Ph.D. Thesis

Preparation of bioactive oxidized ecdysteroid derivatives

Halima Meriem Issaadi

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University of Szeged
Faculty of Pharmacy
Graduate School of pharmaceutical sciences
Department of Pharmacognosy

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Halima Meriem Issaadi

Supervisor
Attila Hunyadi, Ph.D.

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I dedicate this Ph.D. work to my father.

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LIST OF PUBLICATIONS RELATED TO THE THESIS*

I. **H. M. Issaadi**, Y. C. Tsai, F. R. Chang, A. Hunyadi, Centrifugal partition chromatography in the isolation of minor ecdysteroids from *Cyanotis arachnoidea*, *J. Chromatogr. B* **2017**, *1054*, 44-49.

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II. **H. M. Issaadi**, A. Hunyadi, K. Németh, Capillary electrophoresis study on the base-catalyzed formation of bioactive oxidized metabolites of 20-hydroxyecdysone, *J. Pharm. Biomed. Anal.* **2017**, *146*, 188-194.

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Analytical chemistry: Q1

III. **H. M. Issaadi**, J. Csábi, T-J. Hsieh, T. Gáti, G. Tóth, A. Hunyadi, Side-chain cleaved phytoecdysteroid metabolites as activators of Protein Kinase B, *Bioorg. Chem.* **2018**, *82*, 405-413.

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Organic chemistry: Q2 (2017)

*: Latin numerals of the corresponding publications related to this thesis will be referenced in this dissertation

LIST OF ABBREVIATIONS

α	Separation factor
Akt	Protein kinase B
aq.	Aqueous
BGE	Background electrolyte
^{13}C NMR	Carbon nuclear magnetic resonance spectroscopy
CE	Capillary electrophoresis
CI	Combination index
CPC	Centrifugal partition chromatography
COSY	Correlation spectroscopy
γ	Gamma
δ	Delta
1D	One-dimensional
2D	Two-dimensional
DEPT	Distorsionless enhancement by polarization transfer
DS	Degree of substitution
DTT	Dithiolthreitol
20E	20-Hydroxyecdysone
EcR	Ecdysteroid receptor
EGTA	Ethylene glycol tetraacetic acid
equiv.	Equivalent
ESI	Electrospray ionization
gs	Gradient-selected
^{1}H NMR	Proton nuclear magnetic resonance
HESI	Heated electrospray ionization
HMBC	Heteronuclear multiple bond coherence spectroscopy
HPLC	High-performance liquid chromatography
HRMS	High-resolution mass spectroscopy
HSQC	Heteronuclear single quantum correlation
IC_{50}	Fifty percent inhibitory concentration
ICH	International Conference for Harmonization
$K_{(U/L)i}$	Partition coefficient
λ_{max}	Wavelength of maximum absorbance

LD_{50}	Fifty percent lethal dose
LOD	Limit of detection
LOQ	Limit of quantification
MDR	Multiple drug resistance
MS	Mass spectroscopy
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NOESY	Nuclear overhauser effect spectroscopy
NP-HPLC	Normal-phase high pressure liquid chromatography
NP-TLC	Normal-phase thin layer chromatography
τ	Tau
PBS	Phosphate buffer saline
PIDA	(Diacetoxyiodo)benzene
PIFA	[Bis(trifluoroacetoxy)iodo]benzene
PVDF	Polyvinyldene fluoride
ROESY	Rotating-frame overhauser effect spectroscopy
RP-HPLC	Reverse-phase high pressure liquid chromatography
RP-TLC	Reverse-phase thin layer chromatography
RPM	Rotation per minute
Rs	Resolution
RXR	Retinoid X receptor
SDS	Sodium dodecyl sulfate
Sf	Retention of the stationary phase
TLC	Thin-layer chromatography
t_{migr}	Migration time
TOCSY	Total correlated spectroscopy
UHPLC	Ultra-high pressure liquid chromatography
USP	Ultraspiracle protein
UV	Ultraviolet

I. INTRODUCTION

I.1 Ecdysteroids – distribution and chemical diversity

Ecdysteroids were discovered as steroidal hormones responsible for the control of molting and metamorphosis in arthropods [1]. The first ecdysteroid discovered, ecdysone, was isolated from silkworm pupae by Butenandt and Karlson in 1954 [2], and its structure was elucidated by X-ray crystallography by Huber and Hoppe in 1965 [3]. Subsequently, ecdysteroids were also discovered in the Plant Kingdom, when the first phytoecdysteroids, ponasterone A, B and C, were isolated from the leaves of *Podocarpus nakaii* [4]. Almost simultaneously with this, 20-hydroxyecdysone (20E, **1**) and polypodine B (**4**) were obtained from the rhizomes of *Polypodium vulgare* [5, 6], 20E (**1**) from the roots of *Achyranthes fauriei* and the bark of *Podocarpus elatus* [7] and 20E (**1**) along with ecdysone from the pinnae of *Pteridium aquilinum* [8].

These pioneering discoveries provided the starting point for the screening of many hundreds of plant species resulting in the isolation of phytoecdysteroids from more than 100 terrestrial plants belonging to ferns, angiosperms and gymnosperms. It appears that at least 5 to 6% of terrestrial plant species contain significant levels of these compounds [9], which can reach 2 to 3% of dry weight in some species (e.g. seeds of *Rhaponticum carthamoides,* inflorescences of *Serrulata inermis* and roots of *Cyanotis arachnoidea*) [10]. Currently 510 natural analogues are listed in the online phytoecdysteroid database, and 20E (**1**) is recognized as by far the most widespread: among the 176 ecdysteroid-positive plants, 147 species contain 20E (**1**) [11]. The structure of ecdysone and 20E (**1**) are presented in **Fig. 1**.

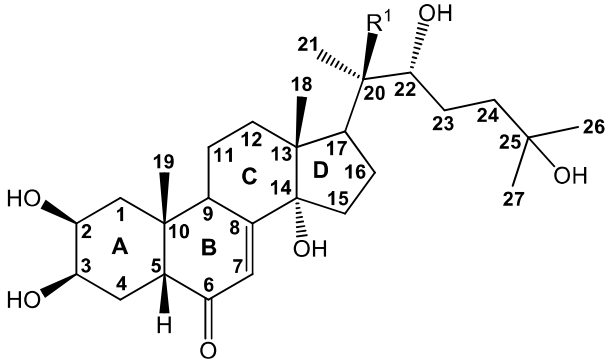

Fig. 1. Common structural features of ecdysteroids
Ecdysone: R^1 = H; 20-hydroxyecdysone (20E, **1**): R^1 = OH

Structurally, ecdysteroids contain a polyhydroxylated cyclopentano-perhydrophenanthrene ring system. They are biosynthetically derived from cholesterol, and thus possess a C_{27} carbon skeleton. However, the number of carbon atoms can vary, they occur as C_{27}, C_{28}, or C_{29} type of molecules if they possess the whole side-chain e.g., abutasterone,

dacrysterone and cyasterone, respectively and as C_{24}, C_{21}, or C_{19} compounds, e.g., sidisterone, poststerone (**7**) and rubrosterone, respectively, as a result of partial or complete metabolic cleavage of the side-chain between C-24 and C-25, C-20 and C-22, or C-17 and C-20. Most of the phytoecdysteroids possess a 7-en-6-one chromophore group in the B-ring responsible for UV absorption with λ_{max} at *ca.* 242 nm in methanol. The A/B ring junction is normally *cis* (5β-H), whereas the B/C and C/D ring junctions are almost always *trans*. Methyl groups are present at C-10 and C-13 with a β-orientation. Most ecdysteroids possess hydroxyl groups at C-2β, C-3β, C-14α, C-20R, and C-22R positions. Thanks to the variability in the number, position, and orientation of these groups, the wide variety of conjugating moieties, and the possibility of forming further rings, ecdysteroids have a remarkable structural diversity [12].

I.2 Pharmacological effects and potential application of ecdysteroids

Ecdysteroids have a very strong hormonal effect on insects, but since they are relatively polar compounds and therefore must be ingested to be active, their application as environmentally sound insecticides did not live up to expectations. On the other hand, bioactivity profile of these compounds suggests their possible use for various indications in mammals including humans.

The range of effects of ecdysteroids in mammals appears to be very wide, and this is accompanied by a negligible acute toxicity: in mice, the LD_{50} value of 20E (**1**) is 6.4 g/kg for intraperitoneal injection and more than 9 g/kg after oral application [13]. While at present our knowledge on the pharmacological effects of ecdysteroids in mammals is somewhat fragmentary, it appears that they do not interact with the vertebrate steroid hormone system, but exert a wide range of bioactivities including anabolic, antidiabetic, anticancer, adaptogenic, hepatoprotective, wound-healing, immunoprotective, and anti-inflammatory [14].

In the course of this Ph.D. work, biological tests related to the anabolic, anti-diabetic and chemo-sensitizing (anticancer) properties of ecdysteroids were performed. Therefore, this introduction will focus mainly on the results obtained in this regard, whereas the other beneficial effects of ecdysteroids will be only briefly summarized.

Anabolic effect. The anabolic effect of ecdysteroids is by far the most investigated among their bioactivities. The first two reports on the pharmacological activity of ecdysteroids in mammals (by Otaka et al. and Okui et al., both in 1968) showed the stimulation of protein synthesis in mouse liver [15, 16]. These initial findings were later confirmed by another study by Otaka et al. [17], where a male mouse received pure ecdysteroids intraperitoneally that led to an increase of the liver protein synthesis. A wide range of studies on the possible anabolic

effect of ecdysteroids in other animals followed. For instance, ecdysteroids increased the protein synthesis in rats and mice [18-24]. Increased protein synthesis upon ecdysteroid treatment was also observed in Japanese quail [25-26] and cattle [27] and a previous study by our group also revealed that 20E (**1**) increases skeletal muscle fiber size in a muscle-specific manner in rats [28].

There are also a few human studies available on the anabolic effects of ecdysteroids, mostly performed during the 1990s by Russian scientists. In 1995, Simakin et al. [29] found that 20E (**1**) treatment of trained athletes taking protein supplement results in a 6 to 7% increase in lean muscle tissue with a nearly 10% reduction in fat. Furthermore, Gadzhieva et al. [30] reported that a 3 weeks supplementation of sportsmen with ecdysteroid-containing products (brand names Ekdisten, Leveton or Prime Plus) during training diminishes fat content and improves total work under conditions of daily aerobic-anaerobic training. Finally, in 1997, Azizov et al. [31] reported that ecdysteroid supplementation contributes to restoring the levels of immunoglobulin A and G to their basic physiological state, which effect was accompanied by a *ca.* 10 to 15% increase in the exercise capacity of athletes. To the best of our knowledge, only one scientific study contradicts the above-mentioned reports: Wilborn et al. [32] found no effect of 20E (**1**) supplementation on training adaptation and/or anabolic/catabolic status in resistance-trained subjects.

The negligible toxicity and apparent anabolic activity of natural ecdysteroids on mammals made ecdysteroid-containing preparations popular among sportsmen and bodybuilders, and gave rise to an unregulated market of such commercial products. A large number of ecdysteroid-containing preparations are commercially available worldwide, originated from different plant species e.g., *Leuzea*, *Cyanotis*, *Ajuga* and *Polypodium* [33]. Several food companies are also showing great interest in adding ecdysteroid-containing plant materials into products as a way of enhancing health claims.

Despite the relatively large number of (*in vitro* and animal) studies on the anabolic effect of ecdysteroids, their exact mechanism of action is only partially understood. In arthropods, their effect is well-characterized: it is mediated via a specific nuclear receptor composed of the EcR and USP proteins [34]. However, neither ecdysteroids nor the EcR protein are endogenous components of mammalian cells. Although it was suggested that ecdysteroids could bind to other nuclear steroid receptors, in particular to the androgen receptor in mammals, this has been disproven [33]. This would imply that the anabolic effect occurs through non-genomic mechanisms. Recently, Gorelick-Feldman et al. [35] found that 20E (**1**) treatment of a mouse skeletal muscle cell line (C2C12) leads to a hasty elevation in intracellular calcium levels,

leading to a maintained protein kinase B (Akt) activation and increased protein synthesis within 2 hours. The same study also showed that removal of free calcium reduces Akt phosphorylation induced by 20E (**1**), and binding free calcium with EGTA also counteracts the increase in protein synthesis. These results suggest that calcium flux is an important mediator of the protein synthesis-inducing effect of 20E (**1**), in which the activation of Akt plays a central role.

The possible role of *in vivo* metabolites of ecdysteroids in the observed anabolic activity has long been part of related scientific discussion. Recently, Kumpun et al. [36] showed that the metabolism of 20E (**1**) involves side-chain cleavage between C-20 and C-22 and dehydroxylation at C-14. These led to the formation of poststerone (**7**), 14-deoxy-20E (**32**) and 14-deoxypoststerone as main primary metabolites, which then underwent further metabolic steps of reduction. In parallel to the present Ph.D. work, our research group recently found that poststerone (**7**) acts as a potent anabolic agent on rat skeletal muscles *in vivo*, implying that it plays an important role in the *in vivo* anabolic activity of its parental compound 20E (**1**) [37].

Anti-diabetic effect. Ecdysteroids were also reported to have potential as anti-diabetic agents. Orally-supplied 20E (**1**) had antihyperglycemic effect in alloxan-induced diabetic rats and mice [38]. It was also shown that 20E (**1**) can reduce glucose production and induce Akt2 phosphorylation in H4IIE cell culture, and that it significantly reduces weight gain, body fat mass, plasma insulin levels and glucose tolerance in mice fed a high-fat diet [39]. This ecdysteroid could also suppress hepatic glucose formation, thus exerting glucose-lowering effect in an insulin-independent manner [40]. A recent study demonstrated that quinoa extract enriched in 20E (**1**), as well as pure 20E (**1**), alleviates the effect of high-fat diet on adipose tissue in mice, without interfering with the body weight gain [41]. The similar anti-obesity effect of the 20E (**1**)-containing quinoa and pure 20E (**1**) also suggests that the role of other phytochemicals present in quinoa is minimal [41].

Concerning the effect of plant extracts containing ecdysteroid mixtures, *Ajuga* species serve as good examples. In 2014, Hsieh et al. [42] reported that *Ajuga nipponensis*, the richest ecdysteroid source among the *Ajuga* species, has the strongest ability for α-glycosidase inhibition and for decreasing glucose uptake. Also, a "cocktail" of ecdysteroids extracted from *Ajuga turkestanica* showed significant hypoglycemic effect on rats with alloxan-induced hyperglycemia, suggesting that the sum of these compounds can be considered as a promising hypoglycemic preparation [43]. Another interesting plant of the *Ajuga* genus is *Ajuga iva*, called "Chendgora" in Morocco and Algeria; this plant is traditionally used as a decoction to "eliminate the cause of diabetes" [44]. The antidiabetic effect of this species was experimentally evidenced by El Hilaly et al. [45] and Hamden et al. [46].

Anticancer effect. The anticancer potential of ecdysteroids was first reported in the beginning of the 1990s, when their dose-dependent cytotoxic activity on leukemia 1210 cells was established [47]. Later, different ecdysteroids were found to be able to strongly inhibit effects on Epstein-Barr virus early antigen induction by the tumor promoter, and particularly cyasterone showed potent antitumor-promoting activities on two-stage carcinogenesis in mouse-skin *in vivo* [48]. Above these early reports, our research group made a substantial contribution to the field in this regard by discovering the chemo-sensitizing effect of certain ecdysteroids. Related studies conducted over the last few years made it clear that ecdysteroids with a relatively lower polarity, and particularly those substituted by 2,3- and 20,22-acetonide groups, can strongly interfere with the drug resistance of various cancer cell lines, including susceptible and multi-drug resistant (MDR) cell lines of various origin [49-51]. The chemo-sensitizing activity was observed in combination with several chemotherapeutics including doxorubicin, paclitaxel, and vincristine, but not with cisplatin [52]. Interestingly, when comparing this effect of ecdysteroids on drug susceptible and ABCB1-transfected MDR cell lines, a strong MDR selectivity was observed even though the compounds were very weak, or practically inactive as functional inhibitors of the efflux transporter responsible for the resistance [53, 54]. Nevertheless, the most dramatic effect up to now was observed on non-MDR SH-SY5Y neuroblastoma cells: very strong synergism was found between ecdysteroids and vincristine, resulting in low picomolar IC_{50} values of the chemotherapeutic agent in the presence of certain ecdysteroids [51].

Other activities. Ecdysteroids are believed to possess adaptogenic, antidepressant and tonic properties i.e., they can safeguard the body from stress, increase resistance to fatigue and improve physical and sexual performance. However, only a few scientific studies confirm the above-mentioned health benefits [55-58].

Ecdysteroids exert protective effects against lipid peroxidation by free radicals, i.e. they have antioxidant activity. The *in vivo* effect of 20E (**1**) was reported to be more potent than that of vitamin D_3 against free radical-induced lipid peroxidation in vitamin D deficient animals [59]. Also, ecdysteroids isolated from *Serratula strangulata* protected human erythrocytes from H_2O_2-induced hemolysis, decreased lipid peroxidation in rat liver microsomes, scavenged galvinoxyl radicals [60], inhibited AAPH-induced hemolysis of human red blood cells, and decreased Fe^{2+} + cysteine-induced lipid peroxidation in liver microsomes [61]. It was also reported that 20E (**1**) has a greater activity than hydroquinone in counteracting Fe^{2+}-initiated free-radical oxidation of mitochondrial lipids, and that it demonstrates antioxidant activity against reactive oxygen species generated in iron- or azo-compound-initiated free-radical

oxidation of lipids [62]. Finally, Hu et al. [63] confirmed the ability of 20E (**1**), inter alia, to reduce oxidative stress, to restore cellular antioxidant potential and to markedly attenuate H_2O_2-induced intracellular calcium level in a concentration-dependent manner.

Ecdysteroids were found to reduce cholesterol levels by reducing its biosynthesis and thus enhancing its catabolism [38]. Ecdysteroids such as integristerone A, 20E (**1**), cyasterone and viticosterone E affected the absorption of $^3H_1$-cholesterol in the small intestine of rats with experimental hypercholesterolemia [64]. Ecdysteroids from *Rhaponticum carthemoides* (Willd) Iljin. and *Ajuga turkestanica* (Rgl.) Brig. enhanced the conversion of cholesterol into bile acids in normal rats [65].

The possible cardioprotective action of ecdysteroids was described in conditions of myocardial pathology. Experimentations on white rats with background of chronic cardiac failure showed that Ecdysterone-80 (herbal preparation containing 20E (**1**), 25*S*-inocosterone and α-ecdysone [55]) decreased lethality and heart muscle hypertrophy, and it had beneficial influence on the ratio of the activity of intravascular vasoconstrictors/vasodilators [66]. Another study indicated a beneficial activity of ecdysteroid supplementation in case of hypertension, with an effect on the cardiac remodelling process [67].

