed all around the world with the purpose of muscular development or for the promise of other health benefits [37, 38]. Description of numerous beneficial effects of 20E supplementation can be found on the internet, though it is hard to evaluate the reliability of these statements, since most of them have no

human studies behind. The relative efficacy and safety of these ecdysteroid-containing products is still not confirmed satisfactorily, and requires further research.

Above its anabolic action, 20E is also considered as a "general strengthening", roborant, or rather adaptogenic substance. Preparations made from *Leuzea carthamoides* – a plant containing high levels of ecdysteroids, especially 20E – were observed to have such an effect. For example a green tea, called '*maralan*' is made from this plant and consumed extensively in Central Europe to improve general well-being, increase appetite and improve digestion [13, 38]. 20E was also shown to exert some immunomodulatory effects in mice, rats and humans [40, 41, 42].

20E was also found to affect the carbohydrate and lipid metabolism. In multiple experiments carried out on rodents, it has been shown, that orally-supplied 20E can reduce induced hyperglycaemia, stimulate glucose incorporation into glycogen in the liver, and enhance glucose utilization by tissues in general by increasing tissue sensitivity to insulin [43-46]. Therefore, ecdysteroids might even find a potential application in type II diabetes. Ecdysteroids also exert hypocholesterolemic effect by reducing the biosynthesis of cholesterol, and enhancing its catabolism, promoting its conversion into bile acids [43, 47].

When applied topically on the skin, ecdysteroids exert wound healing effect which is associated with the stimulation of keratinocyte differentiation [6, 48]. Ecdysteroids also show psoriasis-inhibiting [49] and anti-inflammatory effects [50], which demonstrates a potential use of ecdysteroids as components of cosmetics, and accordingly some patents aiming at this purpose were submitted in the past few decades [51-53].

It is worth noting, however, that even though numerous beneficial biological effects of ecdysteroids on vertebrates can be found in scientific literature, the majority of these findings are limited to *in vitro* and animal studies, and therefore it is difficult to make a sound judgment about how and to what extent ecdysteroids affect the human body. Further clinical studies are required to critically investigate the real value of the above mentioned results and potential applications. Nevertheless, ecdysteroids are certainly an exciting group of natural compounds from many chemical and biological aspects.

1.3. Selected examples for the semi-synthesis of ecdysteroids

Although ecdysteroids of natural origin show great structural variability, this diversity can be even more enhanced by semi-synthetic transformations. Nevertheless, chemical modifications have also been recognized as a reasonable strategy to obtain less accessible, minor natural ecdysteroids from the most widespread phytoecdysteroids (particularly 20E). Numerous publications dealt with chemical transformations of ecdysteroids, as reviewed recently [54]. Hereinafter, those examples are briefly introduced that are related to the subject of this Ph.D. thesis.

Considering that the highly available ecdysteroids in nature (e.g. 20E) which are good candidates for semi-synthesis are relatively polar, and possess several hydroxyl groups in their structure, using protecting groups to ensure selective transformations is frequently applied. Such example is the formation of dioxolane derivatives by protecting the vicinal hydroxyl groups (diols) in the starting material. Usually, for such purpose, a simple ketone (e.g. acetone) is used which under acidic condition – provided by phosphomolybdic acid or tosylic acid – goes into reaction with the susceptible diol (the 2,3- and/or the 20,22-diol in case of 20E) to form a dioxolane ring [9, 54, 55]. However, this kind of reaction may not only serve as an intermediate step – *viz.* protecting diols – in the synthetic procedure, but, through extending the range of the applied reagent to other ketones or aldehydes, it may also increase the number of novel ecdysteroid derivatives available for biological studies. This consideration – amongst others – is manifested in the objectives of this dissertation.

The base-catalyzed autoxidation of 20E was reported by *Suksamrarn et al.* [56], who used a 2% aqueous methanolic NaOH solution for their reaction. Besides several minor products, they identified 9α,20-dihydroxyecdysone and calonysterone as main products. As their goal was to obtain calonysterone in a higher quantity, they did not pay much attention to the other minor side products. The two main products were tested for molting hormone activity, and showed extremely low or no activity.

Using common oxidizing agents (such as $NaIO_4$, Jones reagent, $KMnO_4$, etc.) instead of molecular oxygen, results in a more severe change in the structure of the starting ecdysteroid molecule, causing side-chain cleavage between C-20 and C-22, and gives poststerone (**51**) as major product [56, 57, 58]. As it was mentioned above, this compound was observed as a major metabolite of 20E [32], and therefore its bioactivity may be of particular interest.

Two opposite ends of the ecdysteroid molecule (e.g. 20E) can be targeted by combining side-chain cleavage with dioxolane formation. Such products are obtained by taking poststerone (**51**) into reaction with different aldehydes and ketones under acidic conditions. According to our best knowledge, only one such compound has been synthesized (**56**) so far, which was an intermediate product in the synthesis of ecdysteroid derivatives with an isoxaline ring in their side chain [59]. By preparing other dioxolane derivatives of poststerone (**51**), the combined effects of the removal of the side-chain and the dioxolane formation on biological activity can be observed, and this may lead to further interesting bioactive ecdysteroid derivatives.

Though fluorinated compounds are not common in nature, a large proportion (*ca.* 20-25%) of the commercialized pharmaceutical drugs contains at least one fluorine atom [60]. Special properties of the fluorine atom, such as strong electronegativity, small size and the low polarizability of the C-F bond, can have great impact on the biological activity and frequently also on the metabolic stability of a molecule, therefore fluorination of natural products appears to be an increasingly attractive strategy to obtain

new leads for drug discovery [61]. Nonetheless, only a few fluorinated ecdysteroids have been synthesized so far. Such an example is the fluorination of 20-hydroxyecdysone 2,3;20,22-diacetonide (**20**), performed by *Tomás et al.* aiming to prepare 25-fluoroponasterone A [62]. In their work, the acetonide moieties served as protecting groups and were eventually removed by acidic hydrolysis, and the target compound was tested in an insect bioassay. However, to our best knowledge, fluorinated ecdysteroids have not been reported for their activity on any bioassays related to mammals. A closer inspection of the above mentioned fluorinating reaction, as well as testing the antitumor potential and chemo-sensitizing activity of the synthesized products constitutes a part of this dissertation.

1.4. Previous studies of our group substantiating the present work

The research on phytoecdysteroids has a several decades-long tradition at our department, accounting for the discovery of *ca*. one-fourth of the natural ecdysteroids known to date. Our research group's interest turned towards the problem of multi-drug resistance (MDR) in cancer a few years ago, when 20-hydroxyecdysone 2,3;20,22-diacetonide was suddenly identified as a very strong modulator of the resistance of a murine MDR cancer cell line transfected to express the human ABCB1 transporter, commonly referred to as P-glycoprotein or P-gp [9]. MDR is a major cause of failure in cancer chemotherapy [63]. Drug resistance of cancer cells is often a consequence of the up-regulation of efflux pumps (e.g. the above-mentioned P-gp, as the one with the highest incidence) that excrete the anticancer drug from the cell [64]. Much effort has been put into finding efficient inhibitors – including active natural compounds – of these efflux pumps [65, 66]. Unfortunately, even after decades of intensive research, a clinically effective inhibitor has not been identified, which underlines the need for novel approaches in fighting MDR.

Concerning related bioactivities of ecdysteroids, altogether 58 compounds representing a significant structural diversity, including 39 natural and 19 semi-synthetic derivatives were tested in the above-mentioned study by *Martins et al.* [9]. The compounds were tested for their *in vitro* activity against an L5178 mouse T-cell lymphoma cell line and against its sub-cell line overexpressing the human ABCB1 efflux transporter as a result of transfection with pHa *MDR*1/A retrovirus. Three different tests were performed in order to investigate 1) the anti-proliferative activity of the compounds, 2) how they modulate the efflux of rhodamine 123 mediated by the ABCB1 transporter, and 3) whether they show synergism, antagonism or additive effect when used in combination with doxorubicin, a well-known anticancer drug and substrate of the efflux pump. It was found that the tested compounds showed very weak anti-proliferative or cytotoxic activity, with the majority having IC_{50} values higher than 90 µM. Some less polar ecdysteroids (acetonides) were able to increase the accumulation of rhodamine 123 in the cancer cells, indicating that these compounds can inhibit the efflux function of the ABCB1 pump. Ecdysteroids were tested in combination with doxorubicin by using the checkerboard microplate

method in order to quantitatively describe the interaction on the resistant cell line. Amongst the tested compounds, several “classical”, polar ecdysteroids, including 20E, showed weak antagonism. On the other hand, less polar compounds – mostly acetonides, with special regards to 20-hydroxyecdysone-2,3;20,22-diacetonide (**20**) – showed strong synergistic activity in combination with doxorubicin, representing an MDR reverting effect [9]. It was of particular interest, that potentiation of the cytotoxic activity of doxorubicin did not correlate with the effect on rhodamine 123 accumulation within the cancer cells representing efflux pump inhibition, therefore mechanism of action other than a functional ABCB1 inhibition should likely (also) be attributed to these compounds. This observation highlights the promising potential in the preparation and investigation of further less polar ecdysteroid derivatives, which manifested in the objectives of the Ph.D. work presented in this dissertation.

As it was mentioned above, acetonide groups are widely used in organic synthesis as protecting groups of vicinal diols: an acetonide group is usually very easy to synthesize as well as to subsequently remove it by acidic hydrolysis to obtain the unaltered diol [54]. Accordingly, the acid sensitivity of **20** was also studied in order to answer the question whether a chemo-sensitizing ecdysteroid diacetonide might undergo acidic decomposition after oral administration and thus eventually exert the opposite effect (i.e. increasing resistance as for example 20E). By using near gastric pH (1.48), it was found that although **20** indeed undergoes decomposition (calculated half-life was 7.30 minutes), the primary product is the 20,22-monoacetonide derivative **6** – which also displayed a mild synergism with doxorubicin in the combination studies –, and full decomposition to the antagonistic 20E is minimal with only 5.64% of 20E present after 1 hour of acidic treatment [9]. This suggests that even though such a low pH is indeed unfavorable due to decreasing the activity of ecdysteroid diacetonides, their biological activity could likely be retained with parenteral application or by using e.g. enterosolvent formulations. Concerning the biocompatibility and metabolic stability of acetonide groups, triamcinolone acetonide, a well-known anti-inflammatory steroid drug, has been clearly described for not acting like a prodrug: the acetonide group stays on the molecule throughout the metabolic process and the drug acts as such [68]. Moreover, in a most recent study from our group, **20** was found to have significantly prolonged pharmacokinetics in rats as compared to that of the rapidly eliminating 20E, which suggests an increased metabolic stability [69]. These findings provide further support to the presumption that ecdysteroid dioxolanes should be stable enough to retain their biological activity throughout the metabolic process, and therefore the preparation of novel ecdysteroid dioxolanes deserves further attention.

Based on their findings, *Martins et al.* made the following structure-activity relationship (SAR) interpretations (see **Fig. 3.**) [9]:

- The presence of a hydroxyl group at C-1 increases synergism with doxorubicin.
- The lack of the 2-OH strongly increases synergism.

- Epimerization of the 3-OH group increases synergism.
- Apolar groups (acetate, acetonide) attached to the 2,3-diol significantly enhance synergistic effect.
- In case of the polar compounds, presence of the 5β-OH group counteracts the antagonistic effect.
- Presence of an 11α-OH contributes to antagonism with doxorubicin.
- Epimerization of the 14-OH group enhances the synergistic effect.
- Apolar groups (acetate, acetonide) attached to the 20,22-diol significantly increase synergism.
- Removal or modification of the side-chain results in enhanced synergism.

Making the parent ecdysteroid compound less polar at the 2,3-diol and the 20,22-diol by attaching an apolar moiety to the molecule or by removing / modifying the side chain is beneficial concerning MDR-modulating activity, and might result in enhanced synergism when applied in combination with doxorubicin.

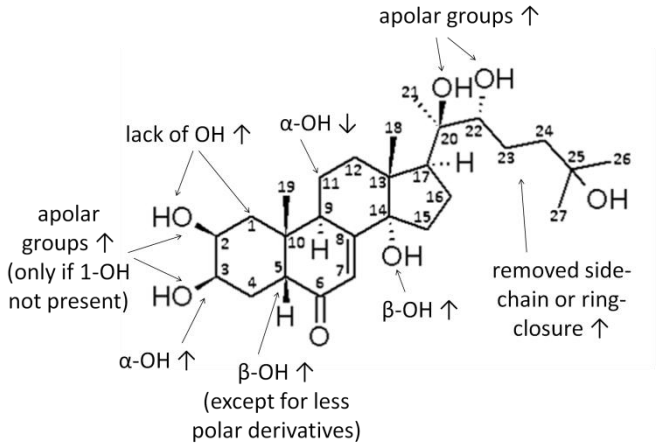


Figure 2 – Structure-activity relationships (SAR) determined by *Martins et al.* [9].
Up/down arrows represent effect of the indicated substituent on the strength of synergism with doxorubicin

The way ecdysteroids exert their activity on MDR has not been identified, but based on the results of *Martins et al.* [9], it could be suspected that a direct ABCB1 inhibition is not or not the only mechanism of action. As it was mentioned earlier, according to *Gorelick-Feldman et al.* [25, 29], PI3K-mediated Akt-activation is connected with the anabolic activity of 20E. Also, *Constantino et al.* demonstrated that muristerone A and ponasterone A – two ecdysteroids showing similar polarity to that of 20E – induce Akt-phosphorylation in a PI3K-dependant fashion [71]. The PI3K/Akt pathway plays a central role in regulating cell death *e.g.* through Akt mediated inhibition of the Bcl-2-associated death promoter (BAD) protein [72]. Moreover, Akt is frequently overexpressed in cancer cells, and it was recently found that its hyper-activation sensitizes cancer cells to oxidative stress induced apoptosis [73, 74]. Altogether, this pathway might be suspected to be involved in the resistance reversal activity of ecdysteroids on the studied MDR cells, while the exact mechanism of action is still to be clarified.

1.5. Objectives

As shown above, there seems to be a great pharmacological potential in particularly the relatively less-polar ecdysteroid derivatives as potential modulators of MDR. Accordingly, the following goals were set up for the Ph.D. work presented in this dissertation:

1. The preparation of new semi-synthetic ecdysteroid derivatives with potent biological activity. In order to increase the chance of finding new active compounds and to obtain further insight into the SAR of ecdysteroids, different kinds of structural modifications and materials were chosen. The proposed structural modifications included the formation of dioxolanes, autoxidation of ecdysteroids, side-chain cleavage and fluorination.

2. Biological evaluation of the obtained ecdysteroids. Scientific co-operations were planned to utilize for studying the biological activity of the synthesized compounds. With these tests, the following bioassays were targeted: 1) the *in vitro* anti-proliferative activity of the synthesized compounds on various sensitive and MDR cancer cell lines, 2) functional ABCB1 efflux pump inhibition, 3) the *in vitro* anti-proliferative activity in combination with doxorubicin or other chemotherapeutics and/or 4) their effect on the Akt-phosphorylation.

3. Establishing new structure-activity relationships (SARs). Based on the results of the biological assays, we intended to investigate how structural modifications on the tested compounds affect their biological activity, in order to set further research directions.

2. MATERIALS AND METHODS

2.1. Standard ecdysteroid samples

20-hydroxyecdysone (20E, **1**, 90% originated from the roots of *Cyanotis arachnoidea*) was purchased from Shaanxi KingSci Biotechnology Co. Ltd. (Shanghai, China), and further purified by crystallization from ethyl acetate : methanol (2:1, *v/v*), so that purity of 20E utilized for the semi-synthesis was 97.8%, by means of HPLC-DAD, maximum absorbance within the range of 220-400 nm.

Natural ecdysteroids – dacryhainansterone (**2**), 2-deoxy-20-hydroxyecdysone (**3**), ajugasterone C (**4**) and polypodine B (**5**) – were previously isolated from Ajuga, Serratula and Silene species and fully characterized by our research group [9, 75-78].

2.2. Semi-synthetic procedure of ecdysteroid derivatives

2.2.1. Preparation of 20,22-monosubstituted ecdysteroid derivatives **6**-**19**

The starting material, 20E (**1**), was dissolved in methanol (10 ml, Merck) to a final concentration of 100 mM or 25 mM in case of compounds **13**-**16**, and the corresponding reagent (**6:** acetone, **7:** butyraldehyde, **8:** valeraldehyde, **9:** 3-pentanone, **10:** methyl isobutyl ketone, **11,12:** vanillin,**13,14:** cinnamaldehyde, **15,16:** 4-benzyloxybenzaldehyde, 10 equiv. each; **17:** acetophenone, 50 equiv.; **18:**

methyl ethyl ketone, 100 equiv..; and **19:** benzaldehyde, 5,0 grams) was added to the solution. Phosphomolybdic acid (1.0 g, or in case of compounds **13, 14**: 0.5 g) was added and the mixture was stirred at room temperature for 10 min (in case of compounds **17**-**19**: 60, 45 and 5 min, respectively). The reaction was terminated by neutralizing the pH with a 5% aq. solution of $NaHCO_3$ (Merck), then methanol was evaporated until only water was present, and the products were extracted with methylene chloride. After evaporating to dryness, the residue was subjected to chromatographic purification.

Compounds **6**, **9** and **10** were recrystallized from acetonitrile without chromatographic purification. For the purification of compounds **7**, **8**, **13**, **14**, **17**, **18**, **19**, rotational planar chromatography (RPC) was applied with gradient elution, using 80 ml solvent in each step. As solvent systems, appropriate mixtures of ethyl acetate : ethanol : water (**7** and **8**) or cyclohexane : ethyl acetate (**13**, **14**, **17**, **18**, **19**) were used. In case of compounds **13** and **14**, after combining the corresponding fractions and drying, they were subjected to reverse phase HPLC purification applying a Zorbax XDB-C8 5µm 9.4 x 250 mm column with 75% aq. methanol as eluent at a flow rate of 3 ml/min. Compounds **11**, **12**, **15** and **16** were first purified by means of column chromatography (CC), applying solvent systems of ethyl acetate : ethanol : water. In the second step, the combined, dried fractions were subjected to RP-HPLC using the previously mentioned column with 70% (**11**, **12**) or 80% (**15**, **16**) aq. methanol as eluent at a flow rate of 3 ml/min. The yields were: **6** (236.6 mg, 45.4%), **7** (116.2 mg, 21.7%), **8** (142.8 mg, 26.0%), **9** (183.5 mg, 33.4%), **10** (71.9 mg, 25.2%), **11** (156.3 mg, 25.4%), **12** (67.0 mg, 10.9%), **13** (27.0 mg, 18.5%), **14** (13.9 mg, 9.4%), **15** (67.3 mg, 39.9%), **16** (33.7 mg, 20.0%), **17** (196.8 mg, 33.8%), **18** (279.1 mg, 52.2%), **19** (292.5 mg, 51.4%).

