Ph.D. Thesis

Analysis of the ecdysteroid profile of *Serratula wolffii* roots

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1. Introduction

1.1. Distribution, application and structural diversity of ecdysteroids

Ecdysteroids were discovered as steroid hormones controlling moulting and metamorphosis in insects. These compounds occur in all classes of arthropods and probably in other classes of invertebrates too. In the mid-1960s, phytoecdysteroids, structurally related compounds, were identified in several plant species. The ready availability of ecdysteroids in plants allows pharmacological studies, which have demonstrated that they influence many physiological functions and are not toxic to mammals. The most pronounced effect of ecdysteroids on mammals is a stimulation of protein synthesis without the adverse side-effects associated with vertebrate steroids or their analogues. However, the mode of action and the metabolism in mammals, including humans, have remained open questions.

The specific effects of ecdysteroids on insects and their low mammalian toxicity formed the basis of development of safe insecticides. Bisacylhydrazines are functional analogues of ecdysteroids, identified as non-steroidal agonists of ecdysteroid receptors.

An ecdysteroid-inducible gene expression system is a new line of biomedical application of ecdysteroids. The absence of ecdysteroid receptor in vertebrate cells has attracted attention to this transgenic system. Importantly, ecdysteroids are neither toxic nor teratogenic to vertebrates and they can easily penetrate into all tissues. Gene-switching systems based on ecdysteroid/ecdysteroid receptor complex have been developed and produced for experimental use.

Although less than 2% of the world's flora has been investigated for the presence of ecdysteroids, over 300 ecdysteroid analogues have been identified in plant sources. The ecdysteroids possess a cyclopentano-perhydrophenanthrene carbon skeleton with β side chain at C-17. Characteristic structural elements of phytoecdysteroids are 7-en-6-one chromophore in ring B and *trans* C/D ring junction. Phytoecdysteroids are highly hydroxylated with 2-8 hydroxy groups. The commonly hydroxylated sites are the 2β, 3β, 14α, 20R, 22R and 25 positions.

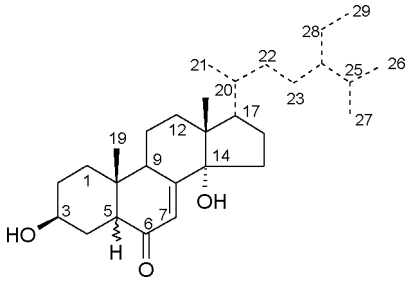


Figure 1. The common structure of ecdysteroids.

1.2. Aims of the study

Scientific investigations of the ecdysteroids comprise a promising and developing area of biomedical chemistry, which includes world flora screening, identification of the most active compounds and study of practical application possibilities. The economic, large-scale extraction of ecdysteroids is the basic purpose behind these studies. However, ecdysteroids (except for 20-hydroxyecdysone) can not be synthetized. The isolation of ecdysteroids from plant sources is the only way to obtain them. The suitable plant materials must contain a large amount of ecdysteroids (>1%), produce sufficient biomass per land surface and have unspecial cultivating requirements. The *Serratula* species meet these requirements, and analysis of the ecdysteroid patterns of some *Serratula* species has been in progress for some time by the research group at the Departement of Pharmacognosy.

Our main aims were as follows:

a) To study the ecdysteroid profile of the roots of *S. wolffii*. This means first the isolation and elucidation of the structures of new native phytoecdysteroids.

b) Further, we set out to achieve the isolation of biologically active compounds:
   - the identification of new ecdysteroids with an 11α-OH group,
   - the isolation of ecdysteroids with high moulting activity, and
   - the preparation of ecdysteroids which are active in gene switching sytems.

c) If the isolated compounds provide such a possibility, our objectives include the analysis of structure-activity relationships.

d) Study of the ecdysteroid pattern helps extend the available knowledge on the species and/or genus to the estimation of chemotaxonomic relations and to the acquisition of information on the biosynthetic pathways.

e) To improve the efficiency of the earlier isolation procedure, to simplify the methodology and to develop a new, rapid isolation process, which is generally applicable to other plant sources too.

2. Materials and methods

2.1. Plant material

Roots of *S. wolffii* Andrae were collected in August, 2003 from Herencsény, Hungary. A voucher specimen (collection number S94) has been deposited at the Department of Pharmacognosy, University of Szeged, Hungary.