Ecdysteroids might also be useful in the treatment of superficial wounds, burns, and psoriasis. In the 1990s, Detmar et al. [68] highlighted that ecdysteroids promote wound healing in the skin with the stimulation of keratinocyte differentiation; and in 2017, a study on ecdysteroids-containing preparation from *Stachys hissarica* confirmed this finding [69].

Ecdysteroids are used in cosmetics [70]; they have also been proposed to stimulate the body's natural defences to protect against solar radiation [71]. They seem to have the ability to inhibit the calf skin collagenase that is an enzyme involved in skin manifestations of aging [72].

The above-listed various bioactivities reported for ecdysteroids demonstrate well that this group of compounds has many promising potential use. Even though the *in vivo*, and particularly the clinical evidence for related health benefits is rather limited, it is clear that ecdysteroids are a rich source of bioactive natural products, and that studies on these compounds raise many fascinating research questions concerning basic research as well as possible practical applications.

I.3 Semi-synthesis, isolation and identification of ecdysteroids

As mentioned above, natural ecdysteroids show a remarkable chemical diversity [12]. To date, the range of chemical permutations detected suggests that probably > 1000 analogues could exist in the Plant Kingdom [14]. Certainly, this chemical variability should necessarily

be closely connected to the pharmacological properties, in other words, the expression profile of the above-mentioned bioactivities must greatly vary among the individual ecdysteroids. Exploring related structure-activity relationships is a great challenge, and it requires a combined chemical approach of chromatographic isolation from natural sources and appropriately selected semi-synthetic transformations. The isolation from crude natural matrices and chemical mixtures, the online detection, the chemical fingerprinting and the quality control of herbal products have recently become much easier achievable thanks to the availability of modern chromatographic techniques of different selectivity. Hereinafter, selected examples are introduced that are closely connected to the subject of this Ph.D. thesis concerning the semi-synthesis, isolation and analysis of ecdysteroids.

I.3.1 Selected examples for the semi-synthesis of ecdysteroids

The use of semi-synthetic strategy can be practical when trying to obtain structural analogues with different and/or enhanced pharmacological properties as compared to the naturally occurring ecdysteroids [73]. Also, the semi-synthesis of a target compound from a more abundant precursor is useful in order to overcome a common major limitation of natural products isolation that is the "poor yield", especially when the active compound is present in extremely low concentration in a natural product extract [74]. In the course of this Ph.D. work, oxidative side-chain cleavage, autoxidation and gamma irradiation of ecdysteroids were performed; literature background to these is summarized below.

Oxidative side-chain cleavage. The oxidative side-chain cleavage between C-20 and C-22 of 20E (**1**), to obtain its *in vivo* metabolite, poststerone (**7**), was previously performed by others. Semi-synthetic procedures for this reaction gave poststerone (**7**) in unstated yields when $NaIO_4$ was used [75], and the yield was 62% with the use of Jones reagent [76]. In a procedure recently published by our group, using (bis(trifluoroacetoxy)iodo)benzene (commonly referred to as PIFA) for the same oxidation, poststerone (**7**) could be obtained from 20E (**1**) in an isolated yield of 57.80% [53]. Studies of our group, performed in parallel to this Ph.D. work, most recently revealed that poststerone (**7**) is an active metabolite that plays a major role in the anabolic activity of its parent compound 20E (**1**) [37]. This would suggest that side-chain cleaved metabolites of ecdysteroids other than 20E (**1**) might also be valuable bioactive compounds, certainly expectable to form *in vivo* after ingestion.

Autoxidation. The base-catalyzed autoxidation of 20E (**1**), using a 2% NaOH solution in aqueous methanol, was reported by Suksamrarn et al. in 1994 [77]; they identified calonysterone (**6**) and 9α,20-dihydroxyecdysone (**42**) as main products. Recently, our research group reported an update to this reaction and revealed the formation of several new, previously

unidentified oxidized derivatives [78]. Also, the autoxidation of 20E 2,3;20,22-diacetonide (**19**) and ponasterone 2,3;20,22-diacetonide in 10% aqueous methanolic NaOH was reported by Savchenko et al. [79]. The results showed a conversion of the starting materials to 9α,20-dihydroxy-5α-ecdysone (**22**) and 9α-hydroxy-5α-ponasterone diacetonides. Considering that the base-catalyzed autoxidation can provide various products with different chemical substitutions mainly in the rings B, C and D, the application of such reaction can result in the preparation of diverse compounds whose bioactivity has previously not been investigated.

Gamma irradiation. The irradiation chemistry is focusing on transformations produced by the absorption of high-energy irradiation. During these inelastic collisions, the particles lose energy with concomitant formation of energy-rich excited or ionized molecules [80]. One of the major types of ionising irradiation is the gamma-ray irradiation. In the radiolysis of water, gamma rays generate high-energy electrons initiating a large variety of reactions that lead to the formation of primary reactive intermediates, i.e. hydroxyl radicals ($HO^{\bullet}$), hydrated aqueous electrons (e^{-}**)** and hydrogen radicals ($H^{\bullet}$) that can transform or destroy organic compounds through various mechanisms [81]. To the best of our knowledge, gamma irradiation of ecdysteroids have not been studied by any other groups. However, some limited studies on the effects of ionizing irradiation on solid state steroids are available. For instance, the gamma irradiation of cholesterol lead to the formation of products oxidized on their rings A and B [82]. Also, the gamma irradiation of corticosteroids were characterized, and modifications were observed on the side chain including its loss to C_{17} ketones, as well as conversion of the C-11 alcohol to the C-11 ketone [83]. Ergosterol and calciferol were found to be sensitive to irradiation-induced oxidation in the presence of oxygen, and the irradiation of calciferol afforded a peroxide and other non-peroxidic substances [84].

I.3.2 Selected techniques for the analysis, isolation and identification of ecdysteroids

The conventional chromatographic techniques for the routine detection of ecdysteroids comprise planar chromatography and particularly TLC that is the fastest and easiest technique to screen samples for their ecdysteroid content with suitable selectivity. Among modern instrumental techniques, NP-HPLC and RP-HPLC [85] are the most popular for both analytical and preparative purposes. In many cases, they allow an unambiguous identification on the basis of the co-migration of the analysed ecdysteroids with reference compounds. However, an extensive clean-up (solid-liquid extraction, filtration, centrifugation, etc.) is essential to avoid any column contamination or overloading resulting in excess peak broadening or distortion. The use of such techniques in the isolation and analysis of ecdysteroids have extensively been reviewed by our group over the past few decades [86].

In the course of this Ph.D. work, two less-common techniques in the isolation and analysis of ecdysteroids were additionally used: a preparative liquid-liquid chromatographic technique, centrifugal partition chromatography (CPC), and an analytical technique, capillary electrophoresis (CE); literature background to these is summarized below.

Centrifugal partition chromatography. Liquid-liquid chromatography is gaining popularity as a viable separation technique for the fractionation of complex mixtures or for the straightforward isolation of pure compounds in natural products chemistry [87-89]. The principle of such technique relies on differences in the physicochemical properties of the targeted analytes, i.e. their solubility, polarity and hydrophobic/hydrophilic character. When compared with traditional solid-liquid techniques, the all-liquid chromatography presents many advantages: i) no irreversible adsorption on solid support; ii) full recovery of the injected sample; iii) minimal tailing; iv) low risk of sample denaturation; v) economic maintenance; vi) low solvent consumption; vii) sample loads ranging from milligrams to grams; viii) aqueous and non-aqueous solvent systems can be used and ix) a wide range of pH is tolerated [89-91].

Centrifugal partition chromatography is based on the use of a constant-gravity field produced by a rotation mechanism that allows maintaining the liquid stationary phase while the mobile phase is pumped through it. An effective CPC separation depends on the selection of a suitable biphasic solvent system and on the use of optimal separation parameters, which can be determined by the following criteria:

1. The partition coefficients ($K_{(U/L)_i}$) and the separation factors (α) values: $K_{(U/L)_i}$ = concentration of a solute in the upper phase / concentration of a solute in the lower phase. Ideally, $K_{(U/L)_i}$ should fall in the range between 0.5-2, but this can be extended to extreme values of 0.25-16. $\alpha = K_{(U/L)_i} / K_{(U/L)_{(i-1)}}$ where $K_{(U/L)_i} > K_{(U/L)_{(i-1)}}$. The separation factor values should be greater or equal to 1.5.
2. The settling time of the selected biphasic solvent system (i.e. the time required for the two phases to settle into two clear layers after mixed in a test tube) should be shorter than 30s in order to provide acceptable retention of the stationary phase (Sf).
3. Nearly equal volumes of each phase of the solvent system to avoid undue waste of solvent.
4. Appropriate Sf value during the "filling step" of the CPC column. Sf = Vc - Ve / Vc where Vc is the total volume of the column and Ve is the expelled volume of the stationary phase. The higher the retention of the stationary phase, the better the peak resolution will be.

The above criteria were thoroughly examined for all the developed CPC solvent systems presented in this dissertation.

Capillary electrophoresis. CE is a considered as a reference micro-analytical method because of the rapid analysis time and the efficiency that it provides [92]. This technique relies on the use of a bare fused silica capillary filled with a conductive fluid at a certain pH value. The compounds are separated by using an electric field. Because of the law of electrical neutrality, the immobile silanol anions will pair with the mobile buffer cations forming a double layer along the wall. The remaining buffer cations are attracted to the negative electrode dragging the bulk buffer solution with them. In one run, negative, neutral and slow positive components can be separated and detected at different velocities depending on their size and charge. The main advantage of this technique is the flat flow profile resulting in no peak broadening: positive charges are all located close to the capillary wall, thus there is no pressure force in the middle of the capillary.

In previous publications comparing CE and HPLC methods for the analysis of plant secondary metabolites [93-96], various advantages of CE were highlighted, including ultra-high separation efficiency, easy change of the separation media, and extremely small volumes of sample and media needed. Also, Prior to a CE analysis, unlike to what is foreseen for HPLC, minor sample pre-purification is required and a wide range of pH is tolerated by the instrument.

II. OBJECTIVES

The research on phytoecdysteroids has a several decades-long tradition in the department of Pharmacognosy, University of Szeged, and this work resulted in the discovery of *ca.* one-fourth of the natural ecdysteroids known to date. Our scientific interest recently turned towards the application of semi-synthetic strategies, with the aim of extending related chemical space towards new bioactive ecdysteroid analogues, and/or to obtain minor natural compounds in sufficient amounts for bioactivity testing. This work also raised new challenges for chromatographic method development. Accordingly, the following objectives were set up for the Ph.D. work presented in this dissertation.

1. The preparation of new semi-synthetic ecdysteroid derivatives. In order to increase the chemical (and, supposedly, pharmacological) diversity of the compounds to obtain, a diverse set of structural modifications of major phytoecdysteroids were selected. These modifications included oxidative side-chain cleavage (specifically targeting the 20,22-diol to obtain expectable *in vivo* metabolites), base-catalyzed autoxidation (primarily targeting the B-ring), and gamma irradiation (targeting the whole molecule with an interesting chemistry never explored for ecdysteroids before).

2. The development of new chromatographic methods for the isolation and analysis of ecdysteroids. Some of the selected chemical transformations were expected to result in complex mixtures whose separation would likely raise new challenges as compared to those connected to the phytochemical work. Therefore, it was also our objective to develop new preparative and/or analytical chromatographic methods in the course of our studies.

3. Biological evaluation of the isolated ecdysteroid derivatives. Bioactivity studies on the prepared compounds were planned in scientific co-operations. It was our objective to study selected ecdysteroids for their effect on the Akt-phosphorylation (related to the anabolic and anti-diabetic effects), or for their potential as chemo-sensitizing (antitumor) agents.

III. MATERIALS AND METHODS

III.1 Natural ecdysteroids 1-4 as starting materials

III.1.1 Standard ecdysteroid samples available

20-hydroxyecdysone (20E, **1**) isolated from the roots of *Cyanotis arachnoidea* with a purity of 90% was purchased from Shaanxi KingSci Biotechnology Co., Ltd. A purification step was performed by recrystallization from ethyl acetate - methanol (2:1, v/v), so that purity of 20E (**1**) utilized was 97.80%, by means of RP-HPLC. 2-Deoxy-20E (**2**) was previously isolated by our research group from *Silene italica ssp. Nemoralis* [97], ajugasterone C (**3**) and polypodine B (**4**) were obtained from a prior isolation from *Serratula wolffii* [98].

III.1.2 Isolation of dacryhainansterone and calonysterone [I][1]

A commercial crude extract of the roots of *Cyanotis arachnoidea* was purchased from Xi'an Olin Biological Technology Co., Ltd. The extract was percolated with methanol and subsequently fractionated through a silica gel column using a stepwise gradient of dichloromethane - methanol increasing the methanol content from 3% to 15% with a final wash with 100% methanol resulting in 6 fractions. Fraction 4 (244.80 g), eluted with dichloromethane - methanol (93:7, v/v), was subjected to further separation through silica column using gradient of *n*-hexane - ethyl acetate - ethanol to obtain 13.16 g of a mixture containing mainly dacryhainansterone (**5**) and calonysterone (**6**), and some impurities (referred as **i** and **ii**). A 1 g aliquot of the above mentioned mixture was purified by CPC with the use of a biphasic solvent system composed of *n*-hexane - ethyl acetate - methanol - water (1:5:1:5, v/v/v/v), in ascending mode through six consecutive injections. The equipment parameters were settled as follows:

[1] reference to own publications related to the thesis

constant pressure of 86 bars, flow rate of 10 ml/min and rotation speed of 2400 rpm. An altogether 95.28% of the initial weight was recovered after the separation and CPC combined fractions 1-8, 9-11, 12 and 13-20 were subsequently investigated by RP-HPLC on a Kinetex XB C18 (5 μm, 250 × 4.6 mm) column with an isocratic solvent system of 30% aq. acetonitrile at a flow rate of 1 ml/min. From the combined fractions 9-11, dacryhainansterone (**5**, 357.33 mg) was reached and from the combined fractions 13-20 calonysterone (**6**, 299.22 mg) was attained.

III.2 Preparation of side-chain cleaved ecdysteroids 7-18 [III]

III.2.1 Preparation of compounds 7-12 by side-chain cleavage

Poststerone (**7**) was synthesized from 20E (**1**) by dissolving 5 g of 20E (**1**) in 400 ml of methanol, then 1 equiv. of PIDA ((Diacetoxyiodo)benzene) was added and the solution was stirred at room temperature for 45 min. The reaction mixture was then neutralized with a 5% aq. solution of $NaHCO_3$, the solvent was evaporated at 40°C under vacuum, the dry residue was re-dissolved in methanol, and 12 g of silica powder was added. Methanol was evaporated from the suspension and the dried extract was transferred into a universal Rf cartridge. For the purification, CombiFlash® Rf+ Lumen apparatus was used with a 80 g HP Silica RediSep® Rf Gold column. Mobile phase A was composed of *n*-hexane - dichloromethane (6:4, v/v), mobile phase B was composed of dichloromethane - methanol (1:1, v/v) and a 40 min gradient program of 0 - 30% B in A was applied at a flow rate of 60 ml/min to yield compound **7** (3.07 g).

Compounds **8-12** were synthesized from compounds **2**, **3**, **4**, **5** and **6** respectively. Each starting material was dissolved in methanol to a final concentration of 10 mM (**2**: 24 mg; **3**: 22.40 mg; **4** and **6**: 20 mg, **5**: 50 mg), and then 1 equiv. of PIDA was added. The mixtures were stirred for 25 min at room temperature in case of compounds **8**, **9** and **11** and 45 min at room temperature in case of compounds **10** and **12**. The reactions were worked up as described above. Compounds **8** (11.43 mg) and **9** (12.03 mg) were purified over semi-preparative RP-HPLC using a Gemini C18 (5 μm, 250 × 10 mm) column with 50% aq. methanol at a flow of 3 ml/min. Compound **10** (10.87 mg) was purified over preparative RP-HPLC applying a Kinetex XB C18 (5 μm, 250 × 21.2 mm) column with 25% aq. acetonitrile at a flow of 16 ml/min. Compound **11** (32.22 mg) was purified by flash chromatography using CombiFlash® Rf + Lumen apparatus with a 4 g HP Silica RediSep® Rf Gold column. Compound **11** was dissolved in methanol and 1 g of silica powder was added. Methanol was removed from the suspension and the dried extract was transferred into a universal Rf cartridge. Mobile phase A was ethyl acetate, mobile phase B was methanol and a 15 min gradient program of 0 - 5 % B in A was applied at

a flow rate of 18 ml/min. For the purification of compound **12** (19.50 mg), CPC instrument was used with a biphasic solvent system composed of *n*-hexane - ethyl acetate - water - methanol (1.5:5:1.5:5, v/v/v/v) in ascending mode. Parameters of the instrument were as follows: constant pressure of 86 bars, flow rate of 10 ml/min and rotation speed of 2600 rpm. The purification was performed through one injection and an altogether 94.42% of the initial weight was recovered after the separation. CPC combined fractions 1-7, 8-11, 12-18 and 19-25 were investigated on RP-HPLC on a Kinetex XB C18 (5 µm, 250 × 4.6 mm) column with an isocratic solvent system of 30% aq. acetonitrile at a flow-rate of 1 ml/min. From the combined fractions 8-11, compound **12** was reached.

III.2.2 Preparation of compounds 12-18 through autoxidation of poststerone

For the synthesis of compounds **12-18**, 450 mg of poststerone (**7**) was dissolved in a mixture of 10 ml of methanol and 80 ml of water, and 500 mg of NaOH dissolved in 10 ml of water was added. The reaction mixture was stirred for 4 hours at room temperature and was terminated by neutralizing the pH with an aq. solution of acetic acid at 9.60%. After the evaporation of the solvent under nitrogen, the residue was extracted through silica using methanol as eluent then dried. The final reaction mixture was fractionated by CPC with a biphasic solvent system previously developed in our laboratory, it was composed of ethyl acetate - water - methanol (20:20:1, v/v/v). The fractionation was performed through four consecutive injections and an altogether 95.43% of the initial weight was recovered after the separation. The fractions were combined based on their NP-TLC fingerprints on silica plates developed in a solvent system of ethyl acetate - ethanol - water (12:1:0.5, v/v/v). The combined CPC fractions were evaporated under vacuum at 40 °C and investigated by RP-HPLC on a Kinetex XB C18 (5 µm, 250 × 4.6 mm) column with an isocratic solvent system of 23% aq. acetonitrile at a flow rate of 1 ml/min. Fractions 6, 7, and combined fractions 11-13 were selected for additional purification steps. Fraction 6 was further purified over semi-preparative RP-HPLC using a Gemini C18 (5 µm, 250 × 10 mm) column with 30% aq. acetonitrile at a flow rate of 3 ml/min to yield compounds **12** (1.50 mg) and **16** (3.76 mg). Fraction 7 was further purified over semi-preparative RP-HPLC applying a Gemini C18 (5 µm, 250 × 10 mm) column with 27% aq. acetonitrile at a flow rate of 3 ml/min to yield compound **17** (10.61 mg). The combined CPC fractions 11-13 were further purified over semi-preparative RP-HPLC utilizing a Luna Phenyl-Hexyl (5 µm, 250 × 10 mm) column with 20% aq. acetonitrile at a flow rate of 3 ml/min to yield compounds **13** (10.20 mg), **14** (5.70 mg), **15** (11.30 mg) and **18** (4.60mg).

