Ph.D. Thesis

University of Szeged
Department of Pharmacognosy

Analysis of the ecdysteroid profile of *Serratula wolffii* roots

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Scientific publications

I. Kalász, H.; **Liktor-Busa, E.**; Janicsák, G.; Báthori, M. 2006. Role of preparative rotation planar chromatography in the isolation of ecdysteroids. *J. Liq. Chromatogr. R. T.* **29**: 2095-2109. IF: 0.825

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

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

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

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1. Introduction

Ecdysteroids were discovered as steroid hormones of arthropods. The first ecdysteroid, ecdysone (E), was isolated from *Bombyx mori* by Butenandt and Karlson (1954) (1). Ecdysteroids regulate the moulting, metamorphosis, reproduction and diapause of insects (2). They probably occur in other classes of invertebrates (3). In the mid-1960s, phytoecdysteroids, structurally related to E, were identified in several plant species (4,5). So far, less than 2% of the world's flora has been investigated for the presence of ecdysteroids (6). In most ecdysteroid-containing species, the levels of ecdysteroids are between 0.1-3% of the dry weight, which is 1000-fold higher than in insects (7). Among the crop plants, spinach (*Spinacia oleracea*) and quinoa (*Chenopodium quinoa*) contain ecdysteroids in significant levels (8,9). The ecdysteroid patterns are usually complex, but the most widespread and main phytoecdysteroid is 20-hydroxyecdysone (20E), which is generally one of the major endogenous ecdysteroids in insects. This fact supports the hypothesis that the biological function of ecdysteroids in plants is to protect against non-adapted phytophagus invertebrates (10). 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. However, the mode of action and the metabolism in mammals, including humans, have remained open questions (11).

The specific effects of ecdysteroids on insects and their low mammalian toxicity formed the basis of development of safe insecticides (12). Nevertheless, the ecdysteroids themselves have limited application in the control of pests because of their high polarity and their environmental instability (13). On the other hand, bisacylhydrazines, functional analogues of ecdysteroids, are successful and selective pest control agents (14).

The mode of action of ecdysteroids in arthropods has been elucidated. Ecdysteroid responses are mediated by the intracellular ecdysteroid receptor (EcR) complex, which modifies the activity of specific gene sets (15). Since the EcR complex is not a natural component of vertebrate cells, genes placed under the control of ecdysteroid-response elements have deserved attention. The low mammalian toxicity and the specificity of the EcR complex indicate that successful gene-switching systems might be developed from this system (16).

1.1. Structural diversity of ecdysteroids

The ecdysteroids possess a cyclopentano-perhydrophenanthrene carbon skeleton, derived biosynthetically from cholesterol or other sterols. The carbon number can vary between C19 and C29. The anellations of the rings are characteristic: the C/D ring junctions are generally *trans*, while the A/B ring junction is normally *cis* (5β-H) and only rarely *trans* (5α-H). Essential structural elements of phytoecdysteroids are a 7-en-6-one chromophore in ring B, resulting in characteristic ultraviolet (UV) absorption, and a β-side-chain at C-17. Phytoecdysteroids are highly hydroxylated with 2-8 hydroxy groups. The commonly hydroxylated sites are the 2β, 3β, 14α, 20R, 22R and 25 positions. The variation of the structures lies in the number, position and orientation of the hydroxy groups. Ecdysteroid derivatives also include esters, ethers and glycosides. Besides these stuctural variations, other modifications can be found: additional unsaturation or the presence of a 5- or 6-membered lactone ring at C-17. **Figure 1** illustrates this structural diversity.

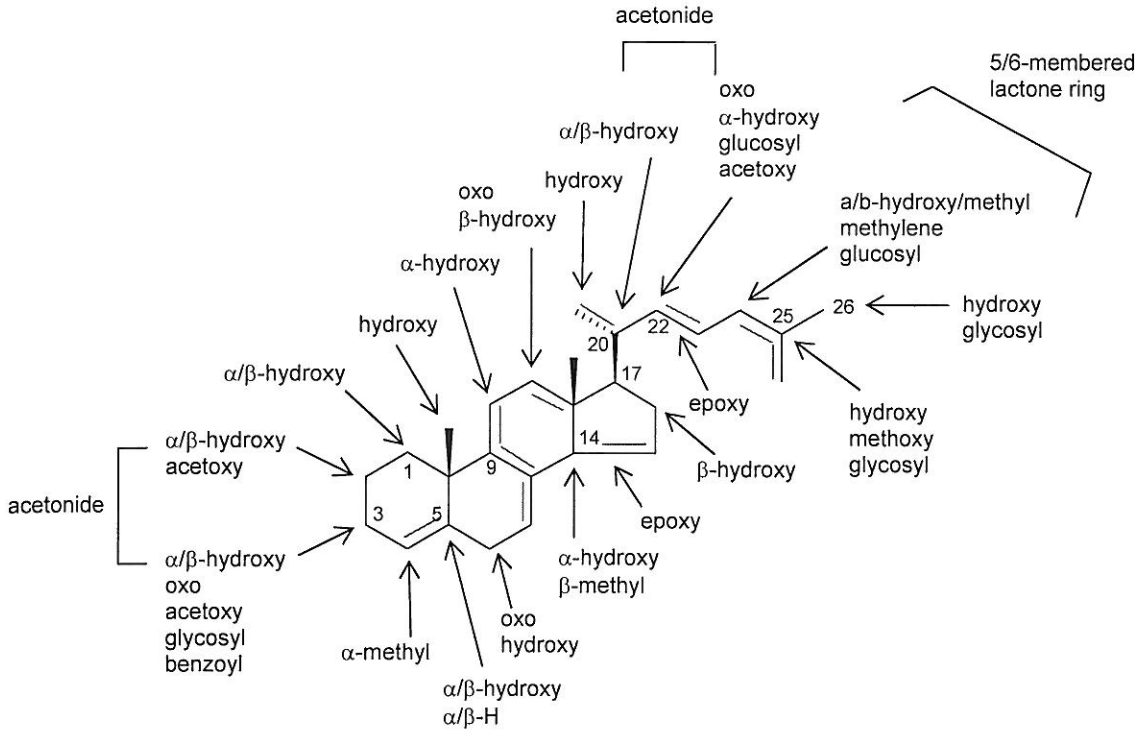


Figure 1. Structural diversity of phytoecdysteroids, showing the numbering of the carbon atoms. The blue bonds indicate possible unsaturation. Arrows mark possible substitution sites and substituents on the ecdysteroid skeleton and on the side-chain.

Over 300 ecdysteroid analogues have been identified in plants so far (17). It has been presumed that more than 1000 possible structures might occur in nature (18). The vast diversity of ecdysteroids found in plants provides natural sources for structure-activity studies. Structure-insect hormone activity relationships have been analysed by comparative molecular field analysis (CoMFA) and 4D-quantitative structure-activity relationship (QSAR) techniques to predict the activity and to design more active analogues (19,20). Further, it has proved possible to put forward a pharmacophore hypothesis for the ligand interaction with the ecdysteroid receptor. The characteristic molecular features of ecdysteroids (oxygen functions at C-2, C-3, C-20 and C-22; a Δ^7-double bond and a 6-keto group) are not essential for the activity, as previously suggested in connection with early empirical structure-activity relationship (SAR) studies. 4D-QSAR has identified functionalities capable of H-bond donation. The predictions drawn from these studies were that a hydroxy group on C-2 acts as an H-bond acceptor, C-3 should be substituted, preferably with a polar negative atom, a hydroxy group on C-20 is an H-bond donor and a hydroxy group on C-22 is an H-bond acceptor (18).

1.2. Pharmacological effects of ecdysteroids in mammals

A large number of papers dealing with the pharmacological activities of ecdysteroids are available in the literature. These studies reveal a wide range of effects; the anabolic, the adaptogenic, the tonic and the roborant properties are the most important activities. Ecdysteroids have very low toxicity in mammals: in the mouse, the LD_{50} of 20E is >6.4 g/kg and >9 g/kg on i.p. and oral administration, respectively (21).

The most pronounced effect of ecdysteroids on mammals is a stimulation of protein synthesis by increasing the level of mRNA translation in the liver polysomes (22). Pharmacological experiments have compared the physical performance or biochemical parameters of animals that have received 20E or other analogues with those of animals treated with anabolic vertebrate steroids (23). These studies indicate a significant anabolic effect of the ecdysteroids. In contrast with vertebrate steroid hormones, adverse androgenic, antigonadotropic and thymolytic side-effects have not been described after the administration of ecdysteroids (24). Study of the structure-anabolic relationship showed that the presence of a 2,3-diol system and the hydroxy groups at C-11 and C-20 are of great importance for the manifestation of the anabolic effect. Turkesterone, bearing an 11α-hydroxy function, was the most potent phytoecdysteroid in this experiment (25).

The ecdysteroids display hypocholesterolaemic effects, through a reduction of cholesterol biosynthesis and an increase of its catabolism (26). They prevent the hepatotoxic action of heliotrine or carbon tetrachloride in animal models (27). 20E restores the normal glomerular filtration rate and suppresses albuminuria in rats treated with a nephrotoxic mixture (28).

20E given orally to rats reduced hyperglycaemia induced either by glucagon or by alloxan treatment (29). Recent studies indicated that the ecdysteroids are able to exert a glucose-lowering effect in hepatocytes, but have no effect on insulin release (30). It is noteworthy that ecdysteroid-containing plants (e.g. *Ajuga iva* and *Morus alba*) are used as antidiabetics in traditional medicine (31,32).

Various effects of ecdysteroids on the CNS have been described. They induce glutamic decarboxylase (an enzyme involved in GABA biosynthesis) and acetyl cholinesterase in the rat brain (33,34). 20E produces antiepileptic effects in spontaneously epileptic rats, by acting on the $GABA_A$ receptors (35).

Ecdysteroids exhibit antioxidant properties, antifungal and antibacterial activity and immunomodulatory effects (36).

The mode of action of ecdysteroids in vertebrates is not fully understood at the present time. They are not expected to bind to nuclear receptors in mammals, because their full cholesterol side-chain prevents any binding to the receptors of the steroid hormones. Rapid actions via membrane receptors have been described. These studies concluded that 20E produces a decrease in the cyclic AMP levels and increases the synthesis of leukotrienes and prostaglandins. Recent investigations found that ecdysteroids are able to potentiate the IL-3-dependent activation of protein-kinase B. This pathway has a central role in the mammalian cell metabolism. These results provide an explanation of many effects of ecdysteroids (hypoglycaemic, anabolic, etc.) in one hypothesis (11,37).

The ecdysteroid metabolism in mammals is not well documented either. All studies to date have shown that ecdysteroids are short-lived in mammals. It was concluded that reduction in ring B, epimerization at C-3 and dehydroxylation at C-14 are general features of the ecdysteroid metabolism (38). Other analysis based on mass-spectroscopic (MS) techniques identified unchanged E and deoxyecdysone derivatives, as major metabolites after the injection of E (39).

Unfortunately, no extensive, systematic trials on any mammalian species have been published. In spite of the limited knowledge, over 250 different preparations containing ecdysteroids for oral application are available in the market. On the other hand, increasing

numbers of patents provide evidence of various benefical medical and cosmetic effects of ecdysteroids (11).

An ecdysteroid-inducible gene expression system is a new line of biomedical application of ecdysteroids. The absence of EcR 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 (24). The EcR has been cloned and characterized from insect species. Mammalian cell lines have been transfected with the EcR and different reporter genes. The EcR regulates gene expression as a dimer (40). One of the most promiscuous heterodimeric partners for the vertebrate nuclear receptors is the retinoid X receptor, which is equivalent to the ultraspiracle in insects (41). In these systems, the general ecdysteroid, 20E, is ineffective as an elicitor, whereas the rare phytoecdysteroids muristerone A and ponasterone A are active (42). Gene-switching systems based on ecdysteroid/EcR complex have been developed and produced for experimental use (43). An ecdysteroid-inducible gene expression system would also have great potential in human therapy (44).

1.3. Plant sources of ecdysteroids

The phytoecdysteroids have been reported to occur in over 100 plant families. Their occurrence in fungi and ferns means that their origin is probably very ancient (6). The distribution of ecdysteroid-containing species shows great heterogeneity within families and even genera. However, four plant families (Caryophyllaceae, Amaranthaceae, Chenopodiaceae and Asteraceae) can be emphasized as families containing ecdysteroid-rich species (45). The frequency of positive species in a family does not appear to correlate with the levels of ecdysteroids. Most plant species biosynthesize one or two major ecdysteroids and series of minor compounds, which provide evolutionary flexibility to respond to predation (46). The composition of the phytoecdysteroids of a species varies with the season, the habitat, the vegetation period and the developmental stage (47).

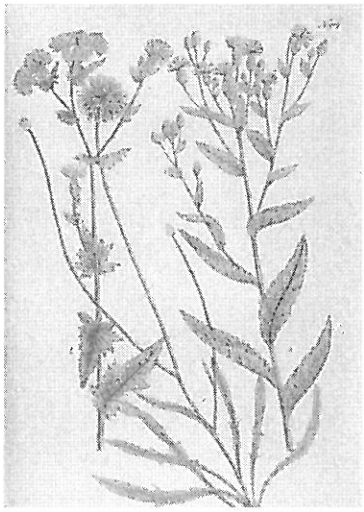
The current knowledge of the biosynthetic pathway is rather limited. The biosynthesis of phytoecdysteroids has been demonstrated to proceed via cholesterol and/or lathrosterol (48). Plants (unlike insects) are capable of biosynthesizing ecdysteroids from mevalonic acid (49).

1.4. Taxonomic classification and botanical characterization of *Serratula wolffii*

Serratula species are perennial herbs with unarmed leaves. *S. wolffii* has a stout, erect, subglabrous stem; its height is 80-150 cm. The basal leaves are irregularly pinnatifid; the

segments are usually elliptic-lanceolate. The leaflets are irregularly serrated, with setulae on the margin and veins. The campanulate capitula measures 25-30 mm; it frames an irregular panicle. The outer bracts are acute and velutinous; the inner ones are rather rigid and long-attenuate. The florets are purple. This species originates from Russia and Romania (50). Four species from the *Serratula* genus, *S. wolffii* Andrae, *S. tinctoria* L., *S. lycopifolia* Vill. and *S. radiata* W. et K., occur in the Carpathian Basin; the latter three species are protected in Hungary.

Taxonomic classification (51):
Division: Magnoliophyta
Class: Magnoliopsida
Subclass: Asteridae
Order: Asterales
Family: Asteraceae
Subfamily: Tubuliflorae
Tribe: Cynareae
Subtribe: Centaureinae
Genus: *Serratula*
Species: *Serratula wolffii* Andrae



Serratula wolffii

1.5. Chemical constituents of *Serratula* species

Phytoecdysteroids are the typical plant steroids of the *Serratula* genus. **Table 1** lists several *Serratula* species which contain 20E. Numerous minor ecdysteroids have been isolated from *S. coronata* L., *S. sogdiana* Bunge, *S. strangulata* Iljin., *S. tinctoria* L. and *S. wolffii* Andrae. **Table 2** details compounds isolated from various *Serratula* species. *S. wolffii* has been found by TLC-densitometric determination to contain amounts of 20E in the range 0.13-0.85% (70). The vegetation dependence of

Table 1. *Serratula* species contain 20E

***Serratula* species**	Reference
S. algida Iljin.	52
S. centauroides L.	53
S. chinensis S.Moore	54
S. inermis Gilib.	52
S. komarovii Iljin.	55
S. lyratifolia Schrenk.	52
S. manshurica Kitag.	56
S. procumbens Regel.	57
S. quinquefolia Bieb.	57
S. xeranthemoides Bieb.	58

the 20E content of *S. wolffii* has been followed. There are two maxima of ecdysteroid content, at the beginning of vegetation (in April and May) and during blossoming (in August) (71).

Further characteristic compounds of *Serratula* species are the flavonoids (72). Apiin, apigenin, luteolin, luteolin glycosides, chrysoeriol, quercetin, quercetin derivatives and kaempferol have been isolated from a few *Serratula* species (73-75). Some *Serratula* species contain lignans, such as arctiin, which seems to be a chemotaxonomic marker of the Cynareae tribe (76, 77). The occurrence of mono- and diterpenoids and sesquiterpene lactones in the genus has also been documented (78, 79).

Table 2. Ecdysteroids isolated from *Serratula* species.

***Serratula* species**	Ecdysteroid	Reference
S. coronata L.	ajugasterone C; 22-deoxy-20E; E; 3-epi-20E; 20E; 20E 22-acetate; polypodine B	59
	Coronasterone	60
	E 22-acetate; (25S)-inokosterone 26-acetate; ajugasterone C 20,22-ethylidene; 20E 20,22-ethylidene; 20E 2-acetate; 20E 3-acetate	61
S. sogdiana Bunge	20E; vitikosterone E	62
	Sogdisterone	63
S. strangulata Iljin.	25-deoxy-11,20-dihydroxy-E; 20E; 20E 20,22-acetonide	64
S. tinctoria L.	3-epi-poststerone; 3-epi-rubrosterone; 20E; 20E 2-acetate; 20E 3-acetate; 20E 22-acetate; 20E 2,22-diacetate; 20E 3,22-diacetate; 5β-hydroxyrubrosterone; makisterone C; 22-oxo-20E; polypodine B; poststerone; pterosterone; rubrosterone	65
	22-epi-20E; gerardiasterone	66
S. wolffii Andrae	ajugasterone C; ajugasterone C 20,22-monoacetonide; integristerone A; 20E; 20E 20,22-monoacetonide; 20E 2,3;20,22-diacetonide; polypodine B; pterosterone	67
	herkesterone; 11α-hydroxypoststerone	68
	ajugasterone C; ajugasterone D; dacryhainansterone; 22-deoxy-20,21-dihydroxyecdysone; 22-deoxy-20E; 20,26-dihydroxyecdysone; 3-epi-20E; 14-epi-20E; 22-epi-20E; 5α-20E; 25-hydroxydacryhainansterone; isovitexirone; makisterone A; makisterone C; turkesterone	69

1.6. Ecdysteroid isolation, identification and structure determination procedures

The complexity of the procedure of ecdysteroid purification depends on three parameters: the scale of the experiment, the concentration and the polarity of the compounds (80). Thanks to the progress in spectroscopic methods, the amount of any pure ecdysteroid required to establish its structure is only 1-2 mg. The level of the major phytoecdysteroid(s) is about 1-2% of the dry mass, and the minor ecdysteroids occur in much lower concentrations (ng/g or pg/g). The major ecdysteroid, 20E, must be removed from the plant extract in the initial steps; otherwise, it may contaminate the later-eluted minor ecdysteroids and the whole chromatographic pattern. The polarity and chromatographic behaviour of ecdysteroids mainly depend on the number and position of the hydroxy groups (36). Typically, there is a complex mixture of chemically similar ecdysteroids to separate and this is complicated by other contaminating compounds (pigments, phenolics, etc.), which makes purification difficult. The purification strategy always involves a multi-step procedure, including extraction, sample preparation and several chromatographic steps. The initial stages of a separation procedure include methods with high loading capacity and inexpensive stationary phases. Subsequent steps employ techniques which require smaller samples and provide effective separation. The optimized combination of preliminary purification and chromatographic methods results in the pure ecdysteroids (81).