*2.2.2. Preparation of 2,3;20,22-disubstituted ecdysteroid derivatives **20**-**28** in one step*

20E (**20:** 50 mg; **21**-**23:** 200 mg; **24**-**28:** 480 mg) was dissolved in acetone (50 ml, compound **20**) or methyl ethyl ketone (20 ml, compounds **21**-**23**) or benzaldehyde (5 ml, compounds **24,25**) or methanol (10 ml, compounds **26**-**28**), and the reagent was added to the solution (**26:** butyraldehyde, **27:** valeraldehyde, **28:** 3-pentanone, 100 equiv. each). Phosphomolybdic acid (**20**-**23:** 20 mg; **24**-**28:** 1.0 g) was added as catalyst, and the mixture was stirred at room temperature for 5 min (in case of compounds **26**-**28:** 30 min). The reaction was worked up as described above in chapter 2.2.1.

Reaction products were subjected to purification by RPC using appropriate mixtures of *n*-hexane : acetone (15:1, 10:1, 4,3:1, 3:1, 2:1 for compounds **20**-**23**) and cyclohexane : ethyl acetate (10:1, 2:1, 1:1,5, 1:4 for compounds **24** and **25**; 1:1, 1:2, 1:3, 1:20 for compound **26**; and 10:1, 2:1, 1:1,5, 1:4 for compounds **27** and **28**). The yields were: **20** (35.1 mg, 60.2%), **21** (15.5 mg, 6.3%), **22** (4.9 mg, 2.1%), **23** (8.4 mg, 3.6%), **24** (36.1 mg, 5.5%), **25** (43.8 mg, 6.7%), **26** (242.4 mg, 41.2%), **27** (134.5 mg, 21.8%), **28** (42.3 mg, 6.9%).

2.2.3. Preparation of 2,3;20,22-disubstituted ecdysteroid derivatives **29**-**36** *in two steps*

Previously obtained 20,22-monosubstituted compounds (**6:** 20.7 mg; **7:** 40.0 mg; **9:** 40.7 mg; **10:** 50.0 mg; **19:** 57.0 mg; **17:** 87.3 mg; **6:** 104.0 mg) were dissolved in methyl ethyl ketone (2 ml, **29, 30**) or in methanol (5 ml) and the reagent (**31**-**35:** acetone, 500 equiv.; **36:** butyraldehyde, 500 equiv.) was added to the solution. Phosphomolybdic acid (**29, 30**: 20 mg; **31**-**36:** 0.5 g) was added to the solution, and the mixture was stirred at room temperature for 5 (**29, 30**) or 60 (**31**-**36**) min. The reactions were worked up according to the method described above in chapter 2.2.1.

The reaction products were purified by means of RPC, applying gradient elution with 80 ml of solvent in each step. Appropriate mixtures of cyclohexane and ethyl acetate were used with the following ratios: 10:1, 2:1, 1:1,5 and 1:4. The obtained yields were as follows: **29** (5.1 mg, 23.1%), **30** (5.1 mg, 23.1%), **31** (10.9 mg, 25.4%), **32** (15.8 mg, 36.2%), **33** (15.5 mg, 28.9%), **34** (24.8 mg, 40.6%), **35** (38.7 mg, 41.5%), **36** (53.0 mg, 46.2%).

2.2.4. Preparation of 2,3-monosubstituted ecdysteroid derivatives **37**-**42**

Compound **37** (27.4 mg, 5.0%) was obtained as a side-product from the synthesis of **28**. In case of compounds **38**-**42**, 20E was dissolved in methanol to a final concentration of 100 mM, then phenylboronic acid (1 equiv.) was added, and the mixture was stirred for 30 min. The reagent (**38**: acetone, 500 equiv.; **39**: propionaldehyde, 3 ml; **40**: valeraldehyde, 3 ml; **41**-**42**: methyl isobutyl ketone, 3 ml) was added to the solution and catalytic amount of *p*-toluenesulfonic acid (or 0.5 g phosphomolybdic acid in case of compound **38**) was added. The mixture was stirred for 60 min, 30 min or 3 days (in case of compounds **38**, **39**, **40**-**42**, respectively) at room temperature. The reaction was terminated by neutralizing the pH with a 5% aq. solution of $NaHCO_3$, then methanol was evaporated until only water was present, and the products were extracted with methylene chloride. After evaporating to dryness, the residue was dissolved in 10 ml ethyl acetate, then 5 ml of a 30% aq. H_2O_2 solution was added. The reaction mixture was stirred for 1 day (or 1 hour in case of compound **38**). Then the reaction mixture was worked up as described in chapter 2.2.1.

For the purification of the compounds, RPC was applied with gradient elution, using 80 ml solvent in each step. In case of compound **38**, mixtures of cyclohexane : ethyl acetate (10:1, 2:1, 1:1.5, 1:4) was used and after combining the corresponding fractions based on TLC check, compound **38** was obtained in the following yield: 13.3 mg (10.2%). To obtain compounds **39**-**42**, RPC was utilized in two consecutive steps, using mixtures of cyclohexane and ethyl acetate in appropriate ratios. In the first step this ratio was 20:1, 8:1, 2:1, 1:2 for compound **39** and 2:1, 1:1, 1:2, 1:5 for compounds **40**-**42**. In the second step the following ratios were applied: 1:2, 1:5 for compound **39**, 2:1, 1:1, 1:2, 1:5 for compound **40** and 1:1, 1:5 for compounds **41** and **42**. After combining the corresponding fractions and evaporating to dryness, yields were the following: **39** (4.5 mg, 0.9%), **40** (15.6 mg, 28.5%), **41** (7.0 mg, 1.3%), **42** (6.3 mg, 1.1%).

2.2.5. Preparation of oxidized ecdysteroid derivatives **43**-**50** *from 20E through base-catalyzed autoxidation and longitudinal study of the reaction*

For the synthesis of compounds **43**-**46**, 1.7 g 20E was dissolved in 50 ml 70% aq. methanol, then 1.0 g NaOH was added. The reaction mixture was stirred for 3 hours, and the reaction was stopped by neutralizing the pH with concentrated acetic acid. The solvent was evaporated under reduced pressure at 40 °C. The dry residue was subjected to CC over silica gel 60 (63-200 µm), eluting with ethyl acetate (22 fractions) and ethyl acetate : ethanol : water 80:2:1 (*v/v/v*, 77 fractions). Fractions 48-70 and 71-99 were combined and evaporated. The first combined fraction (48-70) was subjected to reversed-phase CC on Lichroprep RP-18 (40-63 µm) using a stepwise gradient of 30%, 35%, 40%, 45% and 50% aq. methanol (7 fractions each). Fractions 8-23 were combined and repeatedly purified on the same column with the same gradient; from fraction 10, compound **44** (10.0 mg, 0.6%) was obtained by crystallization from ethyl acetate : methanol (2:1, *v/v*). The combined fractions 11-18 were purified by HPLC on a Zorbax XDB-C8 5µm 9.4 x 250 mm column with 17% aq. acetonitrile as eluent at a flow rate of 3 ml/min, to yield compounds **43** (2.3 mg, 0.1%) and **45** (1.4 mg, 0.1%). The second combined fraction (71-99) obtained from the first silica gel column, was subjected to reversed-phase CC, using a gradient of 30%, 35%, 40% and 50% aq. methanol (34, 5, 5, 5 fractions, respectively). Fractions 21-28, eluted with 30% methanol were combined and subjected to preparative TLC on Kieselgel 60 F_{254} plates with a methylene chloride : methanol : benzene (25:5:5, *v/v/v*) solvent system to obtain compound **46** (5.0 mg, 0.3%).

In case of compounds **47**-**50**, 120 mg 20E was dissolved in 10 ml 10% aq. methanol, then 100 mg NaOH was added. The mixture was stirred for 1 day (**47**, **49**) or 8 or 15 hours (**48**, **50**). Then the reaction was terminated by neutralizing the pH as described above, and the mixture was evaporated to dryness. In case of compounds **47** and **49**, the reaction mixture was fractionated by centrifugal partition chromatography (CPC) in ascending mode, with a solvent system of ethyl acetate : methanol : water (20:20:1, *v/v/v*) and 20 ml fractions were collected. The combined fractions were evaporated at 40 °C, dissolved in 20% aq. acetonitrile and stored under 8 °C for 1 week. In the meantime, two of the fractions completely converted to compound **47**. In case of compound **49**, the reaction mixture was purified by CPC in the same way as in case of compound **47**, and fractions 9-10 were combined and evaporated under vacuum at 40 °C. The residue was further purified by HPLC, using a Zorbax ODS 5 µm 250 x 9.4 mm column, with 30% aq. acetonitrile as eluent at a flow rate of 3 ml/min, yielding compound **49** (2.0 mg, 1.6%). To obtain compound **48**, the reaction mixture was fractionated by CPC the same way as described for compounds **47** and **49**. Fraction 4 was further purified by HPLC using a Zorbax XDB-C8 5 µm 250 x 9.4 mm column with 30% acetonitrile as eluent at a flow rate of 3 ml/min yielding compound **48** (4.4 mg, 3.7%). In case of compound **50**, the reaction mixture was purified by HPLC, using the same column with 35% aq. methanol at a flow rate of 3 ml/min to yield compound **50** (10.0 mg, 8.1%).

For the longitudinal study of the autoxidation reaction, a 120 mg (0.25 mmol) aliquot of 20E was dissolved, and 100 mg of NaOH was added, in three replicates. The reaction mixture was stirred for 2 days, and samples were taken at 0.5, 1, 2, 3, 4, 5, 6, 15, 24 and 48 h. All samples from the three independently performed reactions were analyzed by HPLC-DAD after neutralizing the pH with a 9.6% aq. solution of acetic acid. A gradient system of aq. acetonitrile was used on a Kinetex XB-C18 5 µm, 4.6 x 250 mm column at a flow rate of 1 ml/min. Concentrations of the compounds in each sample were determined from the AUC values of the corresponding peaks at the maximum absorption wavelength (λ_{max}) of the compound (247, 228, 228, 304, 360 and 260 nm for compounds **1**, **43**, **44**, **46**-**48** and **50**, respectively). Serial dilutions with known concentrations of the previously isolated compounds served as calibration.

2.2.6. Preparation of ecdysteroid derivatives **51**-**55** *obtained by side-chain cleavage*

Poststerone (**51**) was synthesized from 20E by dissolving 2.0 g 20E in 50 ml methanol, then adding 2.7 g (1.5 equiv.) [bis(trifluoroacetoxy)iodo]benzene (PIFA) to the solution, and then stirring at room temperature for 60 min. The reaction was stopped by neutralizing the pH with a 5% aq. solution of $NaHCO_3$, and the mixture was dried. The residue was dissolved in ethyl acetate and filtered through silica to absorb remnants of the reagent. To obtain poststerone (**51**) in a pure form, CPC was applied. A mixture of ethyl acetate : water : methanol was used in a ratio of 20:20:1 in ascending mode, with a flow rate of 10 ml/min. Fractions of 20 ml were collected, then corresponding fractions were combined and evaporated to dryness, yielding poststerone (**51**) (872 mg, 57.8%).

Compounds **52**-**55** were synthesized from compounds **2**, **3**, **4** and **5**, respectively. Each starting material was dissolved in methanol to a final concentration of 100 mM (**2, 3:** 46 mg; **4:** 48 mg; **5:** 50 mg), and then 64.5 mg [bis(trifluoroacetoxy)iodo]benzene (1.2 equiv.) was added. The mixtures were stirred for 60 min at room temperature. The reaction was stopped by neutralizing the pH by using a 5% aq. solution of $NaHCO_3$, and the mixture was evaporated to dryness. The residue was dissolved in ethyl acetate and filtered through silica, then dried. Compounds **52** and **55** were further purified by RPC using a gradient elution with cyclohexane : ethyl acetate 1:1, 1:5 and ethyl acetate : ethanol 12:1, 6:1 with 80 ml solvent in each step. From the collected 23 fractions, corresponding fractions were combined based on TLC check, giving the following estimated yields: **52** (21.6 mg, ~60%), **55** (22.7 mg, ~60%). Compounds **53** and **54** were purified by HPLC applying a Kinetex XB-C18 5 µm 250 x 21.2 mm column with 50 % aq. methanol as eluent at a flow rate of 10.0 ml/min, detecting at 254 nm. The isolated yields were as follows: **53** (19.5 mg, 56.4%), **54** (22.7 mg, ~60%).

2.2.7. Preparation of poststerone dioxolanes **56**-**64**

60 mg (0.166 mmol) of poststerone (**51**) was dissolved in 10 ml of methanol, then the reagent was added to the solution (**56**: acetone, 20 ml; **57**: propionaldehyde, 5 ml; **58**, **59**: butyraldehyde, 5 ml; **60**: valeraldehyde, 5 ml; **61**: methyl isobutyl ketone, 5 ml; **62**, **63**: methyl ethyl ketone, 5 ml; **64**: 3-pentanone, 5 ml). Catalytic amount of *p*-toluenesulfonic acid was added, and the mixture was stirred at room temperature for two days in case of compound **56** and one week in case of compounds **57**-**64**. Then the reaction mixture was quenched with 5% aq. solution of $NaHCO_3$ and diluted with water. The mixture was concentrated by vacuum distillation until only water was present, and the aq. solution was extracted with methylene chloride. The combined organic phases were dried with anhydrous Na_2SO_4 and evaporated to dryness. The products were isolated by RPC on silica gel with appropriate eluents composed of ethyl acetate and ethanol. Compounds **57** and **59**-**63** were further purified by semi-preparative HPLC using isocratic elution with aq. methanol (63%, 70%, 68%, 75% for compounds **57**, **59**, **60** and **61**, respectively, and 65% for compounds **62** and **63**) at a flow rate of 3.0 ml/min, by utilizing an Agilent Eclipse XDB-C8 5 µm 250 x 9.4 mm column. The yields were: **56** (39.2 mg, 58.8%), **57** (16.7 mg, 25.1%), **58** (10.0 mg, 14.5%), **59** (7.3 mg, 10.2%), **60** (7.6 mg, 10.7%), **61** (1.5 mg, 2.0%), **62** (8.9 mg, 12.9%), **63** (7.0 mg, 10.2%), **64** (16.2 mg, 22.7%).

2.2.8. Preparation of fluorinated ecdysteroid derivatives **65**-**68**

120 mg (0.25 mmol) of compound **20** was dissolved in 3 ml of anhydrous methylene chloride at -78°C under nitrogen atmosphere, then (diethylamino)sulfur trifluoride (DAST, 34 µl, 1.2 equiv.) was added dropwise to the solution. The reaction mixture was stirred at room temperature for 75 minutes. On completion, the mixture was quenched with 5% aq. solution of $NaHCO_3$, then the mixture was extracted with methylene chloride. The combined organic layers were dried with anhydrous Na_2SO_4, filtered and evaporated *in vacuo*.

The residue was subjected to CC on silica using a gradient of *n*-hexane and ethyl acetate (8:2, 7.5:2.5, 7:3, 6.5:3.5, 6:4, 4:6, *v/v* each). Ten fractions of 10 ml were collected with each composition, and the combined fractions 8-9, 10-13, 16-22 and 41-55 contained compounds **68**, **67**, **65** ad **66**, respectively. Preparative HPLC was applied utilizing a Kinetex XB-C18 5 µm 250 x 21.2 mm column with 90% aq. acetonitrile as eluent at a flow rate of 10.0 ml/min, detection wavelength was 243 nm. The isolated yields were as follows: **65** (41 mg, 35.3%), **66** (20 mg, 16.6%), **67** (20 mg, 17.2%), **68** (4 mg, 3.3%).