In order to obtain a larger amount of the above poststerone (**7**) derivatives, the same reaction using a 500 mg aliquot of poststerone (**7**) was conducted, stirring for 7 hours in this

case. The reaction mixture was worked up and purified as described above. The isolated yields were as follows: compound **13** (27.90 mg), compound **14** (3.04 mg), compound **15** (20.00 mg), compound **16** (20.00 mg) and compound **18** (5.72 mg).

III.3 Preparation of ecdysteroid diacetonide derivatives

III.3.1 Preparation of 20E 2,3;20,22-diacetonide as starting material for semi-synthesis

20E 2,3;20,22-diacetonide (**19**) was synthesized from 20E (**1**) by dissolving 2 g aliquot of 20E (**1**) in acetone to a final concentration of 1 mg/ml and then the same amount of phosphomolybdic acid was added. The mixture was sonicated at room temperature for 4 min, the reaction was stopped by alkalizing with a 10% aq. $NaHCO_3$, and the solution was subsequently concentrated by vacuum distillation until only water was present. Finally, the mixture was submitted to extraction with dichloromethane (5 × 100 ml), Na_2SO_4 was added to the organic phase and the solvent was evaporated under vacuum at 40 °C. The obtained extract was dissolved in methanol, 2.5 g of silica powder was added and then methanol was evaporated, the resulting residue was transferred into a universal Rf cartridge. For the purification, CombiFlash® Rf + Lumen apparatus was used with a 40 g HP Silica RediSep® Rf Gold column. Mobile phase A was dichloromethane, mobile phase B was methanol and a 60 min gradient of 0 - 5% B in A was applied at a flow rate of 60 ml/min to yield compound **19** (1.26 g).

III.3.2 Preparation of compounds 20-24 through autoxidation of 20E 2,3;20,22-diacetonide

For the preparation of compounds **20**-**23**, 450 mg of 20E 2,3;20,22-diacetonide (**19**) was dissolved in a mixture of 95 ml of methanol and 5.3 ml of water, and 960 mg of NaOH dissolved in 5.3 ml of water was added. The reaction mixture was stirred for 8 hours at room temperature. The pH of the final mixture was adjusted to 7 with a diluted aq. solution of acetic acid at 9.60%. After the evaporation of the solvent under nitrogen, the residue was subsequently fractionated by CPC with a solvent system composed of *n*-hexane - dichloromethane - methanol - water (1:0.215:1:1, v/v/v/v) in ascending mode. Parameters of the instrument were the followings: constant pressure of 86 bars, flow rate of 10 ml/min, and rotation speed of 2300 rpm. The fractionation was performed through seven consecutive injections and an altogether 88.89% of the initial weight was recovered after the separation. The fractions were combined based on their NP-TLC fingerprints on silica plates developed in a solvent system of *n*-hexane - ethyl acetate - ethanol - water (16:31:2:1, v/v/v/v) and RP-TLC on C18 plates using the eluting solvent system: methanol - water (0.95:0.5, v/v). The combined CPC fractions were evaporated under vacuum at 40 °C and investigated by RP-HPLC on an Agilent Eclipse XDB C8 (5 μm,

150 × 4.6 mm) column with an isocratic solvent system of 42% aq. acetonitrile at a flow rate of 1 ml/min. CPC fractions 1-12, 13-20 and 23-29 were selected for additional purification steps. The combined fractions 1-12 were further purified over semi-preparative RP-HPLC using an Agilent Eclipse XDB C8 (5 µm, 250 × 10 mm) column with 45% aq. acetonitrile at a flow rate of 3 ml/min to yield compound **22** (15.28 mg). The combined fractions 13-20 were purified over semi-preparative RP-HPLC applying an Agilent Eclipse XDB C8 (5 µm, 250 × 10 mm) column with 55% aq. acetonitrile at a flow rate of 3 ml/min to yield compound **23** (18.04 mg). The combined fractions 23-29 were further purified over semi-preparative NP-HPLC using an Agilent Zorbax SIL (5 µm, 250 × 9.4 mm) column with a solvent system composed of cyclohexane - isopropanol - water (20:2.5:0.1, v/v/v) at a flow rate of 3 ml/min to yield compounds **20** (9.95 mg) and **21** (16.04 mg).

Subsequently, 120 mg aliquot of 20E 2,3;20,22-diacetonide (**19**) was reacted with NaOH using the same reaction setup as above during 15 h. The reaction mixture was purified over RP-HPLC using a Luna Phenyl-Hexyl (5 µm, 250 × 10 mm) column with 55% aq. acetonitrile at a flow rate of 3 ml/min to yield compound **24** (9.34 mg).

III.3.3 Preparation of calonysterone 2,3;20,22-diacetonide

Calonysterone 2,3;20,22-diacetonide (**25**) was obtained from calonysterone (**6**) by dissolving a 120 mg aliquot of calonysterone (**6**) in 40 ml of acetone and then the same amount of phosphomolybdic acid was added. The reaction mixture was sonicated for 5 min at room temperature and stopped by settling the pH to 7 with a 10% aq. solution of $NaHCO_3$. Acetone was evaporated under vacuum at 40 °C, and the remaining aqueous mixture was extracted with dichloromethane (5 × 40 ml). Finally, Na_2SO_4 was added to the organic phase and the solvent was evaporated under vacuum at 40 °C. The residue was dissolved in dichloromethane and 1 g of silica powder was added. After the evaporation of dichloromethane, the dried residue was transferred into a universal Rf cartridge. For the purification, CombiFlash® Rf + Lumen apparatus was used with a 12 g HP Silica RediSep® Rf Gold column. Mobile phase A was *n*-hexane, mobile phase B was ethyl acetate, and a 40 min gradient program of 10 - 50 % B in A was applied at a flow rate of 30 ml/min to yield compound **26** (89.64 mg).

III.4 Preparation of compounds 26-39 by gamma irradiations of 20E

The gamma irradiations of aqueous solutions of 20E (**1**) were performed in collaboration with the group of *Dr. Tünde Tóth* (Centre for Energy Research Hungarian Academy of Sciences, Radiation Chemistry Department, Budapest, Hungary; Budapest University of Technology and Economics, Budapest, Hungary), and preliminary analytical screening was

performed by the group of *Dr. György Tibor Balogh* (Gedeon Richter Plc, Budapest, Hungary) using HPLC-ESI-MS system on a Waters Cortecs C18 (2.7 μm, 150 × 4.6 mm) column and ESI source in positive mode.

Aqueous solutions containing 200 mg of 20E (**1**) at a concentration of 0.5 mmol/dm^3 were prepared. The 20E (**1**) solutions were irradiated at room temperature using a 60Co panoramic type γ-irradiation facility (dose rate = 10,5 kGy/h), the absorbed dose was 1 or 2 kGy. The irradiations were performed in N_2- or N_2O-saturated solution. The final irradiated materials were lyophilised and analysed with HPLC-ESI-MS. The aqueous solutions of 20E (**1**) irradiated in N_2- or N_2O-saturated solution that absorbed a dose of 2 kGy were selected for purification. Due to the low amounts of the residues (180 mg residue **I** of aqueous solution of 20E (**1**) irradiated in N_2-saturated solution and 152 mg residue **II** of aqueous solution of 20E (**1**) irradiated in N_2O-saturated solution), the samples were directly subjected to preparative RP-HPLC using a Kinetex XB C18 (5 μm, 250 × 21.2 mm) column with 20% aq. acetonitrile at a flow rate of 16 ml/min. The purification of residue **I** resulted in the isolation of compounds **26** (4.82 mg), **27** (0.96 mg) and **28** (0.53 mg). The purification of residue **II** allowed the isolation of compounds **27** (3.53 mg), **28** (1.70 mg), **29** and **30** (1.10 mg).

In order to obtain a larger amount of the above products and to isolate other minor compounds, the irradiations were scaled-up. Two aqueous solutions (**I** and **II**) each containing 1 g of 20E (**1**) at a concentration of 2 mmol/dm^3 were prepared. Irradiations were performed similarly as above (dose rate = 10 kGy/h, absorbed dose = 6 kGy), either in N_2-saturated (solution **I**) or in N_2O-saturated solution (solution **II**). Each final irradiated material was lyophilized and 838 mg of residue **I** and 843 mg of residue **II** were obtained. These residues were fractionated by CPC with a solvent system composed of *tert*-butanol - ethyl acetate - water (0.45:0.9:1, v/v/v) in ascending mode. Parameters of the instrument were as follows: constant pressure of 87 bars, flow rate of 10 ml/min and rotation speed of 2900 rpm. The fractionation was performed through one injection of each residue, and an altogether 95.71% and 97.35% of the initial weights of residue **I** and **II**, respectively, were recovered after the separation. The fractions were combined based on their NP-TLC fingerprints on silica plates developed in a solvent system of ethyl acetate - ethanol - water (20:2:0.5, v/v/v). The combined CPC fractions were evaporated under vacuum at 40 °C and investigated by RP-HPLC on a Kinetex Biphenyl (5 μm, 250 × 4.6 mm) column with an isocratic solvent system of 20% aq. acetonitrile at a flow rate of 1 ml/min. For the purification of combined CPC fractions of residues **I** and **II**, preparative (16 ml/min) or semi-preparative (3 ml/min) RP-HPLC techniques using different chromatographic columns were utilized as summarized in **Table 1**.

Table 1 Chromatographic purification of compounds **26-29** and **31-39**

	Fraction	Solid phase	Mobile phase	Flow	Products
N_2	1-12	Kinetex XB C18	20% aq. CH_3CN	16 ml/min	**28** (4.06 mg)
	13-14	Kinetex XB C18	20% aq. CH_3CN	16 ml/min	**31** (6.61 mg) **32** (2.00 mg)
	15-16	Kinetex Biphenyl	20% aq. CH_3CN	16 ml/min	**26**(13.15 mg)
	17-18	Luna Phenyl-Hexyl	15% aq. CH_3CN	3 ml/min	**33** (2.62 mg)
	21-26	-	-	-	**1** (257.96 mg)
	27-30	Kinetex Biphenyl	20% aq. CH_3CN	16 ml/min	**27** (7.79 mg)
	31-39	Kinetex Biphenyl	20% aq. CH_3CN	16 ml/min	**35** (1.20 mg)
	40-43	Luna Phenyl-Hexyl	20% aq. CH_3CN	3 ml/min	**36** (1.48 mg)
N_2O	1-16	Kinetex Biphenyl	20% aq. CH_3CN	16 ml/min	**28** (6.76 mg) **34** (0.90 mg)
	17-20	Kinetex XB C18	20% aq. CH_3CN	16 ml/min	**31** (1.20 mg) **33** (1.00 mg)
	21-27	Luna Phenyl-Hexyl	17% aq. CH_3CN	3 ml/min	**29** (0.70 mg)
	28-31	Luna Phenyl-Hexyl	20% aq. CH_3CN	3 ml/min	**37** (1.50 mg)
	32-36	-	-	-	**1** (387.66 mg)
	37-45	Luna Phenyl-Hexyl	18% aq. CH_3CN	3 ml/min	**27** (18.18 mg) **38** (1.00 mg)
	48-54	Luna Phenyl-Hexyl	15% aq. CH_3CN	3 ml/min	**36** (2.27 mg)
	63-73	Kinetex Biphenyl	20% aq. CH_3CN	16 ml/min	**39** (2.66 mg)

III.5 Longitudinal study of the base-catalyzed autoxidation of 20E by CE [II]

III.5.1 Preparation of reference compounds 6, 41 and 43

For the preparation of compounds **6**, **41** and **43**, base-catalyzed autoxidation of 20E (**1**) was performed as previously published [78], with the minor difference that the final pH of the mixture was set to slightly acidic (pH ≈ 6). The final mixture was fractionated through classical reverse phase column chromatography with a stepwise gradient of methanol - water (3:7 to 6:4, v/v) resulting in 15 combined fractions. Fraction 2 was further purified through CombiFlash® Rf + Lumen apparatus with a 12 g HP Silica RediSep® Rf Gold column. Mobile phase A was *n*-hexane, mobile phase B was acetone, and a 40 min gradient program of 0 - 20 % B in A was applied at a flow rate of 30 ml/min to yield compound **41** (78.20 mg). Fraction 8 was purified by CPC with a solvent system of ethyl acetate - water - methanol (20:20:1, v/v/v) in ascending mode leading to compound **43** (40.58 mg). Fraction 9 was crystallized from ethyl acetate - methanol (2:1, v/v) resulting in the isolation of compound **6** (115.06 mg).

III.5.2 CE setup, evaluation of the CE method applicability and data analysis

The CE analysis was performed with an Agilent Capillary Electrophoresis 3DCE system applying a bare fused silica capillary of 64.5 cm total and 56 cm effective length with 50 μm

I.D. The temperature was set to 25 °C, the samples were injected by 5×10^3 Pa pressure for 6s and the separation voltage was +30 kV. The detection with UV was at the maximum absorption wavelength (λ_{max}) of each compound (222, 228, 228, 247, 260 and 360 nm for compounds **6**, **40**, **41**, **1**, **42** and **43**, respectively). Between measurements, the capillary was rinsed by 0.1 M HCl, 1 M NaOH, 0.1 M NaOH and water for 2 min, then with background electrolyte (BGE) for 3 min. Phosphoric acid buffer (10 mM) at pH 11 was applied as BGE completed with 5 mM sulfobutyl ether β-cyclodextrin (SBEBCD) selector and the pH was adjusted by NaOH.

Applicability of the proposed method was checked according to the recommendations of the Good Laboratory Practice [99]:

1. Linearity of the method was assessed by standards for each material at a minimum of four concentration levels, ranging between 25 and 1200 μg/ml with triplicate injections for each concentration.
2. Limits of detection (LOD) and limits of quantification (LOQ) were calculated for each material, and they were defined as LOD = (b + 3 sx / y) / a and LOQ = (b + 10 sx / y) / a, where a, b and (sx / y) are the slope, the intercept and the residual standard deviation estimated from the linear regression, respectively [99].
3. Intra-day precision was studied by triplicate injections of 1200 μg/ml concentration of 20E (**1**), compounds **42** and **43** and of 600 μg/ml concentration of compounds **6** and **41**. Inter-day precision was determined by repeating the procedure during three consecutive days.
4. Accuracy of the proposed method was established by the determination of the recoveries of the samples by calculating their concentrations including concentrations: close to the quantitation limit, in the middle of the range and at the high end of the calibration curves.

III.5.3 Monitoring the base-catalyzed autoxidation of 20E by capillary electrophoresis

Briefly, 120 mg of 20E (**1**) was dissolved in a mixture of (9:1 v/v) aqueous methanol. The reaction was initiated with the addition of 100 mg of NaOH dissolved in 1 ml water. The reaction was continuously stirred and aliquots from reaction mixture were diluted by 10 times in aqueous methanol solution (9:1, v/v) and then subsequently injected into the CE equipment. Three independent autoxidation reactions were performed during 48h, and the sampling was made each 30 minutes in the first 8 hours and then at 15, 24, 27, 29, 31 and 48 hours.

In order to compare between raw quantitative analytical data obtained previously with RP-HPLC [78] and new data obtained with CE, all data were fitted through an automatic selection of the best-fit model by using the Levenberg-Marquardt based algorithms implemented in OriginPro 9.1 software. In case of RP-HPLC results, best-fit models for nonlinear regression were: one-phase exponential decay function model for 20E (**1**) data,

inverted offset exponential function modified model for compound **41** data, and exponentially modified Gaussian peak function for use in chromatography for compounds **42** and **43** data. In case of CE results, automatically-selected best-fit models were: one-phase exponential decay function model for 20E (**1**) data, exponential function whose exponent is a 2nd order polynomial model for compounds **6** and **41** data, Chesler-Cram peak function for use in chromatography model for compound **42** data and exponentially modified Gaussian peak function for use in chromatography model for compound **43** data.

III. 6 Procedures for structure elucidation

Turbo ion spray tandem mass spectrometry spectra of compounds **8** and **10** were recorded on an API 2000 triple quadrupole tandem mass spectrometer (AB SCIEX, Foster City, CA, USA) in positive mode equipped with ESI ion source. HRMS spectra of compounds **12-18** were recorded on a Waters Acquity I-Class UHPLC system (Waters Co., Milford, MA, USA) coupled with a Thermo Scientific Q Exactive Plus orbitrap mass spectrometer in positive mode equipped with HESI source (Thermo Fisher Scientific, Scoresby, Australia). HRMS spectra of compounds **20-25** were obtained from an Agilent 1100 HPLC system coupled with Thermo Q Exactive Plus orbitrap mass spectrometer in positive mode equipped with HESI source (Waters Co., USA).

NMR investigations of compounds **8**, **10**, **12**, **14-18** and **20-25** were performed in collaboration with *Prof. Gábor Tóth* (Department of Inorganic and Analytical Chemistry, Budapest University of Technology and Economics, Budapest, Hungary).

^{1}H (500.1 MHz) and ^{13}C (125.6 MHz) NMR spectra of the compounds 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 0.7 to 5 mg of compounds were dissolved in 0.1 ml of methanol-d_4 and transferred to 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 (δ_C = 49.1 and δ_H = 3.31 ppm). NMR assignments of compound **18** were also determined in dimethyl sulfoxide-d_6 (δ_C = 39.5 and δ_H = 2.50 ppm). Pulse programs of all experiments 1D (^{1}H, ^{13}C, DEPTQ, DEPT-135, APT, sel-TOCSY, sel-ROE (τ_{mix}: 300 ms) and 2D (^{1}H,^{1}H-COSY, gs-HSQC, edited gs-HSQC, gs-HMBC, band-selective HSQC and band-selective HMBC) were taken from the Bruker software library. Most ^{1}H assignments were accomplished using general knowledge of chemical shift dispersion with the aid of the ^{1}H-^{1}H coupling pattern (^{1}H NMR spectra). ^{1}H-NMR chemical shifts of overlapped signals were identified utilising 1D selective ROESY responses, or from

1D selective TOCSY experiments or by HSQC experiments. The NMR signals of the compounds were assigned by comprehensive 1D and 2D NMR methods using widely accepted strategies [101, 102].