2.2. Reagent and standard ecdysteroids samples

Solvents of HPLC grade were from Merck (Darmstadt, Germany). Solvents of analytical grade were from Reanal (Budapest, Hungary). Reference ecdysteroids were available from earlier isolation work and fully characterized in previous studies. Their identities and purities were verified by NMR and HPLC.

2.3. Procedures for isolation of ecdysteroids

The ecdysteroids were subjected to exhaustive extraction with methanol. The next step involved preliminary purification using fractionated precipitation with acetone and solid-phase extraction on polyamide. The isolation of ecdysteroids from the purified plant extract was based on the optimized combination of chromatographic methods: vacuum RP-CC, RPC and HPLC. Each chromatographic step was monitored by conventional TLC.

2.4. Structure elucidation

The known compounds were identified by direct comparison of their physical and spectroscopic characteristics with those published in the literature. They were also characterized by co-chromatography with pure reference ecdysteroids, using NP- and/or RP-TLC and also NP- and/or RP-HPLC.

In addition to chromatography, all ecdysteroids were characterized by different spectroscopic methods. UV, NMR and MS were utilized to identify ecdysteroids. The NMR and MS spectra provided the basic information on the structures of the compounds. In the course of the structural elucidation of the compounds, the MS and NMR spectra data were evaluated in comparison with those for the main phytoecdysteroid, 20-hydroxyecdysone.

3. Results and discussion

3.1. Isolation of ecdysteroids

With combined chromatographic methods, 10 known and 13 new natural ecdysteroids were isolated from the roots of *S. wolffii* Andrae. Four of the known compounds (ponasterone A, stachysterone B, 22-deoxyintegristerone A and shidasterone) were found in the *Serratula* genus for the first time. **Table 1** and **Figure 2** show the structures of all the isolated ecdysteroids. The compounds discovered in nature for the first time are denoted (*).

3.2. Significance of the isolated ecdysteroids

- Isolation of biologically active compounds

Our investigation confirmed that *S. wolffii* is a good source of 11α-hydroxyecdysteroids. Four of the isolated compounds possess an 11α-OH group; ajugasterone C (**10**) is the main compound among them. 11α-Hydroxyshidasterone (**1**), serfurosterone B (**17**) and 22-dehydro-20-deoxy-ajugasterone C (**19**) are new members of the small 11α-hydroxyecdysteroid subfamily. The structure activity experiments have confirmed that 11α-hydroxy group of ecdysteroids is important in the manifestation of the anabolic activity.

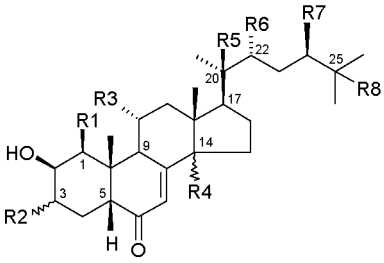
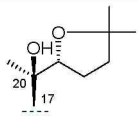
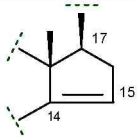
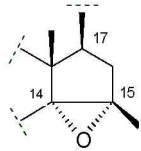
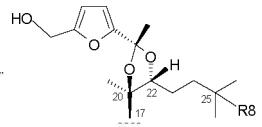
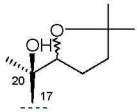
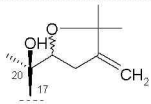
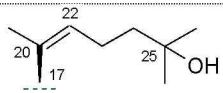
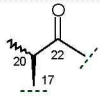
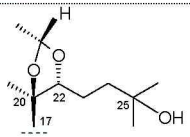
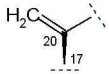
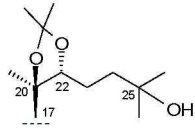
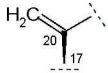
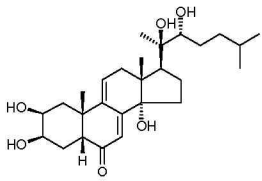


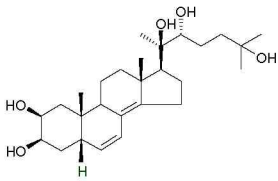
Table 1. Structures of classical ecdysteroids, containing 7-en-6-one chromophore.