1.6.1. Separation methods used for the isolation and analysis of ecdysteroids

The extraction of dried, milled samples is best performed with a polar solvent such as methanol or ethanol. Alternative solvents are acetone, acetonitrile and methanol-water mixtures. After concentration, the extract is subjected to solvent partition to remove non-polar and polar contaminants. Suitable partitioning systems for removing lipids, chlorophylls or other non-polar contaminants are hexane/light petroleum and aqueous propanol/aqueous methanol. The separation of polar impurities from the ecdysteroids can be carried out by partition between water and n-butanol (the ecdysteroids partition into the organic phase) or water and ethyl acetate (the ecdysteroids remain in the organic phase) (82). Other liquid-liquid partition chromatographic methods can be performed by counter-current distribution (CCD) or droplet counter-current chromatography (DCCC). These techniques provide 100% sample recovery and purify samples up to the gram range. The most common solvent systems are chloroform-methanol-water mixtures (83). HSCCC (high-speed counter-current chromatography) was designed as an alternative to DCCC. In spite of its effectiveness, DCCC is rather time-consuming (the processing of a sample requires 1-5 days). The

centrifugal force in HSCCC allows the separation to be achieved in hours rather than days (82).

Further purification is achieved by column chromatography (CC) on silica or alumina, with a step-gradient elution of methanol or ethanol in a chlorinated solvent (chloroform or methylene chloride). Sephadex LH-20 may be used to remove non-polar contaminants, and polyamide is utilized specifically to remove polyphenols and chlorophylls. Reversed-phase flash chromatography with a step-gradient of water in methanol provides a further possibility for separation. Affinity chromatography on immobilized phenylboronic acid is suitable for the selective retention of compounds bearing vicinal diols (84).

Planar chromatography, and particularly TLC, has been used for the qualitative analysis (e.g. monitoring extraction) and isolation of ecdysteroids (85). Many different solvent systems have been described. General mobile phases are chloroform-ethanol or methanol mixtures (86). RP-TLC on bonded phases and paraffin-impregnated silica gel has also been employed for the qualitative analysis of ecdysteroids, involving development with various methanol-water mixtures (87). The most usual detection procedures are fluorescence quenching and use of a vanillin-sulfuric acid spray reagent. The colours produced range from pink and red to dark-green. Overpressure-layer chromatography (OPLC) and high-performance TLC (HPTLC) have also been applied for the characterization of ecdysteroids (82).

HPLC is the most popular technique for ecdysteroid separation, for both analytical and preparative purposes (88). Normal-phase systems generally include silica columns (sometimes aminopropyl- or diol-bonded columns). The strong UV absorbance of ecdysteroids allows the simple and sensitive detection of these compounds. On the other hand, UV detection precludes the use of solvents with a high UV-cut-off (e.g. ethyl acetate, benzene or acetone). Different mixtures of dichloromethane-isopropanol-water (from 125:15:1, v/v/v for non-polar compounds to 125:40:3, v/v/v for glucosides) and cyclohexane-based solvent systems are widely used. Non-polar (trimethylsilane) bonded-phase columns can be developed to give very symmetric peaks. RP-HPLC with a C_{18}-bonded column provides effective separations with methanol-water or acetonitrile-water mixtures. The advantage of the latter mobile phase is the lower viscosity. Water can be replaced with buffer or trifluoroacetic acid (0.1%, v/v) to suppress peak tailing (89). Use of a combination of two NP and two RP systems is recommended for the correct identification of a compound.

Ecdysteroids are too polar and have too little thermal stablility to be suitable for gas chromatography but, through protection of some or all of the hydroxy groups as silyl ethers,

they can be thermally stabilized and their polarity reduced so that they can be chromatographed in the gaseous phase (90). The trimethylsilyl ether derivates can be detected by an electron capture detector, which greatly enhances the sensitivity and selectivity of the detection of ecdysteroids (91). The introduction of fused-silica capillary columns increased the resolution of GC. The difficulties in preparing silyl ethers have discouraged the use of GC in ecdysteroid work.

Other separation methods, such as capillary zone electrophoresis and supercritical fluid chromatography, are very efficient and fast separation methods on an analytical scale (82,92).

Chromatographic techniques coupled with biological (HPLC-RIA) or spectroscopic (TLC-MS and HPLC-MS) methods are frequently used to detect and identify ecdysteroids. TLC combined with either off-line or on-line MS is an appropiate technique with increased identification effectiveness [93]. HPLC coupled with MS can furnish more information for ecdysteroid identification with higher specificity [94,95]. One paper has described the possible use of the combination of HPLC with IR, UV, NMR and MS in the discovery of new ecdysteroids [96].

1.6.2. Structure elucidation of ecdysteroids

The identification of a pure ecdysteroid is based on physical (the physical state of the compound, melting point (m.p.), optical rotation and circular dichroism (CD) measurements) and spectroscopic methods (UV-visible spectra and IR spectra). The HRMS and NMR spectroscopy are used to elucidate the structures of new compounds. In some cases, the final proof of the steric structure is established by using X-ray crystallography (97).

1.7. 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 20E) 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. Reagents and standard ecdysteroid 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 (69). Their identities and purities were verified by NMR and HPLC.

2.3. General experimental procedures

2.3.1. General methods and apparatus

Densitograms were recorded with a Shimadzu CS-9301PC densitometer (Osaka, Japan) operating in the reflectance-absorbance mode at 254 nm. Optical rotations were measured with a Perkin-Elmer 341 polarimeter. The UV spectra were recorded in MeOH and

in DMSO (comp. **17**) with a Shimadzu UV 2101 PC spectrophotometer. NMR spectra were recorded in MeOH-d_4 and DMSO-d_6 (comp. **17**) in a Shigemi sample tube at room temperature with a Bruker Avance DRX-500, a Varian Unity Inova-500 and an Inova-600 spectrometer. Chemical shifts are given on the δ-scale, referenced to the solvents (MeOH-d_4: $\delta_C = 49.15$ and $\delta_H = 3.31$). In the 1D measurements (^1H, ^{13}C and DEPT-135), 64K data points were used for the FID. The pulse programs of all experiments [gs-COSY, phase-sensitive DQF-COSY, gs-HMQC, HMQC-TOCSY (mixing time = 80 ms), edited gs-HSQC; gs-HMBC, 1D NOESY (mixing times = 350, 400, 500 and 600 ms), and 2D gs-NOESY (mixing time = 400 ms)] were taken from the Bruker and Varian software library; the other parameters (pulse length, levels and delays, etc.) were in agreement with the parameters given in our previous work (98,99). The MS measurements were performed on a Finnigan TSQ 7000 tandem mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with a laboratory-built nanoelectrospray ion source. A high voltage of about 1000 V was used in the ion source. The instrument was scanned in the normal MS mode over the mass range 10–1500, with a scan time of 2 s. HRESIMS recordings were made on a Finnigan MAT 95SQ tandem mass spectrometer (Finnigan MAT, Bremen, Germany).

2.3.2. Chromatographic techniques

NP-TLC

NP-TLC was performed on 20 x 20 cm silica plates (Silicagel 60 F_{254}) (E. Merck, Darmstadt, Germany). The plates were developed by an ascending technique in a glass chamber (Desaga, Heidelberg, Germany) at room temperature. The following mobile phases were used:

TLC_1: dichloromethane-methanol-benzene (50:10:6, v/v/v),

TLC_2: ethyl acetate-96% ethanol-water (80:10:5, v/v/v),

TLC_3: toluene-acetone-96% ethanol-25% ammonia (100:140:32:9, v/v/v/v)

TLC_4: methanol-water (4:6, v/v)

TLC_5: methanol-water (7:3, v/v)

TLC_6: acetonitrile-water (35:65, v/v)

After development of the plates, the ecdysteroids were detected directly by fluorescence quenching at 254 nm and by the use of a vanillin-sulfuric acid spray reagent. After spraying, the spots were visualized in daylight and at 366 nm. The whole isolation procedure was controlled by using NP-TLC.

Solid-phase extraction (SPE)

The clean-up used MN-polyamide SC6 (particle size:0.06-0.16 mm) (Woelm, Eshwege, Germany) as the column material (210x145 mm, 688 g). The mobile phases were water (2 L) and aqueous methanol (9:1, 8:2, 7:3, 1:1, v/v) (1 L each).

Vacuum reversed-phase column chromatography (RP-CC)

Vacuum RP-CC was carried out on end-capped octadecyl-silica (0.06-0.2 mm particle size) (Chemie Ueticon-C-gel, C-560, Ueticon, Switzerland) packed into a 400 x 32 mm glass column. Elution was performed with a stepwise gradient of 30%, 35%, 40%, 45%, 50%, 55% and 60% aqueous methanol (1000 mL each). The pressure was less than 1 atm throughout the whole separation.

Rotation planar chromatography (RPC)

RPC was carried out on a Harrison Model 8924 Chromatotron instrument (Harrison Research, Palo Alto, CA). The stationary phase for RPC was silica gel 60 GF_{254} (E. Merck), manually coated on the rotor as a 1 mm layer (for RPC1-1, the thickness of the layer was 4 mm). We used nine mobile phases for development:

RPC1: solvent system 1/A: chloroform-methanol-benzene (50:3:2, v/v/v);
solv. syst. 1/B: chloroform-methanol-benzene (50:5:3, v/v/v);
solv. syst. 1/C: chloroform-methanol-benzene (50:10:6, v/v/v);

RPC2: solv. syst. 2/A: ethyl acetate-ethanol-water (80:2:1, v/v/v);
solv. syst. 2/B: ethyl acetate-ethanol-water (80:5:2, v/v/v);
solv. syst. 2/C: ethyl acetate-ethanol-water (80:10:5, v/v/v);

RPC3: solv. syst. 3/A: ethyl acetate-ethanol-water (80:5:2, v/v/v);
solv. syst. 3/B: ethyl acetate-ethanol-water (80:7:3, v/v/v);
solv. syst. 3/C: ethyl acetate-ethanol-water (80:10:5, v/v/v).

The numbers after RPC denote which solvent system was used. Combinations of numbers (e.g. RPC2-3) denote the numbers of chromatographic procedures.

As the first step of the procedure, the dry stationary phase was completely wetted with the first applied mobile phase (50-100 mL). The samples were dissolved in the first elution solvent and were then introduced through the inlet. The separation by RPC was achieved with gradient elution in three steps. The flow rates were 4-5 $mL \cdot min^{-1}$ (RPC1-1: 10 $mL \cdot min^{-1}$) Thirty fractions (10 mL in each case, except for RPC1-1: 30 mL) were collected.

High-performance liquid chromatography (HPLC)

HPLC analyses were performed with a Jasco Model PU-2080 Pump, Jasco Model UV-2070/2075 Detector.

(a) *NP-HPLC*:

- *Analytical*

NP-HPLC$_1$: Zorbax-SIL column (5 µm, 250 x 4.6 mm i.d.) (DuPont, Paris, France), elution with cyclohexane-isopropanol-water (100:40:3, v/v/v) at 1 or 1.2 mL·min^{-1}.

NP-HPLC$_2$: Zorbax-SIL column (5 µm, 250 x 4.6 mm i.d.) (DuPont, Paris, France), elution with dichloromethane-isopropanol-water (125:40:3, v/v/v) at 1 mL·min^{-1}.

NP-HPLC$_3$: Zorbax-SIL column (5 µm, 250 x 4.6 mm i.d.) (DuPont, Paris, France), elution with dichloromethane-isopropanol-water (125:30:2, v/v/v) at 1 mL·min^{-1}.

NP-HPLC$_4$: Zorbax-SIL column (5 µm, 250 x 4.6 mm i.d.) (DuPont, Paris, France), elution with dichloromethane-isopropanol-water (125:25:2, v/v/v) at 1 mL·min^{-1}.

- *Semipreparative*

NP-HPLC$_5$: Zorbax Sil column (5 µm, 250 x 9.4 mm i.d.) (DuPont, Paris, France), elution with cyclohexane-isopropanol-water (100:40:3, v/v/v) at 2 or 2.5 or 3 mL·min^{-1}.

(b) *RP-HPLC*:

- *Analytical*

RP-HPLC$_1$: Zorbax SB C18 (5 µm, 250 x 4.6 mm i. d.) (DuPont, Paris, France), elution with acetonitrile-water (35:65, v/v) at 0.8 or 1 mL·min^{-1}.

RP-HPLC$_2$: Zorbax SB C18 (5 µm, 250 x 4.6 mm i. d.) (DuPont, Paris, France), elution with acetonitrile-water containing triflouroacetic acid (0.1%) (77:23, v/v) at 0.8 or 1 mL·min^{-1}.

RP-HPLC$_3$: Zorbax SB C18 (5 µm, 250 x 4.6 mm i. d.) (DuPont, Paris, France), elution with methanol-water (6:4, v/v) at 1 mL·min^{-1}.

- *Semipreparative*

RP-HPLC$_4$: Zorbax SB C18 (5 µm, 250 x 9.4 mm i.d.) (DuPont, Paris, France), elution with methanol-water (8:2, v/v) at 2 mL·min^{-1}.

Chromatographic separations were monitored at 242 nm.

2.4. Extraction and isolation

2.4.1. Extraction and prepurification of the crude extract

The fresh roots of *S. wolffii* (4763 g) were washed, milled, and percolated with methanol (20 L) at room temperature. The methanolic extract was evaporated to dryness (208.9 g), using a Rota Wapoor (40 °C, 337 mbar). The dry residue was dissolved in methanol (800 mL), and acetone (400 mL) was added to the solution. The resulting

precipitate was separated by decantation, and then washed three times each with 100 mL of a methanol-acetone (2:1 v/v) mixture. The supernatant and the methanol-acetone solutions were combined and evaporated to dryness. The dry residue (184.6 g) was redissolved in 700 mL of methanol, and acetone (700 mL) was added to the solution. The precipitate was washed three times with 100 mL of a methanol-acetone mixture (1:1, v/v). The supernatant and the methanol-acetone solution were combined and taken to dryness. The dry residue (165.9 g) was dissolved in methanol (600 mL). The precipitation was carried out once again, using 1200 mL of acetone. A methanol-acetone mixture (1:2, v/v; 3 x 100 mL) was used to rinse the precipitate. The final methanol-acetone solution obtained after the fractionated precipitation was evaporated to dryness, and the residue (137.5 g) was dissolved in methanol (300 mL) and adsorbed onto a polyamide stationary phase (344 g). This was added to the top of a column of polyamide (344 g), the stationary phase was previously suspended in water. The ecdysteroids were eluted from the polyamide sorbent with water (2 L) and aqueous methanol (9:1, 8:2, 7:3 and 1:1, v/v) (1 L each). NP-TLC analysis suggested that the fraction eluted with water contained a series of ecdysteroids, which were separated by further chromatographic procedures. The ecdysteroid-containing fraction was evaporated to dryness (24.4 g) and dissolved in methanol (50 mL).

2.4.2. Isolation of ecdysteroids

The fraction eluted with water from the polyamide column was subjected to RP-CC on octadecyl-silica (column 1). The vacuum RP-CC was carried out in two parallel procedures. Half of the sample was applied to the top of a previously packed column of octadecyl-silica (150 g). Vacuum CC was used with gradient elution of 30%, 35%, 40%, 45%, 50%, 55%, and 60% aqueous methanol (1000 mL each) at a flow rate of 5 $mL \cdot min^{-1}$, and 35 fractions (200 mL each) were collected. The fractions containing the same compounds were combined and evaporated to dryness. Six combined fractions were chosen for further purification by RPC on silica.

Fractions 14-19 eluted with 45% aqueous methanol (1.9 g) were dissolved in 15 mL of solv. syst. 1/A and applied to the middle of the rotation plate after equilibration (RPC1-1). The fractions eluted with solv. syst. 1/B were fractionated again by RPC, using solv. syst. 2/A-C (RPC2-2). The repeated RPC resulted in combined fractions eluted with solv. syst. 2/B, which were purified by NP-$HPLC_1$ at a flow rate of 1 $mL \cdot min^{-1}$ to obtain compounds **1** (0.5 mg), **2** (0.8 mg) and **3** (1 mg).

The RP-CC gave fractions 20-23 eluted with 50% aqueous methanol (390 mg), which were evaporated and dissolved in the first RPC mobile phase (1/A; 3 mL). The fraction was separated by RPC, using solv. syst. 1/A-C (RPC1-3). The ecdysteroid-containing fractions eluted with solv. syst. 1/A were used to obtain compound **4** (1 mg) by simple crystallization. Other RPC fractions eluted with the same solv. syst. were purified by RP-HPLC$_1$ at a flow rate of 0.8 mL·min^{-1} to yield compound **5** (1 mg). Fractions eluted with solv. syst. 1/B from RPC1-3 were subjected to repeated RPC, using solv. syst. 2/A-C (RPC2-4). From fractions eluted with solv. syst. 2/A, compounds **6** (4 mg) and **7** (3 mg) were isolated by using NP-HPLC$_5$ at a flow rate of 3 mL·min^{-1}. RPC1-3 resulted in fractions (eluted with solv. syst. 1/C) which were fractionated by RPC2-5. This second RPC step gave fractions eluted with solv. syst. 2/A which were purified by RP-HPLC$_1$ at a flow rate of 0.8 mL·min^{-1} to yield compounds **8** (0.5 mg) and **9** (0.5 mg). Pure compound **10** (14 mg) was obtained by this repeated chromatographic procedure, using solv. syst. 2/B. A combined fraction eluted with solv. syst. 1/C from RPC1-3 was subjected to repeated crystallization to yield pure compound **11** (20 mg). The methanolic solution obtained by regeneration of the rotation plate was further purified by RPC3-6. Fractions eluted with solv. syst. 3/B contained pure compound **12** (3 mg).