Structural characterization of compounds **26-39** by NMR and MS spectroscopic methods were performed in collaboration with *Dr. Zoltán Béni* and *Dr. Miklós Dékány* (Spectroscopic Research Department, Gedeon Richter Plc., Budapest, Hungary).

HRMS analyses were performed on a LTQ FT Ultra or on a Velos Pro Orbitrap Elite (Thermo Fisher Scientific, Bremen, Germany) systems. Prior to direct infusion samples were dissolved in methanol. The ESI method operated in positive ion mode was used in all cases.

NMR data were collected on a Bruker 500 or a Bruker 800 MHz spectrometer, both equipped with a triple resonance Cryoprobe. Chemical shifts were reported in the δ-scale using residual solvent signals ($\delta_C = 3.31$ and $\delta_H = 49.15$) as references. Standards 1D (^1H, ^{13}C) and 2D (COSY, HSQC, HMBC, NOESY/ROESY) spectra were acquired at 25°C in methanol-d_4 using the pulse sequences available in the TOPSPIN 3.5 library. Spectral data analysis and reporting were accomplished in the ACDLab/NMR Workbook 2017.1.3. software suite.

III. 7 Biological evaluation of the compounds

III.7.1 Bioactivity testing on Akt-phosphorylation of compounds 1 and 7-18 [III]

In vitro capacity to increase the Akt-phosphorylation was studied in collaboration with *Dr. Tusty-Jiuan Hsieh* (Graduate Institute of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan).

Mouse C2C12 skeletal myoblasts (BCRC#60083) were purchased from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Taiwan). The cells were seeded in 6-well plates and maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 1% penicillin/streptomycin solution in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. After reaching 100% confluence, C2C12 myoblasts were cultured in differentiation medium (DMEM containing 4500 mg/l D-glucose and 10% horse serum), and the medium was changed every 2 days.

First, poststerone (**7**) was tested in comparison with 20E (**1**) in the phosphorylation of Akt at different concentrations: 0.01, 0.1, 1 and 10 μM: after 6 days of differentiation, the cells became skeletal myotubes and then, the culture medium was changed to serum free normal-glucose DMEM with or without the tested concentrations of 20E (**1**) and poststerone (**7**). Secondly, the ability of compounds **7**-**18** to increase the Akt-phosphorylation was tested: the

cells became skeletal myotubes after 8 days of differentiation, then, the culture medium was changed to serum free normal-glucose DMEM with or without 10 μM of each compound **7**-**18**.

In both studies, after 2 h, the cells were lysed with 500 μl of 1× 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 AKT activation, 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.

III.7.2 *In vitro* antitumor activity testing of compounds 20-23

In vitro antitumor activities of compounds **20**-**23** were experienced in collaboration with *Dr. Gabriella Spengler* (Department of Medical Microbiology and Immunobiology, University of Szeged, Szeged, Hungary).

Mouse T-cell lymphoma (L5178) cell line was purchased from the European Collection of Cell Cultures (ECCAC, Salisbury, UK). $L5178_{MDR}$ cell line was previously obtained by transfecting L5178 cells with pHa MDR1/A retrovirus [103] and it was selected by culturing the infected cells with 60 μg/l colchicine. Cells were cultured in McCoy's 5A medium supplemented with 10% heat inactivated horse serum, L-glutamine, and antibiotics (penicillin and streptomycin), at 37°C in humidified air containing 5% CO_2.

Inhibition of ABCB1 function of compounds **20**-**23** was investigated through the intracellular retention of rhodamine 123, a fluorescent dye, according to our previously published procedures [49]. Briefly, 2×10^6 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 a water bath for 20 min. Following centrifugation (2000 rpm, 2 min), samples were washed twice with 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 nM of tariquidar was used as positive control, and it exerted a complete inhibition of the transporter.

Cytotoxic activity of compounds **20**-**23** alone and in combination with doxorubicin was tested following our published procedures using the checkerboard microplate method [49]. Briefly, 6,000 cells per well were incubated with doxorubicin and the compound to be tested for 48 h at 37 °C under 5% CO_2. Cell viability was determined over MTT staining. The results were evaluated by using the CompuSyn software for the constant ratios and combination index (CI) values were calculated for 50, 75 and 90% of growth inhibition to assess the ecdysteroid-doxorubicin interaction (synergism, additivity or antagonism). Single-treatment activity for each compound was determined from the control lanes of the checkerboard plate.

IV. RESULTS

IV.1 Preparation of the starting materials dacryhainansterone and calonysterone [I]

A commercial extract from the roots of *Cyanotis arachnoidea* was partially purified with multi-steps column chromatography and resulted in the isolation of a mixture containing two main phytoecdysteroids dacryhainansterone (**5**) and calonysterone (**6**) along with two minor components referred to as impurity **i** and **ii** based of RP-HPLC analysis. In order to isolate the two main phytoecdysteroids of interest, a CPC separation method was developed. Of the sixteen tested biphasic systems belonging to the HEMWat (*n*-Hexane - Ethyl acetate - Methanol - Water) family, five solvent systems allowed good partitioning of the targeted compounds between the two phases (see **Table 2**). In order to ensure the separation of the impurities **i** and **ii** from the two targeted phytoecdysteroids, their partition coefficients and separation factors were also taken into account. The solvent system composed of *n*-hexane - ethyl acetate - methanol - water (1:5:1:5, v/v/v/v) provided the best partition coefficients ranging between 0.5 and 2 and gave acceptable calculated separation factor values. The selected solvent system presented the following separation characteristics: a good settling time (27 s), the volume ratio of the upper and lower phases was 0.90 and the retention volume ratio Sf was 0.70.

Table 2 Partition coefficients and separation factors of the tested biphasic systems in separation of compounds **5** and **6** from commercial extract of *Cyanotis arachnoidea*. The best solvent system selected for the preparative separation is highlighted in grey

Solvent systems	Partition coefficients				Separation factors		
***n*-hexane - ethyl acetate - methanol - water (v/v/v/v)**	$K_{(U/L)_5}$	$K_{(U/L)_6}$	$K_{(U/L)_i}$	$K_{(U/L)_{ii}}$	$\alpha_{(5/6)}$	$\alpha_{(i/6)}$	$\alpha_{(5/ii)}$
0:1:0:1	10.916	5.260	6.343	7.880	2.075	1.206	1.385
1:10:1:10	2.699	1.542	1.798	2.283	1.750	1.166	1.182
1:5:1:5	2.042	1.136	1.730	1.307	1.800	1.523	1.562
5:20:5:20	1.341	0.702	0.395	0.887	1.910	1.777	1.512
3:10:3:10	0.965	0.529	0.375	0.566	1.824	1.411	1.705

After six consecutive injections of the 1g aliquot of crude extract in the CPC instrument, dacryhainansterone (**5**) and calonysterone (**6**) were separated in less than 30 min (**Fig. 2**). Combined fractions 1-8 (0-14 min), 9-11 (14-18 min), 12 (18-22 min), and 13-20 (22-30 min) were investigated. From the combined fractions 9-11, dacryhainansterone (**5**) was obtained, and from fractions 13-20, calonysterone (**6**) was isolated. These latter were isolated with a purity of 93.00% for dacryhainansterone (**5**) and 96.00% for calonysterone (**6**). After recrystallizing them from ethyl acetate - methanol (2:1, v/v), their purity reached 99.10% and 99.70% respectively.

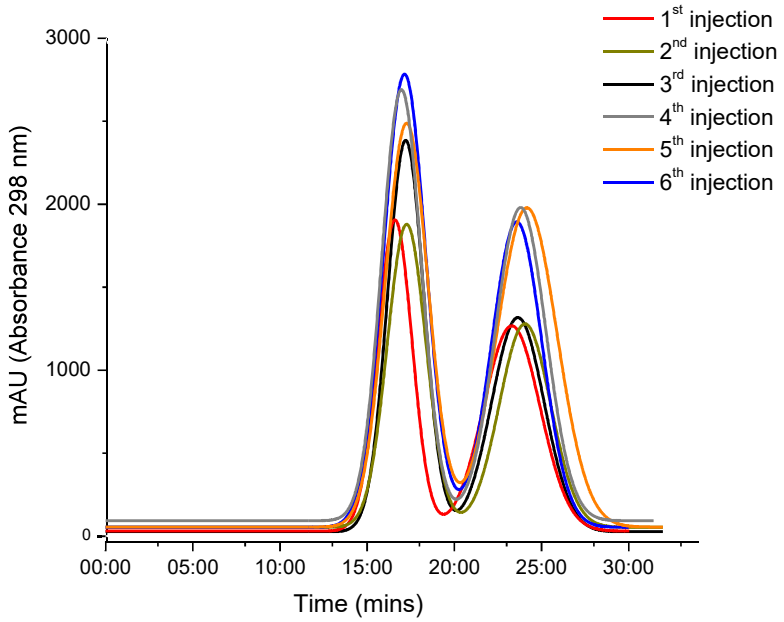

Fig. 2. CPC separations of 1 g of pre-purified commercial extract of *Cyanotis arachnoidea* using six consecutive injections: three were of a concentration of 14 mg/ml and three were of a concentration of 23 mg/ml. Slight shifting of retention times comes from unavoidable variations in the injected solvent composition of each consecutive run

Raw data of chromatograms were extracted from Armen Glider CPC software and were plotted with OriginPro 9.1

IV.2 Preparation of side-chain cleaved ecdysteroids [III]

IV.2.1 Preparation of side-chain cleaved ecdysteroids 7-12

Oxidative side-chain cleavage of different phytoecdysteroids (**1**-**6**) to afford compounds **7**-**12** was performed by using the hypervalent iodine reagent PIDA.

Our recently published procedure, using PIFA for the oxidation of 20E (**1**) to form poststerone (**7**), could provide compound **7** in a yield of 57.80% [53]. In this Ph.D. work, we report an update to this reaction: when using 1 equiv. of PIDA during 45 min in a large scale reaction followed by purification with flash chromatography, poststerone (**7**) could be obtained in a decent isolated yield of 81.41% (3.07 g). The reaction was carried out similarly on other phytoecdysteroids: 2-deoxy-20E (**2**), ajugasterone C (**3**), polypodine B (**4**), dacryhainansterone (**5**) and calonysterone (**6**). Since these compounds were available in lower amounts, smaller scale reactions were performed. Preparative or semi-preparative RP-HPLC was applied to isolate compounds **8**-**10** with the following good yields: **8** (11.43 mg, 68.34%), **9** (12.03 mg, 63.57%) and **10** (10.87 mg, 71.23%) and flash chromatography was used to obtain compound **11** in a high isolated yield of 82.71% (32.22 mg) (see section III.2.1 for detailed purification methods). For the isolation of compound **12** (19.50 mg, 51.86%), a CPC separation method was developed. Six solvent systems belonging to the HEMWat family were tested (**Table 3**) and the system composed of *n*-hexane - ethyl acetate - methanol - water (1.5:5:1.5:5, v/v/v/v) provided

the best partition coefficients and an excellent separation factor. The selected solvent system presented satisfying separation characteristics: a very short settling time (17s), the volume ratio of the upper and lower phases was 0.86, and the retention volume ratio Sf was 0.68. Structures of starting materials **1**-**6** and their side-chain analogues (**7**-**12**) are shown in **Fig. 3**.

Table 3 Partition coefficients and separation factors of the tested biphasic systems in separation of compounds **6** and **12**. The optimal CPC biphasic system selected for the preparative separation is highlighted in grey

Solvent systems	Partition coefficients		Separation factors
***n*-hexane - ethyl acetate - methanol - water (v/v/v/v)**	$K_{(U/L)_6}$	$K_{(U/L)_{12}}$	$\alpha_{(12/6)}$
0:5:0:5	3.145	13.967	4.441
0.5:5:0.5:5	1.200	10.860	9.050
1.25:5:1.25:5	0.784	11.258	14.359
1.5:5:1.5:5	0.460	1.370	2.978
2:5:2:5	0.700	6.302	9.003
2.25:5:2.25:5	0.332	2.190	6.596

After the separation, CPC combined fractions 8-11 and 19-25 were investigated. From the combined fractions 8-11, compound **12** was obtained with a purity of 96.00%. After recrystallization from ethyl acetate - methanol (2:1, v/v), the purity reached 99.00%.

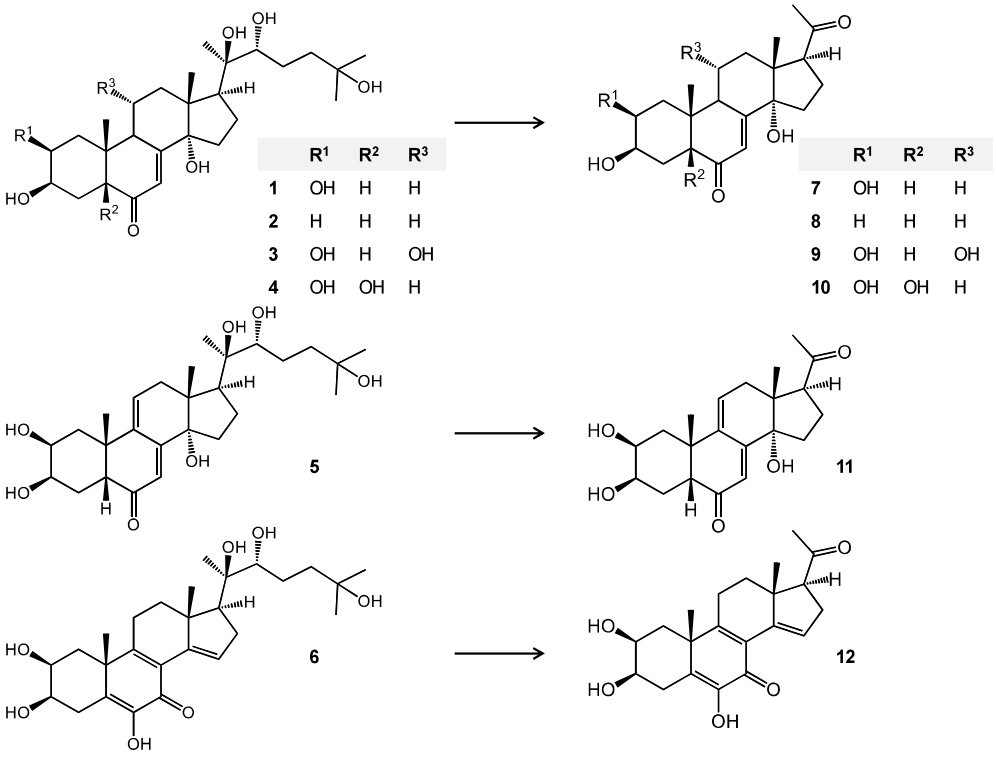

Fig. 3. Starting materials **1**-**6** and their derivatives (**7**-**12**) obtained by side-chain cleavage

IV.2.2 Preparation of compounds 12-18 through autoxidation of poststerone

For the synthesis of poststerone (**7**) derivatives, the starting material was dissolved in methanol - water (9:1, v/v), and subsequently NaOH was added to the mixture as a catalyst. The reaction mixture was stirred for 4 hours, stopped by neutralizing the pH with 9.60% acetic acid, extracted through silica and evaporated under vacuum at 40 °C. The obtained dry residue was fractionated by CPC in ascending mode. The applied biphasic system, composed of ethyl acetate - water - methanol (20:20:1, v/v/v), was previously developed in our laboratory and was found to provide satisfying fractionation of the targeted compounds. Peaks from the combined CPC fractions were collected during subsequent RP-HPLC purifications (see section III.2.2 for detailed methods) and compounds **12-18** were isolated with the following yields: **12** (1.50 mg, 0.34%), **13** (10.20 mg, 2.17%), **14** (5.70 mg, 1.21%), **15** (11.30 mg, 2.42%), **16** (3.76 mg, 0.84%), **17** (10.61 mg, 2.48%) and **18** (4.60 mg, 1.00%). Structures of poststerone (**7**) derivatives (**13-18**) are seen on **Fig. 4** while the structure compound **12** is presented in **Fig. 3**.

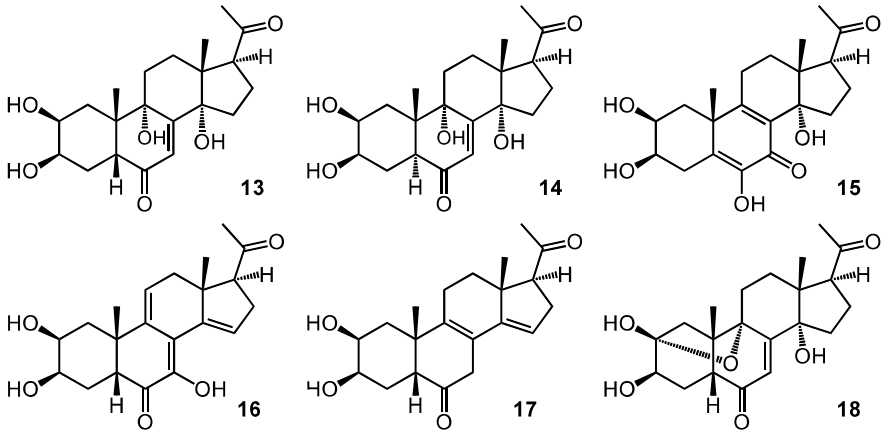

Fig. 4. Ecdysteroid derivatives (**13-18**) obtained from the autoxidation of poststerone (**7**)

Considering our previous observations on the significant bioactivity changes connected to certain autoxidized derivatives 20E (**1**) [78], larger amounts of the above similar derivatives from poststerone (**7**) were required to perform biological activities. Small-scale experiments of the above reaction were performed, and the degradation of poststerone (**7**) and the formation of its oxidised derivatives (**12-18**) were monitored by NP-TLC. A longer reaction time of *ca.* 7-9 hours was found to be preferable in order to obtain the targeted compounds in larger amounts. Consequently, the same reaction was conducted for 7 hours using 500 mg of poststerone (**7**), and subsequently the same purification steps were applied as before. Thanks to the optimized reaction time, yields of compounds **13**, **15**, **16** and **18** increased by 2.46, 1.59, 4.81, 1.10 times respectively. However, as expected based on the previous NP-TLC fingerprints, compound **17** was not detected in this reaction mixture.