Table 1 - Outline of the semi-synthesis and chromatographic purification of compounds **6-36**

Reaction					Chromatographic purification – step 1			Chromatographic purification – step 2			product
starting material	solvent	reagent	catalyst	duration	***method***	solid phase	mobile phase	***method***	solid phase	mobile phase	
1	MeOH	acetone	Phosphomolybdic acid	10 min	***recrystallization from acetonitrile***			-			**6**
		butyraldehyde			***RPC***	silica	EtOAc-EtOH-H_2O				**7**
		valeraldehyde									**8**
		3-pentanone			***recrystallization from acetonitrile***						**9**
		methyl isobutyl ketone									**10**
		vanillin			***CC***	silica	EtOAc-EtOH-H_2O	***RP-HPLC***	Zorbax XDB-C18	70% *aq.* MeOH	**11**
											12
		cinnamaldehyde			***RPC***		cyclohexane-EtOAc			75% *aq.* MeOH	**13**
											14
		4-benzyloxy-benzaldehyde			***CC***		EtOAc-EtOH-H_2O			80% *aq.* MeOH	**15**
											16
		acetophenone		60 min	***RPC***		cyclohexane-EtOAc	-			**17**
		methyl ethyl ketone		45 min							**18**
		benzaldehyde		5 min							**19**
1	acetone		Phosphomolybdic acid	5 min	***RPC***	silica	*n*-hexane-acetone	-			**20**
	methyl ethyl ketone										**21**
											22
											23
	benz-aldehyde						cyclohexane-EtOAc				**24**
											25
	MeOH	butyraldehyde		30 min							**26**
		valeraldehyde									**27**
		3-pentanone									**28**
6	methyl ethyl ketone		Phosphomolybdic acid	5 min	***RPC***	silica	cyclohexane-EtOAc	-			**29**
											30
7	MeOH	acetone		60 min							**31**
9											**32**
10											**33**
19											**34**
17											**35**
6		butyraldehyde									**36**

Table 2 – Outline of the semi-synthesis and chromatographic purification of compounds **37-68**

Reaction					Chromatographic purification – step 1			Chromatographic purification – step 2			product
starting material	solvent	reagent	catalyst	duration	***method***	solid phase	mobile phase	***method***	solid phase	mobile phase	
1	MeOH	3-pentanone	P.M.acid	30 min	***RPC***	silica	cyclohexane-EtOAc	-			**37**
	MeOH + Phenyl-boronic acid	acetone	*p*-toluene sulfonic acid	60 min				***RPC***	silica	cyclohexane-EtOAc	**38**
		propionaldehyde		30 min							**39**
		valeraldehyde		3 days							**40**
		methyl isobutyl ketone									**41**
											42
1	70% *aq*. MeOH	NaOH	-	3 hours	1. ***CC*** 2. ***CC***	1. silica 2. Lichroprep RP-18	1. EtOAc-EtOH-H_2O 2. MeOH-H_2O	***RP-HPLC***	Zorbax XDB-C8	17% *aq.* CH_3CN	**43**
								recrystallization from EtOAc-MeOH			**44**
								RP-HPLC	Zorbax XDB-C8	17% *aq.* CH_3CN	**45**
								prep. TLC	Kieselgel 60 F_{254}	CH_2Cl_2-MeOH-benz	**46**
	10% *aq.* MeOH			1 day	***CPC***	EtOAc-MeOH-H_2O		***recrystallization from 20% aq. acetonitrile***			**47**
				8 hours				***RP-HPLC***	Zorbax XDB-C8	30% *aq*. CH_3CN	**48**
				1 day					Zorbax ODS		**49**
				15 hours	***RP-HPLC***	Zorbax XDB-C8	35% *aq.* MeOH	-			**50**
1	MeOH	PIFA	-	60 min	***CPC***	EtOAc-MeOH-H_2O		-			**51**
2					***RPC***	silica	cHex-EtOAc-EtOH				**52**
3					***RP-HPLC***	Kinetex XB-C18	50% *aq.* MeOH				**53**
4											**54**
5					***RPC***	silica	cHex-EtOAc-EtOH				**55**
51	MeOH	acetone	*p*-toluene sulfonic acid	2 days	***RPC***	silica	EtOAc-EtOH	-			**56**
		propionaldehyde		1 week				***RP-HPLC***	Eclipse XDB-C8	63% *aq.* MeOH	**57**
		butyraldehyde						-			**58**
		valeraldehyde						***RP-HPLC***	Eclipse XDB-C8	70% *aq.* MeOH	**59**
		methyl isobutyl ketone								68% *aq.* MeOH	**60**
		methyl ethyl ketone								75% *aq.* MeOH	**61**
										65% *aq.* MeOH	**62**
											63
		3-pentanone						-			**64**
20	anhydrous CH_2Cl_2	DAST		75 min	***CC***	silica	*n*-hexane-EtOAc	***RP-HPLC***	Kinetex XB-C18	90% *aq.* CH_3CN	**65**
											66
											67
											68

2.3. Procedures for structure elucidation

Melting points were measured with a Boetius apparatus (Dresden, Germany). Optical rotation was determined with a Perkin-Elmer 341 polarimeter. Mass spectra were recorded on an API 2000 triple quadrupole tandem mass spectrometer (AB SCIEX, Foster City, CA, USA) in positive mode with atmospheric pressure chemical ionization (APCI) or electron-spray ionization (ESI). HRESIMS data were recorded on a Waters-Micromass Q-TOF Premier mass spectrometer (Waters, Milford, MA, USA) equipped with an electrospray ion source used in positive mode.

^{1}H (500.1 MHz) and ^{13}C (125.6 MHz) NMR spectra were recorded at room temperature on a Bruker 500 Avance III NMR spectrometer equipped with cryogenic probe head and on a Bruker Avance 500 NMR spectrometer. Amounts of approximately 0.5-10 mg of compounds were dissolved in 0.1 ml of methanol-d_4 and transferred to a 2.5 mm Bruker MATCH NMR sample tube (Bruker BioSpin Corporation, Rheinstetten, Germany). Chemical shifts are given on the δ scale and are referenced to the solvent (methanol-d_4: δ_C = 49.1 and δ_H = 3.31 ppm). Pulse programs of all experiments (^{1}H, ^{13}C, DEPTQ, DEPT-135, sel-TOCSY (mixing time: 80-120 ms), sel-ROE (300 ms), sel-NOE, gradient-selected (gs) ^{1}H,^{1}H-COSY, ROESY, edited gs-HSQC, gs-HMBC (optimized for 10 Hz)) were taken from the Bruker software library (Bruker BioSpin Corporation, Rheinstetten, Germany). In the one-dimensional measurements, 64K data points were used for the FID, sweep widths: 4000 and 30000 Hz, respectively. For two-dimensional measurements, sweep width in F_2 was 4000 Hz, and all data points (t_2 x t_1) were acquired with 2K x 256. In F_1, linear prediction was applied to enhance the resolution. 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). The NMR signals of the products were assigned by one- and two-dimensional NMR methods using widely accepted strategies [79, 80].

For compounds **65**-**68**, ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectra were recorded at room temperature on a Bruker Avance III 600 MHz spectrometer equipped with a cryo probehead. Amounts of approximately 1-10 mg of compounds were dissolved in 0.6 ml of methanol-d_4 and transferred to 5.0 mm Norell XR-55-7 NMR sample tubes. Other parameters of the NMR measurements and structural elucidation were in accordance with what is described above.

2.4. Biological evaluation of the compounds

2.4.1. Cell lines

In the experiments described in the thesis, ten human derived cell lines were used. Breast cancer MCF7 cells and their sub-cell line obtained by adaptation to doxorubicin, $MCF7_{dox}$, as well as MDA-MB-231, MDA-MB-361 and T47D cells, and the neuroblastoma SH-SY5Y cells were cultured in EMEM media supplemented with nonessential amino acids, 1mM Na-pyruvate, and 10% inactivated fetal

bovine serum ($MCF7_{dox}$ was cultured in the presence of 1 µM of doxorubicin each third passage). Prostate cancer cells PC3 and LNCaP cultured in RPMI 1640 media supplemented with 10% inactivated fetal bovine serum (FBS); in case of LNCaP medium also contained 1mM Na-pyruvate, HEPES, and glucose. Epidermal carcinoma cell line KB-3-1 and its subline KB-C-1 obtained by stepwise adaptation to colchicine cultured in RPMI 1640 media supplemented with 10% inactivated FBS. All ten cell lines were cultured at 37°C and 5% CO_2; all media contained nystatin, 2mM L-glutamine, 100 U penicillin and 0.1 mg streptomycin.

Two mouse lymphoma cell lines were also used: a parental L5178 mouse T-cell lymphoma cell line and its multi-drug resistant $L5178_{MDR}$ derivative obtained by transfecting L5178 cells with pHa MDR1/A retrovirus [81], and it was selected by culturing the infected cells with 60 µg L^{-1} colchicine. Cells were cultured in McCoy's 5A medium supplemented with 10% heat inactivated horse serum, L-glutamine, and antibiotics (penicillin and streptomycin). All cell lines were cultured at 37 °C in humidified air containing 5% CO_2.

For the bioactivity testing of oxidized ecdysteroid derivatives (**43, 44**, **46**-**48** and **50**), C2C12 mouse skeletal myoblasts (BCRC#60083) were used as cell line, purchased from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Taiwan). The cells were seeded in 6 cm dishes and maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) FBS and 1% penicillin/streptomycin solution in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. After reaching 100% confluence, myoblasts were cultured in differentiation medium (DMEM containing 450 mg/dl D-glucose and 10% horse serum), and the medium was changed every 2 days. The cells became skeletal myotubes after 8 days of differentiation, and were subjected to bioassays (see chapter 2.4.5.).

2.4.2. Anti-proliferative assay

Anti-proliferative activities of the ecdysteroid dioxolanes were evaluated by serial-dilution method in 96-well flat-bottom microtiter plates; for detailed protocols of each study, please see the publications attached as Annex. Briefly, 6 x 10^3 cells (in case of L5178 and $L5178_{MDR}$) were added to each well or 1 x 10^4 cells were seeded overnight (in case of MCF7, $MCF7_{dox}$, KB-3-1, KB-C-1, MDA-MB-361, SH-SY5Y, PC3 and LNCaP) and serial dilutions of the compounds were added. Results were evaluated using 10% MTT after 48 or 72 h incubation at 37 °C, 5% CO_2. In all cases, the precipitate formed in the MTT reaction [82] was diluted with 10% SDS-HCl after 4 h. Plates were incubated overnight and optical density was measured at 540 and 630 nm using an ELISA reader (Multiskan EX, Lab System, U.S.). Fifty percent inhibitory concentrations (IC_{50}) were calculated using nonlinear regression curve fitting of log(inhibitor) versus normalized response and variable slope with a least squares (ordinary) fit of GraphPad Prism 5 software.

2.4.3. ABCB1 inhibition assay

Inhibition of ABCB1 function was investigated on $L5178_{MDR}$ cells through the intracellular retention of rhodamine 123, a fluorescent dye, evaluated by flow cytometry [9]. Briefly, 2 x 10^6 cells per ml were treated with 2 or 20 µM of each compound. After 10 min incubation, rhodamine 123 (Sigma) was added to a final concentration of 5.2 µM and the samples were incubated at 37 °C in water bath for 20 min. Samples were centrifuged (2000 rpm, 2 min) and washed twice with phosphate buffer saline (PBS, Sigma). The samples were re-suspended in 0.5 ml of PBS and their fluorescence was measured with a Partec CyFlow flow cytometer (Partec, Münster, Germany). 20 µM of verapamil (Sanofi-Synthelabo, Budapest, Hungary) or 50nM of tariquidar (kindly provided by Dr. Milica Pesic – Institute for Biological Research Sinisa Stankovic, Belgrade, Serbia) was used as positive control.

2.4.4. Combination assays with chemotherapeutics

The combined activity of doxorubicin (Teva, Hungary) and ecdysteroids was determined using the checkerboard microplate method according to our previously established protocol [9]. Briefly, cell suspension (10^4 cells per well) was incubated with doxorubicin and the compound to be tested for 48 h at 37 °C under 5% CO_2. Cell growth rate was determined through MTT staining, as described above. The interaction was evaluated by using the CompuSyn software (CompuSyn, Inc., USA) for the constant ratios, and combination index (CI) values are presented for 50, 75 and 90% of growth inhibition. CI values were calculated by means of the median-effect equation [67], where CI < 1, CI = 1 and CI > 1 represent synergism, additive effect (*i.e.*, no interaction), and antagonism, respectively.

Compounds **1**, **6** and **20** were tested (at 50 µM concentration) in combination with doxorubicin, paclitaxel, cisplatin or vincristine according to the same protocol as described for the anti-proliferative assay (chapter 2.4.2.) for the respective cell lines. In each case, statistical analysis was carried out by one-way ANOVA with Bonferroni's post hoc test, and differences were considered significant at *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. Statistically significant potentiation was considered relevant only when at least a two-time decrease in the IC_{50} of the chemotherapeutic agent was observed.

2.4.5. Bioactivity testing on Akt-phosphorylation

The skeletal myotubes obtained from the C2C12 mouse skeletal myoblasts (see above in chapter *2.4.1.*) were further processed as follows. The culture medium was changed to 10% FBS high-glucose DMEM with or without 30 µg/ml of each test compound. The medium was removed after 24 h, and its glucose content was determined in order to calculate the compounds' effect on the glucose consumption. Since no significant activities were found, this was not further evaluated, nor is discussed in the thesis hereinafter. Fresh medium containing the same concentration of compound

was then added to the cells that were incubated for further 2 h for testing the activity on Akt. The cells were then lysed with 700 µl of 1x sample buffer (62.5 mM Tris-HCl, pH 6.8; 10% glycerol; 2% SDS; 50 mM DTT; 0.0025% bromophenol blue), sonicated for 10-15 s, and heated to 95-100 °C for 5 min. For analysis of proteins, the cell lysate was loaded and separated on 10% SDS-polyacrylamide gels. Proteins were then transferred to PVDF membranes and detected using phosphorylated and total AKT antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA).

3. RESULTS

3.1. Preparation of semi-synthetic ecdysteroid derivatives

3.1.1. Preparation of dioxolane derivatives of 20E (see also: I., II.)[1]

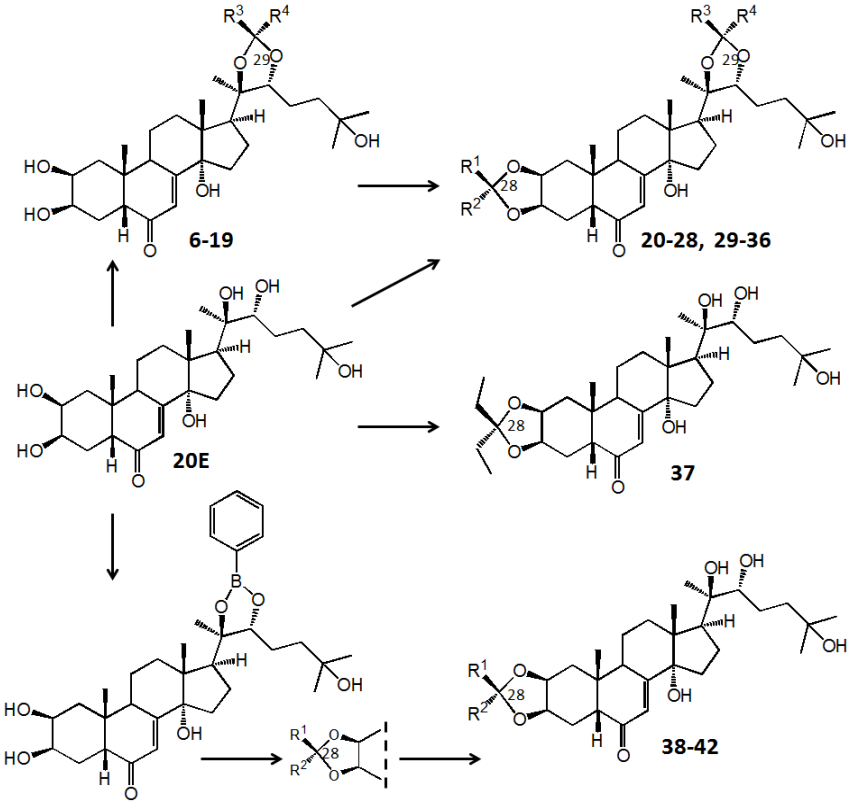
20E (**1**) possesses vicinal diols – one at C-2,3 and another at C-20,22 – that can be involved in acid catalyzed nucleophilic addition (followed by nucleophilic substitution). We carried out this kind of chemical transformation by applying phosphomolybdic acid as a catalyst and various aldehydes or ketones as reagents. As it turned out, from the two diols of 20E, the one at C-20,22 appeared to be more susceptible in this kind of reaction, therefore when less reagent and shorter reaction time were applied, dioxolane formation took place primarily on the side-chain of the starting compound. This way 14 compounds possessing one dioxolane ring in their structure (at C-20,22) were obtained (**6**-**19**) (see **Fig. 3.**). Out of the two substituents connected to C-29, the larger one preferred to take the R^4-position (therefore the stereocenter's configuration is *R*), and lower yield was obtained from the other epimer. For example, compounds **12** and **16** were obtained in 10.9% and 20.0% yields, respectively, in contrast with compounds **11** and **15** (25.4% and 39.9%, respectively) representing the opposite configuration at C-29.

The application of larger amounts of the reagent and/or longer reaction times enabled the formation of 2,3;20,22-disubstituted dioxolane derivatives (**20**-**28**, see **Fig. 3.**). Also in this case, the larger substituent at C-29 preferred to take the R^4-position – constituting *R*-configuration of the stereocenter – , while large moieties at C-28 preferentially took R^1 (with the exception of **24** and **25**, where similar yields were obtained: 5.5% and 6.7%, respectively). For the synthesis of 2,3-substituted dioxolanes, the gradually decreasing reactivity with the increase of the size of the reagent was a limiting factor: larger aldehydes or ketones (mainly those with a substituted aromatic ring) could not be coupled with the C-2,3 diol.

Compounds with two different substituents at C-2,3 and C-20,22 (**29**-**36**, see **Fig. 3.**) could be obtained when a 20,22-monosubstituted dioxolane derivative was subjected to a reaction with another aldehyde or ketone. This second reagent was usually a small compound (*viz.* acetone, or butyraldehyde in case of **36**), therefore steric hindrance didn't affect badly the dioxolane formation, resulting in relatively acceptable yields (see chapter 2.2.3.). Similarly to the synthesis of compounds **20**-**28**, higher amounts of the reagent and mostly longer reaction times were applied in order to countervail the lower reactivity of the C-2,3 diol.

