Preparation of bioactive oxidized ecdysteroid derivatives

Summary of Ph.D. Thesis

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INTRODUCTION

Ecdysteroids occur both as plant secondary metabolites (phytoecdysteroids) and insect moulting hormones (zooecdysteroids). In insects, they regulate moulting and reproduction, whereas as secondary plant metabolites, they appear to have a complex role in plant-insect interactions. Since the isolation of the first phytoecdysteroid, ecdysone, more than 500 natural analogues have been isolated and listed in the online database of herbal ecdysteroids "ecdybase". Among these compounds, 20-hydroxyecdysone (20E, **1**) is recognized as the major and most widespread phytoecdysteroid.

Structurally, ecdysteroids contain a polyhydroxylated cyclopentano-perhydrophenanthrene ring system. Most of the naturally occurring ecdysteroids possess a 7-en-6-one chromophore group in the ring B. The A/B ring junction is normally *cis* whereas the B/C and C/D ring junctions are always *trans*. Methyl groups are present at C-10 and C-13 with a β-orientation, hydroxyl groups are found in C-2β, C-3β, C-14α, C-20R, and C-22R positions.

Numerous, non-hormonal, and typically beneficial pharmacological effects of phytoecdysteroids have been reported in mammals including humans accompanied by a negligible acute toxicity. Among these, the anabolic effect is by far the most investigated. However, the exact mechanism of the observed increased protein synthesis is only partially understood, presuming Akt activation to play a central role. Our research group recently found that poststerone (**7**), one of the *in vivo* metabolites of 20E (**1**), acts as a potent anabolic agent on rat skeletal muscles *in vivo*, implying that it plays an important role in the anabolic activity of its parental compound. Ecdysteroids were also reported to have anti-diabetic properties. There are supporting evidences showing that 20E (**1**) affects glucose metabolism *in vitro* and *in vivo* and reduces hyperglycemia associated with administration of glucagon or alloxan. Recently, it was established that the anti-hyperglycemic effect of 20E (**1**) may be exerted through the PI3K-dependent signalling pathways. Certain relatively less polarity ecdysteroids were recently revealed by our research group to have chemo-sensitizing effect on tumour cells. The applied ecdysteroids strongly interfered with the drug resistance of various cancer cell lines, including susceptible and multi-drug resistant (MDR) cell lines of various origin. Furthermore, ecdysteroids, and 20E (**1**) in particular, were reported to exert adaptogenic, hepatoprotective, wound-healing, immunoprotective, and anti-inflammatory activity.

Vast majority of the available pharmacological studies were performed with major phytoecdysteroids, and mainly the abundant 20E (**1**). However, these compounds show a remarkable chemical diversity, which should necessarily be closely connected to a similar diversity of their pharmacological properties. Exploring related structure-activity relationships

is a great challenge, and requires a combined use of appropriately selected semi-synthetic transformations and new approaches of identification and/or isolation. Accordingly, the following objectives were set up for the Ph.D. work presented in this dissertation.

OBJECTIVES

1. The preparation of semi-synthetized 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, base-catalyzed autoxidation, and gamma irradiation (with an interesting chemistry never explored for ecdysteroids before).

2. The development of new chromatographic methods for the isolation and analysis of ecdysteroids. For the separation of the mixtures resulting from the e of the selected chemical transformations were expected to result in complex mixtures whose separation would likely raise new challenges. Thus, in addition to the conventional chromatographic techniques, two less-common methods were used: a preparative liquid-liquid chromatographic technique, centrifugal partition chromatography (CPC), and an analytical technique, capillary electrophoresis (CE).

3. Biological evaluation of the isolated ecdysteroid derivatives. Bioactivity studies on the prepared compounds were planned for their 1) effect on Akt-phosphorylation (related to the anabolic and anti-diabetic effects) or 2) potential as chemo-sensitizing (antitumor) agents.

MATERIALS AND METHODS

Standard ecdysteroid samples available 1-4

The starting material, 20E (**1**) isolated from the roots of *Cyanotis arachnoidea* with a purity of 90%, purchased from Shaanxi KingSci Biotechnology Co., Ltd. was purified by recrystallization from ethyl acetate - methanol (2:1, v/v) to reach a purity of 97.8%. 2-Deoxy-20E (**2**), ajugasterone C (**3**) and polypodine B (**4**) were previously isolated from *Silene* and *Serratula* species.

Preparation of ecdysteroid derivatives

Isolation of dacryhainansterone and calonysterone as starting materials. A commercial extract from the roots of *Cyanotis arachnoidea* was purchased from Xi'an Olin

Biological Technology Co., Ltd. After partially pre-purification through multi-steps column chromatography, 13.16 g of a mixture containing the two main phytoecdysteroids dacryhainansterone (**5**) and calonysterone (**6**) along with two minor components referred to as impurity **i** and **ii** was obtained. For the straightforward isolation of the targeted phytoecdysteroids **5** and **6**, a preparative CPC method was developed.

Oxidative side-chain cleavage. Compounds **7**-**12** were prepared by oxidative side-chain cleavage using 1 equivalent of the hypervalent iodine reagent (diacetoxyiodo)benzene (PIDA) on corresponding phytoecdysteroids (**1**-**6**, respectively). For the purification, flash chromatography and RP-HPLC were applied for the purification of compounds **7** and **8**-**11** respectively. A novel CPC method was developed for the straightforward isolation of compound **12**.

Autoxidation of poststerone. Compounds **12**-**18** were prepared through autoxidation of poststerone (**7**) by applying 0.5% NaOH as catalyst in aqueous methanol (1:9, v/v) during different timeframes: 4 and 7 hours. The final reaction mixtures were fractionated by CPC in ascending mode and the combined CPC fractions were purified by means of RP-HPLC.

Semi-synthesis of diacetonide compounds. Ecdysteroid diacetonides (**19**-**25**) were obtained by applying acetone under acidic conditions or through base-catalyzed autoxidation. Diacetonide analogues of 20E (**1**) and calonysterone (**6**) (compounds **19** and **25** respectively) were obtained by acid catalyzed reaction provided by phosphomolybdic acid in acetone during 5 min and were isolated by flash chromatography. Diacetonide compounds (**20**-**24**) were prepared through autoxidation of 20E 2,3;20,22-diacetonide (**19**) by applying 1% NaOH as catalyst in aqueous methanol (9.5:0.5, v/v) during different timeframes: 8 and 15 hours. The final reaction mixture stirred during 8 hours was fractionated by a new CPC preparative method in ascending mode. Combined CPC fractions were purified by means of RP-HPLC or NP-HPLC yielding compounds **20**-**23**. The second reaction stirred during 15 hours was purified by means of RP-HPLC to yield compound **24**.