IV.3 Preparation of ecdysteroid diacetonide derivatives

IV.3.1 Preparation of 20E 2,3;20,22-diacetonide

As 20E (**1**) presents vicinal diols at C-2,3 and at C-20,22; the use of an acid i.e. phosphomolybdic acid and a ketone such as acetone as reagent led to diacetonide formation at C-2,3 and C-20,22 on the starting compound resulting in 20E 2,3;20,22-diacetonide (**19**). Flash chromatography was used to isolate the targeted compound **19** (1.26 g, 54.00%) (see section III.3.1 for detailed purification method).

IV.3.2 Preparation of autoxidized derivatives 20-24 of 20E 2,3;20,22-diacetonide

For the preparation of the 20E 2,3;20,22-diacetonide (**19**) derivatives, the starting material was autoxidized in the presence of NaOH two times, using the same reaction setup for different durations. First, 450 mg of 20E 2,3;20,22-diacetonide (**19**) was stirred for 8 hours at room temperature in a methanol - water (95:5, v/v) mixture containing 1% NaOH. After neutralizing the pH of the final mixture by acetic acid 9.60%, the solvent was evaporated under nitrogen stream, and the residue was fractionated by CPC in ascending mode. The new CPC method development involved testing twenty-five solvent systems, among which five gave acceptable partitioning of the targeted compounds between the two phases (see **Table 4**). The solvent system composed of *n*-hexane - dichloromethane - methanol - water (1:0.215:1:1, v/v/v/v) was selected for the separation. This biphasic system presented the best separation characteristics among the tested biphasic systems: a settling time of 32s, the volume ratio of the upper and lower phases was 0.85 and the retention volume ratio Sf was 0.56.

Table 4 Partition coefficients of the tested biphasic systems in separation of compounds **19-23**. The best solvent system selected for the preparative fractionation is highlighted in grey

Solvent systems	**Partition coefficients**				
	Weak to moderate affinity to the organic phase				
***n*-hexane - dichloromethane - methanol - water (v/v/v/v)**	**$K_{(U/L)_{20}}$**	**$K_{(U/L)_{21}}$**	**$K_{(U/L)_{19}}$**	**$K_{(U/L)_{23}}$**	**$K_{(U/L)_{22}}$**
1:0.100:1:1	0.505	0.530	0.224	0.545	1.127
1:0.200:1:1	0. 702	0.608	0.728	0. 972	0.800
	Weak to moderate affinity to the organic phase		**Average affinity**		**High affinity**
1:0.215:1:1	0.878	0.913	1.285	1.284	1.703
	Moderate to high affinity to the organic phase				
	$K_{(U/L)_{20}}$	**$K_{(U/L)_{21}}$**	**$K_{(U/L)_{19}}$**	**$K_{(U/L)_{23}}$**	**$K_{(U/L)_{22}}$**
1:0.225:1:1	1.235	1.114	1.281	2.147	1.370
1:0.250:1:1	1.442	1.214	1.463	2.853	1.500
1:0.300:1:1	2.048	1.763	2.217	3.736	2.252

Subsequent RP-HPLC or NP-HPLC purifications of the combined CPC fractions allowed the isolation of compounds **20**-**23** with the following yields: **20** (9.95 mg, 1.79%), **21** (16.04 mg, 2.88%), **22** (15.28 mg, 2.83%) and **23** (18.04 mg, 3.26%). The second reaction was performed for 15 hours and was stopped by neutralizing the pH as above. Purification over RP-HPLC yielded compound **24** (9.34 mg, 7.83%) (see section III.3.2 for detailed purification methods).

IV.3.3 Preparation of calonysterone 2,3;20,22-diacetonide

Calonysterone 2,3;20,22-diacetonide (**25**) was detected in the above-mentioned mixtures, but in a very low yield. Therefore, this compound was prepared through diacetonide formation from calonysterone (**6**) previously isolated from the extract of *Cyanotis arachnoidea* extract. The same amounts of calonysterone (**6**) and phosphomolibdic acid were sonicated for 5 min at room temperature in acetone. After stopping the reaction and working it up as described in section III.3.3, the final mixture was purified by flash chromatography to yield compound **25** (89.64 mg, 63.91%). Structures of the synthesized 2,3;20,22-diacetonide derivatives (**20**-**25**) are presented in **Fig. 5**.

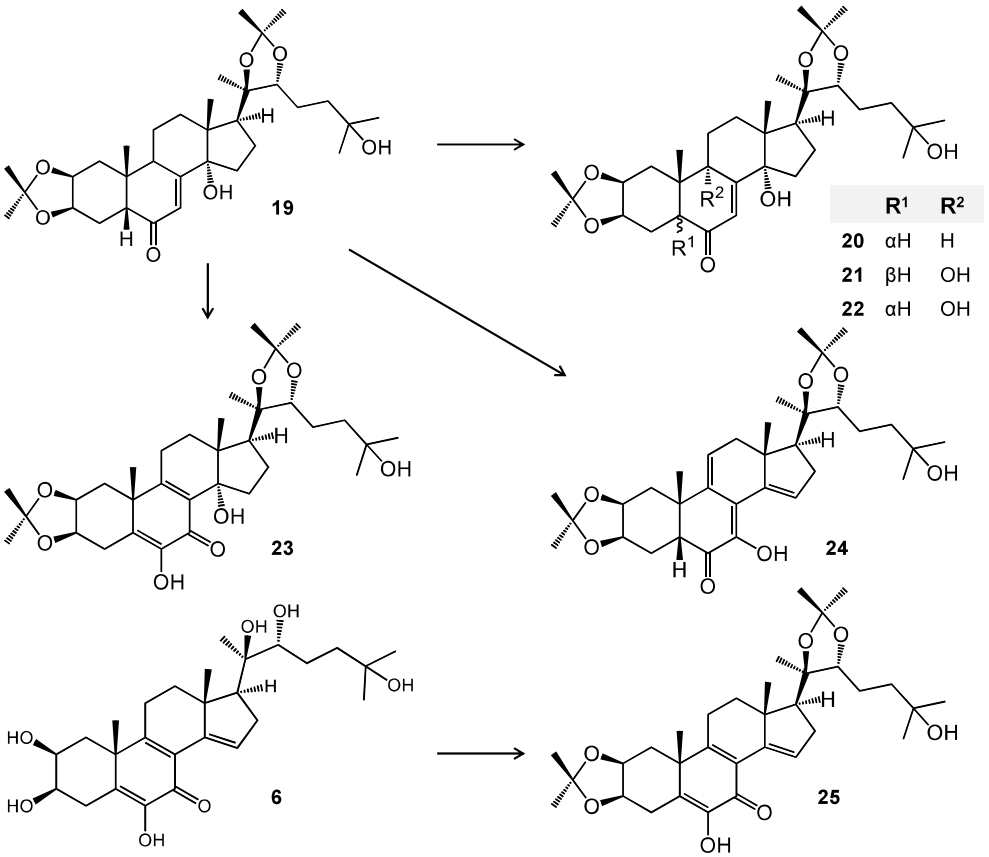

Fig.5. Structures of oxidized ecdysteroid diacetonides (**20**-**25**)

IV.4 Preparation of ecdysteroid derivatives 26-39 from gamma irradiations of 20E

Gamma irradiations of 20E (**1**) were performed targeting structural modifications of the whole starting material with an interesting chemistry never explored for ecdysteroids before.

As a first step, small scale irradiations in N_2- or N_2O-saturated aqueous solutions of 20E (**1**) were performed at dose rate = 10 kGy/h with absorbed doses of 1 or 2 kGy. The preliminary HPLC-ESI-MS analysis of the resulted materials showed that same product patterns were obtained at both absorbed doses; however, the applied gas had an influence on the peak ratio of the observed products. Thus, both irradiations performed in N_2- or N_2O-saturated solutions with an absorbed dose of 2 kGy were selected for purification over preparative RP-HPLC (see section III.4 for detailed purification methods). Common yielded compounds from both materials were compound **27** (N_2-saturation: 0.96 mg, 0.54%; N_2O- saturation: 3.53 mg, 2.35%) and podecdysone B (**28**, N_2-saturation: 0.53 mg, 0.31%; N_2O- saturation: 1.70 mg, 1.16%). 14-perhydroxy-20E (**26**, 4.82 mg, 2.59%) resulted only from the irradiation in N_2-saturated solution, whereas 2-dehydro-20E (**29**) and 2-dehydro-3-epi-20E (**30**) conceded in one single fraction (5:1 ratio, compound **29** being the major compound) were obtained only from the irradiation in N_2O-saturated solution.

Subsequently, larger scale irradiations were performed at dose rate = 10 kGy/h with absorbed dose of 6 kGy in N_2- or N_2O-saturated solutions. The resulting irradiated materials were subjected to fractionation by CPC in ascending mode. In order to find the most suitable CPC biphasic system, nine solvent systems were studied and three of them gave satisfactory partitioning of the targeted compounds between the two phases (see **Table 5**). The solvent system composed of *tert*-butanol - ethyl acetate - water (0.4:0.9:1, v/v/v) was selected for the separation. This latter gave optimal values concerning the settling time (24s), the volume ratio of the upper and lower phases (0.95) and the retention volume ratio Sf (0.52).

Table 5 Partition coefficients and separation factors for the selected biphasic systems in separation of main compounds from the gamma irradiations of 20E (**1**). The solvent system selected for the preparative fractionation is highlighted in grey

Solvent systems	**Partition coefficients**					
	Weak to moderate affinity with the organic phase		**Medium affinity**		**High affinity**	
***tert*-butanol - ethyl acetate - water (v/v/v)**	$\mathbf{K_{(U/L)_1}}$	$\mathbf{K_{(U/L)_{27}}}$	$\mathbf{K_{(U/L)_{33}}}$	$\mathbf{K_{(U/L)_{26}}}$	$\mathbf{K_{(U/L)_{31}}}$	$\mathbf{K_{(U/L)_{28}}}$
0.400:0.9:1	0.702	0.515	1.086	1.381	2.123	10.016
0.425:0.9:1	0.810	0.557	1.090	0.966	2.621	6.133
0.450:0.9:1	0.994	0.634	1.273	1.315	3.624	7.150

The obtained CPC fractions were purified from minor impurities by RP-HPLC (see section III.4 for detailed purification methods). From the irradiation of N_2-saturated aqueous solution of 20E

(**1**), the same compounds attained with small-scale irradiation were obtained with the following yields: 14-perhydroxy-20E (**26**, 13.15 mg, 1.52%), compound **27** (7.79 mg, 0.94%) and podecdysone B (**28**, 4.06 mg, 0.50%). These latter were accompanied by stachysterone B (**31**, 6.61 mg, 0.82%), 14-deoxy-20E (**32**, 2.00 mg, 0.25%), 5α-20E (**33**, 2.62 mg, 0.31%), 2-dehydro-3-deoxy-20E (**34**, 0.90 mg, 0.11%), the 7-11′ hetero-dimer (**35**, 1.20 mg, 0.07%) and compound **36** (1.48 mg, 0.18%). Concerning the irradiation in N_2O-saturated solution, compound **27** (18.18 mg, 2.18%), podecdysone B (**28**, 6.76 mg, 0.83%), 2-dehydro-20E (**29**, 0.70 mg, 0.08%), stachysterone B (**31**, 1.20 mg, 0.15%), 5α-20E (**33**, 1.00 mg, 0.12%), 2-dehydro-3-deoxy-20E (**34**, 0.90 mg, 0.11%), compound **36** (2.27 mg, 0.27%), compound **37** (1.50 mg, 0.18%), 25-hydroxy-dacryhainansterone (**38**, 1.00 mg, 0.37%) and 22-dehydroecdysone (**39**, 2.66 mg, 0.33%) were isolated. Structure of the resulting products from the irradiation in N_2- or N_2O-saturated aqueous solutions of 20E (**1**) are presented in **Fig. 6**.

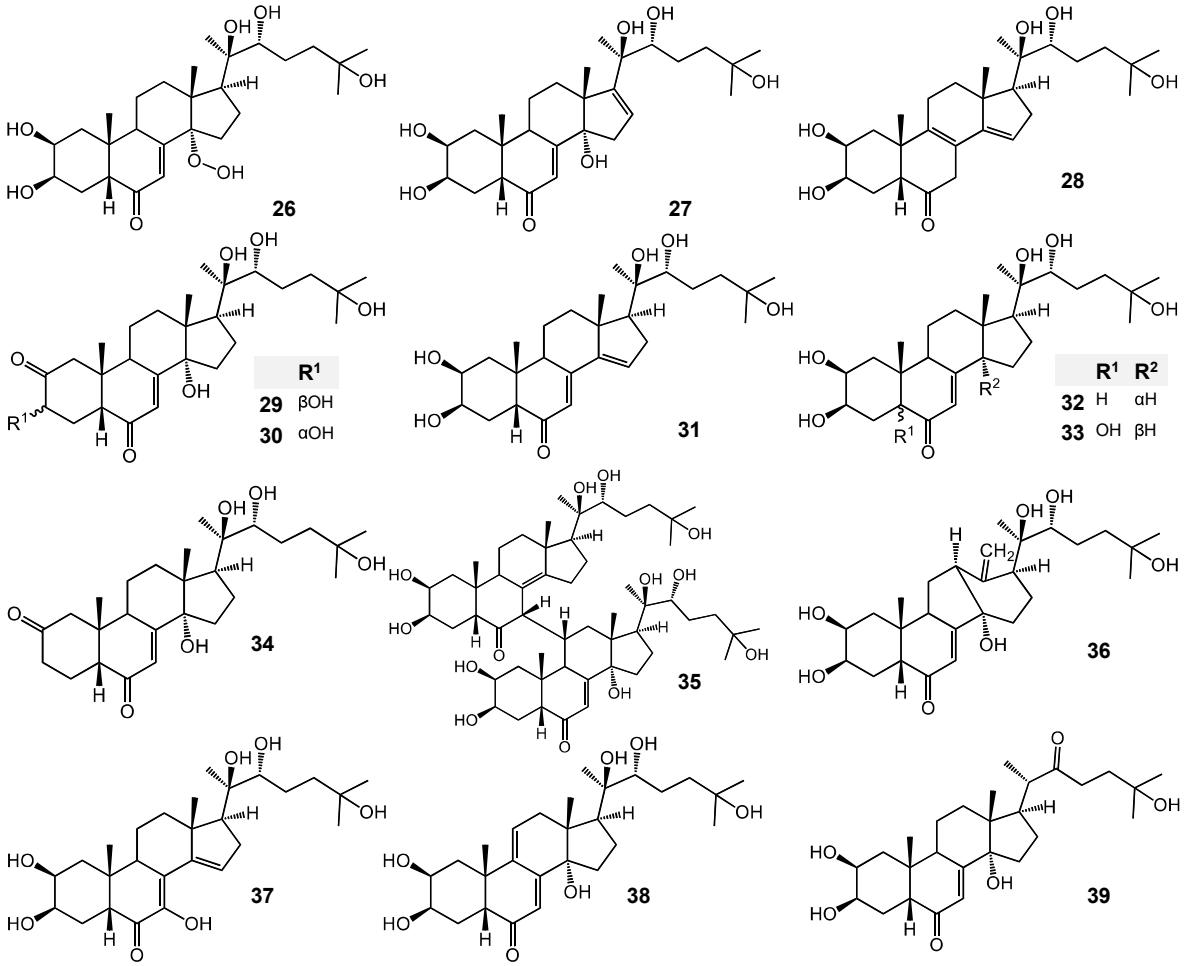

Fig.6. Structures of ecdysteroid derivatives (**26-39**) obtained by gamma irradiations of 20E (**1**)

As seen in **Fig.7** which presents HPLC chromatogram of the crude mixture obtained after gamma irradiation of aqueous solution of 20E (**1**) in N_2O-saturated solution (**A**) and chromatograms of pure compounds obtained after purification steps (**B**), the initial crude

mixture was highly complex with compounds eluting with very closed retention times. Thus, the initial fractionation through CPC allowed to separate several major and minor products, whose isolation would not be possible at once through preparative HPLC.

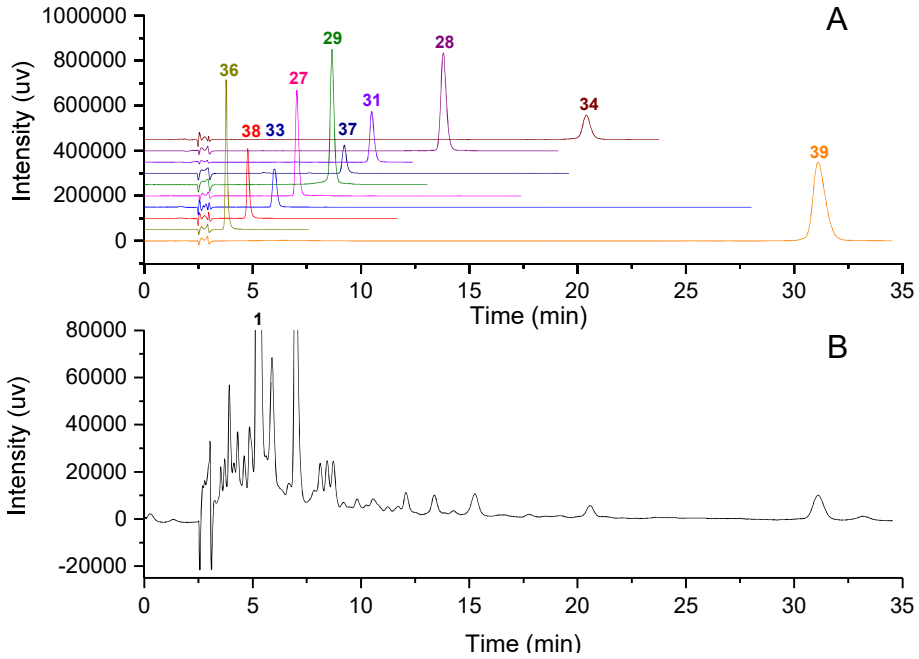

Fig. 7. Chromatograms of (**A**) crude mixture obtained after gamma irradiation of aqueous solution of 20E (**1**) in N_2O-saturated solution and (**B**) pure compounds obtained after purification steps. HPLC fingerprints are presented at λ_{max} (λ= 200 - 650 nm); Column: Kinetex Biphenyl (5 µm, 250 × 4.6 mm); mobile phase: isocratic 20% aq. acetonitrile; flow rate: 1 ml/min

Raw data of chromatograms were extracted from ChromNav software and were plotted with OriginPro 9.1

IV.5 Longitudinal study of the autoxidation of 20E by capillary electrophoresis [II]

The base-catalyzed autoxidation of 20E (**1**) was previously performed and monitored with RP-HPLC by our research group [78]. During the course of this Ph.D. work, further studied of the longitudinal study of the autoxidation of 20E (**1**) by capillary electrophoresis (CE) was achieved. This study was performed through different steps: 1) preparation of reference compounds, 2) optimization of the CE separation of 20E (**1**) and its autoxidized derivatives and evaluation of the applicability of the proposed method and 3) monitoring of the autoxidation by CE and determination of the concentrations of compounds at various times.