Ecdysteroid	R1	R2	R3	R4	R5	R6	R7	R8
11*α*-hydroxyshidasterone (**1**)*	H	*β*OH	*α*OH	*α*OH				
2*β*,3*α*,20R,22R,25-pentahydroxy-5*β*-14*β*-cholest-7-en-6-one (**2**)*	H	*α*OH	H	*β*H	OH	OH	H	OH
2*β*,3*α*,20R,22R,25-pentahydroxy-5*β*-14*α*-cholest-7-en-6-one (**3**)	H	*α*OH	H	*α*H	OH	OH	H	OH
ponasterone A (**5**)	H	*β*OH	H	*α*OH	OH	OH	H	H
stachysterone B (**6**)	H	*β*OH	H		OH	OH	H	OH
14*α*,15*α*-epoxy-14,15-dihydrostachysterone B (**7**)*	H	*β*OH	H		OH	OH	H	OH
makisterone A (**8**)	H	*β*OH	H	*α*OH	OH	OH	CH_3	OH
serfurosterone A (**9**)*	H	*β*OH	H	*α*OH				OH
serfurosterone B (**17**)*	H	*β*OH	*α*OH	*α*OH				H
ajugasterone C (**10**)	H	*β*OH	*α*OH	*α*OH	OH	OH	H	H
20-hydroxyecdysone (**11**)	H	*β*OH	H	*α*OH	OH	OH	H	OH

Ecdysteroid	R1	R2	R3	R4	R5	R6	R7	R8
22-deoxyintegristerone A (**12**)	OH	*β*OH	H	*α*OH	OH	H	H	OH
shidasterone (**13**)	H	*β*OH	H	*α*OH				
24-methylene-shidasterone (**15**)*	H	*β*OH	H	*α*OH				
20,22-didehydrotaxisterone (**16**)*	H	*β*OH	H	*α*OH				
1-hydroxy-20,22-didehydrotaxisterone (**18**)*	OH	*β*OH	H	*α*OH				
22-dehydro-20-deoxy-ajugasterone C (**19**)*	H	*β*OH	*α*OH	*α*OH			H	H
20-hydroxyecdysone 20,22-ethylidene (**20**)	H	*β*OH	H	*α*OH				
1-hydroxy-22-deoxy-20,21-didehydro-ecdysone (**21**)*	OH	*β*OH	H	*α*OH		H	H	OH
20-hydroxyecdysone 20,22-monoacetonide (**22**)	H	*β*OH	H	*α*OH				
22-deoxy-20,21-didehydro-ecdysone (**23**)*	H	*β*OH	H	*α*OH		H	H	OH



dacryhainansterone (**4**)



2*β*,3*β*,20R,22R,25-pentahydroxy-5*β*-cholest-6,8(14)-diene (**14**)*

Figure 2. Structures of ecdysteroid dienes.

Ecdysteroids 7,9(11)-dienes, such as dacryhainansterone (**4**), displayed higher biological activity in the *Drosophila melanogaster* B_{11} cell bioassay than the classical 7-en-6-one ecdysteroids.

Many natural phytoecdysteroids have been screened in gene regulatory systems. Neither ecdysone nor 20-hydroxyecdysone acted as an agonist for the EcR in mammalian cells. In contrast, expression of the reporter gene was increased in cells treated with ponasterone A (**5**). *S. wolffii*, primarily in the Asteraceae family, is a source of this biologically active steroid.

- Ecdysteroids with unprecedented structures

Two compounds, 2*β*,3*α*,20R,22R,25-pentahydroxy-5*β*-14*β*-cholest-7-en-6-one (**2**) and 2*β*,3*β*,20R,22R,25-pentahydroxy-5*β*-cholest-6,8(14)-diene (**14**) are protoecdysteroids, because they differ from the classical ecdysteroid structure. The former molecule is the third ecdysteroid with a *cis* C/D ring junction. The structurally-related compounds 14-epi-20-hydroxyecdysone and 14-epi-ponasterone A 22-glucoside were previously identified in *Serratula wolffii* and *Leuzea carthamoides*, respectively. The surprising discovery that there are 14-epi-ecdysteroids appears to disprove the classical chemical definition of ecdysteroids.

14*α*,15*α*-Epoxy-14,15-dihydrostachysterone B (**7**) is only the second known ecdysteroid containing a 14,15-epoxide ring. The epoxide ring is therefore a rare moiety among ecdysteroids. Five compounds with 14,15- or 22,23-epoxide moieties have previously been isolated from marine microorganisms and fungi. The first ecdysteroid possessing a 14,15-epoxide ring, gymnasterone B, exerts significant cytotoxic activity.

Serfurosterone A (**9**) and B (**17**) are the first ecdysteroids from natural sources known to contain a furan ring: they are acetals of 5-hydroxymethyl-furfural and the ecdysteroids, the 20-hydroxyecdysone (compound **9**), and ajugasterone C (compound **17**). Structurally similar ecdysteroids, the ethylidene acetal derivatives of 20-hydroxyecdysone and ajugasterone C were earlier isolated from other *Serratula* species.