Fractions 28-29 eluted with 55% aqueous methanol from the RP column were combined and taken to dryness (120 mg). These combined fractions were further fractionated by RPC, using solv. syst. 1/A-C (RPC1-7). Fractions eluted with solv. syst. 1/B were purified by NP-HPLC$_5$ at a flow rate of 2 mL·min^{-1} to furnish compounds **13** (2 mg) and **14** (0.7 mg).

The RP-CC resulted in fractions 30-32 (60% aqueous methanol), which were combined, evaporated to dryness (70 mg) and dissolved in solv. syst. 1/A (2 mL). This fraction was separated by RPC1-8. Fractions eluted with solv. syst. 1/A were further purified by RP-HPLC$_1$ at a flow rate of 0.8 mL·min^{-1} to obtain compound **15** (0.5 mg). Fractions eluted with solv. syst. 1/B were fractionated by NP-HPLC$_5$ at a flow rate of 2 mL·min^{-1} to afford compound **16** (0.5 mg). RPC1-8 (solv. syst. 1/C) gave fractions containing compound **17**. RP-HPLC$_1$ at a flow rate of 1 mL·min^{-1} was applied to obtain pure compound **17** (0.5 mg). Methanol was used for regeneration of the rotation plate. This methanolic solution was further fractionated by RPC2-9. Fractions eluted with solv. syst. 2/B contained compound **18,** which was purified by NP-HPLC$_5$ at a flow rate of 2.5 mL·min^{-1} to yield pure **18** (2.5 mg).

Fractions 33-35 (390 mg) were eluted with 60% aqueous methanol from the RP column. The further separation of this combined fraction was carried out in two parallel

chromatographic steps. Half of the combined fraction was dissolved in solv. syst. 1/A (2 mL) and subjected to rotation planar chromatography (RPC1-10). This procedure resulted in fractions eluted with solv. syst. 1/C, which were further fractionated by NP-HPLC$_1$ at a flow rate of 1.2 mL·min^{-1} to give compound **19** (1 mg). The other half of the fraction was subjected to RPC2-11 with solv. syst. 2/A-C. Two fractions eluted with solv. syst. 2/A and solv. syst. 2/C were obtained, which were further purified by NP-HPLC$_1$ at a flow rate of 1.2 mL·min^{-1} to yield compounds **20** (1.3 mg) and **21** (2.7 mg), respectively.

The methanolic solution obtained by regeneration of the RP-column was separated into two parts by repeated RPC (RPC1-12, RPC2-13 and RPC2-14). After evaporation, half of the dry residue (240 mg) was dissolved in solv. syst. 1/A (4 mL). RPC1-12 resulted in the fraction eluted with solv. syst. 1/B, which was taken to dryness and dissolved in solv. syst. 2/A. Fractions eluted with solv. syst. 2/B were further purified by NP-HPLC$_1$ at a flow rate of 1.2 mL·min^{-1} to obtain compound **22** (2 mg). The other half of the dry residue was dissolved in solv. syst. 2/A (4 mL) and was subjected to RPC2-14. The fractions eluted with solv. syst. 2/A were purified by RP-HPLC$_4$ at a flow rate of 2 mL·min^{-1} to yield compound **23** (1 mg). The scheme of the isolation of the pure compounds from the prepurified extract is outlined in **Figure 2**.

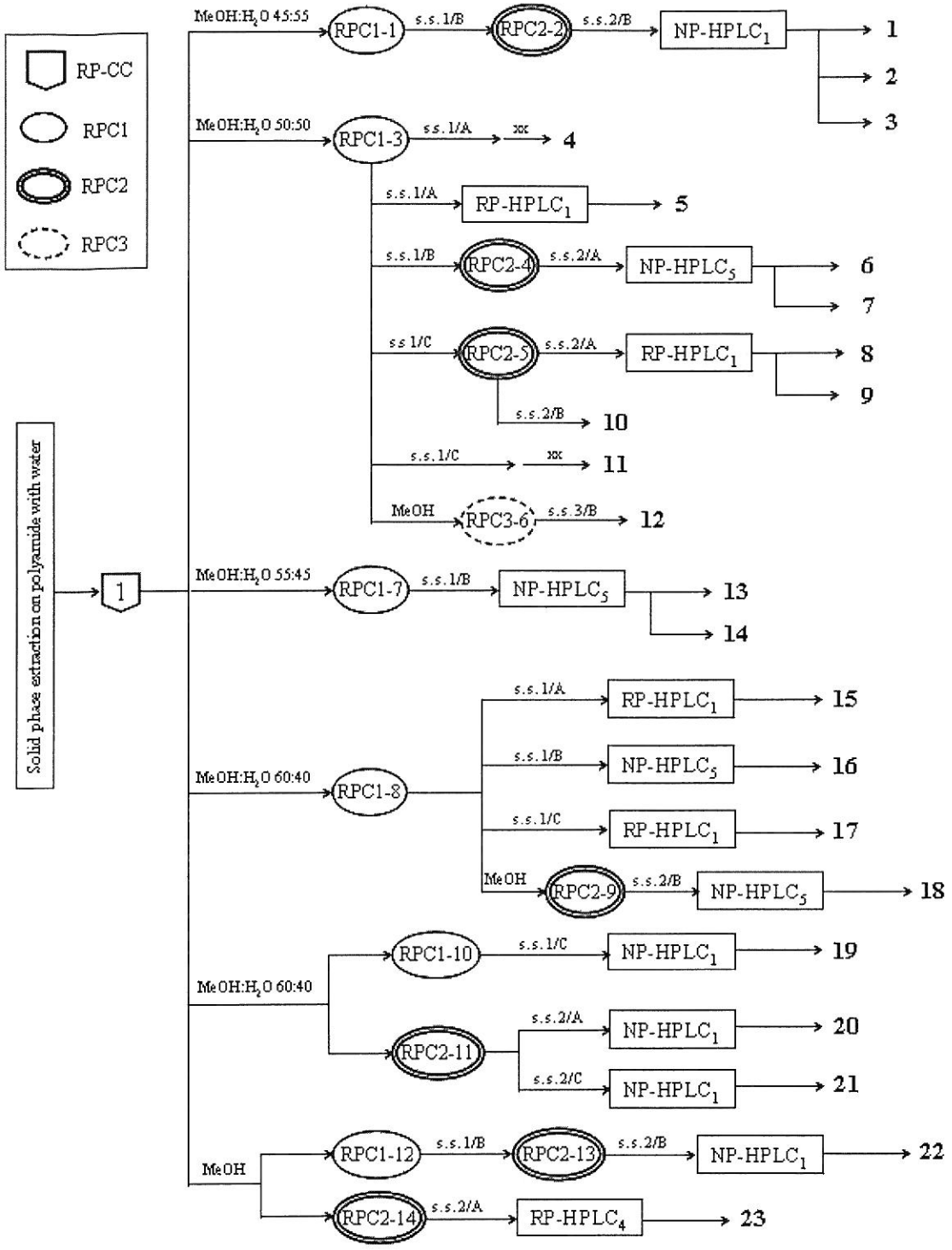


Figure 2. The scheme of the isolation of the pure compounds from the prepurified extract

1: 11α-hydroxyshidasterone, **2**: 2β,3α,20R,22R,25-pentahydroxy-5β-14β-cholest-7-en-6-one, **3**: 2β,3α,20R,22R,25-pentahydroxy-5β-14α-cholest-7-en-6-one, **4**: dacryhainansterone, **5**: ponasterone A, **6**: stachysterone B, **7**: 14α,15α-epoxy-14,15-dihydrostachysterone B, **8**: makisterone A, **9**: serfurosterone A, **10**: ajugasterone C, **11**: 20E, **12**: 22-deoxyintegristerone A, **13**: shidasterone, **14**: 2β,3β,20*R*,22*R*,25-pentahydroxy-5β-cholest-6,8(14)-diene, **15**: 24-methylene-shidasterone, **16**: 20,22-didehydrotaxisterone, **17**: serfurosterone B, **18**: 1-hydroxy-20,22-didehydrotaxisterone, **19**: 22-dehydro-20-deoxy-ajugasterone C, **20**: 20-hydroxyecdysone 20,22-ethylidene, **21**: 1-hydroxy-22-deoxy-20,21-didehydro-ecdysone, **22**: 20-hydroxyecdysone 20,22-monoacetonide, **23**: 22-deoxy-20,21-didehydro-ecdysone

3. Results

3.1. Isolation of ecdysteroids from *Serratula wolffii*

The isolation procedure consists of two main steps: extraction, and the clean-up of the crude extract, followed by a combination of chromatographic methods.

The ecdysteroids were subjected to exhaustive extraction with methanol as solvent, at a methanol:plant ratio of 7:1. The prepurification involved fractionated precipitation and SPE. The precipitation steps removed the majority of the polar contaminants. For this purpose, the crude methanolic extract was mixed with acetone. The extract-acetone volumetric ratios were 2:1, 1:1 and 1:2. The residue of the final methanol-acetone solution was subjected to CC on polyamide. The ecdysteroids were eluted from the sorbent with water. The impurities, mainly phenoloids, remained adsorbed on the polyamide. **Figure 3** presents the extraction and clean-up procedure.

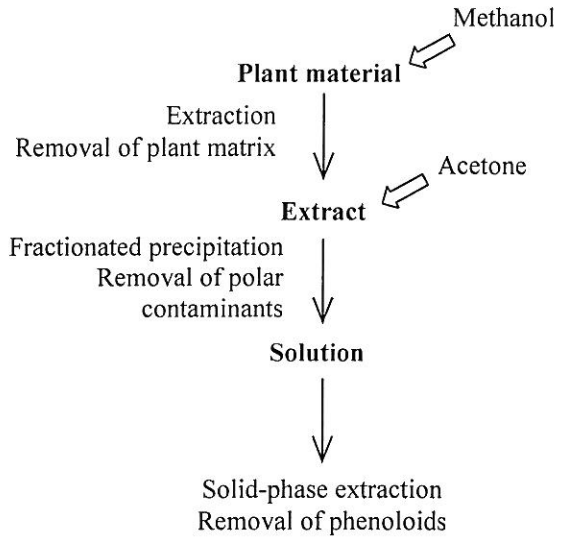


Figure 3. The scheme of extraction and the prepurification of the crude extract

The prepurified extract was fractionated by vacuum RP-CC in two parallel procedures. The sample to stationary phase ratio was 2:5. The chromatographic separation resulted in fractions containing a complex mixture of structurally related ecdysteroids. Fractions eluted with 45, 50, 55, 60 and 100% aqueous methanol were further purified by preparative RPC on silica. Fractionation by RPC was carried out with stepwise gradient elution in three steps.

Compounds **4** and **11** were crystallized from the appropiate RPC fractions to obtain spectroscopically pure compounds. The other fractions were subjected to repeated RPC.

Solvent systems with different selectivities were employed in the two consecutive steps. In this way, compounds **10** and **12** were obtained in pure form. The repeated RPC was completed with NP- or RP-HPLC to give compounds **1-3**, **6-9**, **18** and **22**. The combination of RPC in a single run and NP- or RP-HPLC was used to prepare compounds **5**, **13-17**, **19-21** and **23**. Figures **4** and **5** illustrate recordings on some of the chromatographic steps in the isolation.

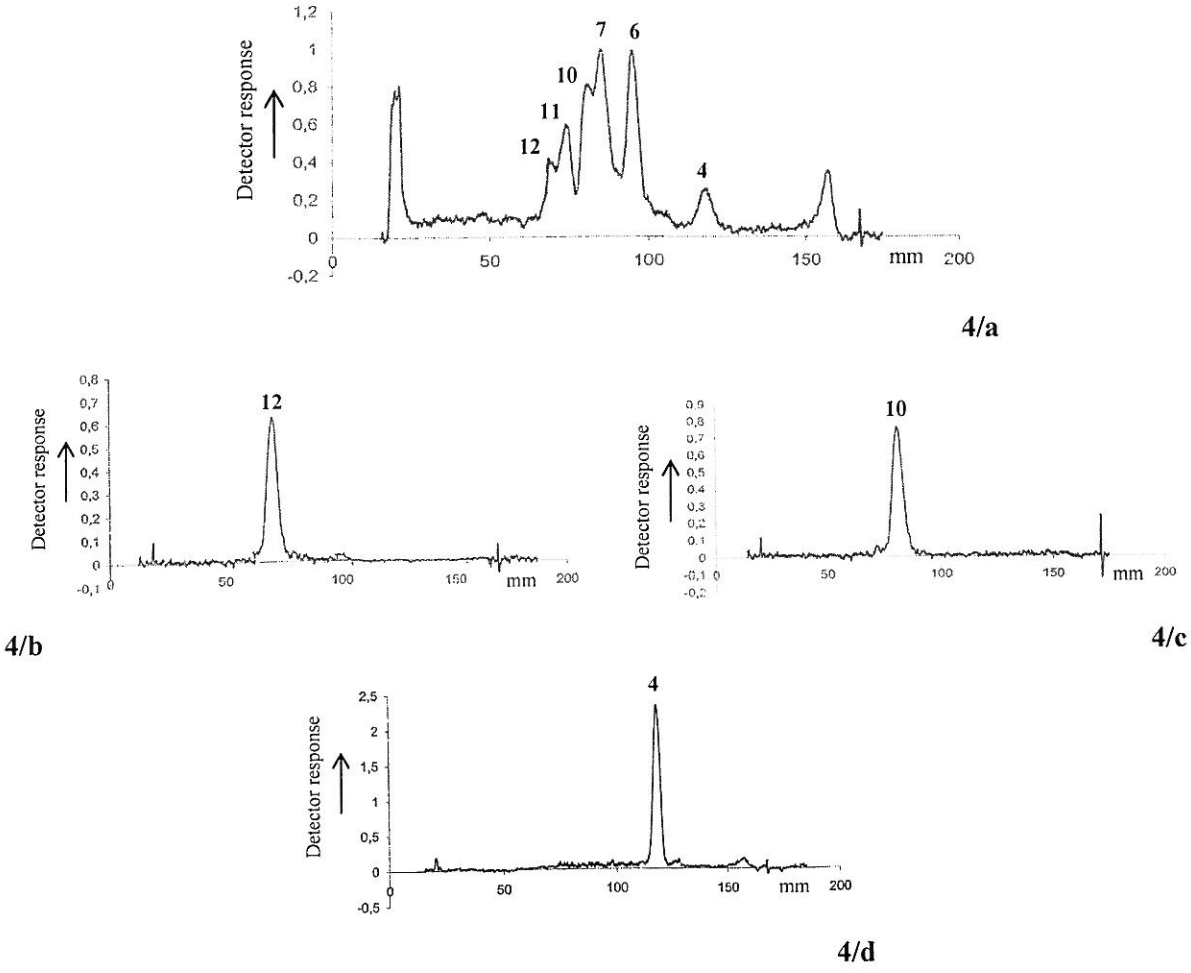
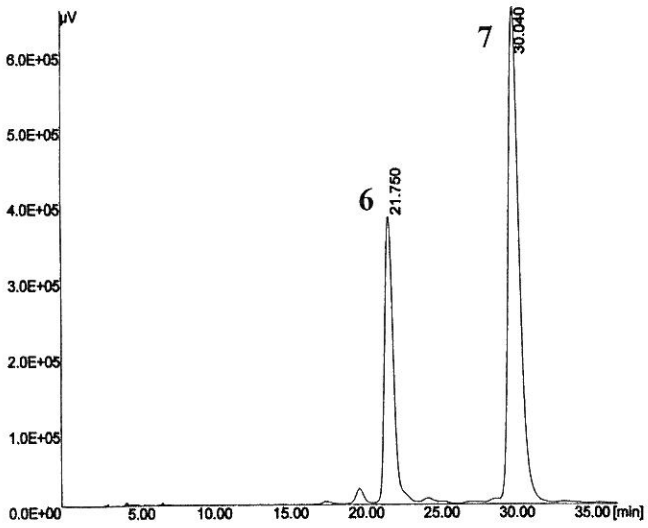
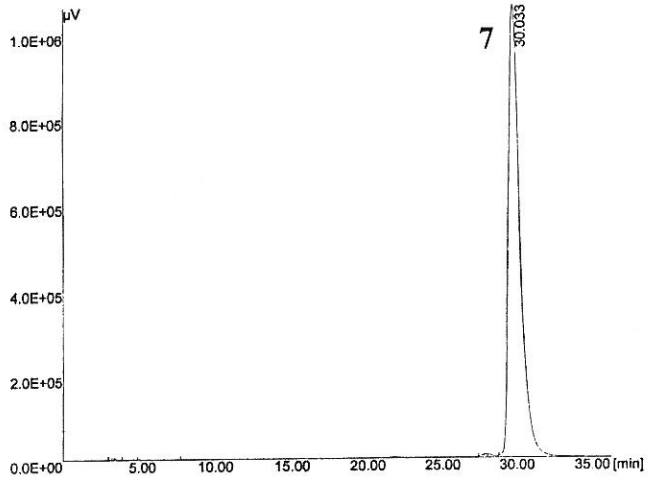


Figure 4/a. Densitogram of the fraction eluted with 50% aqueous methanol from RP-CC. Peaks: 22-deoxyintegristerone A (**12**), 20E (**11**), ajugasterone C (**10**), stachysterone B (**6**), 14α,15α-epoxy-14,15-dihydrostachysterone B (**7**), dacryhainansterone (**4**). **Figure 4/b**. Densitogram of pure 22-deoxyintegristerone A (**12**), obtained by repeated RPC from this fraction. **Figure 4/c**. Densitogram of pure ajugasterone C (**10**), obtained by repeated RPC from this fraction. **Figure 4/d**. Densitogram of pure dacryhainansterone (**4**), obtained by RPC and crystallization from this fraction. Stationary phase: silica gel. Mobile phase: TLC_2



5/a



5/b

Figure 5/a. HPLC chromatogram of the fraction containing stachysterone B (**6**) and 14α,15α-epoxy-14,15-dihydrostachysterone B (**7**). The fraction was obtained by repeated RPC.

Figure 5/b. HPLC chromatogram of pure 14α,15α-epoxy-14,15-dihydrostachysterone B (**7**) obtained by HPLC from this fraction.
HPLC chromatography was carried out on a Zorbax Sil column (5µm, 250 x 9.4 mm i.d.). The mobile phase was cyclohexane-isopropanol-water (100:40:3, v/v/v; at a flow rate of 3 mL·min^{-1}).