IV.5.1 Preparation of reference compounds 6, 41 and 43

For the synthesis of the previously characterized 20E (**1**) derivatives, used here as reference compounds, base-catalyzed autoxidation was performed as previously reported by our research group [78]. Calonysterone (**6**), (5α)-9α,20-dihydroxyecdysone (**41**) and isocalonysterone (**43**) were isolated (see section III.5.1 for detailed methods). Compounds **40**

and **42** (9α,20-dihydroxyecdysone and 14,15-dihydro-14α-hydroxycalonysterone respectively) used during this study were available from our previous work. Structures of the oxidized ecdysteroid derivatives (**40**-**43**) are presented in **Fig. 8** whereas the structure of the starting material 20E (**1**) and calonysterone (**6**) are presented above, in **Fig. 3**.

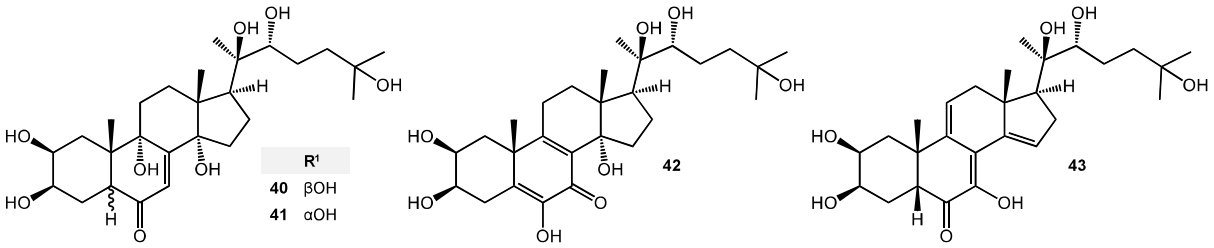

Fig.8. Structures of ecdysteroid derivatives (**40**-**43**) obtained by the autoxidation of 20E (**1**)

IV.5.2 Capillary electrophoresis method development and applicability

In order to optimize the separation conditions and to achieve an effective resolution for all compounds (**1**, **6** and **40**-**43**), a mixture of 20E (**1**) and its oxidized derivatives was prepared in aqueous methanol. The anionic detergent sodium dodecyl sulphate in 50 mM and three anionic cyclodextrin (CD) selectors derivatives with different substituents and different ring cavities were tested: sulfobutyl ether β-cyclodextrin selector (SBEBCD; degree of substitution (DS) = 4), carboxymethyl-β- cyclodextrin (CMBCD; DS = 3.5) and sulfobutyl-ether-γ-cyclodextrin (SBEGCD; DS = 4). The resolution effectiveness of these CDs (i.e. CMBCD, SBEBCD and SBEGCD) were compared and the results are presented in **Table 6**.

Table 6 Migration time (t_{migr}) and resolution (Rs) of 20E (**1**) and its autoxidized metabolites in the presence of the applied CD derivatives. The optimal cyclodextrin derivative selected for the analytical separation is highlighted in grey

CD derivative	**conc.**		**42**	**20E (1)**	**40**	**41**	**43**	**6**
CMBCD	20 mM	t_{migr} (min)	4.46	4.59	4.96	5.00	4.97	5.28
		Rs*		2.80	10.10	0.00	0.00	5.70
CD derivative	**conc.**		**20E (1)**	**42**	**40**	**41**	**43**	**6**
SBEBCD	5 mM	t_{migr} (min)	3.85	4.19	4.60	4.60	4.84	5.45
		Rs*		5.10	1.90	0.00	2.40	7.00
	7.5 mM	t_{migr} (min)	3.69	4.47	4.68	4.68	4.94	5.64
		Rs*		8.70	3.00	0.00	2.70	6.20
	15 mM	t_{migr} (min)	5.07	5.80	6.10	6.10	6.54	7.28
		Rs*		10.50	4.40	0.00	5.00	6.70
SBEGCD	5 mM	t_{migr} (min)	3.68	4.00	4.18	4.18	4.66	5.07
		Rs*		2.20	1.30	0.00	5.10	3.30

** The resolution was defined between two consecutively migrating compounds according to the following equation:* $Rs = 1.18 * (t_1-t_2)/w_{(0.5)1} + w_{(0.5)2}$ *where* t_1 *and* t_2 *are the migration times* $w_{(0.5)1}$ *and* $w_{(0.5)2}$ *are the peak width at half height.*

In the case of CMBCD (20 mM), no baseline separation could be achieved between compounds **40**, **41** and **43**. The use of SBEBCD (5 mM), which has the same cavity size as CMBCD but present different substituents, provided good resolution for 20E (**1**) and compounds **6**, **41**, **42** and **43** and slightly different migration order, but compounds **40** and **41** were overlapping. Increasing the concentration of SBEBCD to 7.5 or 15 mM could not improve the resolution between these two compounds but it resulted only in extended migration times. In the presence of SBEGCD (5 mM) additive, characterized by a relatively larger cavity, weaker resolution could be observed than with SBEBCD.

Under optimized conditions, namely using the suitable selector, SBEBCD at 5 mM at pH 11, and fixing the separation voltage at +30 kV, an effective separation of 20E (**1**) and its bioactive autoxidized derivatives was achieved in 6 min (see **Fig. 9B**). Even though the two 5-epimer compounds **40** and **41** could not be separated, this is hardly a drawback: our previous study revealed that the concentration of compound **40** remains very low through the reaction (maximum detected quantity of 0.62% as compared to the initial amount of 20E (**1**) by means of HPLC [78]), suggesting that its contribution can be considered as negligible.

Applicability of the proposed method was checked according to the recommendations of the Good Laboratory Practice and ICH (International Conference for Harmonization). Thus, the following parameters were taken into account:

1. The linear correlation between the concentrations of all compounds of interest and their corresponding peak areas: the calibration curves exhibited an excellent linear correlation over the concentration ranges with coefficients of determination close to 1.
2. The calculated LOD and LOQ values: even though the LOD and LOQ values were not very low due to the relatively low molar absorption of the analytes, this sensitivity was found perfectly sufficient for reaction monitoring.
3. The intra-day and inter-day reproducibility of the migration times and the peak areas of 20E (**1**) and its derivatives by repeated (n = 3) injections of standard solutions of 1200 µg/ml of 20E (**1**), compounds **42** and **43**, and 600 µg/ml solutions in case of compounds **6** and **41**: the values of relative standard deviations for the intra-day and inter-day precision of all investigated compounds were below 10%, which is the threshold generally considered acceptable for this technique.
4. The accuracy of the method by the investigation of the concentrations' recoveries: good-average recoveries were achieved at the high end of the calibration curves and in the middle of the ranges (99.2% - 102.5% and 97.5% - 101.7%, respectively). Slightly lower average recovery values (93.5% - 100.1%) were obtained for concentrations close to the LOQ.

Consequently, the method demonstrated a good precision. The calculated parameters are presented in **Table 7**.

Table 7 Applicability of the CE method for the separation of 20E (**1**) and its oxidized derivatives

Analytes	**20E 1[a]**	**Compound 41[b]**	**Compound 42[a]**	**Compound 6[b]**	**Compound 43[a]**
LOD (µg/ml)	15.3	21.9	43.0	8.0	70.2
LOQ (µg/ml)	30.1	56.4	171.2	26.3	183.8
Linearity					
Concentration range (µg/ml)	**25-1200**	**25-600**	**75-1200**	**25-600**	**75-1200**
Slope	0.139	0.127	0.079	0.345	0.066
Intercept	1.251	0.918	-0.945	0.064	1.418
Coefficient of determination	1.000	1.000	0.999	1.000	0.999
Precision					
Intra-day repeatability					
RSD (%) (migration time)	0.3	1.3	0.4	0.4	1.5
RSD (%) (area)	7.6	1.1	1.6	5.7	7.7
Inter-day reproducibility					
RSD (%) (migration time)	0.3	3.5	1.1	0.9	2.6
RSD (%) (area)	0.6	5.6	2.8	4.4	9.6
Accuracy					
Recovery at the high end of the calibration curve (%)	105.6 94.8 98.7	100.5 98.4 98.8	104.6 100.8 99.9	104.6 105.1 91.0	92.9 104.1 110.6
Average (%)	99.7	99.2	101.8	100.2	102.5
RSD (%)	5.5	1.1	2.4	7.9	8.7
Recovery in the middle of the range (%)	101.6 105.6 93.0	102.1 106.5 96.5	100.7 96.9 95.0	102.4 100.3 94.1	104.8 102.9 94.7
Average (%)	100.1	101.7	97.5	99.0	100.8
RSD (%)	6.4	4.9	3.0	4.3	5.4
Recovery close to the LOQ (%)	90.6 96.4 93.5	94.2 94.2 105.2	95.7 97.4 97.4	104.8 101.3 94.3	94.8 92.7 96.8
Average (%)	93.5	97.9	96.8	100.1	94.8
RSD (%)	3.1	6.5	1.0	5.3	2.1

[a] *at 1200 µg/ml;*

[b] *at 600 µg/ml;*

Calibration curves are expressed as linear regression lines (y = ax + b).

IV.5.3 Time-dependency study of the autoxidation of 20E

The degradation of the starting material 20E (**1**) and the formation of its autoxidized products **6**, **41**-**43** over various reaction times were studied in three independently performed autoxidation reactions by CE each 30 minutes in the first 8 hours and then at 15, 24, 27, 29, 31 and 48 hours.

A series of electropherograms representing fingerprints obtained at various reaction times are presented in **Fig. 9**. The CE fingerprints of the autoxidation show a clear time dependency. A rapid decrease of the amount of 20E (**1**) can be observed, with most of the starting material transformed after only 3 hours (**Fig. 9B**) and being no more detectable in the mixture after 8 hours (**Fig. 9C**). Among the oxidized metabolites, compound **41** appears to be the only one whose amount keeps slowly increasing over time suggesting that this compound has a relatively higher stability under the strongly alkaline conditions of the autoxidation reaction. Nevertheless, it seems to be clear that the formation of compound **42** is the kinetically most favourable: this compound gives predominant peak at 3 and particularly at 8 hours. Our research group has previously identified compound **43**, the desmotropic pair of calonysterone (**6**), as a major metabolite from this reaction, and found that calonysterone (**6**) itself, forms in significant amounts only when the mixture turns slightly acidic during the neutralization process [78].

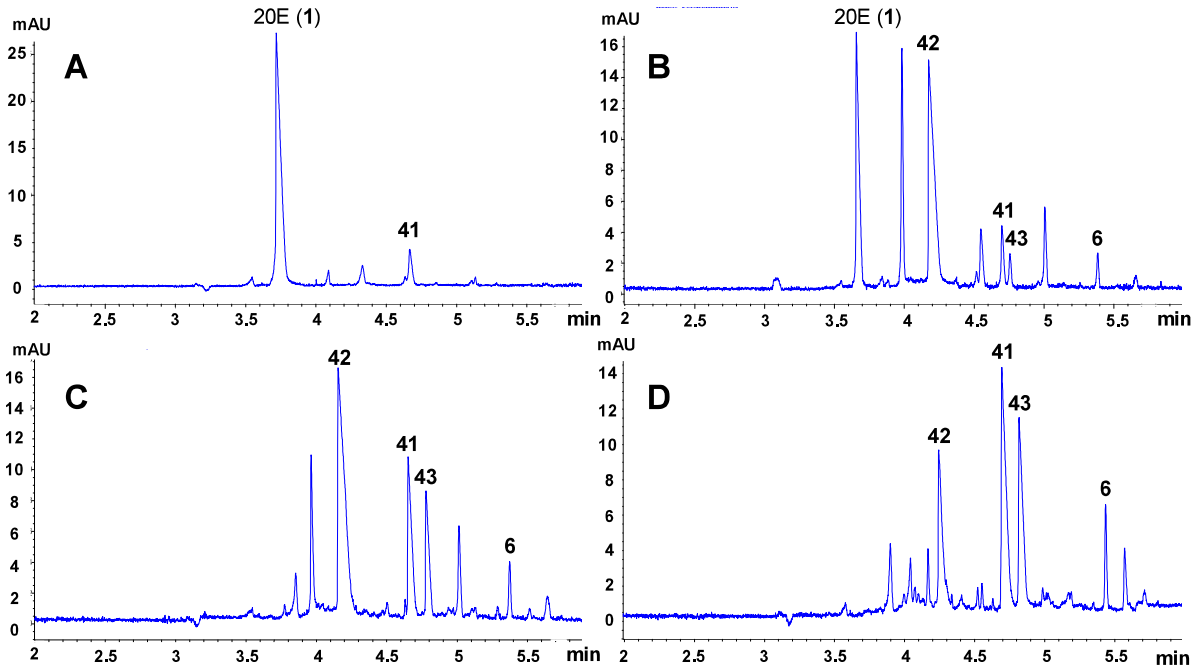

Fig. 9. Electropherograms of crude mixtures obtained after 30min (**A**), 3h (**B**), 8h (**C**) and 29h (**D**) of autoxidation. Electrophoretic conditions: BGE: 5 mM SBEBCD and 10 mM sodium phosphate buffer (pH 11); temperature: 25°C; separation voltage: +30 kV; hydrodynamic injection: 5×10^3 Pa for 6s; detection: 222 nm

The longitudinal study of the autoxidation of 20E (**1**) was previously monitored in our laboratory with HPLC [78]. It must be emphasized that prior to the HPLC injections, each sample had to undergo a neutralization of the pH by the addition of a 9.60% aqueous solution of acetic acid. Raw data obtained from this previous study have been re-evaluated here for a comparative study. Data obtained with the two analyses were fitted using the same Levenberg-Marquardt based algorithms implemented in OriginPro 9.1 software in order to obtain an optimal comparison (see section III.5.3). Relative amounts of the compounds as compared to the initial amount of the starting material 20E (**1**) over time are presented in **Fig. 10**.

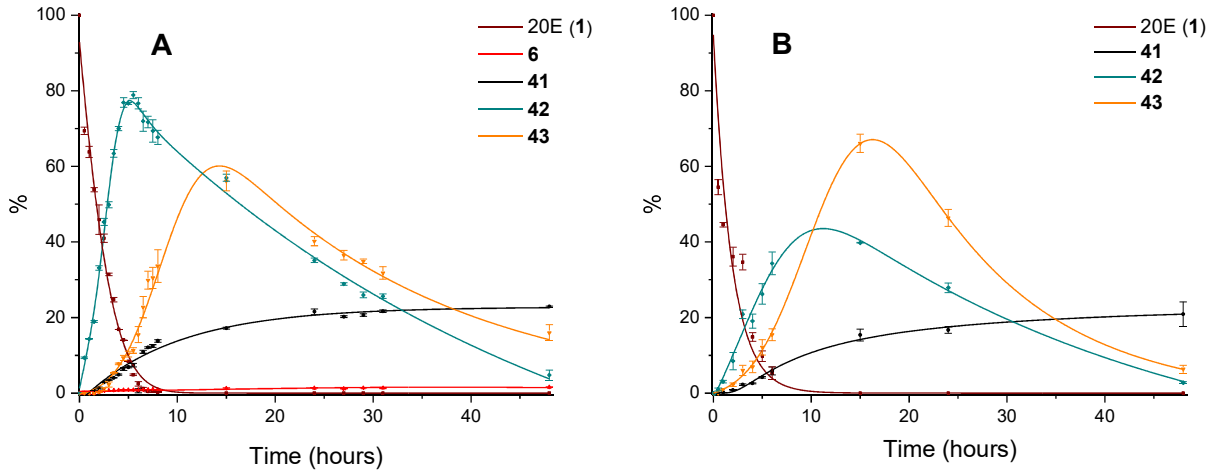

Fig. 10. Amounts of 20E (**1**) derivatives obtained at various times by means of CE (**A**) and HPLC (**B**). Error bars represent standard error of the mean from three independent experiments

As seen from the comparison between **Fig. 10A** and **10B**, no major difference can be observed in the decomposition of 20E (**1**) and the formation of compound **41**: 20E (**1**) underwent a rapid decay with a calculated half-life of 1.39h (HPLC curve analysis) or 1.72h (CE curve analysis), whereas compound **41** increased exponentially reaching a plateau of 24.5% (HPLC curve analysis) or 22.9% (CE curve analysis). This suggests that the neutralization of the samples before each HPLC injection and analysis had no effect on the results of these two compounds.
In contrast with the preceding HPLC results, calonysterone (**6**) was well detectable by CE as a minor primary product, even if at only a very small amount (maximum yield of 1.60%).
A substantial difference in the maximal amounts detected with the two techniques regarding compound **42** must be highlighted: with CE, this compound could be detected with an average maximal amount of 78.90% whereas previously it was observed to reach a maximal value of 54.60% in one single reaction. This shows that compound **42** is formed in great yields during the autoxidation of 20E (**1**), but its chemical structure is sensitive enough to make subsequent sample treatment a critical point for its preparation.

Finally, the short separation time of CE allowed a more frequent sampling and analysis leading to a more accurate determination of the time for maximum yields of each compound than before. Thus, the maximal amount reached by compound **42** occurs at 5 h while the highest yield for compound **43** can be attained at 15 h.

IV.6 Structure elucidation of the prepared ecdysteroids

Compounds **7**, **9** and **11** were previously isolated and fully characterized by our research group; their identification was performed in comparison with our readily available reference compounds [53, 104, 49].

Structure elucidation of the products **8**, **10**, **12-18** was performed by means of MS, HRMS, 1D and 2D NMR spectroscopy. Molecular masses of compounds **8** and **10** were characterized by the presence of m/z signals representing $[M + H]^+$ and $[M + K]^+$. The structural formula of compounds **12-18** was established by means of HRMS (see section III.6). The parental ion $[M+H]^+$ was systematically observed in all the spectra along with numerous signals with characteristic differences of 18 units between them, representing the loss of water from the several hydroxyl groups. Structure determination was achieved by comprehensive NMR methods (see section III.6). Compounds **8-12** resulting from the side-chain cleavage of phytoecdysteroids (**1-6**) presented a characteristic signal (δ_C 212.50 ppm) appearing in the ^{13}C NMR spectra revealing the formation of a carbonyl group at C-20 while the steroid skeleton of the starting compounds remained unchanged.