Gamma irradiations of aqueous solutions of 20E. Compounds **26**-**39** were obtained from gamma irradiations of aqueous solutions of 20E (**1**). Aqueous solutions inclosing 200 mg of 20E (**1**) at a concentration of 0.5 mmol/dm^3 were prepared and were irradiated at room temperature using a 60Co panoramic type γ-irradiation facility (dose rate = 10,5 kGy/h, absorbed dose = 2 kGy). The irradiations were performed in N_2- (solution **I**) or N_2O-saturated solution (solution **II**). The resulting irradiates materials **I** and **II** were subjected to preparative RP-HPLC. The previous 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). The irradiated solutions were fractionated by a novel CPC preparative method in ascending mode. Combined CPC fractions were further purified by means of RP-HPLC.

Longitudinal study of the autoxidation of 20E with CE

The longitudinal study of the autoxidation of 20E (**1**) with CE was completed through different steps:

1) Preparation of reference compounds **6**, **41** and **43**: the autoxidation of 20E (**1**) was performed by applying 1% NaOH in aqueous methanol (1:9, v/v) solvent during 14 hours and the final pH of the mixture was set to ≈ 6. After a fractionation through reverse phase column chromatography, fraction 2 was further purified through flash chromatography to yield compound **41**, fraction 8 was subjected to CPC ensuring the isolation of compound **43** and fraction 9 was crystallized and compound **6** was attained.

2) Optimization of the CE separation of 20E (**1**) and its autoxidized derivatives and evaluation of the applicability of the developed method: 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 was used. The optimization procedure resulted in the application of the following parameters: temperature set to 25 °C, samples injection by 5×10^3 Pa for 6 s, separation voltage of +30 kV and phosphoric acid buffer (10 mM) at pH 11 applied as background electrolyte completed with 5 mM sulfobutyl ether β-cyclodextrin selector. Applicability of the applied method was checked according to the recommendations of the Good Laboratory Practice, the following parameters were taken into account: linearity of the method, limits of detection (LOD) and limits of quantification (LOQ), intra-day and inter-day precision and accuracy.

3) Monitoring of the autoxidation and determination of the concentrations of compounds at various times: 120 mg of 20E (**1**) was dissolved in a mixture of (9:1 v/v) aqueous methanol and 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 and then subsequently injected into the CE equipment. Three independent autoxidation reactions were performed during 48h.

Procedures for structure elucidation

Characterization and structure elucidation of the obtained compounds were performed by means of different spectroscopic methods (MS, HRMS, 1D- and 2D-NMR).

Biological evaluation of the compounds

Effect on Akt-phosphorylation. Poststerone (**7**) was tested in comparison with 20E (**1**) for its potential to activate Akt at different concentrations: 0.01, 0.1, 1 and 10 μM in mouse C2C12 skeletal myoblasts, and compounds **7**-**18** were tested at a concentration of 10 μM.

ABCB1-inhibition and cytotoxic activity. *In vitro* antitumor activities of compounds **20**-**23** were tested on mouse T-cell lymphoma (L5178) cell line and on multi-drug resistant (MDR) cell line obtained by transfecting L5178 cells with pHa MDR1/A. Inhibition of ABCB1 function of compounds **20**-**23** was investigated through the intracellular retention of rhodamine 123 evaluated by flow cytometry. Cytotoxic activity of compounds **20**-**23** alone and in combination with doxorubicin was evaluated. Cell viability was determined through MTT staining. The constant ratios and combination index (CI) values were calculated for 50, 75 and 90% of growth inhibition in order to assess the ecdysteroid-doxorubicin interaction (synergism, additivity or antagonism).

RESULTS AND DISCUSSION

Isolation / Semi-synthesis of ecdysteroid derivatives

Isolation of dacryhainansterone and calonysterone. When starting to explore the chemical composition of commercially available *Cyanotis arachnoidea* extract, significant amounts of dacryhainansterone (**5**) and calonysterone (**6**) were identified. Dacryhainansterone (**5**) was previously detected in the liquid waste left from the extraction of 20E (**1**) from *Cyanotis arachnoidea*, whereas calonysterone (**6**), is reported here as a new ecdysteroid from this species. The targeted phytoecdysteroids 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.

Oxidative side-chain cleavage. Side-chain cleavage between the hydroxylated C-20 and C-22 carbon atoms of various ecdysteroids (**1**-**6**) was performed by applying PIDA as reagent, and compounds **7**-**12** were obtained with the following yields: poststerone (**7**, 81.41%), compounds **8** (68.34%), **9** (63.57%), **10** (71.23%), **11** (82.71%) and **12** (51.86%). When using another iodine compound (bis(trifluoroacetoxy)iodo)benzene (PIFA), the same products were obtained with lower yields. We hypothesize that the lower yields obtained with PIFA are due to decomposition due to by-product TFA that strongly acidifies the reaction medium, while acetic acid released by PIDA provides milder conditions resulting in less complex mixtures and

higher final yields. Structures of the starting compounds (**1-6**) and their analogues (**7-12**) are presented on **Fig.1**.