[1]reference to own publications related to the thesis

Selective dioxolane formation at the less reactive C-2,3 diol required a preceding step of protecting the C-20,22 diol with a removable protecting group (phenylboronic acid). This protecting group coupled selectively to the diol on the ecdysteroid side-chain and could be removed by applying H_2O_2 after the dioxolane ring has been formed at C-2,3 with an aldehyde or ketone. As a result, six 2,3-monosubstituted dioxolane derivatives were obtained from 20E (compounds **36**-**42**, see **Fig. 3.**), with the exception of **37**, which compound arose as a side product from the synthesis of **28** (see chapter 2.2.4.). Structures of the prepared ecdysteroid dioxolanes (**6**-**42**) are shown on **Fig. 3.**



	R^1	R^2	R^3	R^4
6	-	-	-Me	-Me
7	-	-	-H	-*n*-Pr
8	-	-	-H	-*n*-Bu
9	-	-	-Et	-Et
10	-	-	-Me	-*i*-Bu
11	-	-	-H	-3-methoxy-4-hydroxyphenyl
12	-	-	-3-methoxy-4-hydroxyphenyl	-H
13	-	-	-H	-*E*-ethenylbenzyl
14	-	-	-H	-*Z*-ethenylbenzyl
15	-	-	-H	-4-benzyloxyphenyl
16	-	-	-4-benzyloxyphenyl	-H
17	-	-	-Me	-Ph
18	-	-	-Me	-Et
19	-	-	-H	-Ph
20	-Me	-Me	-Me	-Me
21	-Me	-Et	-Me	-Et
22	-Et	-Me	-H	-Me
23	-Me	-H	-Me	-Et

	R^1	R^2	R^3	R^4
24	-Ph	-H	-H	-Ph
25	-H	-Ph	-H	-Ph
26	-*n*-Pr	-H	-H	-*n*-Pr
27	-*n*-Bu	-H	-H	-*n*-Bu
28	-Et	-Et	-Et	-Et
29	-Me	-H	-Me	-Me
30	-Et	-Me	-Me	-Me
31	-Me	-Me	-H	-*n*-Pr
32	-Me	-Me	-Et	-Et
33	-Me	-Me	-Me	-*i*-Bu
34	-Me	-Me	-H	-Ph
35	-Me	-Me	-Me	-Ph
36	-*n*-Pr	-H	-Me	-Me
37	-Et	-Et	-	-
38	-Me	-Me	-	-
39	-Et	-H	-	-
40	*n*-Bu	-H	-	-
41	-Me	-*i*-Bu	-	-
42	-*i*-Bu	-Me	-	-

Figure 3 - Structures of the semi-synthesized ecdysteroid dioxolane derivatives (**6**-**42**)

3.1.2. Autoxidation of 20E and longitudinal study of the reaction (see also: IV., V.)

First, autoxidation was conducted following the method of *Suksamrarn et al.* [56], by stirring 20E (**1**) for 3 h at room temperature in 70% aq. methanol containing 2% NaOH. After carefully neutralizing the pH by acetic acid, the mixture was evaporated under reduced pressure at 40 °C, and separation of the products was initiated by CC over silica gel. Even though a few major products could be observed by TLC, the mixture was highly complex and did not reproduce the previous work [56]. Moreover, a significant amount of unchanged 20E also remained. A multistep procedure including repeated CC and HPLC purification steps was needed to obtain four compounds (**43**-**46**, see chapter 2.2.5.).

Subsequent small-scale experiments were performed, and the amount of 20E was monitored by TLC throughout the autoxidation process. An overnight reaction was found preferable. Following this, a more gentle purification procedure was applied: the neutralized reaction mixture was subjected directly to CPC using a mixture of EtOAc-MeOH-H_2O in ascending mode. The crude, neutralized reaction mixture was also tested by HPLC, and, even though a much less complex mixture was detected than before, the compositions of the combined CPC fractions after vacuum evaporation at 40 °C did not correspond to that of the reaction outcome. The main constituents of certain fractions completely disappeared and apparently were converted to calonysterone (**47**), one of our original target compounds that was otherwise not detected prior to the CPC purification (see chapter 2.2.5.).

In an attempt to isolate the detected sensitive intermediates of **47**, further changes were introduced into the purification process of the reaction. Thus, following the CPC separation of the carefully neutralized aq. solution of the reaction products (with the exception of **50**, where HPLC purification was applied right after terminating the reaction), the fractions were evaporated under a nitrogen stream, peaks were collected during the subsequent HPLC purifications under argon atmosphere, and each compound obtained was subsequently dried under a nitrogen stream (see chapter 2.2.5.). Three further compounds, **48, 49** and **50**, were obtained this way, and, surprisingly, only traces of **47** were detected throughout the entire procedure.

Structures of the prepared oxidized ecdysteroid derivatives are displayed in **Fig. 4.**

Tautomeric interconversion occurring between compounds **47** and **48** was investigated. Both compounds were found to be stable not only in neutral but also in acidic (pH = 3) and basic (pH = 8) media at room temperature. On the other hand, at 80 °C, the aq. solution of **48** was found to yield **47** slowly, so that after 4 days the two compounds were present at around 1:1 ratio. A similar heating of compound **47**, however, led to the appearance of only trace amounts of **48**, suggesting that the known form of calonysterone (**47**) is more stable than its newly identified analogue. Further studies revealed that the key step determining which compound is formed takes place in connection with

the elimination of water from compound **50**. Thus, at pH = 8, **50** quantitatively dehydrated to **48**, while **47** is yielded quantitatively from **50** at pH = 3 (see chapter 4.1.2.).

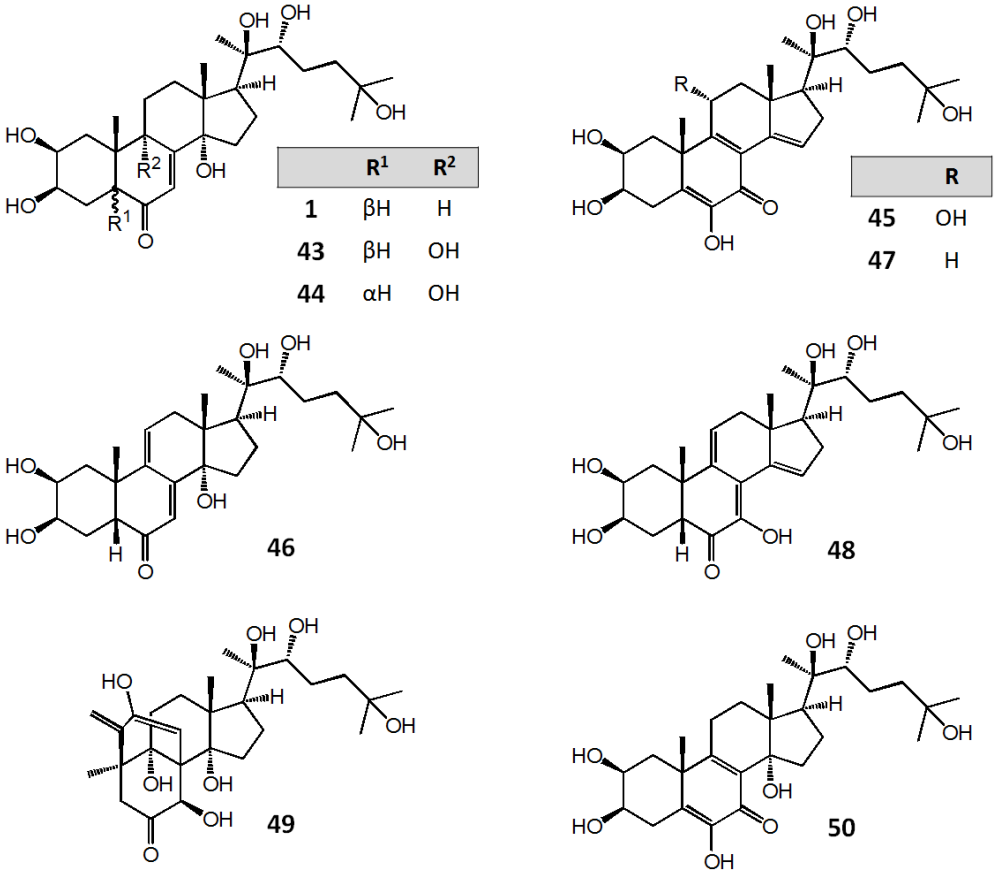


Figure 4 – Oxidized ecdysteroid derivatives (**43**-**50**) obtained from the autoxidation of 20E (**1**)

The formation of each isolated product over various reaction times was studied in three independently performed autoxidation reactions by HPLC at 0.5, 1, 2, 3, 4, 5, 6, 15, 24 and 48 h. Results of this experiment are summarized on **Fig. 5.**

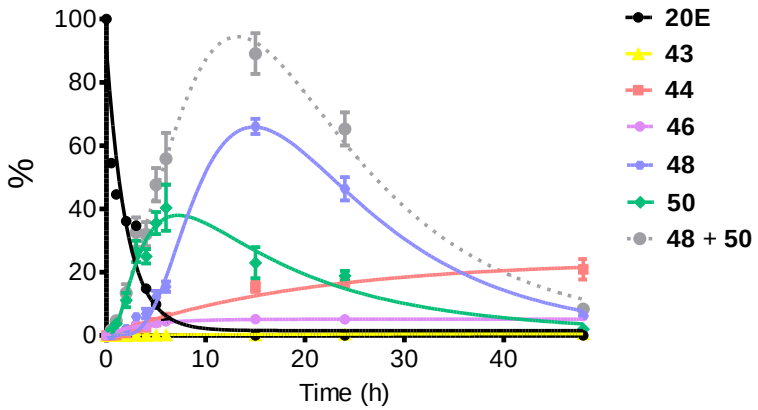


Figure 5 – Amounts of products of 20E obtained at various times as determined by means of analytical HPLC. Error bars represent SEM values from three independent experiments. Nonlinear regression was performed using a one-phase decay (20E, **43**, **44**, **46**) or the log- (Gaussian) (**48** and **50**) exponential models of GraphPadPrism 5. The sum of **48** and **50** and that of their regression curves represent the possible total yields of the desmotropic pair (**47** and **48**).

As seen from the figure, 20E suffers a relatively quick decomposition with a half-life of 1.39 h. Compound **43**, reported previously as a main product under similar conditions [56], was found as a minor component in the presented work, with a maximum detected amount of 0.84%. Compound **44**, its 5α-epimer, was present instead in a significant amount, increasing over a 48 h period, and leading to a plateau of 24.5% based on the regression curve. Since 5α-20E was not detected by HPLC at any time of the reaction, it is suggested that 9α-hydroxylation might be the first step followed by the epimerization at C-5. On using appropriate standards obtained from previous work, the presence of 9β,20-dihydroxyecdysone and 5β,20-dihydroxyecdysone (polypodine B) was also investigated by HPLC, and neither was detected. Due to the very low amounts available and subsequent decomposition, no compound **45** was available as a reference standard for monitoring the time dependence of its formation. Compound **46** was found to be a minor product with a maximum detected amount of 8.72%. Compound **47** could be obtained with a maximum yield of ca. 40% at around 6-7 h by setting the pH as slightly acidic (pH 6-6.5) with a weak acid such as acetic acid prior to evaporation under nitrogen. It was shown by the compositional changes during the reaction that an alkaline pH leads to the elimination of 14-OH group of compound **50** to yield **48** – its highest detected amount was 68.7% at after 15 h. Compound **50** was hypothesized previously as an intermediate to **47** [56], which was confirmed herein.

3.1.3. Preparation of side-chain cleaved ecdysteroids (see also: VIII.)

Since common oxidizing agents (e.g. $NaIO_4$, Jones reagent or $KMnO_4$) proved to give a complex mixture of reaction products in our experiments, we turned our attention to [bis(trifluoroacetoxy)iodo]benzene (PIFA), a hypervalent iodine compound as reagent instead. A relatively large scale reaction was carried out: 2.0 g of 20E (**1**) was dissolved in methanol, 1.5 equivalent of PIFA was added, and the reaction mixture was stirred for an hour, before terminating the reaction and working it up as described in chapter 2.2.6. Purification of the product was performed by means of CPC, and poststerone (**51**) was obtained in a relatively good yield (57.8%): side-chain cleavage took place between the vicinal hydroxyl groups at C-20 and C-22, while the steroid skeleton of the starting compound remained unchanged (see **Fig. 6.**).

The reaction was carried out similarly on other natural ecdysteroids with different substituents on the steroid skeleton, namely dacryhainansterone (**2**), 2-deoxy-20-hydroxyecdysone (**3**), ajugasterone C (**4**) and polypodine B (**5**). Since these compounds were available in lower amounts, smaller scale reactions were performed, and RPC or RP-HPLC was applied to obtain the pure products (**52**-**55**, see **Fig. 6.**) with good yields (see chapter 2.2.6.).

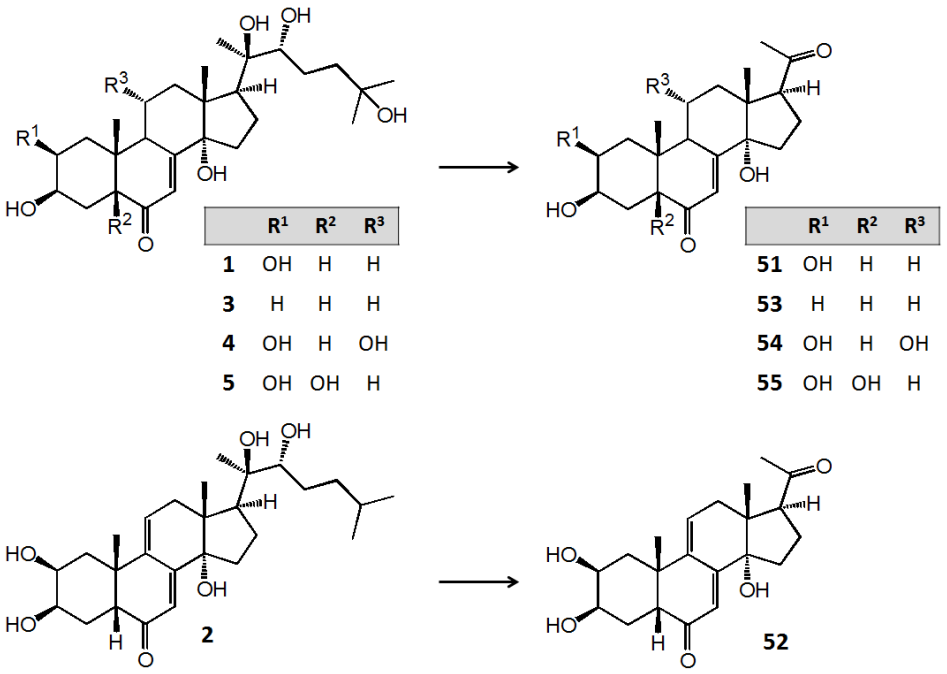
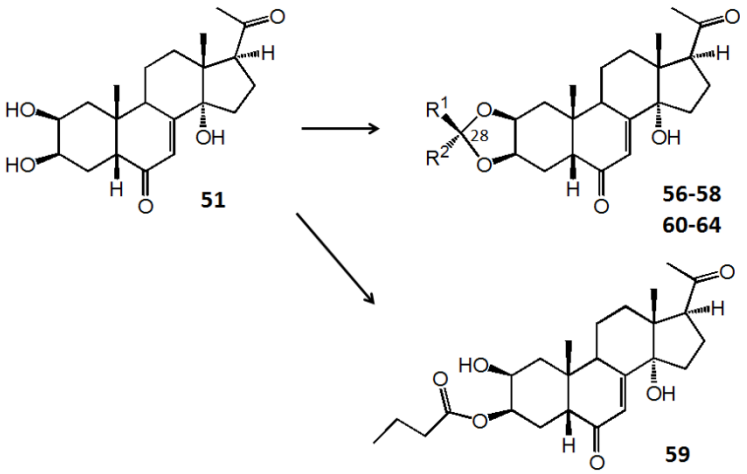


Figure 6 – Ecdysteroid derivatives (**51**-**55**) obtained by side-chain cleavage

3.1.4. Preparation of poststerone dioxolanes (see also: VIII.)

The side-chain cleaved ecdysteroid, poststerone (**51**), has one vicinal diol in its structure at C-2,3, which – based on the observations described in chapter 3.1.1. – is less susceptible to dioxolane formation. Therefore relatively large amounts of reagents were utilized with long reaction times (up to 7 days, see chapter 2.2.7.). Purification of the reaction products was performed by RPC using gradient elution with appropriate mixtures of ethyl acetate and ethanol, and when it was necessary, a second step of purification was applied by means of RP-HPLC with aq. methanol. Unsurprisingly, yields of the products were relatively low (**57**: 25.1%, **58**: 14.5%, **59**: 10.2%, **60**: 10.7%, **61**: 2.0%, **62**: 12.9%, **63**: 10.2%, **64**: 22.7%), with the exception of compound **56**, which – due to the higher reactivity of the applied reagent, acetone – was obtained in a higher yield (58.8%). Amongst the dioxolane derivatives asymmetrically substituted at C-28, larger substituents usually preferred the R^1-position (the stereocenter's configuration is *R*), except for compounds **62** and **63**, where similar yields of the two epimers were obtained (12.9% and 10.2%, respectively). Compound **59**, a poststerone 3-ester, apparently represent an intermediate of the dioxolane formation.

Structures of the synthesized poststerone derivatives (**56**-**64**) are seen on **Fig. 7.**

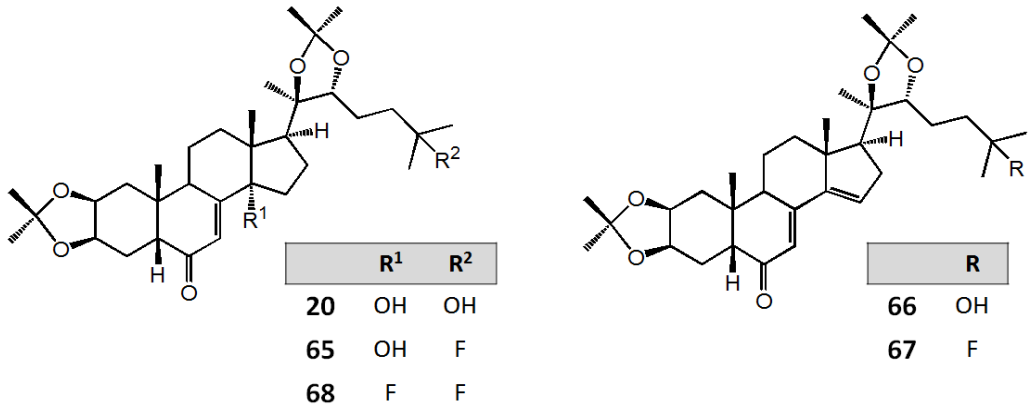


	R¹	R²
56	-Me	-Me
57	-Et	-
58	-*n*-Pr	-
60	-*n*-Bu	-
61	-*i*-Bu	-Me
62	-Me	-Et
63	-Et	-Me
64	-Et	-Et

Figure 7 – Ecdysteroid derivatives (**56**-**64**) synthesized from poststerone (**51**)

3.1.5. Fluorination of 20-hydroxyecdysone-2,3;20,22-diacetonide (see also: VI.)

Following a two-step purification by CC and preparative HPLC, four compounds (**65**-**68**) were obtained from the diethylaminosulfur trifluoride (DAST)-mediated fluorination reaction of **20** (see chapter 2.2.8.). Based on previous reports on fluorination reactions using DAST, it is not surprising, that two kinds of structural modifications took place in this setting: the OH group attached to C-14 was either eliminated as in compounds **66** and **67**, or substituted with fluorine as in compound **68**. Furthermore, fluorine substitution of the 25-OH group led to the production of compounds **65**, **67** and **68**. The obtained yields (**65**: 35.3%, **66**: 16.6%, **67**: 17.2% and **68**: 3.3%) clearly demonstrate that fluorine substitution at position 25 is preferred over fluorination at position 14 – the only product possessing a fluorine substituent attached to C-14 (*viz.* compound **68**) has a meager yield of 3.3%. For the structures of compounds **65**-**68**, see **Fig. 8.**



	R¹	R²
20	OH	OH
65	OH	F
68	F	F

	R
66	OH
67	F

Figure 8 – Ecdysteroid derivatives obtained from the DAST-mediated fluorination of **20**.