3.2. Characterization of the isolated compounds

The chromatographic and physical behaviour of the compounds were examined. **Tables 3-5** show the TLC and HPLC characteristics. Five mobile phases were used for TLC analysis. The stationary phase was either silica with a fluorescence indicator or octadecyl-silica. Triple detection was employed in the case of NP-TLC: dark spots were detected under UV light at 254 nm; after spraying with the vanillin-sulfuric acid reagent, fluorescence spots were visualized under UV light at 366 nm and under daylight. In the event of the absence of the 22-hydroxy group, the spots were orange under UV light and under daylight. NP-HPLC and RP-HPLC systems were used for characterization of the compounds.

Table 3. Colour of phytoecdysteroids after spraying with vanillin-sulfuric acid

Comp.	Colour after vanillin-sulfuric acid		Comp.	Colour after vanillin-sulfuric acid	
	Under UV (366 nm)	Under daylight		Under UV (366 nm)	Under daylight
1	red	red	**13**	violet	green
2	violet	green	**14**	violet	green
3	violet	green	**15**	violet	green
4	red	brown	**16**	orange	orange
5	violet	purple	**17**	dark-red	dark-red
6	violet	purple	**18**	orange	orange
7	violet	green	**19**	red	red
8	violet	purple	**20**	violet	green
9	violet	violet	**21**	orange	orange
10	dark-red	dark-red	**22**	violet	green
11	violet	green	**23**	orange	orange
12	orange	orange			

Table 4. TLC retention factors (R_f x 100) of the isolated compounds

	NP-TLC			**RP-TLC**	
	mobile phases				
Comp.	TLC_1	TLC_2	TLC_3	*TLC_4 / TLC_5	TLC_6
1	35	48	29	52	30
2	34	43	32	53	32
3	37	43	34	54	34
4	63	71	55	10*	21
5	47	74	47	43	16
6	40	53	35	65	42
7	39	49	34	66	44
8	38	40	31	57*	49
9	39	47	33	-	-
10	35	45	34	29*	34
11	30	37	27	47*	56
12	25	36	18	39*	42
13	60	59	44	-	-
14	39	70	32	49	31
15	52	68	44	31	13
16	45	63	39	23	10
17	40	60	44	-	-
18	33	57	26	24	11
19	45	53	49	-	-
20	29	43	37	-	-
21	36	52	27	25	10
22	45	55	37	23	11
23	43	61	38	18	12

Table 5. HPLC retention times of isolated ecdysteroids

	Retention time (min)			
Comp.	NP-HPLC		RP-HPLC	
1	9.7 [a]	14.6 [b]	4.1 [a]	6.7
2	11.2 [a]	13.9 [b]	3.6 [a]	5.7
3	12 [a]	12.4 [b]	3.4 [a]	4.7
4	-	10.4 [c]	6.5 [a]	-
5	6.7 [a]	7.6 [b]	7.5 [a]	9.5
6	21.7 [b]	-	-	-
7	30 [b]	12.9 [b]	3.0 [a]	3.6
8	-	12.7 [a]	7.3 [b]	-
9	13.2 [a]	15.4 [b]	5.5 [a]	-
10	24 [b]	33.3 [c]	-	-
11	22 [a]	15.6 [a]	4.2 [b]	-
12	21.5 [a]	13.8 [a]	9.5 [b]	-
13	17.6 [b]	5.9 [a]	23.3 [b]	-
14	15.6 [b]	13.3 [b]	3.7 [a]	3.1
15	7.7 [a]	7.6 [b]	14.8 [a]	16.6
16	19.9 [b]	9.1 [b]	11.2 [a]	24.3
17	10.5 [a]	12.7 [b]	12 [a]	-
18	19 [b]	10.5 [b]	8.1 [a]	17.2
19	6.8 [a]	8.0 [b]	9.1 [a]	10.2
20	8.2 [a]	35.5*	-	-
21	8.1 [a]	11.5 [b]	11.8 [a]	33.7
22	7.5 [a]	-	-	-
23	8.1 [a]	10.5 [b]	13.4 [a]	17.3
	[a] NP-HPLC$_1$ [b] NP-HPLC$_5$	[a] NP-HPLC$_2$ [b] NP-HPLC$_3$ [c] NP-HPLC$_4$ *dichloromethane-isopropanol-water (125:15:1, v/v/v) at 4 mL·min^{-1}	[a] RP-HPLC$_1$ [b] RP-HPLC$_2$	RP-HPLC$_3$

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, 20E.

3.2.1. Physical properties

The UV spectra provided characteristic information on the 7-en-6-one chromophore. The majority of ecdysteroids possess strong UV absorption spectra with a maximum at 240-245 nm (log $\varepsilon \approx 4$). In compounds **4** and **6,** the conjugated system of the chromophore group is extended, with the result that, in the UV spectra, λ_{max} is shifted to longer wavelength (e.g. **4**, which is a 7,9-diene-6-one, absorbs at 298 nm in methanol). For compounds **9** and **17,** λ_{max} is likewise shifted to longer wavelength (258.7 and 255.7 nm, respectively, in DMSO), which denotes the presence of unusual UV-active groups. The UV spectrum of compound **14** was considerably different because of the lack of the 7-en-6-one chromophore. **Table 6** lists some physical and spectroscopic characteristics for the isolated compounds, such as melting points, optical rotation and UV spectroscopic data.

Table 6. Some physical and UV spectroscopic data on the isolated ecdysteroids

Comp.	M.p. (°C)	$[\alpha]_D^{20}$ (*c*, MeOH)	UV λ_{max}, nm (log ε)
1	-	$[\alpha]_D^{25.5}$ +7° (*c* 0.1)	249 (3.543)
2	-	$[\alpha]_D^{25.5}$ +5° (*c* 0.1)	248 (3.611)
3	-	$[\alpha]_D^{25}$ +14° (*c* 0.05)	246 (3.744)
4	-	$[\alpha]_D^{27}$ +51° (*c* 2.25)	298 (4.152) 235 (3.806)
5	259-260	-	244 (4.093) 326 (2.11)
6	-	-	298 (4.107)
7	-	$[\alpha]_D^{28}$ -5° (*c* 0.1)	240 (3.8)
8	263-265	$[\alpha]_D^{20}$ +60.3±2° (dioxane)	243 (4.09)
9	-	$[\alpha]_D^{25.5}$ +56° (*c* 0.025) DMSO	258.7 (3.925) DMSO
10	216-217	$[\alpha]_D^{18}$ +48.5° (*c* 1.1)	243 (4.014)
11	241-242.5	$[\alpha]_D^{20}$ +58.9±2° (*c* 0.3)	240 (4.103)
12	-	$[\alpha]_D^{26}$ +80° (*c* 0.1)	242 (4.105)
13	245-250	$[\alpha]_D^{25}$ +65.0° (*c* 0.18; $CHCl_3$)	243 (4.04)
14	-	$[\alpha]_D^{28}$ -7° (*c* 0.05)	237 (3.2)
15	-	$[\alpha]_D^{28}$ +3° (*c* 0.05)	241.8 (3.7)
16	231-233	$[\alpha]_D^{28}$ +71° (*c* 0.025)	242 (4.387)
17	-	$[\alpha]_D^{25.5}$ +80° (*c* 0.025) DMSO	255.7 (3.874) DMSO
18	218-220	$[\alpha]_D^{28}$ +10° (*c* 0.05)	241 (3.95)
19	-	$[\alpha]_D^{25.5}$ +9° (*c* 0.1)	241 (3.503)
20	-	-	-
21	-	$[\alpha]_D^{25.5}$ +30° (*c* 0.1)	241.3 (3.852)
22	227-229	$[\alpha]_D^{20}$ +60.1±2° (*c* 1.3)	243 (4.01)
23	-	$[\alpha]_D^{25.5}$ +14° (*c* 0.1)	241.7 (4.094)

3.2.2. Mass spectrometry

The chemical ionization mass spectra (CIMS) and electrospray-ionization mass spectra (ESIMS) are suitable for determination of the molecular masses of ecdysteroids. The disadvantage of electron-impact mass spectra (EIMS) is the low intensity of the signals. The MS of ecdysteroids are charaterized by the appearance of numerous fragments differing from each other by the loss of water from the polyhydroxylated carbon skeleton (**Table 7**). In most cases, the ecdysteroids suffer side-chain cleavage, between C-20 and C-22, and between C-17 and C-20. The mass numbers depend on the extent of hydroxylation of the side-chain and the nucleus. The fragmentation results in two major series of fragments, corresponding to the loss of water from the nucleus and from the side-chain.

Table 7. MS fragmentation of the isolated ecdysteroids

Comp.	M.W.	MS	MS fragments m/z (relative intensity %)
1	478	ESIMS	517 (15) $[M+K]^+$, 501 (25) $[M+Na]^+$, 479 (15) $[M+H]^+$, 461 (5) $[M+H-H_2O]^+$, 443 (20) $[M+H-2H_2O]^+$, 425 (20) $[M+H-3H_2O]^+$, 407 (100) $[M+H-4H_2O]^+$.
	HRESIMS	479.2925 $[M+H]^+$ (calcd for $C_{27}H_{42}O_7$, 479.2919).
2	464	ESIMS	503 (100) $[M+K]^+$, 487 (19) $[M+Na]^+$, 465 (46) $[M+H]^+$, 447 (21.5) $[M+H-H_2O]^+$, 429 (14.5) $[M+H-2H_2O]^+$, 411 (23) $[M+H-3H_2O]^+$.
	HRESIMS	465.3120 $[M+H]^+$ (calcd for $C_{27}H_{44}O_6$, 465.3126).
3	464	EIMS	503 (25) $[M+K]^+$, 487 (100) $[M+Na]^+$, 465 (45) $[M+H]^+$, 447 (46) $[M+H-H_2O]^+$, 429 (17) $[M+H-2H_2O]^+$, 411 (24) $[M+H-3H_2O]^+$.
	HRESIMS	465.3129 $[M+H]^+$ (calcd for $C_{27}H_{44}O_6$, 465.3126).
4	462	CIMS	480 (11) $[M+NH_4]^+$, 463,6 (100) $[M+H]^+$, 447,6 (51) $[M-CH_3]^+$, 445,6 (32) $[M+H-H_2O]^+$, 427 (21) $[M+H-2H_2O]^+$, 362,5 (74), 345,5 (61) $[M-C_{20}-C_{27}]^+$.
5	464	CIMS	482 $[M+H+NH_3]^+$, 465 $[M+H]^+$, 447, 429, 411, 363, 345, 327.
		EIMS	344 (40), 300 (42), 83 (25), 18 (100).
6	462	EIMS	462 $[M]^+$; 444 $[M-H_2O]^+$, 345 (5) $[M-C_{22}-C_{27}]^+$, 327 (7), 99 (86) $[C_{22}-C_{27}]^+$, 81 (45), 43 (100).
		HRMS	444.2861 (for $C_{27}H_{40}O_5$, calc. 444.2815).
7	478	ESIMS	501 (13) $[M+Na]^+$, 479 (100) $[M+H]^+$, 461 (24) $[M+H-H_2O]^+$, 443 (27.5) $[M+H-2H_2O]^+$, 425 (2.6) $[M+H-3H_2O]^+$, 393 (3), 330 (53).
		HRESIMS	479.2924 $[M+H]^+$ (calcd for $C_{27}H_{43}O_7$, 479.2919).
8	494	EIMS	494 $[M]^+$, 363 (100), 345 (78), 131 (24), 113 (59), 95 (30), 70 (100).
9	588	ESIMS	519 (30) $[M+K-R]^+$, 503 (15) $[M+Na-R]^+$, 463 (37) $[M+H-H_2O-R]^+$, 445 (100) $[M+H-2H_2O-R]^+$, 427 (5) $[M+H-3H_2O-R]^+$, 409 (23) $[M+H-4H_2O-R]^+$ $R=C_6H_4O_2$ (in DMSO)
		HRESIMS	589.3393 $[M+H]^+$ (calcd for $C_{33}H_{48}O_9$, 589.3363). (in DMSO)
10	480	EIMS	462 $[M-H_2O]^+$, 379 (5), 361 (16), 343 (64), 335 (1), 325 (39), 317 (2), 299 (6), 281 (4), 145 (23), 127 (6), 109 (21), 101 (2), 83 (25), 43 (100).
11	480	CIMS	498 $[M+H+NH_3]^+$, 481 $[M+H]^+$, 463, 445, 427, 380, 363, 347, 345, 329.
		EIMS	480 (<1) $[M]^+$, 462 (1) $[M-H_2O]^+$, 444 (1) $[M-2H_2O]^+$, 429 (3), 426 (12) $[M-3H_2O]^+$, 411 (2), 408 (3) $[M-4H_2O]^+$, 393 (1), 363 (7) $[M-C_{22}-C_{27}]^+$, 346 (11), 345 (30) $[M-H_2O-C_{22}-C_{27}]^+$, 344 (26), 328 (17), 327 (19), 300 (13), 145 (8), 143 (8) $[C_{20}-C_{27}-H_2O]^+$, 99 (100) $[C_{22}-C_{27}]^+$, 81 (27) $[C_{22}-C_{27}-H_2O]^+$.

		ESIMS	503 (20) $[M+Na]^+$, 481 (50) $[M+H]^+$, 463 (100) $[M+H-H_2O]^+$, 445 (10) $[M+H-2H_2O]^+$, 413 (1), 391(2), 301 (2), 279 (1), 251 (6), 247 (20), 223 (3), 215 (2), 119 (5), 97 (100), 87 (23), 65 (22).
12	480	CIMS	498 $[MH + NH_3]^+$, 481 $[M+H]^+$, 463, 445, 427, 409, 391, 380, 363, 347.
13	462	EIMS	462 $[M]^+$, 405 (1) 363 (19), 345 (50), 327 (15), 99 $[C_{22}\text{-}C_{27}]^+$ (60), 81 (54), 43 (100).
14	448	ESIMS	487 (30) $[M+K]^+$, 471 (26) $[M+Na]^+$, 449 (29) $[M+H]^+$, 431 (40) $[M+H-H_2O]^+$, 413 (100) $[M+H-2H_2O]^+$, 395 (37) $[M+H-3H_2O]^+$.
		HRESIMS	449.3179 $[M+H]^+$ (calcd for $C_{27}H_{45}O_5$, 449.3177).
15	474	ESIMS	497 (10) $[M+Na]^+$, 475 (100) $[M+H]^+$, 457 (72) $[M+H-H_2O]^+$, 439 (5.7) $[M+H-2H_2O]^+$, 421 (3) $[M+H-3H_2O]^+$, 364 (2).
		HRESIMS	475.2975 $[M+H]^+$ (calcd for $C_{28}H_{43}O_6$, 475.2970).
16	446	ESIMS	485 (69) $[M+K]^+$, 447 (93) $[M+H]^+$, 429 (100) $[M+H-H_2O]^+$, 411 (6) $[M+H-2H_2O]^+$, 393 (7) $[M+H-3H_2O]^+$, 347 (6), 320 (4).
		HRESIMS	447.3025 $[M+H]^+$ (calcd for $C_{27}H_{43}O_5$, 447.3021).
17	588	ESIMS	519 (26) $[M+K-R]^+$, 503 (10) $[M+Na-R]^+$, 463 (46) $[M+H-H_2O-R]^+$, 445 (43) $[M+H-2H_2O-R]^+$, 427 (6) $[M+H-3H_2O-R]^+$, 409 (100) $[M+H-4H_2O-R]^+$ R=$C_6H_4O_2$ (in DMSO)
		HRESIMS	589.3389 $[M+H]^+$ (calcd for $C_{33}H_{48}O_9$, 589.3363). (in DMSO)
18	462	ESIMS	501 (26) $[M+K]^+$, 463 (4) $[M+H]^+$, 445 (100) $[M+H-H_2O]^+$, 427 (8) $[M+H-2H_2O]^+$, 408 (2) $[M+H-3H_2O]^+$, 391 (14) $[M+H-4H_2O]^+$, 374 (4), 363 (5), 336 (4)
		HRESIMS	463.2976 $[M+H]^+$ (calcd for $C_{27}H_{43}O_6$, 463.2970).
19	478	-	-
20	506	CIMS	524 $[M+NH_4]^+$, 507 $[M+H]^+$, 489, 471, 463, 445, 427, 411, 409, 391, 370, 346, 279, 272, 246, 299, 187, 160, 143, 124, 99.
21	462	ESIMS	501 (44) $[M+K]^+$, 485 (100) $[M+Na]^+$, 463 (5) $[M+H]^+$, 445 (7) $[M+H-H_2O]^+$, 427 (16) $[M+H-2H_2O]^+$, 409 (9) $[M+H-3H_2O]^+$
		HRESIMS	463.2973 $[M+H]^+$ (calcd for $C_{27}H_{43}O_6$, 463.2970).
22	520	EIMS	520 $[M]^+$, 505 (2), 502 (1), 487 (4), 469 (5), 445 (4), 427 (29), 409 (13), 363 (100), 353 (15), 345 (26), 329 (10), 327 (9), 320 (4), 300 (32), 201 (13), 143 (11), 99 (19), 81 (26).
23	446	ESIMS	486 (100) $[M+H+K]^+$, 470 (50) $[M+H+Na]^+$, 447 (15) $[M+H]^+$, 429 (32) $[M+H-H_2O]^+$, 411 (27) $[M+H-2H_2O]^+$.
		HRESIMS	447.3024 $[M+H]^+$ (calcd for $C_{27}H_{43}O_5$, 447.3021).

3.2.3. NMR spectroscopy

The numbers of C, CH, CH_2 and CH_3 fragments in a molecule were identified from the ^{13}C, DEPT and HMQC spectra. From the ^{13}C chemical shifts, the number of connecting oxygen atoms was established. The singlet methyl signals in the ^1H NMR spectrum aided in their assignments through the characteristic HMBC correlations of these signals over two and three bonds. Identification of the geminal Me-26 and Me-27 groups was unambiguous in consequence of their mutual HMBC correlation. Differentiation between H_3-19 and H_3-18 was achieved by considering the coupling of the latter with C-17, which is also coupled to H_3-21. In accordance with a 6-oxo-$\Delta^{7,8}$-moiety, the olefinic H-7 correlated with C-5, C-9 and C-14 in the HMBC spectra. The hydrogen atoms of ring A form a common spin-system which was analysed by $^1H,^1H$-COSY and HMQC-TOCSY experiments. The ^1H signal

assignments of rings C and D, as well as the side-chain attached to C-17, were obtained in an analogous way. The H_α-9/H_α-2 and H-19/H_β-5 correlations in the NOESY spectrum prove a *cis* type junction of rings A/B. The H_β-12/H_3-18, H_β-12/H_3-21 and H_α-12/H_α-17 cross-peaks verify the *trans* junction of rings C/D. The β-orientation of the H group attached to C-14 was justified by the NOESY correlations between H_3-18/H-14 (**Figure 6**).