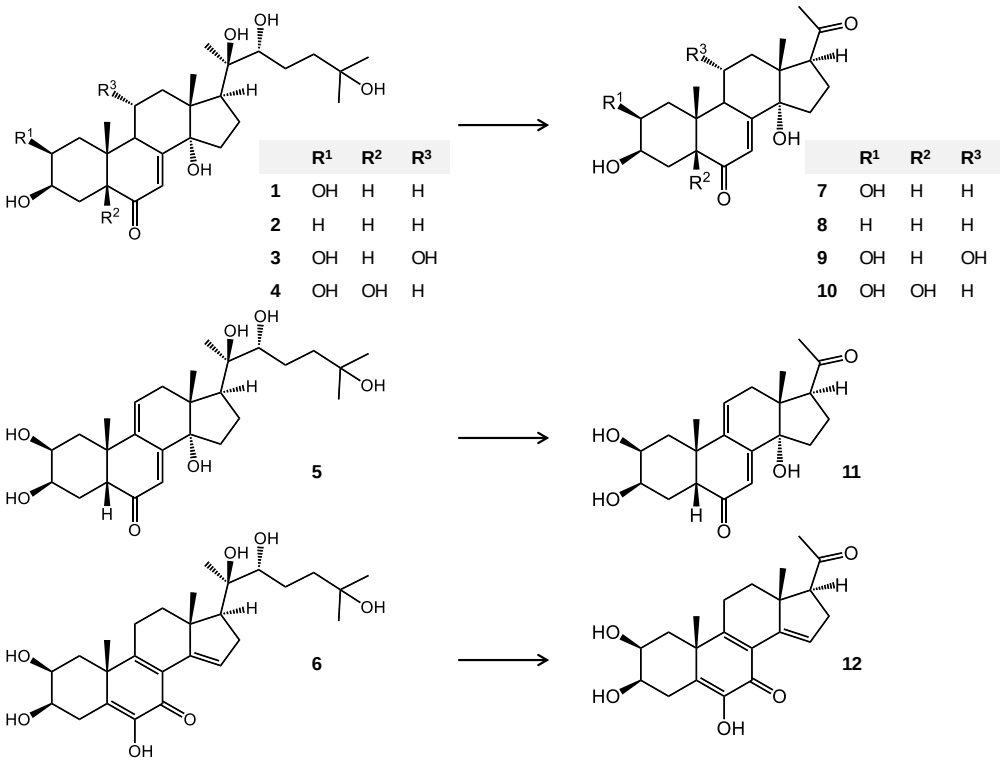

Fig.1. Starting materials **1-6** and their side-chain cleaved derivatives (**7-12**)

Autoxidation of poststerone. The autoxidation of poststerone (**7**) was carried out during 4 hours by applying NaOH as catalyst, and compound **12-18** were isolated with the following yields: **12** (0.34%), **13** (2.17%), **14** (1.21%), **15** (2.42%), **16** (0.84%), **17** (2.48%) and **18** (1.00%). In order to perform biological tests, larger amounts of the previously isolated compounds were required. Small-scale experiments of the above reaction were performed and monitored. A longer reaction i.e *ca.* 7-9 hours was found to be preferable. Thus, the same reaction conducted during 7 hours resulted in compounds **13**, **15**, **16** and **18** with increased yields by 2.46, 1.59, 4.81, 1.10 times respectively. Structures of the synthesized poststerone (**7**) derivatives (**13-18**) are presented on **Fig. 2**.

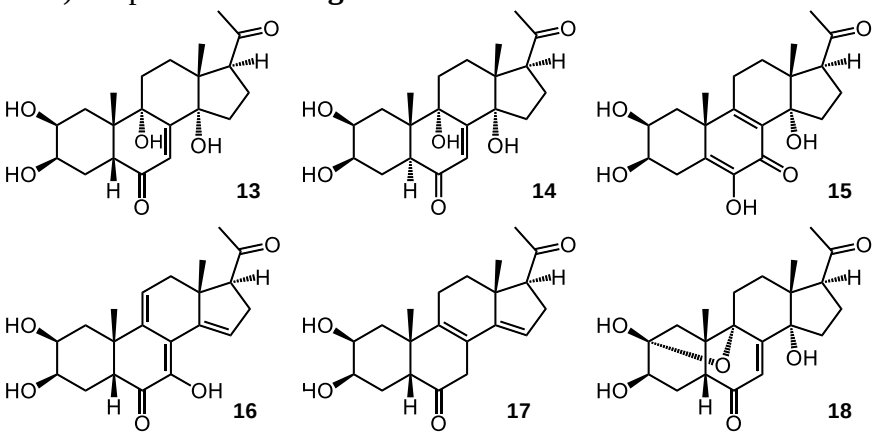

Fig.2. Oxidized ecdysteroid derivatives (**13-18**) obtained from the autoxidation of poststerone

Semi-synthesis of diacetonide compounds. The diols of 20E (**1**) and calonysterone (**6**) at C-2,3 and C-20,22 were targeted in a phosphomolybdic acid-catalyzed reaction to form 20E 2,3;20,22-diacetonide (**19**, 54% yield) and calonysterone 2,3;20,22-diacetonide (**25**, 63.91% yield). The autoxidation of 20E 2,3;20,22-diacetonide (**19**) was carried out twice by applying NaOH as catalyst during 8 and 15 hours, and compounds **20**-**23** and **24** were obtained with the following yields: **20** (1.79%), **21** (2.88%), **22** (2.83%), **23** (3.26%) and **24** (7.83%). Structures of the prepared diacetonide derivatives can be seen on **Fig. 3**.

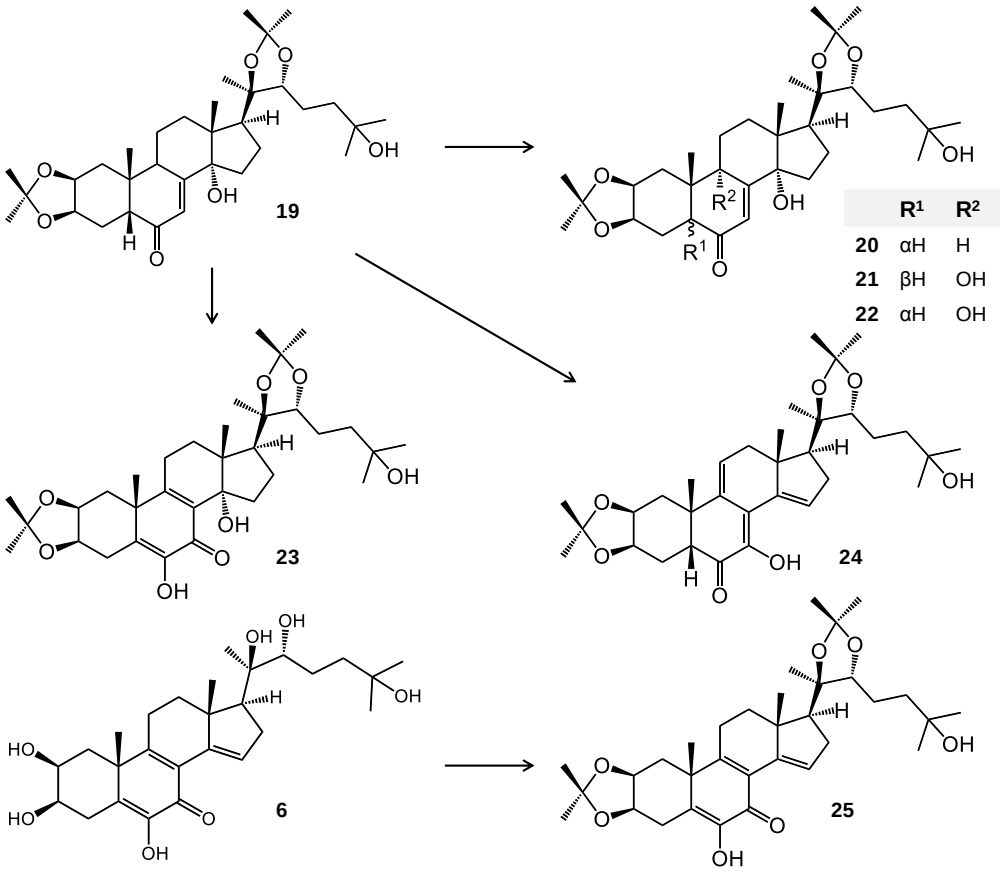

Fig. 3. Structures of oxidized ecdysteroid diacetonides (**19**-**25**)