3.2. Structure elucidation of the prepared ecdysteroids

3.2.1. Structure determination of dioxolane derivatives **6**-**42** (see also: I., II.)

Molecular mass of the prepared ecdysteroid dioxolanes was determined by APCI and ESI measurements in positive mode, as described in chapter 2.3. The mass spectra of these compounds consist of numerous signals with characteristic differences of 18 units between them, representing the loss of water from the several hydroxyl groups. The loss of a fragment from the parent ion $[M+H]^+$ with the molecular mass corresponding to the reagent used for dioxolane formation was also observed in the spectra. Compounds were dissolved in methanol or acetonitrile for the APCI measurements, therefore several signals of adducts with these solvents (32 and 40 units, respectively) could be observed in the MS spectra.

Structure determination of the dioxolane derivatives of 20E was performed by comprehensive one- and two-dimensional NMR methods (see chapter 2.3.), and it is demonstrated here by the example of compound **27**, a 2,3;20,22-disubstituted ecdysteroid derivative (see **Fig. 9.**). The configuration of the 20-hydroxyecdysone core remained unchanged during the formation of dioxolanes. A comparison of the ^{1}H (δ_H 3.84, 3.95, 3.33 ppm) and ^{13}C (δ_C 68.8, 68.6, 78.0, 78.5 ppm) chemical shifts of 20E in positions 2, 3, 20 and 22, respectively, with the corresponding values obtained for compound **27** (^{1}H: δ_H 4.21, 4.11, 3.64 ppm; ^{13}C: δ_C 72.8, 75.0, 84.9, 85.5 ppm) shows a pronounced deshielding. Thereby, the assignment of these signals clearly reveals the position of the dioxolane moieties. In case of **27** , where $R^1 \neq R^2$ and $R^3 \neq R^4$, the C-28 and C-29 atoms are new stereogenic centers, and their configuration was elucidated by two-dimensional ROESY and selective one-dimensional ROESY experiments. Since the molecular mass of **27** (and of all other dioxolane derivatives of **1**) is higher than 500 Da, the signal/noise value of the selective ROE experiments strongly exceeds that of the selective NOEs, which explains the choice of the former method. Assignment of the H-C(28) atoms (δ_H 4.93, δ_C 105.7 ppm) was supported by the H-2/C-28 and H-3/C-28 HMBC correlations, and that of H-C(29) (δ_H 4.91, δ_C 105.4 ppm) by the H-22/C-29 cross peak, respectively. The selective ROESY experiment irradiating at 4.93 ppm showed contacts with the Hα-2 and Hα-3 atoms proving the α-position of the H-28 atom. The ROESY response obtained irradiating the H-29 signal (δ_H 4.91 ppm) on H-22 (δ_H 3.64 ppm) revealed their *cis* arrangement and the *R*-configuration around C-29. The unambiguous assignments of the signals of the two *n*-butyl groups R^1 and R^4 were achieved by selective TOCSY experiments (irradiation at δ_H 4.93 and 4.91, respectively).

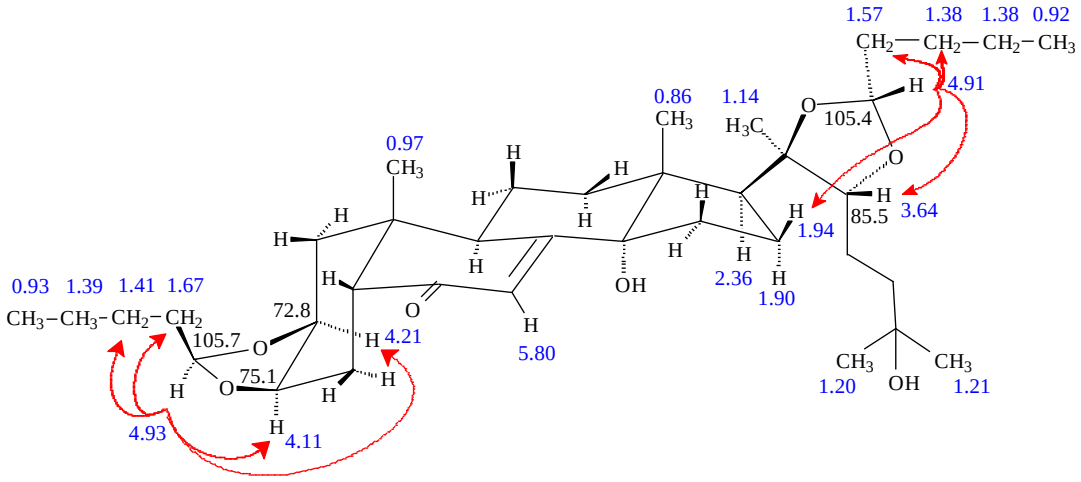


Figure 9 – Stereostructure of **27**. Red arrows indicate the detected ROESY steric proximities, the blue numbers give the characteristic ^{1}H and the black numbers the ^{13}C chemical shifts.

3.2.2. Structure determination of oxidized ecdysteroid derivatives **43**-**50** (see also: IV, V.)

Structure elucidation of the products was performed by means of MS, HRMS and 1D and 2D NMR spectroscopy. Usually, the APCIMS spectrum showed the $[M+H]^+$ peak of the given compound, and HRMS revealed the molecular formula. This latter experiment provided valuable information about the number of new double bonds in the steroid ring system compared to the parental compound 20E.

Two examples of the structure determination are hereby demonstrated with compounds **47** and **44**. In case of compound **47**, LRMS showed a $[M+H]^+$ peak at *m/z* 477, and HRMS revealed the molecular formula $C_{27}H_{40}O_7$. Thus, two new double bonds were incorporated into the steroid ring system as compared to 20E (see the structure in **Fig. 4.**, chapter 3.1.2.). The appearance of a C=O signal at δ_C 181.5 ppm indicated its cross-conjugated arrangement. The HMBC correlations of H_3-19 (δ_H 1.50 / δ_C 42.3 and δ_H 1.50 / δ_C 43.0, respectively) were used to assign the quaternary C-10 and C-1 methylene moieties. In addition, the signals at δ_H 1.50 / δ_C 133.2 and δ_H 1.50 / δ_C 165.3 supported the presence of quaternary sp^2 C= atoms at positions C-5 and C-9, which together demonstrated the B ring as a $\Delta^{5,6}$-7-one-$\Delta^{8,9}$ chromophore. The HMBC cross peak H_3-18 / C-14 (δ_H 1.08 / δ_C 142.6) revealed the splitting of the OH-14α group and the occurrence of a $\Delta^{14,15}$ C=CH ethylene moiety. Compound **47** was identified as calonysterone.

Compound **44** can be characterized, in contrast with the other oxidized ecdysteroid derivatives, with a *trans* A/B ring junction (see **Fig. 10.**). In order to determine the complete ^{1}H and ^{13}C NMR assignments of this compound, the DEPTQ, ed-HSQC, HMBC and 1D selective TOCSY (irradiated at H-5, H-17 and H-22) and selective ROESY (irradiated at H_3-19 and H-5) spectra were measured. The strong H_3-19 / Hβ-4 ROESY (δ_H 1.11 / δ_H 1.75) response, in addition to the H-5 / Hα-1 and H-5 / Hα-3

(δ_H 3.16 / δ_H 2.14 and δ_H 3.16 / δ_H 3.54, respectively) correlations, supported a *trans* A/B ring junction, whereas the H_3-19 / Hβ-11 (δ_H 2.09) and H_3-19 / H_3-18 (δ_H 0.91) steric proximities supported the retained B/C ring junction. Hence, the structure of compound **44** was established as the 5α-epimer of compound **43** [83].

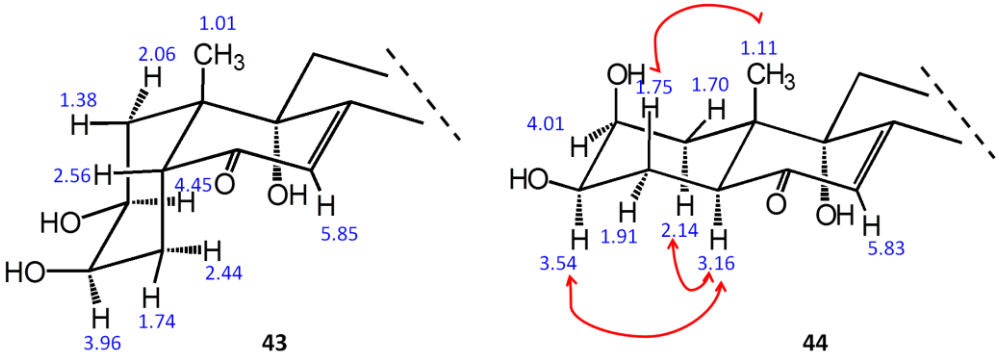


Figure 10 – Comparison of the A/B ring junctions of compound **43** (*cis*) and **44** (*trans*). Red arrows indicate the detected ROESY steric proximities, and blue numbers give the characteristic ^{1}H chemical shifts.

(see also: VIII.)

3.2.3. Structure determination of side-chain cleaved ecdysteroid derivatives **51**-**55** *and* **56**-**64**

In our experiments, side-chain cleavage of ecdysteroids took place between the two hydroxylated carbon atoms C-20 and C-22, and led to the formation of a carbonyl group at C-20 with a characteristic signal (δ_C 212.5 ppm) appearing in the ^{13}C NMR spectra. In case of compounds **51** and **53**-**55**, differences in the presence of a hydroxyl group at C-2, C-5 and C-11 was unambiguously revealed by the interpretation of the corresponding DEPTQ, edHSQC and HMBC spectra. The occurrence of a $\Delta^{9,11}$ C=CH ethylene moiety in compound **52** was unequivocally established by the presence of the signal of H-11 (δ_H 6.34 ppm) in the ^{1}H NMR-, and those of C-9 and C-11 (δ_C 136.4 ppm and δ_C 133.3 ppm, respectively) in the DEPTQ spectra, as well as by the corresponding HMBC correlations. In case of poststerone dioxolanes (**56**-**58** and **60**-**64**), a comparison of the H-2 and H-3 (δ_H 3.86 ppm and δ_H 3.97 ppm), moreover the corresponding C-2 and C-3 (δ_C 68.7 ppm and δ_C 68.5 ppm) values of the parental **51**, with the corresponding ^{1}H and ^{13}C chemical shifts obtained for the dioxolane derivatives (δ_H 4.22-4.29 ppm and δ_H 4.13-4.34 ppm) and (δ_C 72.8-73.6 ppm and δ_C 72.6-75.0 ppm) showed a pronounced deshielding. Similarly to the above mentioned observation with analogous derivatives of **1** (see chapter 3.2.1.), whenever an asymmetrically substituted dioxolane ring was formed, the C-22 atom of the 2,3-dioxolane ring turns into a new stereogenic center. Steric effects made the larger substituent on C-22 to preferentially be situated in the R^1-position, which was confirmed by one-dimensional ROESY experiments. Selective irradiation on the CH_3 signal (δ_H 1.28 ppm) of compound **62** (chosen as example) resulted strong ROESY responses on H-2 and H-3 signals, therefore their *cis* α-positions as well as the *R* configuration of C-22 was established.

*3.2.4. Structure determination of fluorinated ecdysteroid derivatives **65**-**68*** see also: VI.

Compounds **65**-**68** gave parent ion $[M+H]^+$ peaks in their ESI-MS spectra at *m/z* 543, 563, 545, 565, respectively. Appearance of an $[M + H\text{-}H_2O]^+$ peak in the MS spectra of the parental compound **20** and compounds **65**-**66** strongly suggested the presence of at least one remaining hydroxyl group in these compounds, while such peaks were not visible in case of compounds **67** and **68**, suggesting that these latter two compounds possess no free OH-groups. The structures of compounds **65**-**68** were assigned by comprehensive one- and two-dimensional NMR methods, and by also utilizing the spectra of compound **20**.

The mass spectrum of compound **66** suggested that the elimination of a water molecule from **20** took place. This was confirmed by the NMR spectra indicating the presence of a $\Delta^{14,15}$ double bond, represented by the appearance of the chemical shifts of HC-15 (δ_H 6.08 ppm, δ_C 130.1 ppm) and the quaternary C-14 (δ_C 150.4 ppm) observed in the ^{1}H and/or the ^{13}C NMR spectra. Accordingly, the HMQC spectrum revealed seven methylene groups, one less than in compound **20**, while the disappearance of a hydrogen atom from position 15 was also detected in the ^{1}H,^{1}H COSY spectrum leaving only four members in this structural fragment of correlated protons: H-15 (δ_H 6.08 ppm), H_2-16 (δ_H 2.38 ppm and δ_H 2.63 ppm) and H-17 (δ_H 2.08 ppm). These assignments were supported by the HMBC spectrum. Moreover, C-28 and C-29 showed HMBC cross peaks with two methyl groups each analogously to the parent compound **20**, providing further evidence that the acetonide groups at position 2,3 and 20,22 remained intact after the reaction.

In case of compounds **65**, **67** and **68**, evidence for fluorine substitution was found. It is well-known that, as a result of altered substituent increments, the exchange of an sp^3C connected –OH group to a –F manifests in characteristic changes in the NMR spectrum. In the α-position, namely directly on the substituted carbon, a *ca.* 20-25 ppm paramagnetic shift and in the β-position a *ca.* 2-3 ppm diamagnetic shift can be observed, which effect decreases below 1 ppm in the γ-position. In addition to these, both the ^{13}C and the ^{1}H NMR spectra show signal splitting caused by the characteristic direct (~165 Hz), geminal (22-26 Hz) and vicinal (~5 Hz) ^nJ(F,C) and ^3J(F,H) (4.5 Hz) couplings. Based on these, it could be evidenced that compounds **65**, **67** and **68** contain a fluorine atom connected to the C-25, and that compound **68** contains another fluorine substituent also at position C-14. Due to the possible S_N1 or S_N2 mechanism of the reaction, the 14-F substituent in compound **68** can be present in either α or β position. The latter case would also involve a change of the initially *trans* C/D ring junction to *cis*. The effect of such a configurational change on the ^{13}C chemical shifts can well be estimated by comparing the corresponding chemical shifts of 20-hydroxyecdysone and its diastereomer 14-*epi*-20-hydroxyecdysone, where significant, *i.e.* more than 2 ppm differences could be detected on the δC-9 (+2.4), δC-12 (+9.2), δC-13 (+4.0), δC-15 (+9.0), δC-16 (+3.2) and δC-17 (+6.2) (based on unpublished NMR data of our research group for 14-*epi*-20-hydroxyecdysone isolated from

the plant *Serratula wolffii* [75]). Considering that no such changes were detected in case of compound **68**, a retained C-14 configuration could be concluded. Moreover, a detailed analysis of the NOESY spectrum revealed the steric proximity of the H_3C-18 and the H_β-15 hydrogen atoms, which is only possible in case of a *trans* C/D ring junction, providing further evidence for a 14α-F group in compound **68** (see **Fig. 11.**).

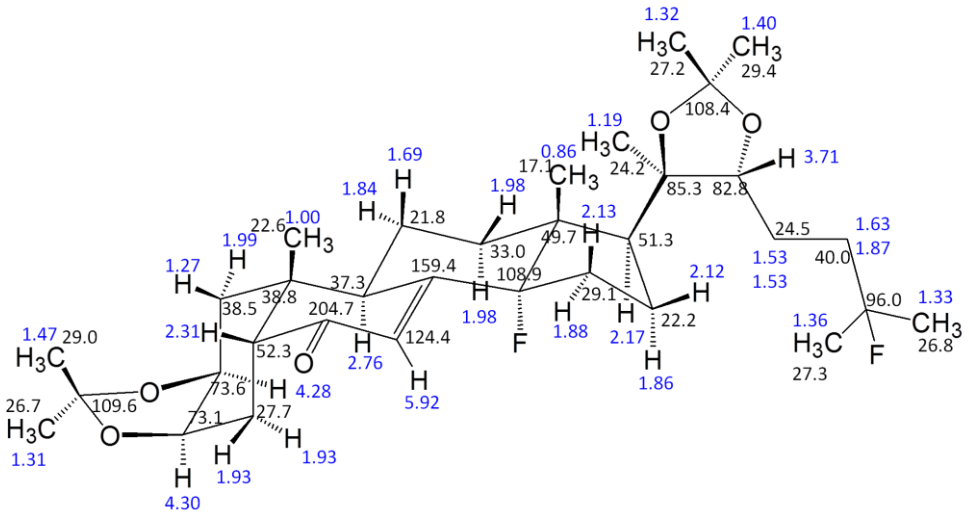


Figure 11 – Stereostructure of compound **68**. Blue numbers give the characteristic ^{1}H chemical shifts, and black numbers represent the ^{13}C chemical shifts. Doublet splitting resulted from ^1J(F,C), ^2J(F,C) or ^3J(F,C) coupling is not shown here.