20,22-Didehydrotaxisterone (**16**) and 1-hydroxy-20,22-didehydrotaxisterone (**18**) without free 22-hydroxy groups were isolated from *S. wolffii*. The biological activities of these compounds were determined via oral aphid (*Acyrthosiphon pisum* (Harris)) tests by our research group. Compound **16** (LC_{50} > 100 ppm on day 4) proved inactive, and compound **18** (LC_{50} = 48.5 ppm) exhibited low oral activity (mortality) against aphid larvae (*Acyrthosiphon pisum* (Harris)) in comparison with the active, main phytoecdysteroid, 20-hydroxyecdysone (LC_{50} = 1.07 ppm). It is again concluded that the 22-oxygen function needs to form an H-bond with the receptor, as otherwise the ecdysteroid loses its moulting activity. 1-Hydroxy-22-deoxy-20,21-didehydro-ecdysone (**21**) and 22-deoxy-20,21-didehydro-ecdysone (**23**), structural isomers of compounds **18** and **16,** respectively, were also isolated from *S. wolffii*. These four compounds are the first ecdysteroids known to possess an extra double bond in the side-chain, at position 20(22) or 20(21).

- Significance of the isolated ecdysteroids from biosynthetical aspects

Nagakari et al. considered that 14α-hydroxylation takes place after the formation of the 5β-H,7-en-6-one system. Compound **2** can therefore be deemed an intermediate of the ecdysteroid biosynthetic pathway, from which the typical ecdysteroids with a 14α-hydroxy group can be formed. However, compound **14** may also be a precursor of biosynthesis. The 7-en-6-one system is likely to derive from steroids with diene structures. Some authors have verified that the last reaction of biosynthesis is the hydroxylation at C-2, C-22 and C-25. We consider that the hydroxylation at C-2, C-22 and C-25 might precede the formation of the 7-en-6-one function group. However, confirmation of this hypothesis demands further evidence.

- Significance of the isolated ecdysteroids from chemotaxonomic aspects

Among the Asteraceae, the search for ecdysteroid-containing species has revealed two positive genera: the genera *Leuzea* and *Serratula*, in which some species (*L. carthamoides, L. integrifolium, S. coronata, S. tinctoria,* etc.) have high ecdysteroid contents. *Leuzea carthamoides* DC [syn. *Rhaponticum carthamoides* (Willd.) Iljin] is cultivated on a large scale for chemical and biological studies,

especially in Eastern Europe. This plant is currently used to obtain various preparations containing ecdysteroids.

The ecdysteroid profiles of *L. carthamoides* and *S. wolffii* display similarity not only in the major ecdysteroids, but also in the minor constituents. Both plants are the good sources of biologically active 11α-hydroxylated ecdysteroids and ecdysteroids with 7,9(11)-diene structures. Several phytoecdysteroids originating from *Leuzea* or *Serratula* species possess unusual units, such as the 14β-OH, the 3α-OH configuration and the *trans* A/B ring junction. Ecdysteroid mono- and diacetonides have also been identified in both plant sources. These molecules are considered to be chemical markers of these species, because the majority of them have not been identified from other plant sources so far. The similarity in the ecdysteroid pattern proves the chemical relationship of the two species. These facts indicate that *S. wolffii* could be an alternative source to *L. carthamoides*. *S. wolffii* would be a suitable plant for phytochemical and pharmacological studies, as well as for manufacture of preparations.