Gamma irradiations of aqueous solutions of 20E. As a first step, small scale irradiations in N_2- or N_2O-saturated aqueous solutions of 20E (**1**) were performed and common yielded compounds from both materials were compound **27** (N_2-saturation: 0.54%; N_2O-saturation: 2.35%) and podecdysone B (**28**, N_2-saturation: 0.31%; N_2O- saturation: 1.16%). 14-perhydroxy-20E (**26**, 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. Concerning the larger scale irradiations, 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**, 1.52%), compound **27** (0.94%) and

podecdysone B (**28**, 0.50%). These latter were accompanied by stachysterone B (**31**, 0.82%), 14-deoxy-20E (**32**, 0.25%), 5α-20E (**33**, 0.31%), 2-dehydro-3-deoxy-20E (**34**, 0.11%), the 7-11′ hetero-dimer (**35**, 0.07%) and compound **36** (0.18%). Concerning the irradiation in N_2O-saturated solution, compound **27** (2.18%), podecdysone B (**28**, 0.83%), 2-dehydro-20E (**29**, 0.08%), stachysterone B (**31**, 0.15%), 5α-20E (**33**, 0.12%), 2-dehydro-3-deoxy-20E (**34**, 0.11%), compound **36** (0.27%), compound **37** (0.18%), 25-hydroxy-dacryhainansterone (**38** 0.37%) and 22-dehydroecdysone (**39**, 0.33%) were isolated. Structure of all the isolated products are shown in **Fig. 4**.

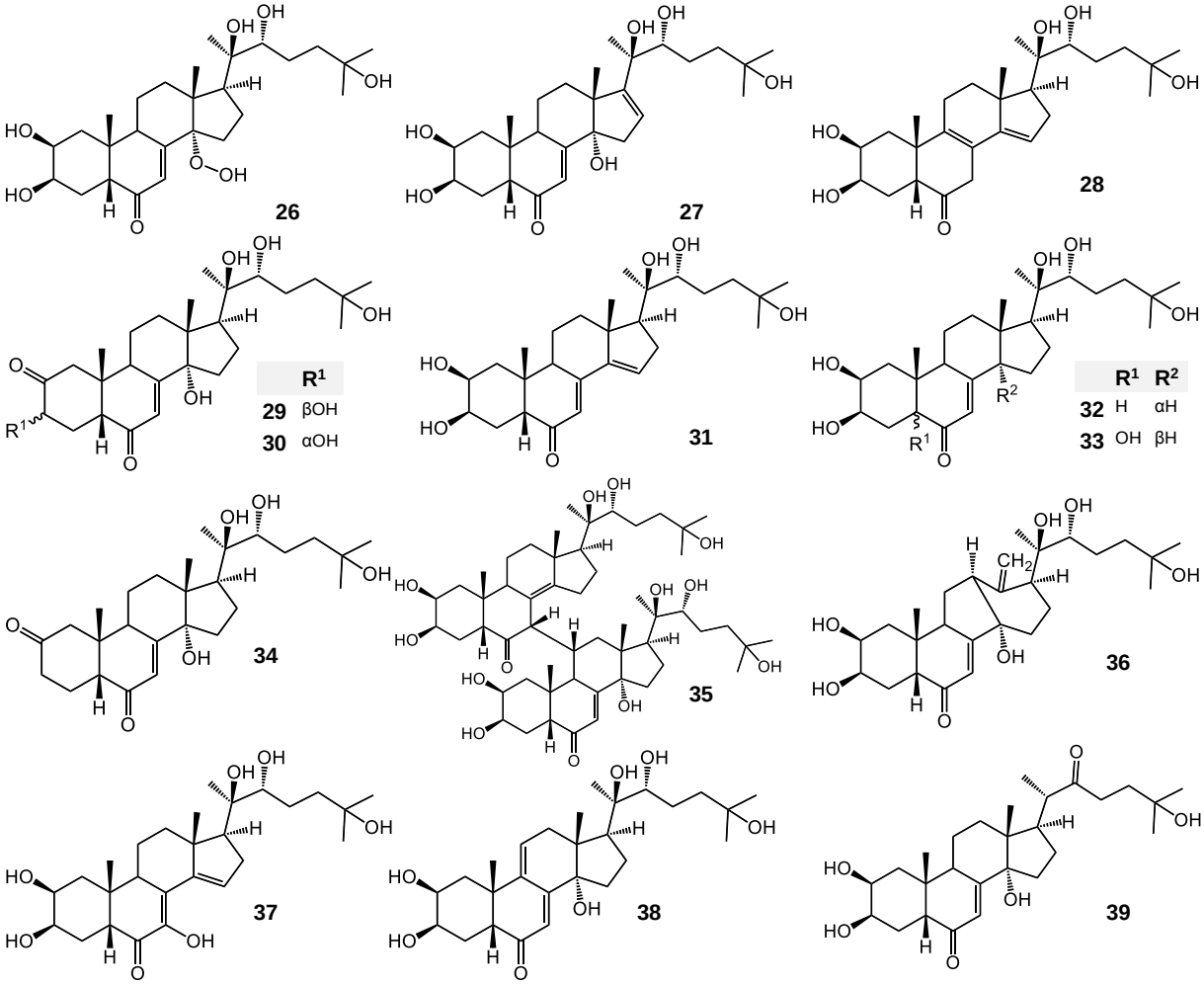

Fig. 4. Structures of ecdysteroid derivatives (**26**-**39**) obtained by gamma irradiation of 20E

To the best of our knowledge, gamma irradiation of ecdysteroids has not been studied before. 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, no side-chain cleavage was observed, the only change affecting the side-chain was the oxidation of the 22-OH group to a keto-group in compound **39**.