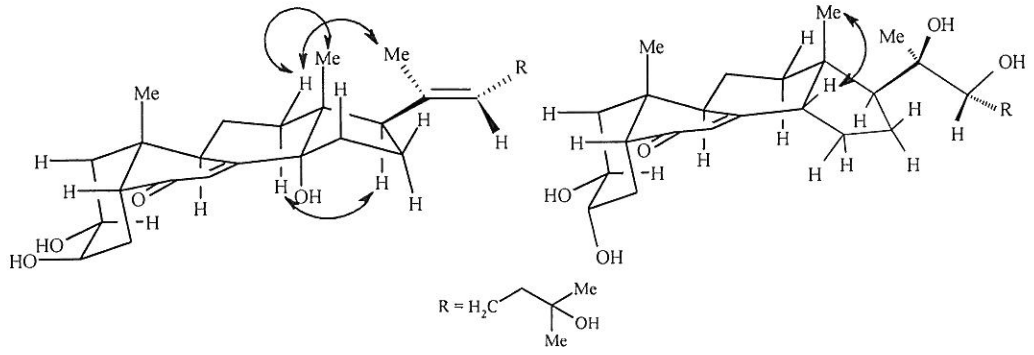


Figure 6. Selected NOESY correlation for structures **A** (C/D *trans*) and **B** (C/D *cis*). Double arrows indicate the characteristic NOESY correlations. **A** = comp. **16**, **B** = comp. **2**

a) The presence of an α-OH group on C-11 as in 11α-hydroxyshidasterone (**1**) was established by the chemical shifts of the H-11 and C-11 signals and by the multiplicity of the H-11 signal.

b) The 3α-OH group of 2β,3α,20R,22R,25-pentahydroxy-5β-14α-cholest-7-en-6-one (**3**) was characterized by the ^{1}H and NOESY spectra: the multiplicity of H-3 is a triplet ($J > 8$ Hz), instead of the usual quadruplet ($J \approx 3$ Hz). The H-3/H-5 NOESY cross-peaks also confirmed the equatorial position of 3-OH.

c) The presence of a 7,9(11)-diene structure was proved on the basis of the HMBC spectra as in dacryhainansterone (**4**): H-7 showed a correlation with a quaternary sp^2 carbon (C-9), and a correlation was detected in the ^{1}H and ^{1}H-COSY spectra between H-11 and H_2-12.

d) The overlapping H-7 and H-15 signals signed out in the HMBC spectrum of stachysterone B (**6**) C-8, C-13, C-14 and C-16 verified the presence of a conjugated $\Delta^{7,8;14,15}$-diene-moiety in the molecule.

e) A CH group (instead of a CH_2 unit) was found at position 15 in 14α,15α-epoxy-14,15-dihydrostachysterone B (**7**). The chemical shift of C-15 (61.3 ppm) indicated the connection of an oxygen atom in this positon. The $^1J_{C\text{-}15,H\text{-}15}$ coupling constant was measured to be 188 Hz by ^{1}H-coupled HMQC, which proved the existence of a 14,15-epoxy group. The presence

of the 14,15-epoxy group was also supported by the chemical shifts of C-14 and C-15 (71.9, 69.0 ppm) in an other 14,15-epoxy ecdysteroid (gymnasterone B) in the literature [100].

f) The H-22/H-28 NOESY correlation in serfurosterone B (**17**) and the chemical shifts of C-20 (83.9 ppm), C-22 (83.4 ppm) and C-28 (96.5 ppm) verified the existence of an acetal-type five-membered ring. Additionally, the H-28/C-29, H-33/C-31 and H-33/C-32 HMBC cross-peaks and the H-28/H-30 NOESY correlations revealed the 5-hydroxymethyl-2-furanoyl substituent at C-28. The characteristic ^{13}C chemical shifts and the low coupling value $^{3}J_{H\text{-}30,H\text{-}31}$ = 3.2 Hz lent further support to the structure [101]. The similar chemical shifts and signal multiplicity of H-28, H-30, H-31, H-33 and C-22 also indicate the presence of the furan unit in serfurosterone A (**9**).

g) 22-Deoxyecdysteroids, such as 22-deoxyintegristerone A (**12**), were characterized by the lack of the H-22 signal in the hydroxymethine zone, a small downfield shift of H-21 (+ 0.08-0.1 ppm in MeOH-*d*4), and small upfield shifts of H-18 and H-17 (-0.04-0.08 ppm in MeOH-*d*4).

h) In 2*β*,3*β*,20R,22R,25-pentahydroxy-5*β*-cholest-6,8(14)-diene (**14**), H-7 gave a COSY correlation with an olefinic hydrogen at position 6. H-6 marked out a quaternary olefinic carbon atom (C-8) in the HMBC spectrum, proving the existence of a conjugated $\Delta^{6,7;8,14}$-diene moiety.

i) In 24-methylene-shidasterone (**15**), the geminal Me-26 and Me-27 groups indicated a quaternary sp^2 carbon atom at 158.3 ppm, proving the attachment of the terminal methylene group to C-24. H_3-21 gave a correlation only to three carbon atoms, in contrast with the other methyl groups. H_3-21/C-17 HMBC responses were also detected for this compound. The ^{13}C chemical shifts of C-22 (82.4 ppm) and C-25 (83.2 ppm) prove the presence of an OR (R ≠ H) subtituent in 24-methylene-shidasterone (**15**). The strong H-22/H-26 NOESY response may indicate the existence of a five-membered ring. In the NOESY spectrum of this compound, the detected H_3-21/H_β-16, H-22/H_β-16, H-22/H_3-21 and H-22/H_3-18 cross-peaks indicate the high mobility of the side-chain, which prevented determination of the absolute configuration at C-20 and C-22.

j) In 1-hydroxy-20,22-didehydrotaxisterone (**18**) and 1-hydroxy-22-deoxy-20,21-didehydroecdysone (**21**), the *β*-orientation of the OH groups attached to C-1 was justified by two reasons. First, H_α-2 is axial and its multiplicity and coupling constant (t; 3.1 Hz) preclude the axial orientation of H-1 because of the absence of an axial/axial coupling constant (9-10 Hz). Second, many ^{1}H and ^{13}C NMR signals of the atoms in ring A and methyl-19 are broad,

as opposed to the corresponding signals of compounds **18** and **21**, indicating the hindered conformational motion of ring A.

k) The *trans* arrangement of C-21 and H-22 in 20,22-didehydrotaxisterone (**16**) and 1-hydroxy-20,22-didehydrotaxisterone (**18**) was proved by the NOESY correlations H-22/H_α-16, H-22/H_β-16, H-22/H_α-17, H_3-21/H_β-12 and H_3-21/H_3-18.

l) In 22-dehydro-20-deoxy-ajugasterone C (**19**), the chemical shift of C-22 indicated the presence of an oxo group in the side-chain. Determination of the absolute configuration of C-20 in 22-dehydro-20-deoxy-ajugasterone C failed, due to the strong overlapping of the H_α-17/H-20/H_β-23 and H_β-16/H_2-24 signals.

m) The acetonide group in position 20,22, as in 20-E 20,22-monoacetonide (**22**) was identified on the basis of the ^{13}C spectra: the chemical shifts of C-20 and C-22 appear at 85.8 and 83.3 Hz instead of the value near 77 ppm for 20E.

Tables 8-14 contain the ^1H and ^{13}C chemical shifts (in ppm), J (^1H, ^1H) couplings (in Hz), 2D ^1H,^1H scalar couplings (COSY), characteristic ^{13}C, ^1H long-range correlations (HMBC) and spatial proximities (NOESY) on the new ecdysteroids isolated.

11α-hydroxyshidasterone

No.		^{13}C	^{1}H	m; *J* (Hz)	COSY	HMBC	NOESY
1	α	39.3	2.58	ddd; 12.8, 4.5, 0.7		69.1	4.01, 1.05
	β		1.37	overlapped			
2	α	69.1	4.005	ddd; 12.1, 4.2, 3.3	3.95, 2.58, 1.37	-	3.95, 3.15, 2.58
3	β	68.7	3.95	q; 2.7	4.00, 1.77, 1.69	-	4.01, 1.78, 1.69
4	α	33.5	1.78				
	β		1.69				
5	β	52.9	2.33	dd; 13.1, 3.7	1.77, 1.69	26.2	1.75, 1.69, 1.38, 1.05
6		?	-	-	-	-	-
7		122.9	5.80	dd; 2.7, 0.7	3.145	85.0, 43.1	
8		165.9	-	-	-	-	-
9	α	43.1	3.145	dd; 8.8, 2.7	5.80, 4.09, 3.11?	165.9, 69.6	
10		?	-	-	-	-	-
11	β	69.7	4.09	m	3.145, 2.22, 2.13	-	2.13, 1.05, 0.83
12	α	43.8	2.23	dd; 12.5, 10.4	4.09	-	2.40, 1.59
	β		2.13	dd; 12.4, 6.0	4.09	70.2, 48.7	4.09, 1.235, 0.83
13		48.3-7?	-	-	-	-	-
14		85.0	-	-	-	-	-
15	α	32.0	1.59		3.50?, 1.96?	72.7?	1.85
	β		1.98				
16	α	21.9	1.82				
	β		1.97				
17	α	51.8	2.40	dd; 9.6, 8.6	1.99	49.2, 22.0, 19.4	2.23, 2.01, 1.76-1.90, 1.235
18	β	19.1	0.83	s	-	85.2, 52.4, 48.3, 43.9	4.09, 2.13, 1.97, 1.235
19	β	24.8	1.05	s	-	53.4, 43.0, 41.4, 38.9	4.09, 2.58, 2.33, 1.38
20		77.4	-	-	-	-	-
21		20.9	1.235	s	-	86.0, 77.4, 51.9	2.40, 2.13, 2.00, 1.75, 0.83
22		85.7	3.92	dd; 8.2, 6.1	1.90?	-	2.01, 1.91, 1.76, 1.248, 1.235
23	a	28.63	1.76	overlapped			
	b		1.91	overlapped			
24	a	39.8	1.75	overlapped			
	b		1.75	overlapped			
25		82.0	-	-	-	-	-
26		28.54	1.248	s	-	82.0, 39.9, 29.1	3.92, 1.75
27		29.1	1.252	s	-	82.0, 39.9, 28.5	1.75

24-methylene-shidasterone

No.		^{13}C	^{1}H	m; *J* (Hz)	HMBC	NOESY
1	α	37.5	1.795	d (HMQC)		
	β		1.43	dd; 13.1, 12.5	68.9, 35.3	2.38, 0.96
2	α	68.9	3.84	ddd; 12.1, 4.3, 3.3	-	3.95, 3.15, 1.79, 1.74
3	α	68.7	3.95	q; 2.9	-	3.84, 1.76, 1.70
4	a	33.2	1.72			
	b		1.76			
5	β	51.9	2.38	dt; 8.1, 4.7	-	1.71, 1.43, 0.96
6		206.6?	-	-	-	-
7		122.3	5.81	d; 2.7	85.4, 51.9, 35.4	1.97, 1.62, 0.96, 0.84
8		168.1	-		-	-
9	α	35.3	3.15	ddd; 11.3, 7.1, 2.7	168.1	3.84, 2.16, 1.80, 1.74
10		39.4	-	-	-	-
11	α	21.6	1.80	d (COSY)		
	β		1.67	t (COSY)		
12	α	32.45	2.165	td; 13.3, 4.8	18.4	3.15, 2.42
	β		1.84			
13		48.4	-	-	-	-
14		85.4	-	-	-	-
15	α	31.8	1.62			
	β		1.97	(NOESY)		
16	α	22.0	1.83			
	β		2.00			
17	α	52.0	2.42	dd; 9.5, 8.2	48.3, 22.3, 18.4	3.91, 2.55, 2.17, 1.85, 1.21
18	β	18.35	0.84	s	85.4, 52.1, 48.4, 32.5	3.91, 1.95-1.99, 1.84, 1.69, 1.21, 0.96
19	β	24.5	0.96	s	51.9, 39.4, 37.5, 35.3	2.38, 1.80, 1.67-1.71, 1.43, 0.84
20		76.8	-	-	-	-
21		20.7	1.21	s	82.4, 76.8, 52.0	3.91, 2.55, 2.42, 1.99, 1.84, 0.84
22		82.4	3.91	t; 8.1	-	2.545, 2.42, 2.00, 1.85, 1.28, 1.21, 0.84
23	a,b	35.6	2.545	dt; 8.2, 2.2	158.3, 104.1, 83.1, 82.4	4.90, 3.91, 2.42, 1.85, 1.21
24		158.3	-	-	-	-
25		83.2	-	-	-	-
26	*Z*	27.9	1.28	s	158.3, 83.2, 29.2	4.80, 3.91
27	*E*	29.2	1.33	s	158.3, 83.2, 27.9	4.80, 2.55
28	a	104.1	4.805	t; 2.3	83.2, 35.6	-
	b		4.895	t; 2.2	83.2, 35.6	-

Table 8. NMR data on 11α-hydroxyshidasterone (**1**) and 24-methylene-shidasterone (**15**)

Multiplicity of signals: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet

2β,3α,20R,22R,25-pentahydroxy-5β-14β-cholest-7-en-6-one							2β,3α,20R,22R,25-pentahydroxy-5β-14α-cholest-7-en-6-one						
No.		^{13}C	^{1}H	m; *J* (Hz)	HMBC	NOESY	No.		^{13}C	^{1}H	m; *J* (Hz)	HMBC	NOESY
1	α	42.7	2.10	dd; 13.9, 4.7	40.2, 52.1, 75.5	3.62, 2.81,1.09	**1**	α	43.1	2.09	dd; 13.3, 3.3	39.6, 57.5, 72.2, 75.4	0.96, 1.09, 2.66, 3.34, 3.62
	β		1.09	dd; 13.9, 11.9	37.7, 72.2	3.62, 2.10, 2.07		β		1.09	dd; 13.9, 11.8	40.3, 72.2	0.96, 2.08, 2.09
2	α	72.2	3.62		-	2.81	**2**	α	72.2	3.62	ddd; 11.3, 8.7, 5.1	-	1.47, 2.09, 2.66
3	β	75.5	3.36		-	2.07, 1.97	**3**	β	75.4	3.34	ddd; 11.7, 8.7, 4.5	-	1.09, 1.47, 1.78, 2.08
4	α	34.1	1.41		-	2.81, 1.97	**4**	α	34.1	1.47	td; 13.1, 11.3	57.5, 75.4	1.78, 2.08, 2.66, 3.34, 3.62
	β		1.79			3.36, 2.0, 1.41		β		1.78	m	-	1.47, 2.09, 3.34
5		57.2	2.07	dd; 13.4, 3.8	-	3.36,1.97, 1.09, 0.91	**5**		57.5	2.08	dd; 13.8, 4.3	39.6, 75.4, 204.2	0.96, 1.09, 1.78, 3.34
6			-	-	-	-	**6**		204.2	-			
7		127.2	5.84	d; 2.5	59.2, 37.7	-	**7**		122.3	5.65	t; 2.1	40.3, 57.2	0.83, 0.96, 1.59, 1.61, 1.70, 2.22
8		~ 170.2	-	-	-	-	**8**		168.5	-			
9	α	37.7	2.81	ddd; 9.7, 6.1, 2.2	170.2, 21.9	3.62, 1.84, 1.41	**9**	α	40.3	2.66	ddd; 11.6, 6.9, 2.4	168.5	1.47, 1.77, 1.91, 2.09, 2.22, 3.62
10		40.2	-	-	-	-	**10**		39.6	-			
11	α	21.9	1.84		-	2.81, 2.10	**11**	α	23.1	1.91			0.96, 1.61, 1.77, 2.09, 2.31, 2.66
	β		1.63		-			β		1.77	m		0.83, 0.96, 1.91, 2.09, 2.31, 2.66
12	α	39.9	1.59		-		**12**	α	40.65	1.61		14.8, 47.0	0.83, 2.22, 2.31, 2.66, 5.65
	β		1.81		-			β		2.31	ddd; 13.1, 4.0, 3.0	-	0.83, 1.61, 1.23 , 1.91
13		45.8	-		-	-	**13**		47.0	-			
14		59.2	2.46	dd; 11.7, 7.2	-	1.77, 1.83, 1.27	**14**		57.2	2.22	ddd; 11.7, 6.2, 1.6	-	0.83, 1.61, 1.70, 1.84, 2.66, 5.65
15	α	34.1	1.71		-		**15**	α	23.6	1.59		-	0.83, 1.70, 1.99, 5.65
	β		1.77		-	2.46		β		1.70	m	47.0, 56.4	1.84, 1.91, 1.99, 2.22, 5.65
16	α	28.2	1.83		-	1.98	**16**	α	22.8	1.99			0.83, 1.59, 1.70/1.705, 1.84, 3.32
	β		1.83		-	2.46, 1.98		β		1.705		47.0, 56.4	1.84, 1.99
17	α	56.3	1.98	t; 8.9	39.9, 45.8	1.62, 1.23, 1.83	**17**	α	56.4	1.84	t; 9.2	14.8, 47.0	1.99, 2.22
18		24.9	1.27	s; -	39.9, 45.8, 56.3, 59.2	2.46	**18**		14.8	0.83	s	40.65, 47.0, 56.4, 57.2	1.61, 1.77, 1.99, 2.31, 5.65
19		23.9	0.91	s; -	37.7, 40.2, 42.7, 57.1	2.07	**19**		24.3	0.96	s	57.5, 43.1, 40.3, 72.2, 39.6	1.09, 1.77, 1.91, 2.09, 5.65
20		78.1	-	-	-	-	**20**		77.8	-			
21		20.4	1.23	s; -	56.3, 78.1, 78.8	1.98	**21**		21.1	1.23	s	56.4, 77.8, 78.4	2.31
22		78.8	3.41	dd; 10.4, 1.6	-	1.82, 1.44	**22**		78.4	3.32		21.1, 77.8	1.31, 1.65, 1.705, 1.99
23	a	27.4	1.35		-		**23**	a	27.4	1.65	ddd; 12.0, 4.3, 1.6	-	3.32, 5.65
	b		1.62		-	1.98		b		1.31	t	42.5	3.32
24	a	42.5	1.44		-	3.41 1.19, 1.22	**24**	a	42.5	1.43	t	27.4, 71.5	1.09, 1.21, 1.65, 2.09
	b		1.82		-	3.41, 1.19 1.22		b		1.80	m	29.0, 30.0, 71.5	0.83, 1.21, 1.31
25		71.5	-	-	-	-	**25**		71.5	-			
26		29.0	1.19	s; -	30.0, 42.5, 71.5	1.82, 1.44	**26**		29.0	1.19	s	30.0, 42.5, 71.5	-
27		30.0	1.22	s;-	29.0, 42.5, 71.5	1.82, 1.44	**27**		30.0	1.21	s	29.0, 42.5, 71.45	1.43, 1.80, 2.31