The molecular formula of compounds **20-25** was established by means of HRMS (see section III.6). The parental ion $[M+H]^+$ was observed for compounds **20**, **21**, **24** and **25** and the appearance of $[M + H - H_2O]^+$ peak in the spectra of compounds **21**, **22** and **23** strongly suggested the presence of at least one remaining hydroxyl group in these compounds. Structure determination of the diacetonide derivatives of 20E 2,3;20,22-diacetonide (**19**) was performed by comprehensive 1D and 2D NMR methods (see section III.6). The spectra showed that the configuration of the starting material core changed during the autoxidation reaction while preserving the diacetonide moieties. The presence of the diacetonide moieties was established by comparing the ^1H and ^{13}C chemical shifts of 20E (**1**) (^1H: δ_H 3.84, 3.95, 3.33 ppm) (^{13}C: δ_C 68.80, 68.60, 78.00, 78.50 ppm) and 20E 2,3;20,22-diacetonide (**19**) (^1H: δ_H 4.23, 4.26, 3.65 ppm) [105] (^{13}C: δ_C: 73.50, 73.13, 85.83, 83.30 ppm) [11] in positions 2, 3, 20 and 22, respectively, with those obtained for the diacetonide derivatives. When comparing with 20E (**1**) chemical shifts', clear deshielding was observed whereas when comparing with 20E 2,3;20,22-diacetonide (**19**) chemical shifts', the values were approximately the same. Concerning the

modifications obtained in the steroidal skeleton, they were recognized by comparing ^{1}H and ^{13}C chemical shifts of the new derivatives **20-25** with the ones characterizing the previously obtained 20E (**1**) autoxidized derivatives. ^{1}H and ^{13}C-NMR data correlated well with the values reported earlier [78, 104] signifying that same structural analogues were attained from both reactions.

Structure determination of the products (**26-39**) resulting from the gamma irradiations of aqueous solutions of 20E (**1**) in N_2- or N_2O-saturated solutions was achieved similarly as described above. Among the fourteen investigated compounds: compounds **27**, **29** and **35-37** were new (see section III.6).

IV. 7 Results of the biological assays

IV.7.1 Bioactivities on the Akt-phosphorylation [III]

A dose-dependency study was performed to test poststerone (**7**), the side-chain cleaved analogue of 20E (**1**) in comparison with its parental compound for their ability to increase the phosphorylation of Akt in murine C2C12 skeletal myotubes. The presented results in **Fig.11** were reached from six parallel experiments.

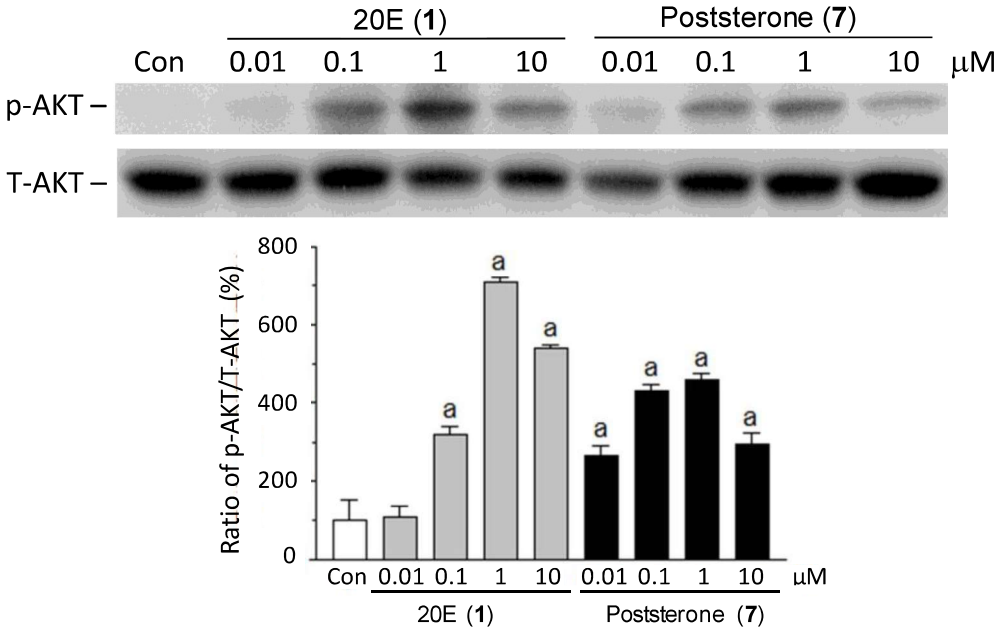

Fig. 11. Dose-dependency comparison of the Akt activation exerted by 20E (**1**) and poststerone (**7**). Error bars represent standard error of the mean from six parallel experiments. a: $p<0.001$ as compared to Con (control)

When comparing 20E (**1**) and poststerone (**7**) for their ability to activate the protein kinase B, it was found that poststerone (**7**) acts as a weaker activator (293.30 ± 68.30% vs. 537.10 ± 25.30% activation for 20E (**1**)) at a concentration of 10 μM. Interestingly, however, poststerone

(**7**) was still active at low as 10 nM concentration (263.40 ± 69.60% activation), while 20E (**1**) was already inactive.

Based on the above results, side-chain cleaved analogue obtained during this Ph.D. work i.e., side-chain cleaved analogues (**7**-**12**) of 20E (**1**), 2-Deoxy-20E (**2**), ajugasterone C (**3**), polypodine B (**4**), dacryhainansterone (**5**) and calonysterone (**6**), respectively, and side-chain cleaved compounds resulting from the autoxidation of poststerone (**7**), i.e. compounds **13**-**18** were tested for their capacity to influence the Akt phosphorylation/activation in murine C2C12 skeletal myotubes when applied at a concentration of 10 µM. The observed activities are presented in **Fig. 12**.

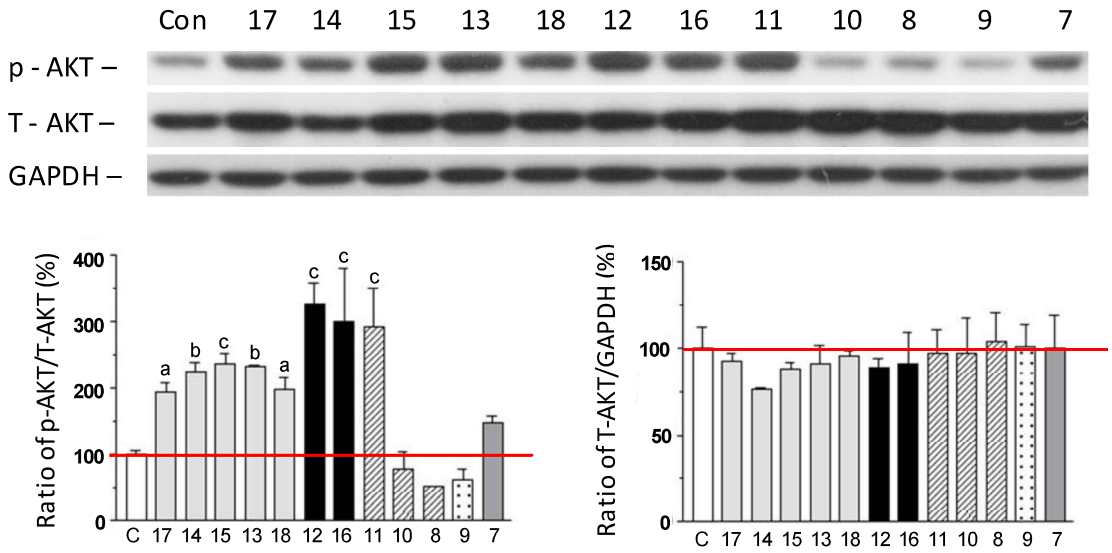

Fig. 12. Activity of compounds **7**-**18** as compared to the control (C) on the Akt phosphorylation. Quantification of Western blots was made by ImageJ; error bars represent standard error of the mean from four parallel experiments. a: $p<0.05$; b: $p<0.01$; c: $p<0.001$

With the exception compounds **8**, **9** and **10**, all tested compounds demonstrated the ability to increase the activation of Akt. In particular, the side-chain cleaved analogues of dacryhainansterone (**5**), calonysterone (**6**) and isocalonysterone (**43**) (i.e. compounds **11**, **12** and **16**, respectively) showed the strongest activity in this regard. All of these compounds express higher degree of unsaturation due to further conjugation extended to their D and/or C rings conjugated with those already present in the ecdysteroid B-ring.

IV.7.2 ABCB1-inhibition and cytotoxic activity

Compounds **20**-**23** were tested for their ability to interfere with ABCB1-mediated drug efflux and for their cytotoxic activity. The compounds exerted negligible inhibition of the ABCB1 efflux transporter; only compounds **20** could be considered as a weak inhibitor, demonstrating a *ca.* 40% inhibition at 20µM (data not presented).

Compounds **20**-**23** were tested for their *in vitro* cytotoxic activity against a mouse lymphoma cancer cell line pair (L5178 and its multi-drug resistant sub-cell line $L5178_{MDR}$ expressing the human ABCB1 transporter), both alone and in combination with doxorubicin. **Table 8** displays the compounds' activity when applied alone while **Table 9** shows results of the combination experiments.

Table 8 Cytotoxic activity of compounds **20**-**23** against a susceptible/resistant mouse lymphoma cell line pair

	IC_{50} (µM)	
Compound	**L5178**	**$L5178_{MDR}$**
20	26.47 ± 2.08	32.12 ± 3.00
21	39.73 ± 2.22	51.64 ± 3.51
22	31.12 ± 3.25	22.72 ±1.97
23	15.74 ± 2.18	20.92 ± 2.80

Table 9 Cytotoxic activity of compounds **20**-**23** in combination with doxorubicin at different compound vs. doxorubicin ratios[a]. Combination index (CI) values[b] are presented at 50%, 75% and 90% of inhibition on the susceptible/MDR mouse lymphoma cell line pair. $0 < CI < 1$, $CI = 1$ and $CI > 1$ represent synergism, additivity and antagonism, respectively. Dm, m, and r represent antilog of the x-intercept (IC_{50}), slope, and linear correlation coefficient of the median-effect plot, respectively [106]. $CI_{avg} = (CI_{50} + 2 \times CI_{75} + 3 \times CI_{90}) / 6$

			CI value						
Compound	**Cell line**	**Ratio (comp:dox)**	**CI_{50}**	**CI_{75}**	**CI_{90}**	**CI_{avg}**	**Dm**	**m**	**r**
20	$L5178_{MDR}$	17.4:1	0.36	0.28	0.23	0.27	4.950	2.993	0.986
	L5178	69.6:1	0.66	0.58	0.51	0.56	6.164	3.164	0.943
21	$L5178_{MDR}$	17.4:1	0.36	0.28	0.23	0.27	4.950	2.993	0.986
	L5178	69.6:1	0.56	0.51	0.46	0.50	5.925	3.002	0.952
22	$L5178_{MDR}$	17.4:1	0.39	0.30	0.24	0.28	4.942	3.401	0.987
	L5178	69.6:1	0.67	0.59	0.52	0.57	5.245	3.024	0.954
23	$L5178_{MDR}$	34.8:1	0.54	0.39	0.29	0.37	5.29	3.720	0.991
	L5178	69.6:1	0.64	0.60	0.56	0.59	4.54	3.068	0.989

[a] *The strongest activity observed on the combination plate is given for each checkerboard assay;*

[b]*Molar drug ratios are given; serial dilutions of doxorubicin were initiated from a commercially available injection of 2 mg/ml (doxorubicin hydrochloride, Teva).*

Based on our results, compounds **20**-**23** demonstrated the capability to act in synergism with doxorubicin on both investigated cell lines, with a moderate selectivity towards the multi-drug resistant one.

V. DISCUSSION

V.1 Preparation of ecdysteroid derivatives

V.1.1 Isolation / Semi-synthesis of ecdysteroid derivatives

Isolation of dacryhainansterone and calonysterone [I]. When starting to explore the chemical composition of commercially available *Cyanotis arachnoidea* extract, significant amounts of dacryhainansterone (**5**) and calonysterone (**6**) were identified. *Cyanotis arachnoidea* extracts are extensively utilized worldwide as ecdysteroid rich materials for various purposes, e.g. as food supplements, use in agriculture and aquaculture, etc. Dacryhainansterone (**5**) has been previously detected by others in the liquid waste left from the extraction of 20E (**1**) from *Cyanotis arachnoidea* [107] while the presence of calonysterone (**6**) is reported by us for the first time from such an extract. It is worth noting that since calonysterone (**6**) can be obtained from the autoxidation of 20E (**1**) [78], it might be an artefact that forms during the production, and not a natural constituent of this species. Regardless of its origin, however, its presence in food supplements makes calonysterone (**6**) an ecdysteroid derivative whose bioactivity is important to study.

Oxidative side-chain cleavage [III]. A recent study of our research group on the *in vivo* bioactivity of poststerone (**7**) revealed this compound as an active metabolite of 20E (**1**), apparently playing a major role in the anabolic activity of its parental compound [37]. This would allow the assumption that side-chain cleaved metabolites of other phytoecdysteroids might also arise *in vivo*, and that such derivatives are valuable bioactive compounds. Therefore, it was one of the objectives of this Ph.D. work to synthetize such derivatives from other ecdysteroids. The recently published procedure of our research group, using the hypervalent iodine reagent PIFA for the oxidative side-chain cleavage between C-20 and C-22 of 20E (**1**), afforded poststerone (**7**) in a yield of 57.80% [53]. In this dissertation, an update of this particular oxidation on 20E (**1**) and other phytoecdysteroids is presented with the use of another iodine compound PIDA. Less complex mixtures were obtained this way, and the expected products were isolated with increased yields. The use of hypervalent iodine (III) reagents for the transformation of natural products and complex molecules is known to promote the efficient and chemo-selective oxidation of alcohols to highly functionalized carbonyl compounds [108]. The trivalent iodine compounds can typically be described as $ArIX_2$, in which the two X ligands serve as good leaving groups in both the initial and the last step of the reductive elimination involving conversion of hypervalent iodine (III) to iodine (I). On the whole, stoichiometric use of $ArIX_2$ results in the co-production of at least an equimolar amount of ArI and HX from the ligands as waste products [108]. Based on this assessment, we hypothesize that the weaker

yields obtained with PIFA are due to decomposition due to the by-product TFA that strongly acidifies the reaction medium, while acetic acid released by PIDA provides milder conditions leading to less complex mixtures and higher final yields of the target compounds.

Autoxidation of 20E, poststerone and 20E 2,3;20,22-diacetonide. Base-catalyzed autoxidation of 20E (**1**) [77, 78] in 2% or 1% aqueous methanolic NaOH solution, and of 20E 2,3;20,22-diacetonide (**19**) in 10% aqueous methanolic NaOH solutions [79] were previously reported and discussed. In the present Ph.D. work, similar reactions were carried out not only with 20E (**1**), but also with poststerone (**7**) and 2,3;20,22-diacetonide (**19**) as starting materials. In several cases, the solvent composition, the time of the reaction and/or the amount of the catalyst NaOH were different as compared to those published previously.

Base-catalyzed autoxidation of 20E (**1**) was performed as previously published [78], with the difference that the final pH of the mixture was set to ≈ 6. Purification of the reaction resulted in the isolation of calonysterone (**6**) in the highest amount as compared to the other products (see section IV.5.1). This outcome confirms the studies performed previously in our laboratory on the formation of the desmotropes **6** and **43** [78] revealing that at acidic pH, compound **6** is the preferred form among the two desmotropes and can directly be formed from the intermediate compound **42** [II].

Base-catalyzed autoxidation of poststerone (**7**) was performed in a solvent composed of methanol - water (9:1, v/v) using 0.5% NaOH as a catalyst during 4 or 7 hours. Analogues corresponding to the autoxidized 20E (**1**) derivatives [78] were obtained, with the exception of compounds **16** and **17**. The monitoring of the reaction with NP-TLC and RP-HPLC showed that compound **17** was not present anymore after 4 hours suggesting that this compound could be decomposing over the reaction, leading to the formation of secondary, tertiary, etc. metabolites [III].

Base-catalyzed autoxidation of 20E 2,3;20,22-diacetonide (**19**) was performed in methanol - water (95:5, v/v) containing 1% NaOH during 8 or 15 hours. This reaction, using different conditions, was previously published by others and resulted in the isolation of the 9α-hydroxy-5α-ecdysteroid **22** in 68% yield [79]. In our study, this product was isolated in much lower yields (2.83%) and was accompanied by other derivatives, i.e. 5α-20E 2,3;20,22-diacetonide (**20**, 1.79%), 9α,20-dihydroxyecdysone 2,3;20,22-diacetonide (**21**, 2.88%) and compound **23** (3.26%). The same reaction stirred for a longer time also resulted in the isolation of isocalonysterone 2,3;20,22-diacetonide (**24**, 7.83%). Calonysterone 2,3;20,22-diacetonide (**25**) was detected as a minor product in both reaction mixtures but could not be isolated. It is worth noting that the much lower yields observed in our study as compared to that published in

the literature [79] is not really a drawback, since at this point our aim was to maximize chemical diversity for the bioactivity testing, instead of maximizing yields. Since the reaction is base-catalyzed, it is reasonable to assume that the amount of base and/or the presence or absence of water play a crucial role in the reaction; in the previous work the amount of NaOH used was 10 times more than in our experimental system and only methanol was used as solvent [79]. The autoxidation of 20E 2,3;20,22-diacetonide (**19**) was performed similarly to that of 20E (**1**) [78] with the exception of the solvent ratio of water - methanol due to solubility limitations, and the same structural analogues were obtained in both reactions.

Gamma irradiation of 20E. To the best of our knowledge, gamma irradiation of ecdysteroids has not been studied before. The most abundant ecdysteroid 20E (**1**) was used as starting material to explore the possible new products resulting from such chemical modification. The gamma irradiations of aqueous solutions of 20E (**1**) in N_2- or N_2O-saturated solutions resulted in the isolation of fourteen compounds of which five are new. A close observation to the irradiated products shows that major modifications took place in the B, C and D rings of the starting material. For example, only compounds **29**, **30** and **34**, presented modifications in the ring A where the hydroxyl group of C-2β was oxidized to a keto-group. Also, in a related study when corticosteroids were subjected to gamma irradiation, side-chain cleavage was observed [83]. Such modification was not identified in our case, the only change affecting the side-chain was the oxidation of the 22-OH group to a keto-group. It is also worth mentioning that almost all the products were isolated at very low yields. This can partially be explained with chromatographic overlapping: a large number of minor compounds were observed accounting for a high amount of irradiated mixture.