3.3. Results of the biological assays

3.3.1. Anti-proliferative activity of the compounds (see also: I., VI, VIII.)

Ecdysteroid dioxolanes – derivatives of 20E (**6**-**42**), of poststerone (**56**-**64**), and compounds obtained from the fluorination of 20-hydroxyecdysone-2,3;20,22-diacetonide (**65**-**68**) – were tested for their anti-proliferative activity. Compounds were tested on mouse T-cell lymphoma (L5178 and $L5178_{MDR}$) cell lines, while, in addition to this, breast cancer (MCF-7, MDA-MB-231, MDA-MB-361 and T47D) and neuroblastoma (SH-SY5Y) cell lines were also utilized for the bioactivity testing of compounds **65**-**68**. Cells were prepared as described in chapter 2.4.1., and serial dilutions of each compound were applied and evaluated by the MTT assay [82]. IC_{50} – (concentration of compound that inhibits 50% of cell growth) was calculated and presented as the average of 3 independent experiments (see **Tables 3a** and **3b**). While the parent compound **1** showed no anti-proliferative activity (IC_{50} > 150 µM not presented in the table), most of its tested derivatives appeared to more or less inhibit cell growth in our experiments. 2,3-monosubstituted derivatives (**37**-**42**) showed no anti-proliferative activity (data not presented on table). As seen from the table, several compounds exerted much lower activity on the $L5178_{MDR}$ cell line as compared to the parental L5178 cell line, while other compounds showed similar activities on both cell lines. Compounds **65**-**68**, obtained from the fluorination of the dioxolane compound **20**, were tested on breast cancer and neuroblastoma cell

lines (see **Table 3b**), and, in general, showed higher anti-proliferative activity compared to that of the parent compound.

Table 3a – IC_{50} values of ecdysteroid derivatives (**6**-**36** and **56**-**68**) tested on the parental L5178 and multi-drug resistant $L5178_{MDR}$ cell line, presented as the average of 3 independent experiments ± standard error of the mean (SEM).

compound	IC_{50} (μM)	
	L5178	$L5178_{MDR}$
1 (20E)	> 150	> 150
6	> 150	> 150
7	40.6 ± 3.6	> 150
8	21.4 ± 1.4	57.8 ± 5.7
9	77.0 ± 1.5	92.7 ± 2.0
10	18.5 ± 1.8	35.7 ± 0.5
11	92.3 ± 23.1	> 150
12	98.9	> 150
13	36.7 ± 0.8	89.4 ± 14.4
14	45.0 ± 9.6	> 150
15	31.6 ± 5.3	43.2 ± 2.7
16	41.0 ± 6.8	59.5 ± 5.8
17	34.1 ± 3.3	38.8 ± 0.4
18	68.9 ± 2.1	> 150
19	50.0 ± 3.4	> 150
20	82.9 ± 1.0	106.1 ±3.3
21	51.2 ± 1.5	56.0 ± 6.4
22	99.6 ± 5.2	> 150
23	52.6 ± 1.5	87.8 ± 10.9
24	20.3 ± 0.8	22.4 ± 0.8
25	30.2 ± 1.2	38.3 ± 1.1
26	19.5 ± 2.6	30.6 ± 1.4

compound	IC_{50} (μM)	
	L5178	$L5178_{MDR}$
27	19.9 ± 0.03	25.5 ± 3.4
28	52.6 ± 12.9	49.7 ± 3.9
29	> 150	> 150
30	> 150	> 150
31	75.2 ± 12.1	64.4 ± 13.7
32	77.5 ± 20.7	75.9 ± 3.1
33	72.2 ± 9.8	66.8 ± 4.7
34	42.0 ± 18.9	42.7 ± 2.6
35	41.6 ± 6.7	46.5 ± 7.0
36	62.6 ± 16.8	64.7 ± 7.3
56	95.7 ± 0.6	> 150
57	91.1 ± 4.2	> 150
58	74.3 ± 2.3	> 150
59	97.8 ± 2.1	> 150
60	72.2 ± 0.6	> 150
61	59.6 ± 0.8	55.4 ± 4.9
62	92.5 ± 1.0	85.7 ± 1.1
63	95.2 ± 1.4	87.7 ± 1.1
64	97.0 ± 5.8	91.6 ± 7.0
65	48.3 ± 0.2	50.1 ± 2.1
66	14.6 ± 0.3	19.3 ± 0.5
67	14.1 ± 0.8	17.4 ± 1.2
68	88.6 ± 1.1	82.3 ± 1.4

Table 3b – IC_{50} values of the ecdysteroid derivatives **65**-**68** compared to that of the parental compound **20**, tested on breast cancer (MCF-7, MDA-MB-231, MDA-MB-361 and T47D) and neuroblastoma (SH-SY5Y) cell lines. Values are presented as the average of 3 independent experiments ± standard error of the mean (SEM).

compound	IC_{50} (μM)				
	MCF-7	T47D	MDA-MB-231	MDA-MB-361	SH-SY5Y
20	75.1 ± 3.4	84.7 ± 3.9	106.1 ± 7.2	69.2 ± 6.0	126.8 ± 9.8
65	63.1 ± 2.3	70.3 ± 1.3	48.9 ± 2.0	30.9 ± 2.6	70.7 ± 4.1
66	30.1 ± 0.8	10.9 ± 0.3	38.5 ± 3.8	13.8 ± 0.4	20.8 ± 0.3
67	43.8 ± 1.7	17.4 ± 1.0	50.2 ± 2.6	11.8 ± 1.2	18.8 ± 1.2
68	127.5 ± 4.3	49.2 ± 5.0	98.4 ± 17.2	53.8 ± 5.7	125.6 ± 13.2

3.3.2. ABCB1-inhibition (see also: I., VI, VIII.)

Accumulation of rhodamine 123 by MDR mouse lymphoma cells was evaluated by flow cytometry in the presence of compounds **6**-**36** and **56**-**68** in order to study their capacity to inhibit the ABCB1 pump and therefore prevent the efflux of the dye, which was consequentially retained inside the MDR cell. Parental mouse lymphoma cells were used as control for dye retention inside the cell while

MDR cells alone do not retain rhodamine 123 at the concentration employed. The efflux pump inhibitor verapamil was used as positive control. All the compounds were dissolved in DMSO, which was also evaluated for any effect on the retention of the fluorochrome. DMSO concentration in the assay was 0.2%. For each compound, the fluorescence activity ratio (FAR), which measures the amount of rhodamine 123 accumulated by the cell in presence of the compound was calculated as follows:

$$FAR = (FL_{MDRtreated}/FL_{MDRuntreated})/(FL_{PARtreated}/FL_{PARuntreated})$$

where FL is the mean of the fluorescence. The obtained results showed marked differences in the compounds' capacity to inhibit the efflux of rhodamine 123 in this bioassay (see **Table 4**). While some compounds (e.g. **10**, **16**, **17**, **25**, **27, 67** and **68**) showed strong inhibitory activity, others were very weak or rather practically inactive at both applied concentrations (**1, 9, 12**-**14, 18, 19, 56**-**60** and **62**-**64**). The inhibition was usually dose dependent, while in some cases, applying the compound in 20 µM resulted in the appearance of cytotoxic activity and therefore made it irrelevant to calculate the FAR value. It is noteworthy that all dioxolane derivatives of poststerone (**56**-**64**, with the exception of compound **61**) proved to be inactive in respect of inhibiting the ABCB1 efflux pump.

Table 4 – Fluorescence activity ratio (FAR) values in presence of 2 and 20 µM of compounds (**6**-**36** and **56**-**68**). Asterisk (*) represent that the compound showed cytotoxicity at this concentration and it was irrelevant to calculate the FAR value. FAR values of the positive control verapamil (20.4 µM) and the negative control DMSO (0.2%) were 5.73 and 0.72, respectively.

compound	FAR	
	2 µM	20 µM
1 (20E)	1.70	1.76
6	-	1.53
7	1.02	4.04
8	2.40	40.76
9	1.42	1.40
10	1.53	98.74
11	0.87	7.85
12	0.87	0.87
13	0.77	1.01
14	0.76	0.91
15	1.11	*
16	1.00	109.40
17	1.21	94.56
18	0.95	1.04
19	1.04	1.69
20	3.33	11.28
21	29.96	45.35
22	6.47	53.49
23	10.00	55.81
24	51.97	*
25	1.08	82.68
26	43.81	*

compound	FAR	
	2 µM	20 µM
27	114.64	*
28	14.71	*
29	3.88	14.07
30	1.41	17.67
31	1.68	60.67
32	2.21	68.46
33	51.67	75.17
34	10.98	67.78
35	61.67	*
36	3.47	63.22
56	1.06	1.54
57	1.20	1.25
58	1.02	4.24
59	0.84	1.02
60	0.95	2.95
61	2.63	30.85
62	0.84	1.16
63	0.73	0.87
64	0.75	1.10
65	1.59	38.62
66	1.28	57.36
67	31.15	109.37
68	11.02	89.78

3.3.3. Combination assays with chemotherapeutics (see also: I., III., VI., VII., VIII.)

The synthesized ecdysteroid dioxolanes (**6**-**42** and **56**-**68**) were tested for their chemo-sensitizing activity on MDR mouse lymphoma cells ($L5178_{MDR}$) towards doxorubicin, using the checkerboard microplate method, according to the procedure described in chapter 2.4.4. Combination indices for the different constant ratios of compounds **6**-**36** and **65**-**68** *vs.* doxorubicin were determined by using the CompuSyn software to plot four to five data points to each ratio. CI values were calculated by means of the median-effect equation, where 0 < CI < 1, CI = 1, and CI > 1 represent synergism, additive effect (*i.e.* no interaction) and antagonism, respectively. Considering that in case of chemotherapy the desirable outcome is a complete eradication of the tumor, a weighted average CI value (where CIs at higher inhibition rates count more) has been suggested as an important measure for the relevance of synergism or antagonism on cancer cell lines [67]. Selected CI values, representing the strongest activities observed, are presented in **Table 5**; for the complete dataset, including further data concerning each fitting, please see the articles' [II., VI.] full text provided in the Annex. As seen from the table, in contrast with 20E, all compounds acted synergistically with doxorubicin. The 2,3;20,22-disubstituted ecdysteroid dioxolanes (**20**-**36** and **65**-**68**) generally exerted more pronounced chemo-sensitizing activity than the 20,22-monosubstituted derivatives (**6**-**19**).

Table 5 – Combination index (CI) values at 50%, 75% and 90% of inhibition at the most active constant ratio of compounds **1**, **6**-**36** and **65**-**68**, combined with doxorubicin on the $L5178_{MDR}$ cell line. 0 < CI <1, CI = 1, CI > 1 represent synergism, additivity and antagonism, respectively, where 0.1 < CI < 0.3 is strong, CI < 0.1 is very strong synergism [67]. $CI_{avg} = (CI_{50} + 2 \times CI_{75} + 3 \times CI_{90})/6$, r represents the linear correlation coefficient of the median-effect plot (for further details, see: II. and VI.)

com-pound	drug ratio	CI values at			CI_{avg}	r
		ED_{50}	ED_{75}	ED_{90}		
20E	20.4 : 1	2.00	2.02	2.04	***2.03***	0.997
6	20.4 : 1	0.84	0.54	0.35	***0.49***	0.955
7	81.6 : 1	0.91	0.31	0.11	***0.31***	0.989
8	40.8 : 1	0.56	0.58	0.78	***0.68***	0.870
9	40.8 : 1	0.50	0.26	0.14	***0.24***	0.959
10	81.6 : 1	0.70	0.44	0.28	***0.40***	0.936
11	20.4 : 1	0.95	0.53	0.34	***0.51***	0.945
12	40.8 : 1	0.86	0.48	0.27	***0.44***	0.958
13	81.6 : 1	0.82	0.68	0.74	***0.73***	0.950
14	40.8 : 1	0.92	0.81	0.94	***0.89***	0.991
15	20.4 : 1	0.66	0.61	0.70	***0.66***	0.954
16	20.4 : 1	0.20	0.25	0.33	***0.28***	0.946
17	20.4 : 1	0.63	0.47	0.61	***0.57***	0.820
18	20.4 : 1	0.92	0.59	0.41	***0.55***	0.968
19	40.8 : 1	0.84	0.81	0.91	***0.87***	0.967
20	40.8 : 1	0.26	0.14	0.08	***0.13***	0.996
21	20.4 : 1	0.31	0.25	0.24	***0.25***	0.999
22	20.4 : 1	0.23	0.16	0.15	***0.16***	0.956
23	20.4 : 1	0.31	0.22	0.17	***0.21***	0.978
24	20.4 : 1	0.70	0.69	0.74	***0.71***	0.998
25	20.4 : 1	0.54	0.40	0.31	***0.38***	0.947
26	20.4 : 1	0.44	0.32	0.27	***0.31***	1.000
27	20.4 : 1	0.39	0.38	0.39	***0.39***	0.979
28	40.8 : 1	0.26	0.30	0.35	***0.32***	0.992
29	20.4 : 1	0.31	0.18	0.15	***0.19***	0.962
30	20.4 : 1	0.34	0.18	0.15	***0.19***	0.956
31	20.4 : 1	0.21	0.12	0.07	***0.11***	0.956
32	20.4 : 1	0.20	0.13	0.10	***0.13***	0.958
33	20.4 : 1	0.23	0.08	0.06	***0.09***	0.984
34	20.4 : 1	0.12	0.10	0.12	***0.11***	0.963
35	20.4 : 1	0.18	0.12	0.12	***0.13***	0.999
36	20.4 : 1	0.15	0.13	0.14	***0.14***	0.999
65	20.4 : 1	0.19	0.19	0.19	***0.19***	0.908
66	20.4 : 1	0.54	0.44	0.37	***0.42***	0.989
67	20.4 : 1	0.48	0.36	0.28	***0.34***	0.983
68	20.4 : 1	0.21	0.12	0.07	***0.11***	0.957

Considering that neither the 2,3-dioxolane derivatives of 20E with retained 20,22-diol (**37**-**42**), nor those of poststerone (**56**-**64**) exerted detectable intrinsic cytotoxic activity on the MDR mouse lymphoma cell line ($L5178_{MDR}$), calculating the combination indices was not feasible in case of these compounds. However, they showed a marked chemo-sensitizing activity on mouse lymphoma cells towards doxorubicin, manifested in the decrease of the IC_{50} values of this anticancer agent (see **Table 6** – results for compounds **37**-**42** are incomplete and are not listed). As seen from the table, all the tested derivatives of poststerone (**51**) exerted higher chemo-sensitizing activity than **51** – with compound **61** being the most potent –, and this difference was more pronounced in case of the resistant $L5178_{MDR}$ cell line. Based on the comparison of results obtained for poststerone dioxolanes (**56**-**64**) to those of 2,3-monosubstituted derivatives of 20E (compounds **37**-**42**), it was observed, that from compounds that have the same dioxolane moiety at position 2,3, those without a side-chain appeared to exert much higher activity. This was reflected in the fold sensitization towards doxorubicin calculated for the corresponding compounds: **57** (3.11) > **39** (0.83), **60** (7.57) > **40** (2.11), **61** (27.26) > **42** (1.44) and **64** (7.42) > **37** (2.01).

Table 6 – Chemo-sensitizing activity of poststerone (**51**) and its derivatives (**56**-**64**) on L5178 and $L5178_{MDR}$ cell lines. Values are presented as the average of 3 independent experiments ± standard error of the mean (SEM). With the exception of 10 µM of compound **51** (either cell line), and **56**, **57** and **63** (L5178), all IC_{50} values differ from that found for doxorubicin alone (control) at $p < 0.01$ by means of one-way ANOVA followed by Dunnett's post-hoc test.

compound	IC_{50} (µM) of doxorubicin			
	L5178		$L5178_{MDR}$	
	10 µM	25 µM	10 µM	25 µM
control	0.41 ± 0.02	0.41 ± 0.02	11.83 ± 0.64	11.83 ± 0.64
51	0.37 ± 0.01	0.29 ± 0.01	13.22 ± 0.44	7.55 ± 0.56
56	0.34 ± 0.02	0.23 ± 0.01	3.80 ± 0.10	1.77 ± 0.08
57	0.33 ± 0.01	0.15 ± 0.004	2.74 ± 0.66	1.39 ± 0.06
58	0.26 ± 0.02	0.17 ± 0.007	1.74 ± 0.11	0.87 ± 0.08
59	0.28 ± 0.02	0.20 ± 0.01	6.81 ± 0.33	3.00 ± 0.09
60	0.21 ± 0.002	0.15 ± 0.01	1.56 ± 0.08	0.79 ± 0.05
61	0.18 ± 0.003	0.12 ± 0.002	0.43 ± 0.02	0.17 ± 0.005
62	0.19 ± 0.01	0.11 ± 0.003	2.60 ± 0.11	1.40 ± 0.08
63	0.34 ± 0.02	0.26 ± 0.04	2.23 ± 0.14	1.47 ± 0.08
64	0.24 ± 0.02	0.23 ± 0.02	1.59 ± 0.02	0.97 ± 0.03

Selected compounds (**1**, **6**, **20**) were tested on other, human derived cancer cell lines as well: breast cancer cell lines included the MCF7 and its ABCB1 expressing cell line $MCF7_{dox}$ adapted to doxorubicin, and prostate cancer cell lines including the steroid dependent LNCaP and the nonsteroid dependent PC3. The effect of 50 µM of compounds was tested on the cytotoxic activity of chemotherapeutics with distinct mechanisms of action, such as doxorubicin (intercalates DNA and inhibits topoisomerase II, ABCB1 substrate), paclitaxel (stabilizes microtubule polymer, ABCB1

substrate) and cisplatin (alkylating agent, non-ABCB1 substrate). It is important to mention that even though the tested compounds exert negligible intrinsic cytotoxic activity at the applied concentration, this approach is still a simplification as compared to an appropriate calculation of synergism, and, as such, it could lead to false positive results. Considering this, we decided to accept only those results as relevant sensitizing activity, where at least a two-time decrease in the IC_{50} value of the chemotherapeutic could be observed. Results of these experiments are shown in **Table 7**.