It is worth mentioning that almost all the products were isolated at very low yields (between 0.1 and 2.2%); this can partially be explained with chromatographic overlapping: a large number of minor compounds were observed accounting for a significant quantity of the amount of the irradiated 20E (**1**). As seen from **Fig. 5**, the crude mixture was highly complex with compounds eluting with very closed retention times (**Fig. 5A**). Thus, it can be concluded that the initial fractionation through CPC allowed the separation of products whose isolation would not be possible at once through preparative HPLC.

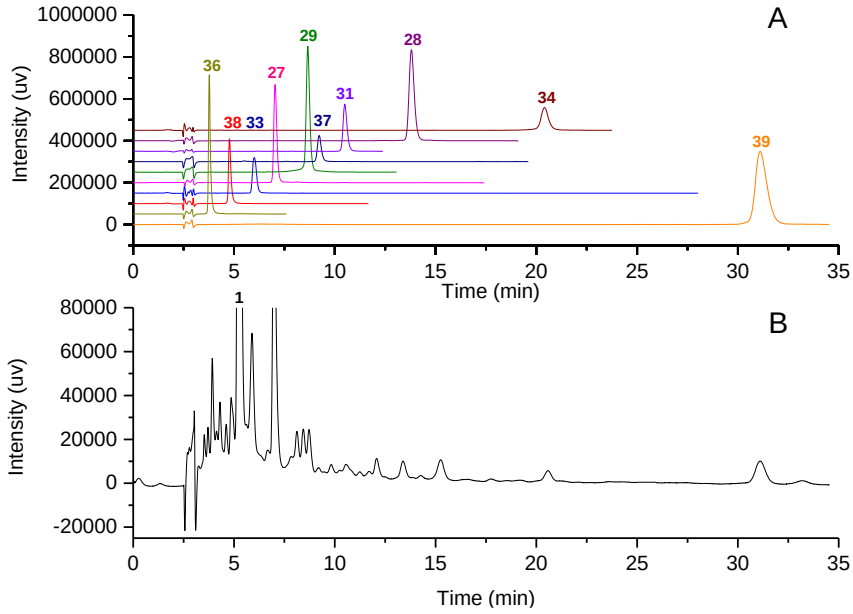

Fig. 7. Chromatograms of (**A**) crude mixture obtained after gamma irradiation of aqueous solution of 20E (**1**) in N_2O-saturated solution and (**B**) pure compounds obtained after purification steps. HPLC fingerprints are presented at λ_{max} (λ= 200 - 650 nm); Column: Kinetex Biphenyl (5 µm, 250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

Development of new CPC methods

Isolation of dacryhainansterone and calonysterone. Of the sixteen tested biphasic solvents belonging to the HEMWat (*n*-Hexane - Ethyl acetate - Methanol - Water) family, five biphasic systems allowed partitioning of dacryhainansterone (**5**) and calonysterone (**6**). The biphasic system ethyl acetate - water (1:1, v/v) was selected as starting point of the optimization procedure and a stepwise addition of *n*-hexane and methanol was made in order to adjust the polarity of the entire system. The solvent system composed of *n*-hexane - ethyl acetate - methanol - water (1:5:1:5, v/v/v/v) provided suitable partitions coefficients ($K_{(U/L)_i}$) values ranging between 0.5 and 2 and acceptable separation factors (α) ≥ 1.5 (see **Table 1**). The selected solvent system presented satisfying separation characteristics: a good settling time (27 s), the volume ratio of the upper and lower phases was 0.90 and the Sf ratio was 0.70.

Table 1 Partition coefficients and separation factors of the selected biphasic system in the isolation of compounds **5** and **6**

Solvent system	Partition coefficients				Separation factors		
***n*-hexane – ethyl acetate – methanol – water (v/v/v/v)**	$K_{(U/L)_5}$	$K_{(U/L)_6}$	$K_{(U/L)_i}$	$K_{(U/L)_{ii}}$	$\alpha_{(5/6)}$	$\alpha_{(i/6)}$	$\alpha_{(5/ii)}$
1:5:1:5	2.042	1.136	1.730	1.307	1.800	1.523	1.562

The separation was performed in ascending mode, at constant pressure of 86 bar, flow rate of 10 ml/min and rotation speed of 2400 rpm in six consecutive injections of the crude extract (**Fig. 6**). An altogether 95.28% of the initial weight was recovered after the separation, and separation of dacryhainansterone (**5**) and calonysterone (**6**) was achieved in less than 30 min.

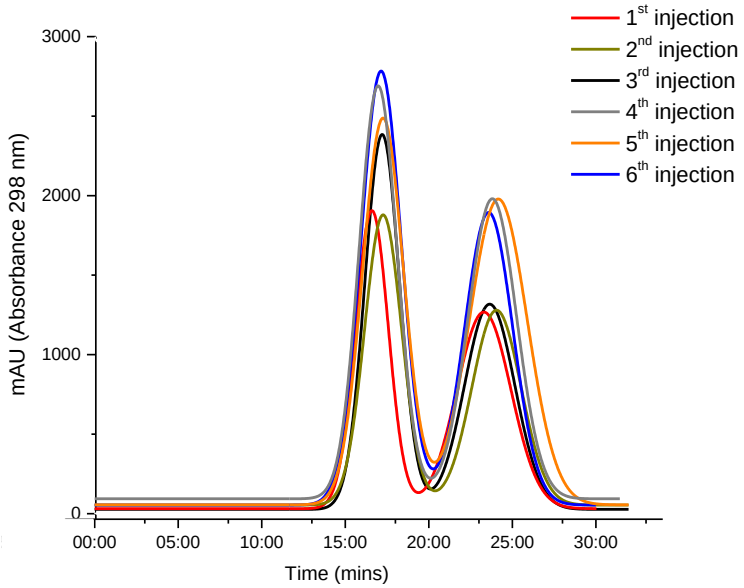

Fig.6. CPC separation of 1 g of crude commercial extract of *Cyanotis arachnoidea* using six consecutive injections of 8 ml sample volume. Slight shifting of retention times comes from unavoidable variations in the injected solvent composition of each consecutive run

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

Isolation of the side-chain cleaved analogue of calonysterone: compound 12. The two ecdysteroids **6** and **12** exerting quiet different polarities, their partition in the eight biphasic systems belonging to the HEMWat family studied was readily achievable. However, many of the applied solvent systems presented very high separation factors (from 4.441 to 14.359) which would led to compounds eluting after a too long separation time resulting in a large solvent consumption. The selected solvent system composed of *n*-hexane - ethyl acetate - methanol - water (1.5:5:1.5:5, v/v/v/v) was the best in terms of solvent consumption while ensuring a good separation of the two ecdysteroids of interest (see **Table 2**). Also, the selected solvent system

presented excellent separation characteristics: the settling time was very short (17s), the volume ratio of the upper and lower phases was 0.86, and the retention volume ratio Sf was 0.68.