Table 9. NMR data on 2β,3α,20R,22R,25-pentahydroxy-5β-14β-cholest-7-en-6-one (**2**) and 2β,3α,20R,22R,25-pentahydroxy-5β-14α-cholest-7-en-6-one (**3**)

Table 10. NMR data on 14α,15α-epoxy-14,15-dihydrostachysterone B (7)
Multiplicity of signals: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet

14α,15α-epoxy-14,15-dihydrostachysterone B

No.		^{13}C	^{1}H	m; *J* (Hz)	HMBC	NOESY
1	α	37.1	1.82	dd; 13.5, 4.3	68.7, 68.5, 52.0, 39.7, 24.5	3.84, 2.84, 1.46, 1.01
	β		1.46	t; 12.8	68.7, 68.5, 52.0,39.7, 39.1, 24.5	2.41, 1.82, 1.01
2	α	68.7	3.84	ddd; 12.1, 4.2, 3.3	68.5, 37.1	3.96, 2.84, 1.82
3	α	68.5	3.95	ddd; 3.0	-	3.84, 1.66
4	a	32.8	1.66	dd; 7.8, 3.0	68.7, 68.5, 52.0, 39.7	3.96, 3.84, 2.84, 2.41
	b					
5		52.0	2.41	dd	205.9, 68.5, 39.7, 39.1, 32.8, 24.5	1.66, 1.46, 1.01
6		205.9	-	-	-	-
7		124.0	5.89	d; 2.8	73.3, 52.0, 39.1	-
8		159.8	-	-	-	-
9	α	39.1	2.84	ddd; 10.2, 6.9, 2.8	-	3.84, 1.82, 1.93, 1.66
10		39.7	-	-	-	-
11	α	21.7	1.93			2.84, 1.81
	β		1.81			1.93, 1.01, 1.02
12	α	35.6	1.81			2.20, 1.71
	β		2.20	dd; 9.0, 2.8	73.3, 42.4, 39.1, 16.5	1.81, 1.19, 0.9
13		42.4	-	-	-	-
14		73.3	-	-	-	-
15	β	61.3	3.97	s	159.8, 73.3, 48.4, 42.4, 27.9	1.94
16	α	27.9	1.92		73.3, 61.3, 48.4, 42.4	3.29, 1.71
	β		1.94		73.3, 61.3, 48.4, 42.4	3.29, 3.97, 1.02
17	α	48.4	1.71	dd; 10.6, 7.0	77.0, 42.4 35.6, 27.9, 16.5	2.20, 1.92, 1.55, 1.19
18		16.5	1.02	s	73.3, 48.4, 42.4, 35.6	1.94, 2.20, 1.81, 1.19
19		24.5	1.01	s	68.7, 52.0, 39.7, 39.1, 37.1	2.44, 1.85, 1.68, 1.58
20		77.0	-	-	-	-
21		20.8	1.19	s	77.0, 78.4, 48.4	3.29, 1.71, 2.20, 1.55
22		78.4	3.29	dd; 10.5, 1.8	78.2, 42.5, 20.8	1.94, 1.92, 1.55, 1.44
23	a	27.4	1.28	td; 11.8, 4.4	78.4	1.79, 1.55, 1.44
	b		1.55	tdd; 11.8, 4.4, 1.8		3.29, 1.71, 1.44, 1.28, 1.19
24	a	42.3	1.44	td; 12.4, 4.2	71.4, 78.4, 29.0, 30.0, 27.4	3.29, 1.79, 1.55, 1.28
	b		1.79	td; 12.6, 4.6	71.4, 78.4, 29.0, 30.01, 27.4	1.44, 1.28, 1.20
25		71.4	-	-	-	-
26		29.0	1.19	s	71.4, 42.3, 30.0	1.44
27		30.0	1.20	s	71.4, 42.3, 29.0	1.79

Table 11. NMR data on serfurosterone A (**9**) and serfurosterone B (**17**)

Multiplicity of signals: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet

serfurosterone A						serfurosterone B						
No.		^{13}C	^{1}H	m; *J* (Hz)	HMBC	No.		^{13}C	^{1}H	m; *J* (Hz)	HMBC	COSY	ROESY
1	α	37.3	1.79	dt; 13.2, 4.2		1	α	38.21	2.46	dd; 12.5; 4.1	-	1.15	3.87; 3.785; 2.98; 1.15; 0.90
	β		1.42	t; 12.9			β		1.15	t; 11.9	66.9	-	2.46; 2.135
2	α	68.5	3.84	dt; 12.0, 3.4		2	α	66.9	3.785	t; 3.6	-	1.15; 2.46	2.98; 2.46; 1.62
3	α	68.3	3.95	s		3	α	66.5	3.76	s (q)	-	2.461	1.48; 1.62; 2.46
4	α	32.7	1.75			4	α	32.0	1.62	td	-	-	
	β		1.71				β		1.46	overlapped			
5	β	52.1	2.39	dd; 12.6, 4.2		5	β	51.1	2.135	dd; 13.1; 3.8	-	5.63	3.785; 2.135; 0.90
6		?	-	-		6		?	-	-	-	-	
7		122.0	5.82	d; 2.5		7		121.0	5.63	d; 2.6	41.2; 82.6	2.98; 2.135	0.69; 0.89/90; 1.84; 2.98
8			-	-		8		162.6	-	-	-	-	
9	α	35.0	3.15	ddd; 8.9, 2.7, 10.8		9	α	41.2	2.98	dd; 8.9; 2.6	38.1; 66.9; 67.2; 162.6	5.63; 3.87	2.08; 3.785; 2.46; 2.135; 2.08; 0.69
10		39.1	-	-		10		40.5	-	-	-	-	
11	α	31.7	1.62			11	β	67.2	3.87	ddd; 10.5; 9.1, 6.0	-	2.98; 2.135; 2.08	0.69; 0.90; 1.93; 2.46
	β	31.7	1.62										
12	α	32.2	2.11	td; 12.6, 4.2		12	α	42.0	2.08	t; 11.45	66.9; 67.2; 82.9	-	2.98; 2.30; 1.93
	β		1.85				β		1.93	dd; 12.1; 5.7	-	-	3.87; 2.08; 1.20; 0.69
13		48.0	-	-		13		46.6	-	-	-	-	
14		85.4	-	-		14		82.7	-	-	-	-	
15	α					15	α	30.2	1.535	d			
	β						β		1.85	overlapped			
16	α					16	α	21.3	1.84	overlapped			
	β						β		1.84	overlapped			
17	α	51,1	2.41	t; 9.1		17	α	49.2	2.30	t; 8.6	21.4	1.85; 1.83	3.69; 2.08; 2.30; 0.69
18	β	17.5	0.86	s	32.3, 48.2, 85.4, 51	18	β	17.5	0.69	s	42.4; 46.6; 48.5; 82.7	-	5.74, 5.63, 3.87; 2.30; 1.93; 1.84; 1.20; 0.90
19	β	24.1	0.96	s	37.1, 35.0, 39.1, 52.1	19	β	24.08	0.90	s	38.0; 40.5; 50.9	-	5.63, 3.87; 3.41; 2.98; 2.46; 2.135; 1.15
20		85.6	-	-		20		83.9	-	-	-	-	
21		23.6	1.30	s		21		21.2	1.20	s	49.2; 83.9	-	2.30; 1.93; 0.69
22		85.7	3.79	dd; 9.4, 2.3		22		83.4	3.69	dd; 9.4; 2.6	-	-	5.74, 2.30; 1.84, 1.40 1.25
23	a					23	a	26.0	1.44	-	-	-	
	b						b		1.51	-	-	-	
24	a	41.7	1.53	td; 13.2, 4.2		24	a	35.8	1.25	-	-	0.89; 0.90	
	b		1.53	td; 13.2, 4.2			b		1.40	-	-	-	
25		71.4	-	-		25		27.5	1.58	dt; 13.3; 6.6	22.5	-	
26		28.7	1.20	s	29.3, 42.2, 71.1	26		22.33	0.88	d	22.44; 27.5; 35.9	1.58	1.58; 1.39; 1.26
27		29.2	1.21	s	28.7, 42.2, 71.1	27		22.44	0.89	d	22.33; 27.5; 35.9	1.58	1.58; 1.39; 1.26
28		98.2	5.79	s		28		96.5	5.74	s	150.3	-	0.69; 3.69; 6.45
29			-	-		29		150.3	-	-	-	-	
30			6.42	d; 3		30		109.9	6.45	d; 3.2	-	4.37; 6.25	0.69; 1.20; 5.74; 6.25
31			6.28	d; 3		31		107.5	6.25	d; 3.2	-	4.37; 6.45	4.37; 6.45
32			-	-		32		156.1	-	-	-	-	
33	a,b	57.2	4.51	s		33		55.7	4.37	s	107.5; 156.1	6.27	6.25

2β,3β,20R,22R,25-pentahydroxy-5β-cholest-6,8(14)-dien						
No.		^{13}C	^{1}H	m; *J* (Hz)	HMBC	NOESY
1	α	37.4	1.70	dd; 13.3, 4.4	70.5, 69.0, 39.5-7, 37.4-6, 36.5-9	-
	β		1.59	t; HSQC		
2	α	69.0	3.66	ddd; 12.5, 4.3, 2.9	-	3.90, 2.39, 1.68, 1.48
3	α	70.5	3.90	q; 2.6	-	3.66, 1.86, 1.48
4	α	36.9	1.48	t; HSQC		
	β		1.86	dt; 14.8, 3.9	70.5, 69.0, 39.5-7, 37.4-6	3.90, 2.09
5	β	39.7	2.09	d; HSQC	130.7, 125.6, 37.4-6, 36.5-9, 23.7	
6		130.7	5.53	dd; 10.0, 5.5	125.9, 39.5-7, 37.4	6.09, 2.09, 1.86
7		125.6	6.09	d; 9.9	147.6, 125.9, 39.5-7, 36.5-9	5.53, 2.41
8		125.9	-	-	-	-
9	α	36.5	2.39			
10		37.6	-	-	-	-
11	α	20.8	1.66			
	β		1.60			
12	α	39.5	1.46	t; HSQC		
	β		2.22	dt; 12.4, 3.0	45.5, 36.5-9, 20.8	1.58, 1.23, 1.13
13		45.5	-	-	-	-
14		147.6	-	-	-	-
15	α	25.4	2.41	d; HSQC		
	β		2.29	t; HSQC	-	2.03
16	α	22.9	1.65			
	β		2.04	t; HSQC		
17	α	56.4	1.59			
18	β	21.5	1.13	s	147.6, 56.4, 45.5, 39.5-7	2.29, 2.21, 2.04, 1.60, 0.77
19	β	23.7	0.77	s	69.0, 39.5-7, 37.4-6, 36.5-9	
20		77.9	-	-	-	-
21		21.0	1.23	s	78.4, 77.9, 56.4	2.22
22		78.4	3.39	dd; 10.5, 1.3	77.2, 42.5, 20.8-21.0	2.03, 1.65, 1.58, 1.42
23	a	27.4	1.27			
	b		1.59			
24	a	42.5	1.42			
	b		1.79	td; 12.8, 4.8	78.4, 71.4, 30.0, 28.9, 27.4	-
25		71.4	-	-	-	-
26		28.9	1.17	s	71.4, 42.5, 30.0	1.79, 1.59
27		30.0	1.20	s	71.4, 42.5, 28.9	1.79, 1.59, 1.42

22-dehydro-20-deoxy-ajugasterone C						
No.		^{13}C	^{1}H	m; *J* (Hz)	HMBC	NOESY
1	α	39.2	2.56	overlapped (d; HMQC)		
	β		1.357	t; 12.4	69.0, 43.1, 40.0	2.33, 1.03
2	α	69.0	3.99	ddd; 11.3?, 4.0, 3.4	-	3.11, 2.56
3	α	68.7	3.95	overlapped	-	1.76, 1.68
4	α	33.45	1.76	ddd; 14.0, 13.2, 2.5	-	3.95, 3.11, 2.34
	β		1.68	dt; 14.1, 3.7	-	3.95, 2.34
5	β	53.0	2.325	dd; 13.0, 4.0	-	1.76, 1.68, 1.36, 1.03
6		?	-	-	-	-
7		122.8	5.80	d; 2.7	84.7, 53.0, 43.1	1.93, 1.61, 1.03, 0.66
8		165.4	-	-	-	-
9	α	43.1	3.11	dd; 8.9, 2.7	165.4, 69.5, 40.0, 24.7	3.97-3.99, 2.02, 1.76
10		40.0	-	-	-	-
11	β	69.5	3.97	overlapped	-	3.11?, 1.43, 1.03, 0.66
12	α	42.4	2.02	t; 11.7	69.5, 47.7, 17.9	3.11, 2.61
	β		1.43	dd; 12.0, 5.7	84.7, 69.5, 43.1	3.97-3.99, 1.57?, 0.66
13		47.6	-	-	-	-
14		84.6	-	-	-	-
15	α	31.7	1.61			
	β		1.93	26.1	5.80, 1.47, 0.66	
16	α	26.1	2.054			
	β		1.47			
17	α	48.4	2.61	overlapped		
18	β	17.9	0.66	s	84.6, 48.5, 47.56, 42.4	3.95-3.99, 2.58-2.61, 1.94, 1.48, 1.43, 1.03
19	β	24.7	1.03	s	53.0, 43.1, 40.0, 39.2	3.95-3.99, 2.57, 2.33, 1.36, 0.66
20		49.5	2.595	overlapped		
21		17.6	1.08	d; 6.4	218.2, 49.56, 48.5	2.61, 2.58, 2.05, 1.48
22		218.2	-	-	-	-
23	a	40.0	2.54	overlapped	218.3	
	b		2.62	overlapped	218.1	
24	a	33.6	1.46	overlapped	-	
	b		1.46	overlapped	-	
25		29.0	1.57	dq; 13.3, 6.6	33.6, 23.0	1.46?, 0.93
26		23.0	0.93	d; 6,6	33.6, 29.0, 23.0	2.62, 2.58, 1.60, 1.47
27		23.0	0.93	d; 6,6	33.6, 29.0, 23.0	2.62, 2.58, 1.60, 1.47

Table 12. NMR data on 2β,3β,20R,22R,25-pentahydroxy-5β-cholest-6,8(14)-dien (**14**) and 22-dehydro-20-deoxy-ajugasterone C (**19**)

20,22-didehydrotaxisterone							1-hydroxy-20,22-didehydrotaxisterone						
No.		^{13}C	^{1}H	m; *J* (Hz)	HMBC	NOESY	No.		^{13}C	^{1}H	m; *J* (Hz)	HMBC	NOESY
1	α	37.6	1.79	dd; 13.4, 4.7	68.7-9, 52.0, 39.5	3.84, 3.18, 0.96	**1**	α	76.6	3.82	s	-	3.10, 1.72, 1.07
	β		1.43	t; 12.8	68.7-9, 39.5, 35.4, 24.6	2.38, 0.96							
2	α	68.85	3.84	ddd; 12.0, 4.2, 3.3	-	3.96, 3.18, 1.75-1.81	**2**	α	68.6	3.88	t; 3.1	-	4.04, 3.10, 1.82, 1.78, 1.72
3	α	68.67	3.955	q; 2.9	-	3.84, 1.72-1.78	**3**	α	71.1	4.04	s	-	3.88, 1.84, 1.77
4	α	33.0	1.76	td; 12.9, 2.0	-	3.95, 3.18	**4**	a	33.67	1.78			
	β		1.70	dt; 14.2, 4.0	-	2.38		b		1.83			
5	β	52.0	2.385	dd; 12.7, 4.5	206.7, 35.4, 33.0	1.72, 1.43, 0.96	**5**	β	47.0	2.61	dd; 12.8, 4.5	-	1.77, 1.07
6		206.7	-	-	-	-	**6**			-	-	-	-
7		121.9	5.81	d; 2.6	85.0, 52.0, 35.4	2.03, 1.67	**7**		122.0	5.835	d; 2.5	85.0, 47.0, 36.0	2.05, 1.66, 1.07, 0.585
8		167.9	-	-	-	-	**8**			-	-	-	-
9	α	35.4	3.175	ddd; 11.2, 7.2, 2.6	167.9, 21.8	3.84, 2.09 , 1.83, 1.78, 1.76	**9**	α	36.0	3.10	t; ~8.8	-	3.88, 3.82, 2.06, 1.82, 1.71
10		39.5	-	-	-	-	**10**		44.0	-	-	-	-
11	α	21.8	1.84				**11**	α, β	22.17	1.69-72			
	β		1.63				**12**	α	31.1	2.06			
12	α	31.1	2.09	(t/d)d; 13.0, 8.0	49.0, 21.8, 17.7	3.175, 2.90		β		1.52	dt; ~15 (HMQC), 3.4	-	1.70, 0.585
	β		1.535	ddd; 12.8, 5.3, 1.7	85.0, 49.1, 35.4	1.85, 0.57	**13**		48.9	-	-	-	-
13		48.9	-	-	-	-	**14**		84.8	-	-	-	-
14		85.0	-	-	-	-	**15**	α	32.3	1.66	t (HMQC)	-	5.80, 1.07?
15	α	32.3	1.66					β		2.05			
	β		2.02		24.1?	5.81, 1.82, 0.57	**16**	α	24.0	1.82			
16	α	24.1	1.83					β		1.945	tdd; ~12, 9.1, 2.2	32.3?	5.30, 2.89, 1.69, 0.585
	β		1.945	tdd; ~12, 9.0, 2.3	32.3	5.30, 1.67, 0.57	**17**	α	54.3	2.89	t; 9.2	134.8, 127.7, 48.9, 31.1, 24.0, 18.4, 17.7	5.30, 2.06, 1.95 , 1.82, 1.69, 0.585
17	α	54.3	2.90	t; 9.2	134.9, 127.7, 48.9, 31.1, 24.1, 17.7	5.30, 2.09, 1.83, 1.69	**18**	β	17.7	0.585	s	84.9, 54.3, 48.9, 31.1	5.30 , 2.06, 1.95, 1.70, 1.52, 1.07
18	β	17.7	0.57	s	85.0, 54.3, 48.9, 31.1	5.81, 5.30, 2.03, 1.95, 1.69, 1.63, 1.54, 0.96	**19**	β	20.2	1.07	s	76.6, 47.0, 44.0, 36.0	3.82, 2.61, 1.71, 0.585
19	β	24.6	0.96	s	52.0, 39.5, 37.6, 35.4	2.38, 1.80, 1.64 , 1.43, 0.57	**20**		134.8	-	-	-	-
20		134.9	-	-	-	-	**21**		18.4	1.68	s	134.8, 127.7, 54.3	2.89, 2.13, 1.95 , 1.52, 0.585
21		18.4	1.69	s	134.9, 127.7, 54.3	2.90, 2.13, 1.94, 1.76-1.88, 1.54, 0.57	**22**		127.7	5.30	t; 7.0	54.3, 44.9, 24.3, 18.4	2.89, 2.13, 1.95, 1.50, 1.20, 0.585
22		127.7	5.30	t; 7.1	54.3, 45.0, 24.4, 18.4	2.90, 2.13, 1.95, 1.50	**23**	a, b	24.3	2.125	ABX	134.8, 127.7, 44.9	5.30, 1.49, 1.20
23	a, b	24.4	2.13	ABX	134.9, 127.7, 45.0	5.30, 1.69, 1.20	**24**	a, b	44.9	1.495	ABX	127.7, 71.5, 29.3, 24.3	5.30, 2.15, 1.20
24	a, b	45.0	1.50	ABX	127.7, 71.5, 29.3, 24.4	5.30, 1.20	**25**		71.5	-	-	-	-
25		71.5	-	-	-	-	**26,27**		29.3	1.20	s	71.5, 44.9, 29.3	1.50
26, 27		29.3	1.20	s; I = 6	71.5, 45.0, 29.3	2.13, 1.50							