Irradiations in N_2- or N_2O-saturated solutions resulted in complex mixtures presenting somewhat similar fingerprints but with different main products formed. From the irradiations in N_2-saturated solutions: 14α-hydroperoxy-20E (**26**), compound **27** and stachysterone B (**31**) were found to be main products, while from the irradiations in N_2O-saturated solutions: compound **27** and podecdysone B (**28**) were main products, whereas only traces of the 14-hydroperoxy derivative (**26**) were observed.

V.1.2 CPC method developments

While the preparative chromatographic purification of complex mixtures was a key step of obtaining ecdysteroid derivatives during this Ph.D. work, most of these separations followed well established protocols of our research group. Centrifugal partition chromatography was an exception to this, and since the use of this technique required extensive method development, it is discussed here in a separate sub-section.

Centrifugal partition chromatography is a modern solid support-free technique used for the fractionation of complex mixtures or for the straightforward isolation of pure compounds in natural product chemistry. The separation is based on the partitioning of the analytes between two immiscible solvents at equilibrium. The selection strategies of the suitable biphasic solvent system during method development are classified into three main classes: theoretical, semi-empirical, and empirical [110]. Concerning the theoretical approach, it attempts to preclude the need for partitioning by calculating the solubility of an analyte in both phases independently, based on the structure of the analyte, the solvents, and the solvent composition of each phase. Hopmann et al. [111] developed a quantum chemical method combined with statistical thermodynamics to predict the $K_{(U/L)_i}$ values. Semi-empirical solvent system selection strategy uses partitioning experiments with an attempt to reduce the number of empirical steps by predicting the $K_{(U/L)_i}$ values for target compounds. Dubant et al. [112] reported the combination of a statistical approach and fast HPLC analysis to generate a three-dimensional partition coefficient map and rapidly predict an optimal solvent system. Han et al. [113] reported the use of a mathematical approach by the application of equations to describe the relationship between the solvent ratio and the $K_{(U/L)_i}$ values. While the theoretical and semi-empirical strategies present the advantage of being less time consuming than the purely empirical approach (see below), they have their own limitations. Concerning the theoretical approach, the $K_{(U/L)_i}$ calculation requires at least three different commercial software platforms, which may somewhat limit the overall applicability of the approach, and even the highest level *in silico* predictions need experimental verification. As for the semi-empirical approach, even if it reduces the need for multiple partitioning experiments, it cannot entirely replace the experimental approach. Moreover, the applicability of this method was only established for common solvent system families but not for the research of new solvent systems. Finally, because any predictive *in silico* approaches require structural information of the analytes, such techniques can have only a very limited use in natural product chemistry that very frequently aims at discovering previously unknown molecules.

The well-settled CPC methods presented in this Ph.D. work are based on the use of the empirical strategy that is an iterative process. Even if this method is labour intensive and requires skills and experience in order to determine the consecutive steps of the iterative process, and to decide when the process can be terminated with success, it does present several advantages. In our case, the most important advantage was the flexibility: from commercial standards to crude extracts, also the $K_{(U/L)_i}$ of many different analytes could simultaneously be

determined in a particular solvent system without any preliminary information on the chemical structures. Finally, results from empirical methods could well be transferred into larger scale separations concerning the $K_{(U/L)_i}$ values, resolution, and solvent system applicability.

The biphasic systems in liquid-liquid chromatography may be obtained from two to five or even more solvents. This makes the possibilities for solvent selection almost limitless. Different solvent system families have previously been developed with varied ratio of solvents that ensure stable biphasic systems. Among these families, the HEMWat family [89] is by far the most widespread because it covers a wide range of hydrophobicity. During this Ph.D. work, the HEMWat family was used for the isolation of dacryhainansterone (**5**) and calonysterone (**6**) from commercially available extract of *Cyanotis arachnoidea*, and also during the preparation of side-chain cleaved analogues, i.e for the isolation of compound **12**, the side-chain cleaved analogue of calonysterone (**6**). Different systems of the HEMWat family were tested and their applicability was evaluated based on different criteria (see Introduction).

Isolation of dacryhainansterone and calonysterone [I]. The biphasic system composed only of ethyl acetate - water (1:1, v/v) was selected as starting point to our optimization procedure, and a stepwise addition of *n*-hexane and methanol was performed in order to shift the polarity of the entire system. In the *n*-hexane - ethyl acetate - methanol - water (0:5:0:5, v/v/v/v) solvent system, the $K_{(U/L)_i}$ values were elevated indicating the high affinity of all the analytes to the upper organic phase, meaning that they would elute closer to the solvent front in the ascending mode. The solvent system *n*-hexane - ethyl acetate - methanol - water (0.5:5:0.5:5, v/v/v/v) did not improve considerably the $K_{(U/L)_i}$ values. For the solvent systems *n*-hexane - ethyl acetate - methanol - water (1.25:5:1.25:5, v/v/v/v) and (1.5:5:1.5:5 v/v/v/v), moderately low values of the $K_{(U/L)_i}$ were obtained, indicating that the compounds would elute after a too long running time. Finally, the results suggested the biphasic system composed of *n*-hexane - ethyl acetate - methanol - water (1:5:1:5, v/v/v/v) as the best choice, providing suitable $K_{(U/L)_i}$ values ranging between 0.5 and 2, and acceptable α values (see section IV.1). These ensured a good separation of the two ecdysteroids of interest in 30 min running time.

Isolation of the side-chain cleaved analogue of calonysterone: compound 12 [III]. Based on the fact that calonysterone (**6**) and its side-chain cleaved analogue (compound **12**) present quite different polarities, their partition was readily achievable. Indeed, based on the $K_{(U/L)_i}$ values presented in the **Table 3** (section IV.2.1), all the applied HEMWat solvent systems provided remarkably high separation factors predicting an excellent resolution between the peaks during the separation. However, using too high separation factors (from 4.441 to 14.359

in our case) would undeniably led to compounds eluting too far away from each other, causing an unnecessarily high consumption and waste of solvent. Also, all the tested biphasic systems, except for the one used for the separation, presented very high $K_{(U/L)_{12}}$ values indicating the high affinity of compound **12** to the upper organic phase, thus this latter would elute too close to the solvent front in ascending mode. Finally, the selected biphasic system: *n*-hexane - ethyl acetate - methanol - water (1.5:5:1.5:5, v/v/v/v) presented the best separation parameters concerning $K_{(U/L)_i}$ values and an excellent α value of 2.978 guaranteeing not only an excellent separation of the two compounds of interest but also an optimal running time.

Fractionation of the crude mixture of 20E 2,3;20,22-diacetonide autoxidation products. The selection of the biphasic system was performed by the use of the so called "best solvent" approach [110]. Based on the low polarity of the targeted compounds, dichloromethane was chosen as one of the solvents to dissolve as much of the crude mixture as possible. Methanol, which can also solve the crude extract was chosen as the mutual solvent, while water, served to make up a two-phase system. Several solvents (i.e. heptane, *n*-hexane, ethyl acetate, cyclohexane, etc.), were tested to find out the best modifier solvent which would lead to optimal $K_{(U/L)_i}$ values; the final choice was *n*-hexane. However, when different ratios of the solvent system *n*-hexane - dichloromethane - methanol - water ($0.1 \leq x \leq 1$:1:1:1, v/v/v/v) were tested, all compounds were systematically concentrated in the lower phase rich in dichloromethane. Thus, in order to decrease the high solubility of the compounds in the organic phase, another approach was tested. The ratio of dichloromethane was gradually decreased whereas the ratio of *n*-hexane was continuously increased in order to maintain equal volumes of the two phases. At a ratio of (1:0.5:1:1, v/v/v/v) *n*-hexane - dichloromethane - methanol - water, the distribution of the compounds was attained. However, solvent systems composed of *n*-hexane - dichloromethane - methanol - water ($1:0.5 \leq y \leq 0.225$:1:1, v/v/v/v), even if provided a distribution, presented high $K_{(U/L)_i}$ values (see section IV.3.2) whereas the extreme decrease of the dichloromethane ratio led to a complete changeover of the $K_{(U/L)_i}$ values' tendency. Finally, a compromise could be reached with the use of the solvent system composed of *n*-hexane - dichloromethane - methanol - water (1:0.215:1:1, v/v/v/v). However, the significant decrease of the ratio of dichloromethane in the biphasic system caused a reduction in the solubility of the crude mixture and thus increasing the number of injections during the purification which led to wastes during the working process. This was most likely the reason why the purification resulted in an unusually low total recovery of the initial weight (88.89%).

Fractionation of crude mixtures of 20E irradiations. Our previous observations regarding the behaviour of different ecdysteroids in different biphasic systems suggested their mild partition between ethyl acetate and water. Thus, the partition of the irradiated materials was first studied between these two solvents: a large proportion of the targeted compounds was more concentrated in the lower aqueous phase. Based on our observations, the enrichment of the upper organic phase with an alcohol changes drastically the partition of this class of compounds. Thus, different alcohols were tested (i.e. methanol, ethanol, isopropanol, *tert*-butanol and *n*-butanol); *tert*-butanol was the only alcohol that led to an improvement of the partitioning. The stepwise increase of *tert*-butanol from a ratio of 0.1 to 0.5 continuously enhanced the partition of the compounds between the two phases, until their almost complete shift from the lower aqueous phase to the upper organic phase. Finally, minor adjustments of the ratios of the three selected solvents to obtain nearly equal volumes of each phase without altering the partitioning of the compounds led to the optimized solvent system composed of *tert*-butanol - ethyl acetate - water (0.4:0.9:1, v/v/v). The use of CPC in this part of the work allowed the fractionation and subsequently the isolation of minor compounds whose isolation would not be possible at once through preparative HPLC because of the huge complexity of the initial crude mixtures. To the best of our knowledge, *tert*-butanol containing solvent system was previously never used for the liquid-liquid chromatography of ecdysteroids; and our solvent system is also a unique composition for natural product isolation since usually the three other isomeric structures of butanol are used for such purposes [89].

V.2 Longitudinal study of the autoxidation of 20E by capillary electrophoresis [II]

In the course of our studies on autoxidized ecdysteroid derivatives, it was one of our objectives to re-evaluate the autoxidation process of 20E (**1**) with a technique that does not require any sample preparation, i.e. capillary electrophoresis (CE).

The autoxidation of 20E (**1**) in presence of a strong base was previously suggested to be initiated by the 6-enolate form [77]. The oxo-enol tautomeric equilibrium of 20E (**1**) is largely shifted towards the more stable “oxo” form in aqueous methanol solution, with only negligible amounts of the enol present under such conditions. The lowest p*Ka* values of 20E (**1**) in “oxo” form and its derivatives are above 13 (based on the predictions by MarvinSketch 16.3.28); on the other hand, the p*Ka* value of the 6-enol group in the 6,8(9)-diene tautomer is 9.28. Since the enolate of this tautomer is required to initiate the autoxidation of 20E (**1**), in our first experiments we attempted to use only phosphate buffer at pH 11 as BGE. Under such conditions, 20E (**1**) migrated in the endo-osmotic flow, possibly due to a relatively slow

formation of the enol limited by the high energy barrier. Therefore, as the analytes behaved as essentially neutral at the pH of the analysis, neither the detection nor the resolution could be carried out without BGE additive(s). The use of cyclodextrin selectors was preferred as they are usually applied for chiral resolutions and for the analysis of metabolites carrying small differences in the substituents [113-115]. The use of neutral cyclodextrin derivatives was excluded because they cannot provide resolution and the pK_a values of cationic cyclodextrin selectors, are roughly around 9.5, so they are also mainly uncharged at pH 11. Therefore, three anionic cyclodextrin derivatives with different substituents and ring cavities were tested and sulfobutyl ether β-cyclodextrin selector at 5 mM was chosen (see IV.5.2 for detailed procedure).

The use of CE proved to be highly preferable over HPLC for such longitudinal study because: 1) it allowed a real "in situ" analysis of the autoxidation of 20E (**1**) with direct injections of the product mixtures (i.e. without any sample preparation steps) into the instrument after a simple dilution, 2) it permitted the use of as highly alkaline pH for the analysis as that used during the reaction, 3) it provided a very short analysis time (6 min vs. 30 min for HPLC) with a high resolution between 20E (**1**) and its oxidized derivatives 4) the short separation time allowed more frequent sampling and analysis leading to a more accurate determination of the time for maximum yield of each compound, and 5) it allowed substantial time saving and lower consumption of mobile phase.

V.3 Biological activity of the compounds obtained

V.3.1 Effect on the Akt-phosphorylation [III]

Based on most recent results of our research group, the side-chain cleaved analogue of 20E (**1**), poststerone (**7**), exerts a potent *in vivo* anabolic activity on rat skeletal muscles, and this activity seems to be connected to the phosphorylation of protein kinase B (Akt). When comparing 20E (**1**) and poststerone (**7**) in this regards, it was found that poststerone (**7**) acts as a weaker activator than for 20E (**1**) at a concentration of 10 μM. However, poststerone (**7**) was still active at as low as 10 nM concentration where 20E (**1**) was already inactive. This suggests that Akt activation may also play a key role in the observed bioactivity of poststerone (**7**), similarly to that of its parent compound 20E (**1**). Based on this, the effect on the Akt phosphorylation was chosen as a simple way to evaluate related bioactivities of selected compounds.

Among the side-chain cleaved compounds **7**-**18**, almost all could highly increase the phosphorylation of Akt (see IV.7.1), confirming our original assumption concerning the potent bioactivity of side-chain cleaved metabolites of ecdysteroids other than 20E (**1**). Side-chain

cleaved analogues presenting higher degree of unsaturation showed the strongest ability in the Akt phosphorylation. This suggests that further double bonds conjugated with those present may positively impact the bioactivities of these compounds in mammalian cells, and that such compounds might have stronger anabolic, antidiabetic and anti-apoptotic activities than the more saturated ones including the abundant 20E (**1**) and its known *in vivo* metabolites. The well-known role of the PI3K-Akt pathway in driving cancer [117, 118], however, should also be taken into account when interpreting such results. At this point it is impossible to make a sound judgment whether or not a stronger *in vitro* activation of this protein kinase would have *in vivo* relevance, and particularly if this would confer ecdysteroid metabolites any pro-cancerous risk. Experiences with the apparent safety of ingestion of large, up to several grams of ecdysteroids by sportsmen at least do not seem to raise concerns [14, 119], even though long-term safety of such a practice has never been studied in humans. Nevertheless, our results clearly show that side-chain cleaved metabolites of ecdysteroids accompanying 20E (**1**) within plant extracts have all the potential to significantly contribute to the complex bioactivity.

V.3.2 *In vitro* antitumor activity

Multi-drug resistance (MDR) is a major cause of failure of cancer chemotherapy. Our research group recently discovered diacetonide ecdysteroid derivatives can strongly sensitize cancer cells to chemotherapeutics (i.e. “chemo-sensitizing” activity) [49]. This sensitization towards various chemotherapeutics could be observed both on MDR and drug susceptible cancer cell lines [52]. Accordingly, semi-synthetized diacetonide derivatives (**20**-**23**) were subjected for related bioactivity testing. First, they were tested for their cytotoxic activity and their ability to inhibit the ABCB1 efflux transporter whose over-expression is among the major reasons for a multi-drug resistant phenotype in cancer cells [120]. While only compound **20** exerted some inhibition of the ABCB1 transporter, each of compounds **20**-**23** acted in synergism when tested in combination with doxorubicin against the studied cancer cell lines. Nevertheless, the synergism was MDR-selective (i.e. while synergism was also observed in a susceptible cell line, it was stronger on its ABCB1-transfected counterpart). This is in agreement with our previous results on the chemo-sensitizing properties of non-ABCB1 inhibitor ecdysteroids derivatives [53, 54]. While the synergism was strong in case of **20**, **21**, and **22** (CI_{avg}<0.3 [106], see section IV.7.2), each compound acted weaker in this regard than their parent compound 20E 2,3;20,22-diacetonide (**19**).

VI. SUMMARY

The main goals of the Ph.D. study presented in this dissertation was to further extend the chemical diversity of ecdysteroids through the semi-synthesis of novel derivatives, to develop new chromatographic techniques for their analysis and/or isolation, and to investigate their biological effect. Our results may be summarized as follows:

1. Preparation of semi-synthetic ecdysteroid derivatives. A series of various ecdysteroid derivatives have been synthesized from different natural ecdysteroids through various chemical approaches. A total of thirty-seven compounds were prepared through oxidative side-chain cleavage, diacetonide formation, base-catalyzed autoxidation or gamma irradiation, as per the followings:

- Six compounds (**7**-**12**) through side-chain cleavage of various phytoecdysteroids.
- Seven compounds (**12**-**18**) through base-catalyzed autoxidation of poststerone.
- Two diacetonide derivatives **19** and **25** from 20E and calonysterone, respectively.
- Five compounds (**20**-**24**) through base-catalyzed autoxidation of 20E 2,3;20,22-diacetonide.
- Fourteen compounds (**26**-**39**) through gamma irradiations of aqueous solutions of 20E in N_2- or N_2O-saturated solutions.
- Three compounds (**6**, **41** and **43**) through base-catalyzed autoxidation of 20E.

Structures of these compounds were determined in collaboration, by means of comprehensive MS and NMR studies. Seventeen of the isolated compounds are new.

2. Development of new analytical and preparative methods for the separation of ecdysteroids. Development of new chromatographic techniques for the successful isolation and/or fractionation using centrifugal partition chromatography, and detection of major and minor compounds using capillary electrophoresis were successfully achieved:

- Two new centrifugal partition chromatography methods were developed for the straightforward isolation of ecdysteroids were developed.
- Two centrifugal partition chromatography methods were developed for the fractionation of highly complex crude mixtures.
- A new capillary electrophoresis method was developed, which allowed a real “in situ” analysis of the time dependency of the base-catalyzed autoxidation of 20E.

3. Biological evaluation of the obtained ecdysteroids. The following results on the bioactivity of the synthesized ecdysteroid derivatives were achieved in research collaboration:

- Effect on the Akt-phosphorylation: compounds **7**-**18** demonstrated the ability to increase the activation, i.e. the phosphorylation of Akt, the compounds with higher

degree of unsaturation showed stronger activity than those with less double bonds in these rings. Related bioactivity testing of compounds **26**-**28**, **30**-**32** and **39** prepared during this Ph.D. work is currently ongoing.

- Inhibition of ABCB1 efflux transporter function: among compounds **20**-**23** tested for this activity, only compound **20** could be considered as a weak inhibitor, and compounds **21**-**23** exerted negligible activity in this regard.
- Cytotoxic activity in combination with doxorubicin: compounds **20**-**23** were tested against a susceptible/multi-drug resistant mouse lymphoma cancer cell line pair, either alone or in combination with doxorubicin, each of these compounds acted in synergism with doxorubicin, and they demonstrated MDR-selectivity in the strength of the synergism.

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