Table 2 Partition coefficients and separation factor of the optimal biphasic systems in separation of compounds **6** and **12**

Solvent system	Partition coefficients		Separation factor
***n*-hexane – ethyl acetate – methanol – water (v/v/v/v)**	$K_{(U/L)_6}$	$K_{(U/L)_{12}}$	$\alpha_{(12/6)}$
1.5:5:1.5:5	0.460	1.370	2.978

The separation was performed in ascending mode and the instrument parameters were as follows: constant pressure of 86 bar, 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. Compound **12** was obtained with a purity of 96.00%. After recrystallizing from ethyl acetate - methanol (2:1, v/v), the purity reached 99.00%.

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. 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 and methanol as its mutual solvent. Water served to make up a two-phase solvent system. Several apolar solvents were tested to find out the best modifier to afford optimum $K_{(U/L)_i}$ values, finally, *n*-hexane was selected. In order to balance the partition of the compounds between the aqueous and the organic phased and to maintain equal volumes of the two phases, the ratio of dichloromethane was gradually decreased whereas the ratio of *n*-hexane was continuously increased. The biphasic system composed of dichloromethane - methanol - water (1:1:1, v/v/v) was selected as starting point of the optimization procedure. Good partition of the compounds could be reached with the solvent system composed of *n*-hexane - dichloromethane - methanol - water (1:0.215:1:1, v/v/v/v) (see **Table 3**). The significant decrease of the ratio of dichloromethane in the mixture lead to a decrease of solubility of the crude mixture and thus to an increase of the number of injections into the CPC instrument during the purification (7 injections). Such high number of injections led to wastes during manipulations and is most likely the reason why our purification resulted in an unusual low yield (88.89%) of the initial weight recovered after the separation. The selected biphasic system presented best separation characteristics among the tested biphasic systems: a settling time of 32 s, the volume ratio of the upper and lower phases was 0.85 and the retention volume ratio Sf was 0.56.

Table 3 Partition coefficients of the selected tested biphasic systems in separation of compounds **19-23**

Solvent systems	Partition coefficients				
***n*-hexane - dichloromethane - methanol - water (v/v/v/v)**	$K_{(U/L)20}$	$K_{(U/L)21}$	$K_{(U/L)19}$	$K_{(U/L)23}$	$K_{(U/L22)}$
1:0.215:1:1	0.878	0.913	1.285	1.284	1.703

The separation was performed in ascending mode and the instrument parameters were as follows: constant pressure of 86 bars, flow rate of 10 ml/min and rotation speed of 2300 rpm.

Fractionation of crude mixtures resulting from 20E irradiations. Previous observations regarding the behaviour of different ecdysteroids in different biphasic systems suggested their mild partition between ethyl acetate and water. When the biphasic system composed of ethyl acetate - water (1:1, v/v) was selected as starting point of the optimization procedure, a large proportion of the irradiated products was more concentrated in the lower aqueous phase. Considering that the enrichment of the upper organic phase by an alcohol changes drastically the partition of the ecdysteroids, different alcohols were tested and *tert*-butanol was able to provide an improvement of the partitioning. 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 resulted in the use of the solvent system composed of *tert*-butanol - ethyl acetate - water (0.4:0.9:1, v/v/v) (see **Table 4**). To our best knowledge, not such solvent system was used for the separation of ecdysteroids before; usually, solvent systems containing the three other isomeric structures of butanol are used for purification of the naturally occurring compounds. The selected biphasic solvent, 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 4 Partition coefficients for the selected biphasic systems in separation of main compounds from the gamma irradiation of 20E (**1**).

Solvent system	Partition coefficients					
***tert*-butanol - ethyl acetate – water (v/v/v)**	$K_{(U/L)_1}$	$K_{(U/L)_{27}}$	$K_{(U/L)_{33}}$	$K_{(U/L)_{26}}$	$K_{(U/L)_{31}}$	$K_{(U/L)_{28}}$
0.450:0.9:1	0.994	0.634	1.273	1.315	3.624	7.150

The fractionations were performed in ascending mode and the instrument parameters were as follows: constant pressure of 87 bars, flow rate of 10 ml/min and rotation speed of 2900 rpm. An altogether 95.71% and 97.35% of the initial weights of solution **I** and **II**, respectively, were recovered after the fractionations.

Longitudinal study of the autoxidation of 20E (1) with CE.

A novel capillary electrophoretic method was developed for the analysis and monitoring of the base-catalyzed autoxidation of 20E (**1**).

1) The base-catalyzed autoxidation of 20E (**1**) was carried during 14 hours by applying NaOH as catalyst in low concentration, and compounds **6**, **41** and **43** were isolated, compounds **40** and **42** used for this study were available in sufficient amounts in our laboratory. Structures of the oxidized derivatives (**40**-**43**) are seen on **Fig. 7**.

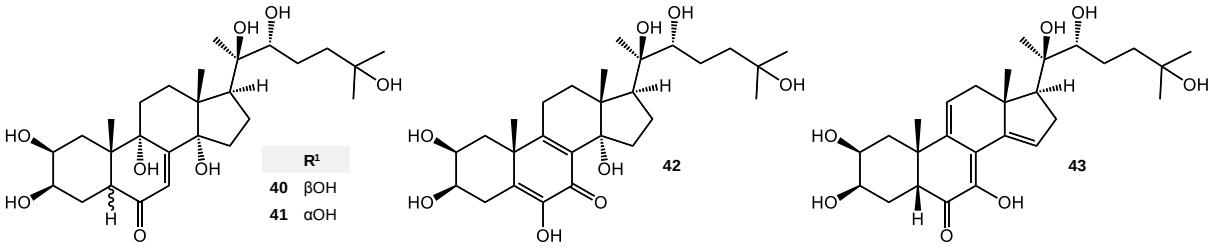

Fig.7. Structures of ecdysteroid derivatives (**40**-**43**)

2) Under optimized conditions, namely using the suitable selector, sulfobutyl-beta-cyclodextrin 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. 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, 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. Thus, the following parameters were taken into account:

1. The linear correlation: calibration curves exhibited an excellent linear correlation over the concentration ranges with coefficients of determination close to 1.
2. The calculated LOD and LOQ: The LOD was below 71 μg/ml and LOQ was below 183 μg/ml, 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: the values of the relative standard deviations of all compounds were below 10%, which is the threshold generally 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.