Table 13. NMR data on 20,22-didehydrotaxisterone (**16**) and 1-hydroxy-20,22-didehydrotaxisterone (**18**)

1-hydroxy-22-deoxy-20,21-didehydro-ecdysone

No.		^{13}C	^{1}H	m; J (Hz)	HMBC	NOESY
1	α	76.6	3.82	s	-	3.11, 1.72 , 1.76
2	α	68.6	3.88	t; 3.1	-	4.05, 3.11, 1.78
3	α	71.1	4.05	s	-	3.88, 1.83, 1.78
4	α	33.7	1.82	t?; 13.5?	-	4.05, 3.88, 3.11
	β		1.77			
5	β	47.0	2.615	dd; 12.8, 4.3	205.9, 35.9, 33.7	1.78, 1.08
6		205.9	-	-	-	-
7		122.0	5.84	d; 2.6	85.1, 47.0, 35.9	2.07, 1.67
8		166.9	-	-	-	-
9	α	35.9	3.11	t; 8.2	166.9	3.88, 3.82, 1.76-1.82, 1.71
10		44.0	-	-	-	-
11	α	22.2	1.77			
	β		1.71			
12	α	31.3	2.11	overlapped	48.5, 17.6	2.99
	β		1.56	d; HMQC		
13		48.5	-	-	-	-
14		85.1	-	-	-	-
15	α	32.3	1.665			
	β		2.06			
16	α	25.3	1.91	t; 8.6	150.3, 85.1, 51.1, 48.5, 32.3	4.91, 2.99, 2.06, 0.61
	β		1.91		150.3, 85.1, 51.1, 48.5, 32.3	4.91, 2.99, 2.06, 0.61
17	α	51.1	2.99	t; 9.2	150.3, 111.2, 48.5, 39.9, 32.3, 31.3, 25.3, 17.6	4.91, 2.12, 2.05, 1.91, 1.57, 1.50
18	β	17.6	0.61	s	85.1, 51.1, 48.5, 31.3	4.91, 2.06, 1.91, 1.72, 1.56, 1.08
19	β	20.2	1.075	s	76.6, 47.0, 44.0, 35.9	3.82, 2.62, 1.72, 0.61
20		150.3	-	-	-	-
21	a	111.2	4.91	s	150.3, 51.1, 39.9	1.91, 0.61
	b		4.95	s	150.3, 51.1, 39.9	2.03-2.09, 1.59, 1.49-1.52
22	a	39.85	2.03	overlapped	150.3, 111.2, 51.1, 24.5	
	b		2.09	overlapped	150.3, 111.2, 51.1, 24.5	
23	a	24.5	1.50			
	b		1.58			
24	a	44.7	1.425	td; 12.5, 4.7	71.6, 29.3-4, 24.5	1.59
	b		1.49	overlapped	71.6, 29.3-4, 24.5	
25		71.6	-	-	-	-
26		29.32	1.18	s	71.6, 44.7, 29.36	1.44-1.52
27		29.36	1.18	s	71.6, 44.7, 29.32	1.44-1.52

22-deoxy-20,21-didehydro-ecdysone

No.		^{13}C	^{1}H	m; *J* (Hz)	COSY
1	α	37.5	1.79		3.85
	β		1.43		3.85
2	α	68.8	3.85	ddd; 12.5, 4.1, 3.4	3.96, 1.79, 1.43
3	α	68.7	3.96	q; 2.6	3.85, 1.72-7
4	α	33.0	1.77		
	β		1.72		
5	β	52.0	2.39	dd; 12.8, 4.7	1.70-1.79
6			-	-	-
7		122.0	5.82	d; 2.7	3.18
8			-	-	-
9	α	35.3	3.18	ddd; 10.2, 7.1, 2.5	5.82, 1.84, 1.65
10			-	-	-
11	α	21.8	1.84		
	β		1.65	t (COSY)	
12	α	31.3	2.14	td; 12.4, 4.4	1.57
	β		1.57		
13			-	-	-
14			-	-	-
15	α	32.2	1.66		
	β		2.05		
16	α	25.4	1.91		2.99, 1.66
	β		1.91		2.99, 1.66
17	α	51.0	2.99	t; 9.2	1.91
18	β	17.7	0.61	s	
19	β	24.6	0.96	s	
20			-	-	-
21	a	111.2	4.91	s	
	b		4.95	s	
22	a	39.9	2.04		
	b		2.08		
23	a	24.6	1.49		
	b		1.57		
24	a	44.7	1.43		
	b		1.49		
25			-	-	-
26		29.3	1.19	s	
27		29.4	1.19	s	

Table 14. NMR data on 1-hydroxy-22-deoxy-20,21-didehydro-ecdysone (**21**) and 22-deoxy-20,21-didehydro-ecdysone (**23**)

3.2.4. Structures of the isolated ecdysteroids

Table 15 and **Figure 7** show the structures of all the isolated ecdysteroids. The IUPAC names of the new compounds are given in **Table 16**. The compounds discovered in nature for the first time are denoted (*).

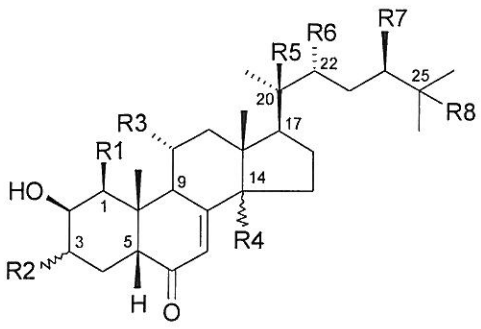
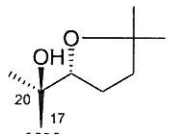
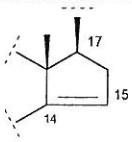
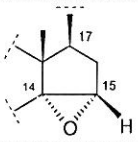
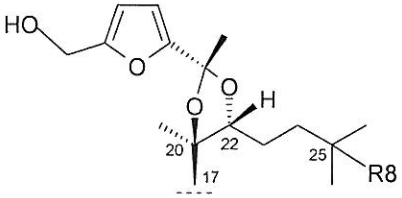
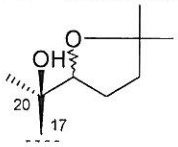
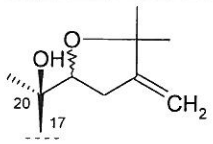
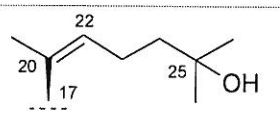
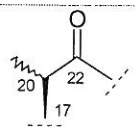
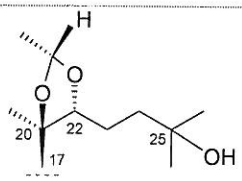
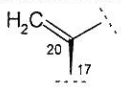
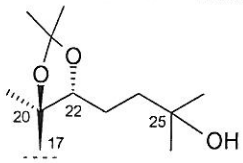
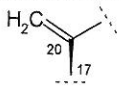
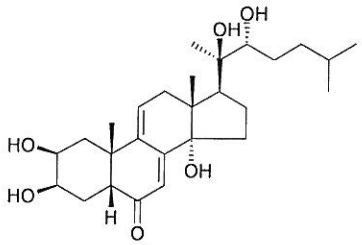


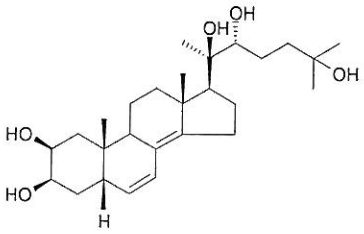
Table 15. Structures of ecdysteroids, containing the classical ecdysteroid characteristics (e.g. 7-en-6-one chromophore)

Ecdysteroid	R1	R2	R3	R4	R5	R6	R7	R8
11α-hydroxyshidasterone (1)*	H	βOH	αOH	αOH				
2β,3α,20R,22R,25-pentahydroxy-5β-14β-cholest-7-en-6-one (2)*	H	αOH	H	βH	OH	OH	H	OH
2β,3α,20R,22R,25-pentahydroxy-5β-14α -cholest-7-en-6-one (3)*	H	αOH	H	αH	OH	OH	H	OH
ponasterone A (5)	H	βOH	H	αOH	OH	OH	H	H
stachysterone B (6)	H	βOH	H		OH	OH	H	OH
14α,15α-epoxy-14,15-dihydrostachysterone B (7)*	H	βOH	H		OH	OH	H	OH
makisterone A (8)	H	βOH	H	αOH	OH	OH	CH_3	OH
serfurosterone A (9)*	H	βOH	H	αOH				OH
serfurosterone B (17)*	H	βOH	αOH	αOH				H
ajugasterone C (10)	H	βOH	αOH	αOH	OH	OH	H	H
20-hydroxyecdysone (11)	H	βOH	H	αOH	OH	OH	H	OH
22-deoxyintegristerone A (12)	OH	βOH	H	αOH	OH	H	H	OH
shidasterone (13)	H	βOH	H	αOH				

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



dacryhainansterone (4)



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

Figure 7. Structures of ecdysteroid dienes

Table 16. Trivial and IUPAC names of the new compounds

Trivial names of new ecdysteroids	IUPAC names of new ecdysteroids
11α-hydroxyshidasterone (**1**)*	22,25-epoxy-2β,3β,11α,14α,20R-pentahydroxy-5β-cholest-7-en-6-one
-	2β,3α,20R,22R,25-pentahydroxy-5β-14β-cholest-7-en-6-one (**2**)*
3-epi-14-deoxy-20-hydroxyecdysone	2β,3α,20R,22R,25-pentahydroxy-5β-14α-cholest-7-en-6-one (**3**)*
14α,15α-epoxy-14,15-dihydrostachystrone B (**7**)*	14α,15α-epoxy-2β,3β,20R,22R,25-pentahydroxy-5β-cholest-7-en-6-one
serfurosterone A (**9**)*	(20R, 22R)-20,22-O-(5'-hydroxymethyl-furfurylidene)-2β,3β,14α,25-tetrahydroxy-5β-cholest-7-en-6-one
-	2β,3β,20R,22R,25-pentahydroxy-5β-cholest-6,8(14)-diene (**14**)*
24-methylene-shidasterone (**15**)*	24-methylene-22,25-epoxy-2β,3β,14α,20R-tetrahydroxy-5β-cholest-7-en-6-one
20,22-didehydrotaxisterone (**16**)*	2β,3β,14α,25-tetrahydroxy-5β-cholest-7,20(22)-diene-6-one
serfurosterone B (**17**)*	(20R, 22R)-20,22-O-(5'-hydroxymethyl-furfurylidene)-2β,3β,11α,14α-tetrahydroxy-5β-cholest-7-en-6-one
1-hydroxy-20,22-didehydrotaxisterone (**18**)*	1β,2β,3β,14α,25-pentahydroxy-5β-cholest-7,20(22)-diene-6-one
22-dehydro-20-deoxy-ajugasterone C (**19**)*	22-dehydro-2β,3β,11α,14α-tetrahydroxy-5β-cholest-7-en-6-one
1-hydroxy-22-deoxy-20,21-didehydro-ecdysone (**21**)*	1β,2β,3β,14α,25-pentahydroxy-5β-cholest-7,20(21)-diene-6-one
22-deoxy-20,21-didehydro-ecdysone (**23**)*	2β,3β,14α,25-tetrahydroxy-5β-cholest-7,20(21)-diene-6-one

4. Discussion

The ecdysteroids are rather polar compounds, and therefore a polar solvent such as methanol is the most suitable for the extraction. Although this solvent is very effective for the extraction of ecdysteroids, it is not selective for them. The extract contained large amounts of other compounds. The next step involved preliminary purification of the crude extract with the aim of removing polar and non-polar contaminants. The fractionated precipitation with acetone was used for the elimination of polar impurities. The resulting acetone-methanol

solution contained the ecdysteroids. The following prepurification step was solid-phase extraction, which was performed on polyamide. This method provides a group separation between ecdysteroids and flavonoids. Ecdysteroids possessing alcoholic hydroxy groups were eluted with water, in contrast with the flavonoids, which remained adsorbed on the stationary phase. The elution of phenoloids needed a higher solvent force.

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. The TLC behaviour (R_f values and colours after spraying with vanillin-sulfuric acid) of the compounds reflects the numbers of free OH groups and the presence of a double bond in the side-chain.

The first step of fractionation was vacuum RP-CC. Chemically bonded octadecyl-silica was employed as stationary phase and elution was performed with a step-gradient. The separation was based on a hydrophobic interaction. The adsorption of ecdysteroids on the stationary phase was eliminated on octadecyl-silica.

The general ecdysteroid isolation procedure was further improved by the introduction of preparative RPC into the purification process. Earlier isolation methods consisted of several adsorption chromatographic steps. RPC has several advantages over these techniques. 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 adsorbent layer for a 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 (102, 103). RP-CC and RPC applied in consecutive steps provided different selectivity and an improved resolution. For example, RPC gave almost complete separation of two ecdysteroids (compounds **4** and **11**) in a single run from the fractions obtained by prepurification and RP-CC of the crude extract. The partial separation in one pass was completed by a repeated pass with another optimized mobile phase, with different selectivity.

The final stage of the isolation was carried out by NP- or RP-HPLC. In NP systems, cyclohexane-isopropanol-water mixtures were generally applied instead of a dichloromethane-based solvent system. Dichloromethane-based solvents suffer from a high UV cutoff, which reduces the sensitivity of detection. Ecdysteroids bind NP silica strongly. Small amounts of water can decrease the adsorption, although it generates deactivation of the stationary phase. RP- HPLC with C_{18}-bonded columns provided efficient separation. Water-

miscible organic solvents (methanol and acetonitrile, as organic modifiers) were used as mobile phases.

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 (17). 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. *Syrov et al.* confirmed that 11α-hydroxyecdysteroids exert strong anabolic effects (25).

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 (104).

Many natural phytoecdysteroids have been screened in gene regulatory systems. Neither E nor 20E 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. *Saez et al.* observed that this compound was a potent inducer of gene expression *in vitro* and *in vivo* alike (105). To study this compound in gene expression systems, ponasterone A has been prepared from *Taxus* and *Podocarpus* species with multi-step chromatographic methods (17). The sophisticated preparation, the lack of good raw material and the lack of synthetic methods increase the price of ponasterone A (1 mg costs $ 43 (106)). *S. wolffii*, primarily in the Asteraceae family, is a source of this biologically active compound. The simplicity of the isolation technique indicates that *S. wolffii* would be a suitable material for the preparation of ponasterone A.

24-Methylshidasterone was isolated from *Vitex* species, as the first shidasterone derivative (17). Two further derivatives, 11α-hydroxyshidasterone (**1**) and 24-methylene-shidasterone (**15**), have now been obtained from *S. wolffii*.

14α,15α-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 (17). The first ecdysteroid possessing a 14,15-epoxide ring, gymnasterone B, exerts significant cytotoxic activity (100). Stereoselective synthesis of this antitumour steroid has already been achieved by *Li et al.* (107).

Serfurosterone A (**9**) and B (**17**) are the first two ecdysteroids from natural sources known to contain a furan ring: they are acetals of 5-hydroxymethyl-furfural and 20E in the case of compound **9,** and ajugasterone C in the case of compound **17**. Structurally related ecdysteroids with an acetal function in the side-chain, 20-hydroxyecdysone 20,22-ethylidene and ajugasterone C 20,22-ethylidene, were earlier isolated from *S. coronata* (61).

A number of 22-O-acyl ester derivatives were prepared by *Suksamrarn et al.* to determine their moulting hormone activities. Among these derivatives, the furan-2-carboxylate moiety was connected to position 22 of 20E. This product was only slightly less active than the parent compound. Their results indicated that a free 22-hydroxy group is not an essential feature for an ecdysteroid to exhibit high moulting activity (108). 20,22-Didehydrotaxisterone (**16**) and 1-hydroxy-20,22-didehydrotaxisterone (**18**) without free 22-hydroxy groups were isolated from *S. wolffii*. 20,22-Didehydrotaxisterone (**16**) and 1-hydroxy-20,22-didehydrotaxisterone (**18**) exhibit unique hydroxylation patterns. They do not have the 20,22-diol structure which is characteristic for ecdysteroids. 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, 20E (LC_{50} = 1.07 ppm). It is again concluded that a free 22-hydroxy group is not an essential feature for biological activity, but 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)

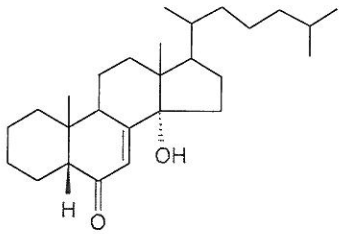


Figure 8/a. Common structure of ecdysteroids

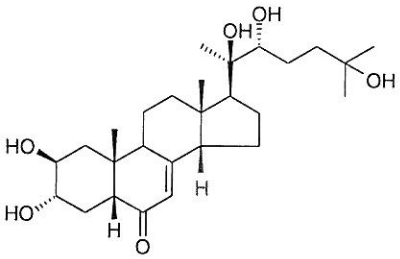


Figure 8/b. Structure of compound **2**

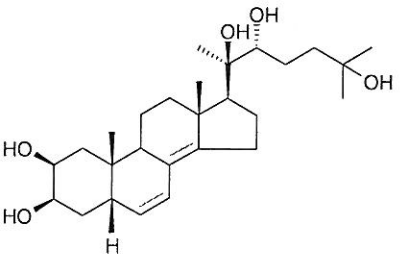


Figure 8/c. Structure of compound **14**

or 20(21).