3.3. Importance of the study from methodology aspects

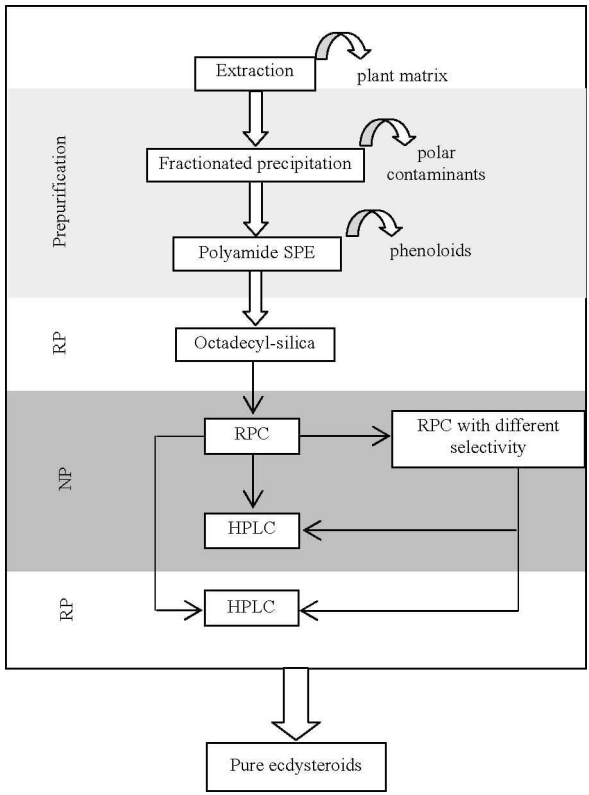
A new isolation process has been developed. After the effective clean-up, the use of only four chromatographic steps proved sufficient to obtain pure ecdysteroids. The adsorption chromatographic steps employed in earlier isolation methods have been eliminated. **Figure 3** illustrates the general scheme of this isolation method.

The general ecdysteroid isolation procedure was further improved by the introduction of preparative rotation planar chromatography (RPC) into the purification process. RPC has several advantages over the adsorption chromatographic steps.

- It is easier to carry out than conventional preparative TLC.
- The forced-flow method driven by a centrifugal force provides faster and better separation.
- The ecdysteroids are in contact with the absorbent layer in shorter time than in TLC. Thus, the problems associated with adsorbent-assisted decomposition are reduced.

- Alteration of the layer thickness, the solvent flow rate and the solvent systems permitted achievement of the best separation.
- RP-CC and RPC applied in consecutive steps provided different selectivity and improved resolution.

Figure 3. The general scheme of the isolation process.



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List of publications

The thesis is based on the following publications

I. Kalász H., **Liktor-Busa E.**, Janicsák G., Báthori M.: Role of preparative rotation planar chromatography in the isolation of ecdysteroids. J. Liquid. Chrom. R.T. 2006, 29, 2095-2109. **IF: 0,825**

II. **Liktor-Busa E.**, Simon A., Tóth G., Fekete G., Kele Z., Báthori M: Ecdysteroids from *Serratula wolffii* Roots. J. Nat. Prod. 2007, 70, 884-886. **IF: 2.418**

III. Simon A., Tóth G., **Liktor-Busa E.**, Kele Z., Takács M., Gergely A., Báthori M.: Three new steroids from the roots of *Serratula wolffii*. Steroids. 2007, 72, 751-755. **IF: 2.849**

IV. **Liktor-Busa E.**, Simon A., Tóth G., Báthori M.: The first two ecdysteroids containing a furan ring from *Serratula wolffii*. Tetrahedron Lett. 2008, 49, 1738-1740. **IF: 2.509**

Other publications

I.a **Liktor-Busa E.**, Szendrei K. Gyógynövény alkalmazások a Kárpát-medencében: Mit ér a fekete ribiszke? I. rész Gyógyszerészet 2007, 51, 618-622, 626-627.

I.b **Liktor-Busa E.**, Szendrei K. Gyógynövény alkalmazások a Kárpát-medencében: Mit ér a fekete ribiszke? II. rész Gyógyszerészet 2007, 51, 681-687, 691-692.

Presentations and published abstract

Liktor-Busa E., Simon A., Tóth G., Gergely A., Praszna L., Máthé I., Báthori M.: *Serratula wolffii*, mint jelentős ekdiszteroid nyersanyagforrás.
XI. Magyar Gyógynövény Konferencia, Dobogókő, 2005.

Liktor-Busa E., Simon A., Tóth G., Máthé I., Báthori M.**:**
Ekdiszteroidok izolálása kombinált folyadékkromatográfiás módszerekkel.
Fiatal analitikusok XX. előadói napja, Budapest, 2005.

Liktor-Busa E., Simon A., Tóth G., Máthé I., Báthori M.:
Új dimenziók az ekdiszteroidok izolálásában.
Congressus Pharmaceuticus Hungaricus XIII., Budapest, 2006.

Liktor-Busa E.:
Serratula wolffii, mint új ekdiszteroidok forrása.
VIII. Clauder Ottó Emlékverseny, Budapest, 2007.

Liktor-Busa E., Simon A., Tóth G., Máthé I., Báthori M.:
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Sesiunea Stiintifica Jubiliara, Marosvásárhely, 2007.

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