3) Nonlinear curve fitting on the data obtained with a previous HPLC analysis and the new CE analysis was performed by OriginPro 9.1 software in order to obtain an optimal comparison between these datasets. 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 9.6% aqueous solution of CH_3COOH. Relative amounts of the compounds as compared to the initial amount of 20E (**1**) are presented in **Fig. 9**.

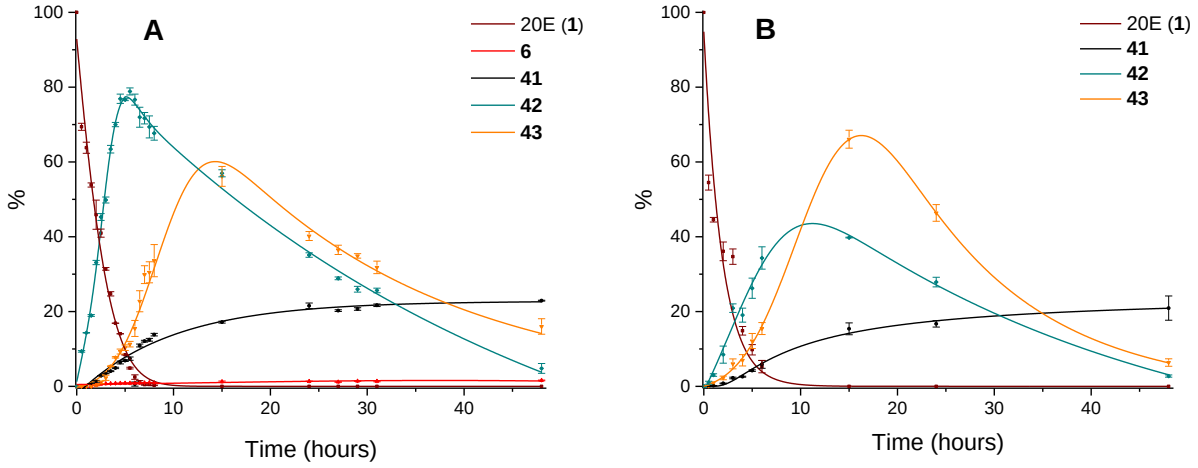

Fig. 9. Amounts of 20E derivatives obtained at various times by means of CE (**A**) and HPLC (**B**). Error bars represent standard error of the mean values from three independent experiments.

As seen from the comparison between **Fig. 9A** and **9B**, no major difference can be observed in the decomposition of 20E (**1**) and the formation of compound **41**: 20E (**1**) underwent a rapid decay whereas compound **41** increased exponentially. This suggests that the sample pre-treatment before HPLC analysis had no effect on the results obtained for 20E (**1**) and compound **41**. Calonysterone (**6**) was well detectable by CE, even if at only a very small amount. A substantial difference in the maximal amounts detected with the two techniques regarding compound **42** must be highlighted suggesting that this latter 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.

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 time dependency of the autoxidation of 20E (**1**) with direct injections of the product mixtures into the instrument after a simple dilution, 2) it permitted the use of similarly high 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) 4) the short separation time allowed more frequent analysis with an accurate determination of the time for maximum yield of each compound, and 5) it afforded large time saving and lower consumption of mobile phase.

Biological activities of the obtained compounds

Effect on Akt-phosphorylation. A dose-dependency study was performed to test poststerone (**7**) in comparison with 20E (**1**) for their ability to increase the phosphorylation of Akt in murine C2C12 skeletal myotubes. The activities are presented in **Fig. 10**.

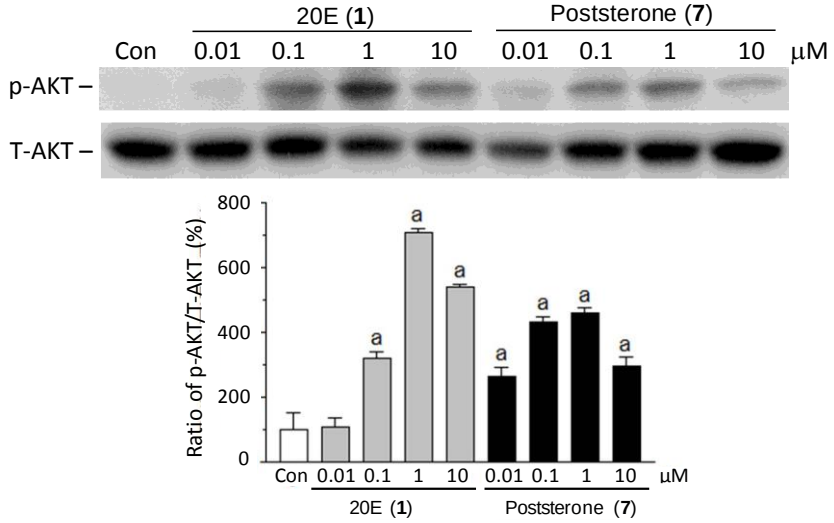

Fig. 10. Dose-dependency comparison of the Akt activation exerted by 20E (**1**) and poststerone (**7**). Error bars represent standard error of the mean from six parallel experiments. a: $p<0.001$ as compared to the control (Con)

The dose-dependency study showed that that poststerone (**7**) acts as a weaker activator than 20E (**1**) at a concentration of 10 μM. Interestingly, however, poststerone (**7**) was still active at as low as 10 nM concentration, where 20E (**1**) was already inactive.

Based on the above results, side-chain cleaved compounds (**7**-**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 shown in **Fig. 13**.