Table 7 – The effect of 50 μM of compounds **1**, **6** and **20** on the IC_{50} values of chemotherapeutics in various cell lines. Values are presented as the average of 3 independent experiments ± standard error of the mean (SEM). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by means of one-way ANOVA followed by Bonferroni post hoc test as compared to that of the chemotherapeutic agent alone (control); n.r.: statistically significant, but not relevant (less than two-fold) sensitization.

cell line	chemothera-peutic agent	IC_{50} (μM) of chemotherapeutics			
		control	1	6	20
L5178	doxorubicin	0.23 ± 0.01	0.12 ± 0.002 (n.r.)	0.07 ± 0.006 ***	0.04 ± 0.002 ***
	paclitaxel	1.00 ± 0.21	0.63 ± 0.04	0.21 ± 0.07 **	0.03 ± 0.02 **
	cisplatin	4.93 ± 0.46	6.94 ± 0.33	7.65 ± 1.05	10.05 ± 0.95 **
$L5178_{MDR}$	doxorubicin	3.54 ± 0.44	2.04 ± 0.16 (n.r.)	1.64 ± 0.08 **	0.05 ± 0.004 ***
	paclitaxel	1.34 ± 0.22	0.71 ± 0.13	0.53 ± 0.12 *	0.23 ± 0.07 **
	cisplatin	4.06 ± 0.99	7.86 ± 1.03	8.73 ± 1.03	11.04 ± 2.10 *
MCF7	doxorubicin	2.14 ± 0.30	1.49 ± 0.24	1.01 ± 0.19 *	0.87 ± 0.07 *
	paclitaxel	0.009 ± 0.0005	0.004 ± 0.0004 ***	0.003 ± 0.0002 ***	0.002 ± 0.00007 ***
	cisplatin	6.58 ± 0.33	13.09 ± 0.39 *	11.69 ± 0.69*	15.60 ± 1.60 **
$MCF7_{dox}$	doxorubicin	-	-	-	-
	paclitaxel	18.64 ± 0.18	16.33 ± 0.43	16.59 ± 0.18	15.41 ± 0.87 (n.r.)
	cisplatin	26.13 ± 1.23	55.38 ± 0.83 ***	50.64 ± 1.87 ***	43.95 ± 2.46 ***
LNCaP	doxorubicin	0.18 ± 0.04	0.18 ± 0.03	0.09 ± 0.01	0.03 ± 0.002 *
	paclitaxel	0.06 ± 0.003	0.04 ± 0.002 (n.r.)	0.03 ± 0.005 **	0.02 ± 0.005 ***
	cisplatin	15.13 ± 1.83	20.77 ± 1.40	14.17 ± 1.82	24.61 ± 1.27 *
PC3	doxorubicin	2.36 ± 0.06	1.93 ± 0.09 (n.r.)	1.90 ± 0.10 (n.r.)	1.12 ± 0.08 ***
	paclitaxel	0.14 ± 0.02	0.10 ± 0.02	0.08 ± 0.02	0.04 ± 0.01 *
	cisplatin	10.42 ± 0.50	15.37 ± 0.60 ***	16.96 ± 0.22 ***	10.90 ± 0.65

From the three tested compounds, **20** exerted the most significant sensitization effect on all cell lines when applied together with doxorubicin or paclitaxel, which is in agreement with our previous experimental results shown in **Table 5**. Results with doxorubicin could not be determined on the highly resistant $MCF7_{dox}$ cell line, since that chemotherapeutic agent had to be applied in such high doses that the measurements were disturbed by its own color. The 20,22-acetonide compound **6** showed tendencies for an activity pattern similar to the diacetonide **20**, but with much weaker activities. Interestingly and somewhat unexpectedly, compound **1** was also found to show significant and relevant sensitizing activity in case of one cell line, MCF7, when coadministered with paclitaxel (in other cases, the difference was not significant compared to the control). On the other hand, the tested ecdysteroids showed an obvious general tendency to decrease the activity of cisplatin in all

cell lines, especially in the two breast cancer cell lines (MCF7 and $MCF7_{dox}$) where all compounds significantly elevated its IC_{50} values. From the human derived cancer cell lines, the steroid dependent LNCaP appeared to be most sensitive towards the applied chemotherapeutics.

Based on the results of an *in silico* physicochemical characterization combined with *in vitro* PAMPA screening, which studies were performed in collaboration and which themselves do not serve as the basis of this dissertation, compounds **20**, **31**, **33**, **34** and **36** were identified as candidates potentially able to penetrate the blood-brain barrier (BBB) through passive diffusion. These compounds (applied in 2.5, 5 and 10 µM concentration) were tested for their ability to chemo-sensitize a central nerve system (CNS) originated tumor cell line, SH-SY5Y neuroblastoma towards vincristine. All the tested compounds exerted a remarkably strong, dose dependent chemo-sensitizing activity. SH-SY5Y cells were sensitive to vincristine (IC_{50} = 39.5 ± 2.9 nM), which sensitivity could still greatly be enhanced with the addition of these relatively apolar ecdysteroids. When applied at 10 µM, compound **31** could decrease the IC_{50} value of vincristine to as low as 0.056 ± 0.03 nM, which represents a dramatic, three orders of magnitude increase in the cytotoxic activity. Compound **20** was also found to exert a strong activity in the experiments: a marked decrease in the IC_{50} of vincristine to 4.1 ± 0.10, 1.6 ± 0.10 and 1.0 ± 0.41 nM was observed when this ecdysteroid was applied in 2.5, 5 and 10 µM concentration, respectively, corresponding to 9.6, 24.7 and 39.5 times sensitization, respectively.

3.3.4. Effect on Akt-phosphorylation (see also: IV.)

The oxidized ecdysteroid derivatives (**43**-**50**, with the exception of **45** and **49**) obtained from the autoxidation of 20E, were tested for their capacity to influence the Akt-phosphorylation in C2C12 myotubes (see **Fig. 12.**).

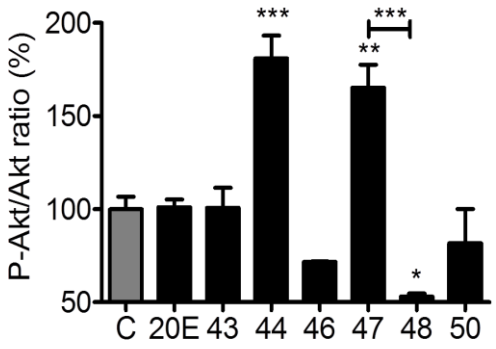


Figure 12 – Activity of compounds **43**-**44**, **46**-**48** and **50** on the Akt-phosphorylation in murine skeletal muscle cells. Quantification of Western blots was performed by ImageJ; error bars represent SEM; **: $p < 0.01$, ***: $p < 0.001$ by one-way ANOVA followed by Bonferroni's post hoc test; *: $p < 0.05$ by one-way ANOVA performed in a planned comparison of **47** and **48** to the control (C) by using Dunnett's post hoc test; n = 2-5.

Contrary to our expectations (based on [25]) on the effect of 20E, this compound did not cause relevant increase in the phosphorylation of Akt, while compounds **44** and **47** showed favorable activities over that of 20E, suggesting their possible more potent anabolic, antidiabetic, and antiapoptotic (cytoprotective) activities as well. Moreover, as an unexpected outcome of our study, the two desmotropes **47** and **48** showed significant differences in their bioactivity. In fact, when both were applied at 30 μg/ml, **48** exerted an opposite effect of that of **47** and decreased the phosphorylation of Akt as compared to the control. This finding highlights that these two tautomers can be stable even in a biological environment. To the best of our knowledge, this provides the first direct proof of a pair of desmotropes exhibiting different bioactivities.

4. DISCUSSION

4.1. Preparation of the ecdysteroid derivatives

4.1.1. Dioxolane formation

20E (**1**) can be considered as a good candidate for semi-synthesis, particularly when less polar compounds are to be prepared: it is available in high quantities, and apolar groups can simply be coupled to its several hydroxyl groups. For example, the diols at C-2,3 and C-20,22 can participate in acid catalyzed nucleophilic addition reactions. This takes place, when a single reactant (an aldehyde or ketone) gets protonated by an acid catalyst (phosphomolybdic- or tosylic acid) and attacked by one of the lone electron pairs of an oxygen atom in a hydroxyl group. Provided that two hydroxyl groups are in a vicinal position (*viz.* being a diol), this nucleophilic attack can happen twice in a row, resulting in the formation of a 1,3-dioxolane ring. In the hereby presented work, we found that from the two diols of 20E, the one at C-20,22 is more reactive than the C-2,3 diol, most probably because of steric effects. Therefore, the formation of 20,22-monosubstituted dioxolanes is favorable, and such compounds can be selectively prepared when smaller amount of the reagent and/or shorter reaction time is applied. Most assuredly also out of steric effects, the larger substituent connected to C-29 preferentially takes the R^4-position, which was reflected by the difference in the yields of the two epimers (*e.g.* compounds **16** and **12** or **15** and **11**, see chapter 3.1.1.). The configuration of the stereogenic center at C-29 was unequivocally clarified by the observed correlation between the H-29 and H-22 signals in the selective ROE experiments. Compounds **20**-**28** were obtained by applying larger amounts of the reagent and longer reaction times, while compounds **29**-**36** were synthesized in two consecutive reactions (see chapters 2.2.2 and 2.2.3.). In both cases, a new stereogenic center was created at C-28 with an *R*-configuration, similarly to the case at C-29. Due to the lower reactivity of the C-2,3 diol, in order to selectively form a dioxolane ring at this position, a preceding step of protecting the C-20,22 diol with a removable protecting group was required, which, after the

dioxolane-formation at the C-2,3 diol, could be removed by applying H_2O_2 (compounds **38**-**42**, see **Fig. 3.** in chapter 3.1.1.). The need for this step of protecting is supported by the fact that out of the 2,3-monosubstituted ecdysteroid dioxolanes, only one compound (**37**) was obtained directly from 20E, being a side product with a low yield (see chapter 2.2.4.). Since poststerone (**51**) has only one vicinal diol in its structure, dioxolane formation at C-2,3 is straightforward in its case (see chapter 2.2.7.). Reactions were monitored by regular TLC checks, and, as it turned out, long reaction times (*e.g.* one week) were needed to obtain reasonable yields, thus giving further proof to the lower reactivity of the C-2,3 diol. For the configuration of the newly obtained stereogenic center at C-22, not surprisingly, the same preference of *R*-configuration could be observed as in case of C-28 in the 2,3-substituted dioxolane derivatives (**20**-**42**).

4.1.2. Base-catalyzed autoxidation of 20E

Based on the observations of *Suksamrarn et al.* [56], the oxidation of 20E by molecular oxygen can be catalyzed by using a base (such as NaOH) in relatively low concentration (see chapters 1.3. and 2.2.5.). The reaction was carried out and monitored, and the formation of each isolated product over various reaction times was studied (as described in chapters 2.2.5. and 3.1.2.). Considering that compound **43** – reported by *Suksamrarn. et al.* [56] as a main product under similar conditions – was found as a minor product (0.84%), and compound **44** – its 5α-epimer –, was detected instead in a significant amount (24.5%), one can only assume that temperature and/or the amount of methanol might play important roles in the stereoselectivity of C-5, since the ambient temperature used was 30-32 °C in the previous work, and no information was provided on the ratio of the "aqueous methanol" as solvent [56]. The diacetonide derivative of compound **44** has been reported as the main product of a similar reaction of 20-hydroxyecdysone-2,3;20,22-diacetonide (**20**) [84]. However, 5α-20E was not detected at any time of the reaction (see chapter 3.1.2.), it is suggested that 9α-hydroxylation should be the first step followed by the epimerization at C-5. The tautomeric interconversion occurring between compounds **47** and **48** and their formation from compound **50** was investigated as described in chapter 3.2.2. On the basis of our observations, these two compounds represent a very rare case of isomeric compounds occurring as desmotropes. Desmotropy or desmotropism, a type of tautomerism where both forms may be isolated, is closely linked with tautomers in the solid state, and it is unusual to find two isolated desmotropes that are stable without the detectable coexistence of the other tautomer when dissolved in the same solvent [85]. A possible reaction mechanism for the autoxidation of 20E is proposed based on the present results and shown in **Fig. 13.**

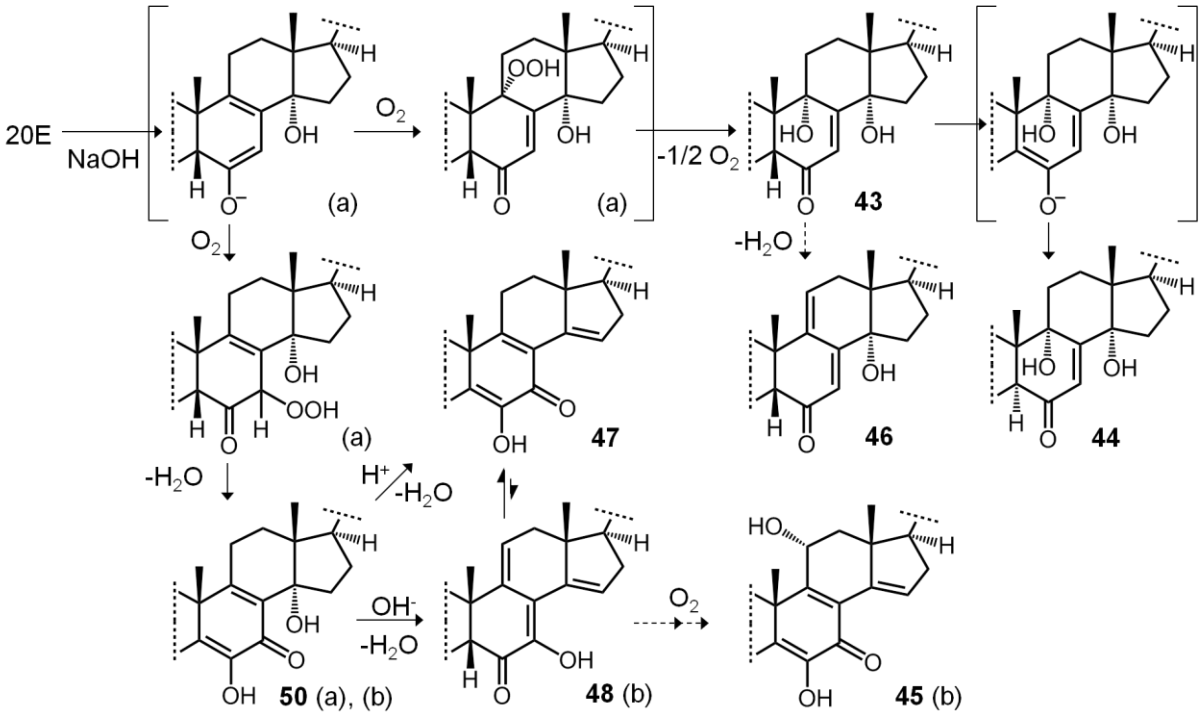


Figure 13 – Proposed reaction mechanism for the formation of compounds **43**-**48** and **50**.
(a) – intermediates hypothesized previously [56], (b) – new compounds, - → – minor products

4.1.3. Side-chain cleavage

Since the most abundant natural ecdysteroid, 20-hydroxyecdysone (20E, **1**) is known to undergo a side-chain cleavage between C-20 and C-22 during its *in vivo* metabolism to yield poststerone (**51**) [32], it seems reasonable to speculate that similar side-chain cleaved metabolites can also be formed from other ecdysteroids with a non-substituted diol at this position. *In* vitro, side-chain cleavage requires a more potent oxidizing agent than molecular oxygen (used for autoxidation, described in the previous chapter), therefore common oxidizing agents – such as $NaIO_4$, Jones reagent and $KMnO_4$ – were put to the test. However, these reagents proved to give a complex mixture of reaction products in our experiments, hence we used a hypervalent iodine compound (PIFA) instead as reagent, which showed to provide good yields of the desired reaction products (see chapter 2.2.6.). In these reactions, structural modifications affected the side-chain with a good selectivity – cleaving it between C-20 and C-22, and leading to the formation of a carbonyl group at C-20 –, leaving the starting compound's skeleton unchanged. This finding was supported by the investigation of the corresponding NMR spectra, as described in chapter 3.2.3.

4.1.4. Fluorination

Based on the promising aspects of fluorination of natural compounds (described in chapter 1.3.), we set a task to prepare fluorinated derivatives of 20-hydroxyecdysone-2,3;20,22-diacetonide (**20**), an ecdysteroid with particularly strong chemo-sensitizing properties. For the reaction, a commonly

used fluorinating agent, DAST, was applied, and resulted in fluorine substitution and/or water elimination in the parent compound. The presence of a fluorine atom attached to C-14 and/or C-25, as well as of a $\Delta^{14,15}$ double bond was proved by comprehensive NMR measurements (described in chapter 3.2.4.). According to our findings, fluorine substitution at C-14 did not result in the change of configuration, therefore the S_N1 reaction mechanism is suggested for this substitution, which mechanism is also more likely whenever steric effects (*e.g.* due to a rigid steroid skeleton, as in our case) prevent an S_N2 attack.

4.2. Biological activity and SAR of the obtained compounds

4.2.1. Anti-proliferative activity and related SAR of ecdysteroid dioxolanes

Ecdysteroid dioxolanes (**6**-**42** and **56**-**68**) were tested on their ability to inhibit cell growth on parental (L5178) and multi-drug resistant ($L5178_{MDR}$) mouse lymphoma cell lines (as described in chapters 2.4.2. and 3.3.1.). As it was found, – in contrast with the parental 20E, which shows a very high structural similarity with the anti-apoptotic muristerone A [86] –, the majority of the tested compounds showed anti-proliferative activity in the bioassays. However, in most cases, this activity proved to be weak on the parental cell line, and often somewhat even less pronounced on its resistant counterpart. It appears, that from the two possible substitutions of 20E leading to dioxolane formation, the one taking place at C-20,22 has greater effect on anti-proliferative activity, while the impact of 2,3-substitution is negligible. For example, the 2,3-monosubstituted derivatives of 20E (compounds **37**-**42**) – with an intact side chain – showed no anti-proliferative activity at up to 150 µM on either cell lines, just like 20E. However, dioxolane formation at C-20,22 might lead to marked increase in cell growth inhibition, which becomes more pronounced when an apolar phenyl group is part of the substituent (as in case of compounds **13**-**16**, **24**, **25**, **34** and **35**). Removal of the side-chain – though it slightly decreases the polarity of the parent compound – does not appear to contribute significantly to the anti-proliferative activity of the compounds, as many of the dioxolane derivatives of poststerone (compounds **56**-**64**) showed meager or no activity in this regard. Even though dioxolane formation at C-20,22 might not always lead to marked anti-proliferative activity (as seen in case of compounds **11**, **12**, **22**, **29** and **30**), structural modifications on the side-chain – and therefore change in its polarity – appears to play a role in the observed effect of the tested compounds.