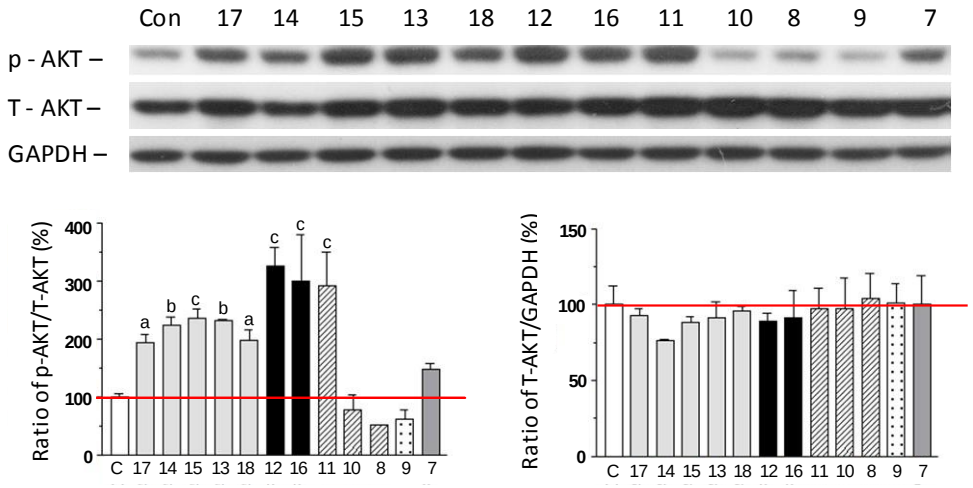

Fig. 13. Activity of compounds **7**-**18** as compared to the control (C) on the Akt phosphorylation. Quantification of Western blots was performed by ImageJ; error bars represent standard error of the mean from four parallel experiments. a: $p<0.05$; b: $p<0.01$; c: $p<0.001$

With the exception compounds **8**, **9** and **10**, all tested compounds demonstrated the ability to increase the activation of Akt. In particular compounds **11**, **12** and **16**, presenting higher degree of unsaturation due to further conjugation extended to their D and/or C rings showed the strongest activity in this regard. This suggests that further double bonds conjugated with those already present may positively impact the bioactivities of these compounds in mammalian cells.

ABCB1-inhibition and cytotoxic activity. Compounds **20-23** exerted negligible inhibition of the ABCB1 efflux transporter; only compound **20** could be considered as a weak inhibitor with a *ca.* 40% inhibition at 20µM (data not presented). The same compounds were also tested for their *in vitro* cytotoxic activity both alone and in combination with doxorubicin (see **Table 7** and **Table 8**).

Table 7 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 8 Cytotoxic activity of compounds **20-23** in combination with doxorubicin at different compound vs. doxorubicin ratios[a]. 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. $CI_{avg} = (CI_{50} + 2 \times CI_{75} + 3 \times CI_{90}) / 6$

			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 ability to act in synergism with doxorubicin on both cell lines, with a moderate selectivity towards the multi-drug resistant one.

While only compounds **20** and **22** exerted some inhibition of the ABCB1 transporter, each of compounds **20**-**23** acted in synergism with doxorubicin against the studied cancer cell lines. Nevertheless, the synergism was MDR selective. While the synergism was strong in case of **20**, **21**, and **22** (CI_{avg}<0.3), each compound acted weaker in this regard than their parental compound 20E 2,3;20,22-diacetonide (**19**).

SUMMARY

The main goals of the presented Ph.D. study were 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 total of thirty-seven ecdysteroid derivatives including seventeen new compounds have been synthesized from different natural ecdysteroids through various chemical approaches:

- 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 **26** from 20E and calonysterone, respectively,
- Five compounds (**20**-**25**) through base-catalyzed autoxidation of 20E 2,3;20,22-diacetonide,
- Fourteen compounds (**26**-**39**) through gamma irradiations of aqueous solutions of 20E under N_2 or N_2O atmospheres,
- Three compounds (**6**, **41** and **43**) through base-catalyzed autoxidation of 20E.

2. Development of new analytical and preparative methods for the separation of ecdysteroids. Development of new chromatographic techniques for the 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 purification of ecdysteroids.
- Two centrifugal partition chromatography methods were developed for the fractionation of highly complex mixtures.

- A new capillary electrophoresis method was developed, which allowed a real "in situ" analysis of the time dependency of the base-catalysed autoxidation of 20E.

3. Biological evaluation of the obtained ecdysteroids.

- 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. Evaluation of compounds **26**-**28**, **30**-**32** and **39** on the same pharmacological model is currently in process.
- 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, 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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THE THESIS IS BASED ON THE FOLLOWING PUBLICATIONS:

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* 1054 (2017) 44-49.

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*. 146 (2017) 188-194.

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.* 82 (2018) 405-413.

PRESENTATIONS RELATED TO THE THESIS:

1. A. Hunyadi, B. Dankó, J. Csábi, M. Vágvölgyi, **H.M. Issaadi**, L. Fási, Z. Zoofishan: A brief overview of our compound library available for collaborative studies. *4th Workshop of COST Action CM1106, Chemical Approaches to Targeting Drug Resistance in Cancer Stem Cells*, Chioggia, Italy, 10-11 March, 2016.
2. A. Hunyadi, B. Dankó, J. Csábi, M. Vágvölgyi, **H.M. Issaadi**, L. Fási, Z. Zoofishan: What we can provide for collaboration: an overview of our available compound library. *2nd meeting of COST Action CM1407, Challenging Organic Syntheses Inspired by Nature*, Madrid, Spain, 4-5 April, 2016.
3. **H.M. Issaadi**, Z. Kele, G. Tóth, A. Hunyadi: Synthesis of novel oxidized ecdysteroid metabolites. *Fiatal Gyógynövénykutatók Fóruma: A Magyar Gyógyszerésztudományi Társaság Gyógynövény Szakosztályának tudományos konferenciája*, Budakalász, Magyarország, 24 June, 2016.
4. **H.M. Issaadi**, K. Németh, A. Hunyadi: Direct analysis of the formation of autoxidized derivatives of 20-hydroxyecdysone by capillary electrophoresis. *Trends in Natural Product Research – PSE Young Scientists' Meeting Lille 2017 Natural Products in Health, Agro-Food and Cosmetics*, Lille, France, June 28th-July 1st 2017.
5. **H.M. Issaadi**, J. Csábi, K. Németh, A. Hunyadi: Comparative HPLC and CE studies on the formation of 20-hydroxyecdysone metabolites from base-catalyzed autoxidation and Fenton reaction. *65th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA)*, Basel, Switzerland 3-7 September, 2017.
6. **H.M. Issaadi**, J. Csábi, Z. Kele, T-J. Hsieh, G. Tóth, A. Hunyadi: Preparation of side-chain cleaved phytoecdysteroid metabolites activating protein kinase B. *COST ACTION CM1407 4th Meeting: Challenging organic synthesis inspired by nature – from natural products chemistry to drug discovery*, Lisbon, Portugal, 21-22 September, 2017.

OTHER PRESENTATION:

7. O. Bensebia, **H.M. Issaadi**, V. Andrea, K. Allia: Effects of drying temperature on the total phenolic, flavonoids contents and antiradical activity of dried sage leaves. *Joint Natural Products Conference 2016*, Copenhagen. Denmark, 24-27 July, 2016.