In case of compounds **20** and **65**-**68**, cell growth inhibition was tested on other cell lines as well (human breast cancer and neuroblastoma cells, see chapters 2.4.2. and 3.3.1.). In general, fluorine substitution at C-25 or C-14 and C-25 appears to have little effect on the very mild anti-proliferative activity of compound **20**, as seen from a comparison of the results obtained for compounds **20**, **65** and **68**. Moreover, a comparison of the activities of compounds **66** and **67** leads to the same conclusion. On the other hand, a $\Delta^{14,15}$ double bond, formed by the elimination of the 14-OH group,

markedly increased the anti-proliferative activity of compounds **66** and **67**, as compared to compounds **20** and **65**, respectively. It is worth noting that, even though all of the above structural changes led to more lipophilic compounds, their activity did not show any correlation to the log*p* values, which were calculated as 4.01, 4.91, 4.61, 5.50 and 5.80 for compounds **20** and **65**-**68**, by using ChemAxon's web based resource available at http://chemicalize.org [87]. The most relevant cell line specific differences in the anti-proliferative potential of compounds **20** and **65**-**68** were observed between the MCF-7 and T47D cells. Information on the observed structure-activity relationships concerning the anti-proliferative activity of ecdysteroid dioxolanes are summarized at the end of the discussion in **Fig. 14.**

4.2.2. Inhibition of the ABCB1 efflux pump and related observations on SAR

Since multi-drug resistance in cancer cells is often a consequence of the up-regulation of the ABCB1 (P-glycoprotein 1) efflux pump, ecdysteroid dioxolanes (**6**-**36** and **56**-**68**) were tested for their efflux-inhibiting activity. As described in chapter 3.3.2., marked differences were observed amongst the activity of the compounds, some showing high inhibiting effect, while others being completely inactive. Usually, applying higher concentration (20 μM) of the compound led to more potent pump-inhibition, and in some cases resulted in cytotoxicity, making it impossible to measure the accumulation of rhodamine 123 within the cell. The observed results were in good correspondence with the ones seen in the cell growth inhibiting assay (see chapter 3.3.1.), as many of the compounds that showed relatively high anti-proliferative activity (e.g. **10**, **15**-**17**, **24**-**27**, **67**) also proved to show significant inhibition of the ABCB1 pump (or cytotoxicity), especially when applied in higher concentration. In case of compounds **65**-**68**, elimination of 14-OH, as well as fluorination at C-14 or C-25, manifested in a significant increase in the ABCB1 inhibitory activity. It seems that side-chain cleavage does not lead to the appearance of pump inhibition, as neither 20E, nor the poststerone derivatives (**56**-**64**, with the exception of **61**) showed any activity in this bioassay. **Fig. 14** summarizes the observed SARs.

4.2.3. Chemo-sensitization of cancer cells to doxorubicin and related SAR

With the exception of compounds **37**-**42**, the checkerboard microplate approach was utilized for performing combination studies between 20E or its dioxolane derivatives and doxorubicin against multi-drug resistant mouse lymphoma cells, and all ecdysteroid dioxolanes showed synergistic activity with this chemotherapeutic agent indicating a sensitizing effect (see **Table 5** in chapter 3.3.3.). This effect was more pronounced in case of the 2,3;20,22-disubstituted dioxolanes compared to that observed for 20,22-monosubstituted ones. Based on the comparison of the C-28 and C-29 epimer pairs, it was found that at C-28, the larger substituent needs to take the R^2-position (**24** *vs*.

25), while at C-29 the R^3-position for a stronger activity (see **11** *vs*. **12** and **15** *vs.* **16**). As concerns the compounds with a dioxolane moiety only at the C-20,22 position, increasing the length of the substituent coupled to C-29 led to a significant increase in the synergistic activity with doxorubicin till the length of three carbon atoms (compound **7**), however a longer alkyl substituent (compound **8**) appeared to be less preferable. Introducing larger aromatic groups did not lead to stronger activities, although further substituents on the aromatic ring (compounds **11**, **15**) were able to increase activity as compared to the case when a non-substituted phenyl group was present (compound **19**). Addition of a β-methyl group to C-29 could, however, significantly increase the activity as compared to that of the 29α-phenyl substituted derivatives (see **17** *vs.* **19**, respectively).

Compounds **37**-**42** and **56**-**64** – although being dioxolane derivatives – did not show considerable anti-proliferative activity on their own, and therefore combination indices (CIs) were not calculated for them. However, they were still tested in combination with doxorubicin, and their sensitizing activity was measured in comparison with that of doxorubicin, when it was used alone (see **Table 6** in chapter 3.3.3.). These compounds exerted significant effect in the bioassay, which was even more pronounced in case of the side-chain cleaved poststerone derivatives. This indicates the beneficial role of this structural modification on the chemo-sensitizing activity. Generally, with larger substituents connected to C-2,3, higher activity was observed, which appeared to be most significant in case of an *iso*-butyl group (compound **41** amongst the 2,3-monosubstituted derivatives of 20E, and compound **61** amongst such derivatives of poststerone). Moreover, from comparing the activities of compounds **41** *vs*. **42** and **62** *vs*. **63**, it can also be seen that, in case of these epimers, the larger substituent is preferred to be in the α-position for a stronger sensitizing activity, which correlates well with the findings described earlier for compounds **24** *vs.* **25**.

Taking into consideration that some 20,22-monosubstituted dioxolane derivatives of 20E (namely compounds **7**, **9**, **12** and **18**), as well as most derivatives of poststerone (compounds **57**-**64**) showed strong sensitization of the multi-drug resistant mouse lymphoma cells towards doxorubicin, while having very low FAR values (see **Tables 4**, **5** and **6**), these compounds should exert their effect in a way other than a functional inhibition of the ABCB1 efflux pump. This finding particularly increases the value of these compounds, since they might provide another tool for handling MDR rather than directly inhibiting the otherwise physiologically important P-gp pump. However, the exact mechanism of action of the tested ecdysteroid derivatives is still to be clarified and needs further experimental work. The observed structure-activity relationships are seen on **Figure 14**.

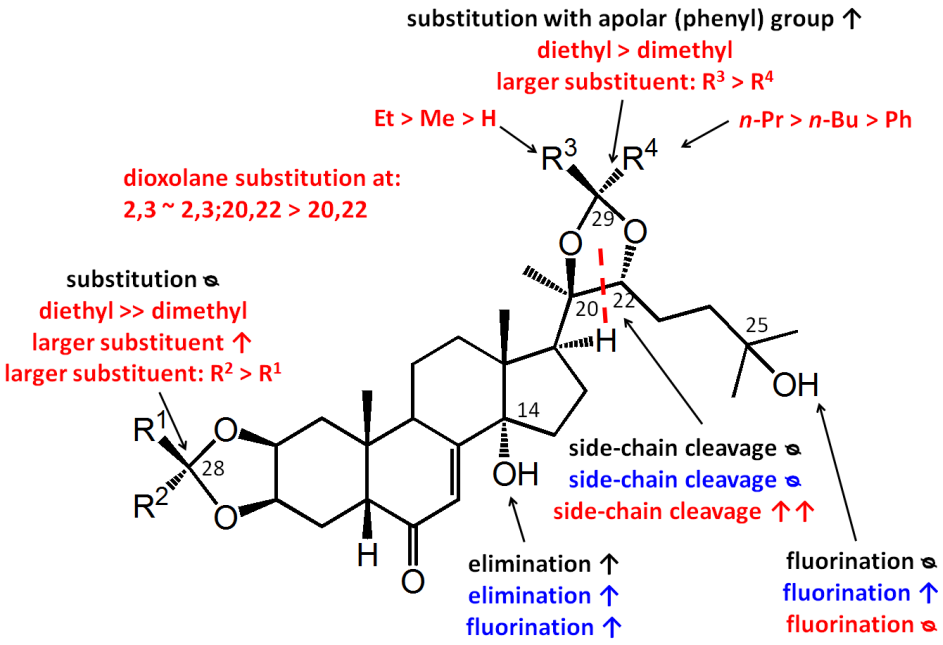


Figure 14 – SAR summary for ecdysteroid dioxolanes (**6**-**42** and **56**-**68**). Colors represent: **black – effect on anti-proliferative activity**, **blue – effect on ABCB1-inhibiting activity**, **red – effect on chemo-sensitizing activity to doxorubicin**. "Greater than" symbols denote stronger synergistic activities, ↑ refers to increase in activity, and ø means no effect.

4.2.3. Chemo-sensitization of various cancer cell lines to chemotherapeutics

Three selected compounds, 20E and its 20,22-mono- (**6**) and 2,3;20,22-diacetonide (**20**) derivatives were tested on other, human derived cell lines (two breast cancer and two prostate cancer cell lines) beside the mouse lymphoma cells, in combination with other chemotherapeutics than doxorubicin. In this study, our aim was to extend our studies towards a more diverse set of cell lines (using various resistant and susceptible ones) as well as to chemotherapeutics of different mechanism of action. The results obtained from these experiments (see **Table 7** in chapter 3.3.3.) support our assumption that less polar ecdysteroids do not or not exclusively act as ABCB1 inhibitors: compound **20** could effectively sensitize non-MDR human cell lines with no detectable (MCF7, [88, 89]) or very low (LNCaP, [90]) expression of the ABCB1 transporter, and the sensitizing effect was also observed on susceptible mouse lymphoma cell line (L5178). In accordance with the observations on the SAR of ecdysteroid dioxolanes in the previous chapter, compound **6**, the monoacetonide derivative of 20E, showed less potent activity than the diacetonide compound **20**, while in most cases, the activity of compound **1** was considered to be irrelevant (see chapter 3.3.3.). An interesting outcome of the experiments was that all three compounds decreased the sensitivity of the cells towards cisplatin. This raises concerns of a possible interference with actual chemotherapy with this anticancer agent, considering that a large number of food supplements containing high amounts of

ecdysteroids (mainly compound **1**) are available on the market. On the other hand, *Konovalova et al.* have previously found that 10 mg/kg of compound **1** could potentiate the activity and decrease the toxicity of cisplatin in P388 leukemia or B16 melanoma bearing mice, and the authors suggested that beneficial metabolic and immune system modulatory effects of this compound might be the reason for this phenomenon [91]. Such mechanisms could indeed overwrite an otherwise antagonistic effect observed in our experimental *in vitro* setup. Nevertheless, the strong potentiating activity of compound **20** on the activity of doxorubicin and paclitaxel is highly promising.

Some dioxolane derivatives (**20, 31, 33, 34, 36**) – potentially being able to penetrate the BBB – were also tested on the SH-SY5Y neuroblastoma cell line towards vincristine. All the tested compounds showed strong dose-dependent sensitizing activity, and compound **31** proved to be most potent, with an IC_{50} value of vincristine as low as 0.056 ± 0.03 nM. This dramatic increase in the observed cytotoxic activity suggests the high sensitivity of this neuroblastoma cell line towards such treatment with the combination of vincristine and ecdysteroids.

4.2.4. Effect on Akt-phosphorylation

Although it is known that 20E can increase the Akt-phosphorylation leading to a marked increase in the protein synthesis in the same skeletal muscle cell line used in this study [25], this could not be detected in our experimental setup (for details of the experiment, see chapter 2.4.5.). There are major differences between our protocol and that previously utilized for 20E: we used much higher doses than *Gorelick-Feldman et al.* and, in our case, the cells were not serum-starved overnight but pretreated with the ecdysteroid. As seen on **Fig. 12.** in chapter 3.3.4., compounds **44** and **47** significantly increased Akt-phosphorylation, while compound **48** – the desmotropic pair of **47** – showed the opposite effect at the applied concentration. These findings are of interest due to the fundamental role of the Akt kinase in cellular metabolism and survival. Considering that the apoptosis of β-cells play a crucial role in the pathologic mechanism of type II diabetes [92] and that decreased Akt kinase activity is connected to insulin resistance via impaired translocation of the glucose transporter GLUT4 [93], compounds **44** and **47** have both anabolic and antidiabetic potential. Moreover, hyperactivation of the pro-survival kinase Akt, which is generally up-regulated in cancer, has been suggested as a potential new anticancer target, since it can effectively sensitize cancer cells to oxidative apoptosis [73, 74]. As such, compounds **44** and **47** are also potential candidates in combination studies with pro-oxidant anticancer agents, which may lead to reduced toxicity issues attributed to existing drugs that target the PI3K/Akt pathway [74]. On the other hand, the significant difference between the activities of the two desmotropes (**47** and **48**) is a most unusual, puzzling phenomenon, and clarification of the underlying mechanisms is of particular interest for the future studies.

5. SUMMARY

The primary goal of the Ph.D. study presented in this thesis was to synthesize ecdysteroid derivatives, to investigate their biological effect, and to establish novel structure-activity relationships. Our results may be summarized as follows.

1. Preparation of semi-synthetic ecdysteroid derivatives

Altogether 63 ecdysteroid derivatives have been synthesized directly or in multiple steps from 20-hydroxyecdysone (20E) or other natural ecdysteroids, including 46 new compounds. These compounds were obtained by dioxolane formation, base-catalyzed autoxidation, side-chain cleavage or fluorination, including:

- 14 dioxolane derivatives (**6**-**19**) of 20E, substituted only at the 20,22-diol
- 17 compounds (**20**-**36**) derived from 20E with two dioxolane rings in their structure
- 6 derivatives (**37**-**42**) obtained from 20E, substituted only at the 2,3-diol
- 8 compounds (**43**-**50**) yielded by the base-catalyzed autoxidation of 20E, which reaction was also monitored in a longitudinal study, and compounds **47** and **48** proved to be a desmotropic pair
- 5 compounds (**51**-**55**) derived from various ecdysteroids by removal of the side-chain
- 9 dioxolane derivatives (**56**-**64**) of poststerone, obtained by dioxolane formation at the 2,3-diol and
- 4 compounds (**65**-**68**) yielded by the fluorination of 20-hydroxyecdysone-2,3;20,22-diacetonide.

Structures of these compounds were determined by means of comprehensive MS and NMR measurements, and, for their vast majority, a complete NMR signal assignment was achieved.

2. Biological evaluation of the obtained ecdysteroids

- Anti-proliferative activity: Ecdysteroid dioxolanes (**6**-**42**, **56**-**64**) were tested for their anti-proliferative activity on L5178 mouse T-cell lymphoma cells and on its multi-drug resistant $L5178_{MDR}$ counterpart. Compounds **65**-**68** were tested on breast cancer and neuroblastoma cell lines as well. Most of the dioxolane compounds showed low or no anti-proliferative activity, which was especially true for the 2,3-monosubstituted derivatives of 20E (**37**-**42**) and those of poststerone (**56**-**64**).
- ABCB1 inhibition: Compounds **6**-**36** and **56**-**68** were tested for their ability to inhibit this efflux transporter: while some compounds showed marked inhibition, most of them were weak in this regard; others, especially the side-chain cleaved compounds **56**-**60** and **62**-**64** proved to be completely inactive.
- Combination with chemotherapeutics: Tested compounds (**6**-**42**, **56**-**64**) were found to act synergistically with doxorubicin on mouse T-cell lymphoma cells, with more pronounced activity

in case of disubstituted dioxolanes and poststerone derivatives. Selected compounds were tested on a more diverse cell line panel (various breast, cervix, and –prostate cancer and a neuroblastoma), and combinations with paclitaxel, cisplatin or vincristine (anticancer agents with different mechanism of action than that of doxorubicin) were also evaluated. Synergistic activity was observed in case of combinations with all chemotherapeutics, but cisplatin (showing antagonistic effects). SH-SY5Y neuroblastoma was identified as a cell line showing particular sensitivity towards the chemo-sensitizing activity of ecdysteroid dioxolanes when combined with vincristine.

- Mechanism of action: Our SAR studies on ecdysteroid dioxolanes revealed that they can be engineered to become completely inactive as functional ABCB1 inhibitors, while retaining a strong chemo-sensitizing activity with a strong selectivity towards the ABCB1-transfected cancer cells. As such, the underlying mechanism of action can be presumed to somehow be connected to the machinery of the efflux transporter. However, this mechanism is still to be clarified and requires further studies.
- Effect on the Akt-phosphorylation: Oxidized ecdysteroid derivatives (**43**-**50**) were tested for their activity on the Akt-phosphorylation in C2C12 myotubes, and significant differences were observed in their bioactivity. Amongst these, the difference between the bioactivities of a desmotropic pair (**47** and **48**) is of particular interest. To our best knowledge, our work represents the first report of such a phenomenon.

3. Establishing new structure-activity relationships (SARs)

Further structure-activity relationships were discovered concerning the anti-proliferative, ABCB1-inhibiting and chemo-sensitizing activity of ecdysteroid dioxolanes (**6**-**42** and **56**-**68**) on multi-drug resistant mouse T-cell lymphoma cells. The role played by the substitution at the 2,3- and 20,22-diol, the size of the substituent, elimination and fluorination, as well as the impact of side-chain cleavage was investigated. In general, dioxolane formation and side-chain cleavage increases chemo-sensitizing activity and makes the tested cancer cells more susceptible towards treatment with doxorubicin.

Based on these findings, poststerone dioxolanes (**56**-**64**) can be highlighted as the most prospective leads for further research, as non-cytotoxic, non-efflux pump inhibitor, yet MDR selective adjuvant anticancer agents.

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