According to the classical chemical definition, ecdysteroids are steroids, whose nucleus bears a *cis*-fused A/B ring junction, a 7-en-6-one chromophore and a 14α-OH (109). **Figure 8/a** presents these chemical characteristics in red. This definition is not fully satisfactory, since several biogenetically related compounds with closely similar structures have been identified. These ecdysteroid-related molecules are named protoecdysteroids (46). 2β,3α,20R,22R,25-Pentahydroxy-5β-14β-cholest-7-en-6-one (**2**) and 2β,3β,20R,22R,25-pentahydroxy-5β cholest-6,8(14)-diene (**14**) are new members of this group. The structures of these compounds are given in **Figure 8/b** and **8/c**.

In compound **2,** the lack of the 14-OH and the anellation of rings C/D would demand a new definition of ecdysteroids. The structurally-related compounds 14-epi-20-hydroxyecdysone and 14-epi-ponasterone A 22-glucoside were previously identified in *S. wolffii* and *L. chartamoides*, respectively (69, 110).

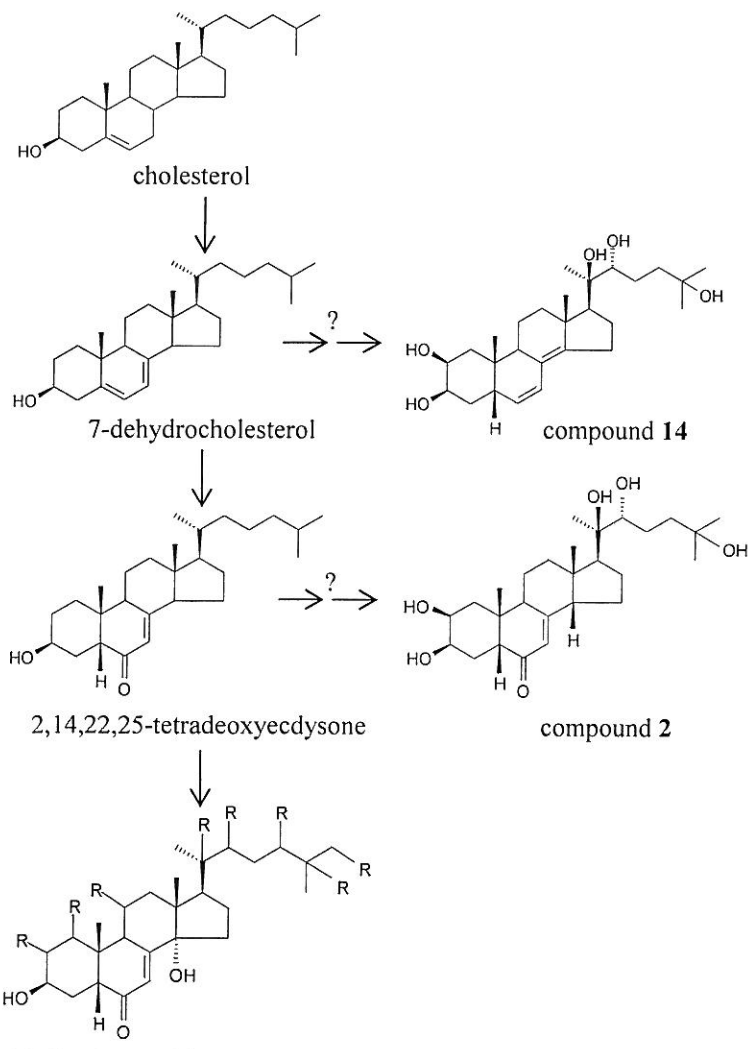


Figure 9. The biosynthetic pathway of ecdysteroids

Nagakari et al. considered that 14α-hydroxylation takes place after the formation of the 5β-H,7-en-6-one system (111). 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 ecdysteroid biosynthesis. The 7-en-6-one system is likely to derive from steroids with diene structures (112). Some authors have verified that the last reaction of biosynthesis is the hydroxylation at C-2, C-22

and C-25 (113). This observation is in contrast with our hypothesis. We consider that the hydroxylation at C-2, C-22 and C-25 might precede the formation of the 7-en-6-one functional group system. However, confirmation of this hypothesis demands further evidence. **Figure 9** illustrates the biosynthetic pathway of ecdysteroids in plants.

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. *L. 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 high content of phytoecdysteroids in the roots or seeds of *Leuzea* and the large structure variability of minor compounds make this species the most important ecdysteroid source (11,114).

Table 17. Ecdysteroids originating from *S. wolffii* and *L. carthamoides*

		S. wolffii L.	Ref.	*L. carthamoides* DC.	Ref.
Main ecdysteroids		ajugasterone C (**10**), 20E (**11**), makisterone A (**8**), polypodine B	71	ajugasterone C, 20E, makisterone A, polypodine B	115
Minor ecdysteroids	11α - hydroxyecdysteroids	isovitexirone, turkesterone 11α-hydroxyposterone	116 68	isovitexirone rapisterone B	117 118
	3-epi-ecdysteroids	3-epi-20E 2β,3α,20R,22R,25-pentahydroxy-5β-14α-cholest-7-en-6-one (**3**)	116 -	3-epi-20E rapisterone D	119 120
	5α-ecdysteroids	5α-20E	116	5α-20E	119
	22-oxo-ecdysteroids	22-dehydro-20-deoxy-ajugasterone C (**19**)	-	22-oxo-20E	119
	7,9(11)-diene-6-ones	dacryhainansterone (**4**) 25-hydroxydacryhainansterone herkesterone	- 69 68	dacryhainansterone	17
	ecdysteroid-acetonides	ajugasterone C 2,3;20,22-diacetonide, ajugasterone C 20,22-monoacetonide, 20E-2,3;20,22-diacetonide, 20E-20,22-monoacetonide	67	20E-2,3-monoacetonide, 20E-20,22-monoacetonide, 20E-2,3;20,22-diacetonide, polypodine B 20,22-monoacetonide	117
	14-epi-ecdysteroids	14-epi-20E 2β,3α,20R,22R,25-pentahydroxy-5β-14β - cholest-7-en-6-one (**2**)	69 -	14-epi-ponasterone A 22-glucoside	110

Table 17 presents a comparison between the compounds of *L. carthamoides* and those of *S. wolffii*. The ecdysteroid profiles of the two plants display similarity not only in the major ecdysteroids, but also in the minor constituents. Both plants are 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 pattern of compounds 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 the manufacture of preparations.

5. Summary

Our results may be summarized as follows:

1.

- Twenty-three ecdysteroids were isolated and characterized from the roots of *S. wolffii*.
- Thirteen of the compounds have been discovered for the first time in a natural source.
- Four of the known ecdysteroids have been found in the *Serratula* genus for the first time.

2. Structures and biological significance of the isolated ecdysteroids.

- Four ecdysteroids contain an 11α-OH group. C-11 hydroxylation improves the anabolic effect.
- Dacryhainansterone, which is the most active compound in insect hormonal bioassays, was identified in the roots of *S. wolffii*.
- The biological activities of 1-hydroxy-20,22-didehydrotaxisterone and 20,22-didehydrotaxisterone confirmed that the 22-oxygen function plays an important role as an H-bond acceptor in the moulting hormone activity.
- *S. wolffii* is a new plant source of ponasterone A, which is employed in gene regulation systems in increasing amounts.
- The second ecdysteroid with a 14,15-epoxide ring (14α,15α-epoxy-14,15-dihydrostachystrone B) has been isolated from nature.

- The first two ecdysteroids (serfurosterone A and B) containing a furan ring have been obtained from *S. wolffii*.
- Two compounds (2β,3α,20R,22R,25-pentahydroxy-5β-14β-cholest-7-en-6-one and 2β,3β,20R,22R,25-pentahydroxy-5β-cholest-6,8(14)-diene) 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 surprising discovery that there are 14-epi-ecdysteroids appears to disprove the classical chemical definition of ecdysteroids. Both compounds provide a possibility to devise a theory of new biosynthetic pathways.

3. Significance of the study from methodology aspects.

- A new isolation process has been developed. After the 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.
- RPC was introduced and successfully used in the isolation procedure to obtain pure ecdysteroids. This on-line preparative chromatographic method is an effective and inexpensive tool for the separation of ecdysteroids in complex mixtures with low solvent usage and less time consumption.

4. *S. wolffii* as a good source of ecdysteroids

- The high ecdysteroid content of *S. wolffii* and the chemical similarity demonstrate that *S. wolffii* could be an equivalent source to *L. carthamoides*, which is one of the most important ecdysteroid-containing plants.

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Role of Preparative Rotation Planar Chromatography in the Isolation of Ecdysteroids

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Abstract: A reliable isolation procedure is elaborated for the purification of ecdysteroids from *Serratula wolffii*. The procedure is also applicable to other plant sources. The general ecdysteroid isolation procedure was improved by the introduction of preparative rotation planar chromatography (RPC) to the purification process. Effective and simple cleanup and vacuum reversed-phase column chromatographic separation was completed with RPC, or repeated RPC, to obtain pure ecdysteroids (ajugasterone C, dacryhainansterone, 22-deoxy-integristerone A, 20-hydroxyecdysone, and 2 new ecdysteroids) from the crude extract. This paper discusses the advantages of this method as the final step of ecdysteroid isolation.

Keywords: Ecdysteroids, Plant material, *Serratula wolffii*, Rotation planar chromatography

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INTRODUCTION

Ecdysteroids occur in plants in multicomponent mixtures of structurally related compounds.[1,2] Their isolation generally involves a complex combination of a series of preparative-scale chromatographic procedures, such as thin-layer chromatography, normal- and reversed-phase column chromatography, flash chromatography, droplet counter-current chromatography (DCCC), gel chromatography, and high performance liquid chromatography (HPLC).[3–5]

There has recently been a great interest in ecdysteroids because of their uses in both traditional and modern medicine and agriculture.[6,7] The ecdysteroids exert molting hormone activity to insects and various forms of significant pharmacological activities to mammals, including humans. Their anabolic action without thymolytic and androgenic side-effects is the most thoroughly investigated and scientifically proved[8] aspect of their pharmacological behavior.

Ecdysteroids have also attracted interest as inducers of gene-regulation systems based on the ecdysone receptors of insects. Ecdysteroid 7,9(11)-dienones seem to be suitable ecdysteroid receptor agonists, as they exert high hormone activity on insects binding with high affinity to the ecdysteroid receptor.[9]

The pharmacological and biological importance of ecdysteroids has initiated attempts to improve their isolation procedures. The *Serratula* plant species are rich sources of ecdysteroids. *S. wolffii* is characterized by a high accumulation and wide structural diversity of ecdysteroids. These species biosynthesize a series of biologically active ecdysteroids, among them 11-hydroxylated ecdysteroids, and ecdysteroids with 7,9(11)-dienone structures. 11-Hydroxylation is important for manifestation of the anabolic action of ecdysteroids.[10]

The aim of the present work was to improve and simplify the procedure of ecdysteroid isolation and to study the role of rotation planar chromatography in this process. This paper follows the nomenclature of planar chromatography outlined by Nyiredy et al.,[11] so RPC is the abbreviation of rotation planar chromatography, not reversed-phase chromatography. The effective cleanup and optimized combination of vacuum reversed-phase column chromatography on octadecyl silica and repeated preparative rotation planar chromatography on silica resulted in pure, biologically active ecdysteroids, such as ajugasterone C, dacrychainansterone, 22-deoxy-integristerone A, 20-hydroxyecdysone, and two earlier unknown ecdysteroids. The ecdysteroids were identified using thin-layer chromatography (TLC) and mass spectrometry (MS).

EXPERIMENTAL

Plant Material

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

TLC

TLC was carried out on 20 × 20 cm glass plates coated with silica gel F_{254} (E. Merck, Darmstadt, Germany). The plates were developed by the ascending technique in an unsaturated glass chamber (Desaga, Heidelberg, Germany) at room temperature. The following mobile phases were used:

- dichloromethane-methanol-benzene (50:10:6 v/v/v);
- ethyl acetate-96% ethanol-water (80:10:5 v/v/v).

The plates were developed to a distance of 148 and 150 mm (used for the separation of ajugasterone C). After development of the plates, the ecdysteroids were detected either directly by fluorescence extinction at 254 nm or by the use of vanillin-sulfuric acid spray reagent. After spraying, the spots were observed either in daylight or at 366 nm.

Densitograms were recorded using a Shimadzu CS-9301PC densitometer (Osaka, Japan) in the reflectance-absorbance mode at 254 nm.

RPC

A Harrison Model 8924 Chromatotron instrument (Harrison Research, Palo Alto, CA) was used. The stationary phase was silica gel 60 GF_{254} (E. Merck), manually coated on the rotor as a 1 mm layer. Previous TLC experiments served as a tentative guide for mobile phase selection. Stepwise development was used with nine mobile phases (100 mL each):

- System 1/A: chloroform-methanol-benzene (50:3:2 v/v/v);
- System 1/B: chloroform-methanol-benzene (50:5:3 v/v/v);
- System 1/C: chloroform-methanol-benzene (50:10:6, v/v/v);
- System 2/A: ethyl acetate-ethanol-water (80:2:1, v/v/v);
- System 2/B: ethyl acetate-ethanol-water (80:5:2, v/v/v);
- System 2/C: ethyl acetate-ethanol-water (80:10:5, v/v/v);
- System 3/A: ethyl acetate-ethanol-water (80:5:2, v/v/v);
- System 3/B ethyl acetate-ethanol-water (80:7:3, v/v/v);
- System 3/C: ethyl acetate-ethanol-water (80:10:5, v/v/v).

Before applying the sample, the dry stationary phase was completely wetted with the firstly applied mobile phase (either solvent system 1/A or 2/A, 3/A, 50 mL in each case), and a further 5 min was allowed for equilibration. The solution of the sample (see below) dissolved in the first elution solvent (3 mL) was introduced through the inlet. The mobile phase flow rates were 4–5 mL min^{-1} (see below); in each case, thirty 10 mL fractions were collected. Therefore, the total elution times were 60 min and 70 min. The sorbent layers were regenerated with 50 mL of methanol. The separation

was monitored by observing under ultraviolet (UV) illumination at 254 nm and by TLC, and the separation of the fractions was verified by normal-phase TLC.

Vacuum Reversed Phase Chromatography (RP-CC)

Vacuum RP-CC was carried out on end-capped octadecyl silica (0.06–0.2 mm particle size) (Chemie Urticon-C-gel, C-560, Ueticon, Switzerland) packed into a 400 × 32 mm glass column. Stepwise gradient elution was done using methanol-water (30:70, 35:65, 40:60, 45:55, 50:50, 55:45, and 60:40 v/v); 1,000 mL each.

Solid Phase Extraction (SPE)

The stationary phase MN-polyamide SC 6 for column chromatography, 0.06–0.16 mm (Woelm, Eshwege, Germany) was used for the cleanup. The ecdysteroids were eluted with deionized water.

Ecdysteroid Isolation

The fresh roots of *S. wolffii* (4763 g) were washed, milled, and percolated with 20 L of methanol at room temperature. The extract was evaporated, the dry residue (208.9 g) was dissolved in 800 mL of methanol, and 400 mL of acetone was then added to the solution. The formed precipitate was separated by decantation and was washed three times with 100 mL of methanol-acetone (2:1 v/v) mixture. The methanol-acetone solutions were combined and evaporated to dryness, and the residue was dissolved in 700 mL of methanol. The precipitation was repeated twice, using 700 mL and 1,200 mL of acetone. The final residue of the methanol-acetone solution, obtained after the fractionated precipitation (137.5 g), was dissolved in 300 mL of methanol, adsorbed onto polyamide stationary phase, and packed into the top of a column containing 344 g polyamide (210 × 145 mm). The ecdysteroids were eluted from the polyamide with 2 L of water. The aqueous solution was evaporated, and the dry material (24.4 g) dissolved in 50 mL of methanol.

Vacuum RP-CC was carried out in two parallel procedures. Half of the methanolic solution was applied to the top 150 g of end-capped octadecyl silica packed in the column. Elution from the column was carried out with stepwise gradients of 30%, 35%, 40%, 45%, 50%, 55%, and 60% aqueous methanol (1,000 mL each) at a flow rate of 5 mL · min^{-1}, and 200 mL fractions were collected. Fractions 18–23 were combined and evaporated to dryness. The dry residue (0.39 g) was dissolved in 3 mL of RPC developing

solvent 1/A and applied to the middle of the rotation plate after equilibration. The plate was developed stepwise with three mobile phases (solvents 1/A, 1/B, and 1/C). The effluents were collected manually in test tubes.

The contents of each fraction were checked using conventional TLC. The fractions containing the same composition were combined and evaporated to dryness. The number of combined fractions was at least nine, and these were again investigated by TLC. The combined fractions A, C, E (derived from fractions 5–6, 10–14, and 24–25, respectively) were further purified by crystallization.

The combined fractions B (derived from fractions 7–8, dry residue 17 mg) and D (derived from fractions 18–23, dry residue 48 mg) were fractionated again by RPC using solvent systems 2/A, 2/B, and 2/C. The RPC separation was carried out similarly as above, but the mobile phase flow rate was now 4 mL min^{-1}. The contents of each fraction were controlled by conventional TLC. The fractions containing the same compounds were combined and evaporated to dryness. In the first case (combined fractions B), the repeated RPC resulted in pure dacryhainansteron (1 mg) in fraction 2. In the second case (combined fractions D), two pure ecdysteroids were obtained, a new ecdysteroid (ecdysteroid 1, 15 mg) and ajugasterone C (14 mg). According to the TLC measurements, these ecdysteroids were present in fractions 3–6 and 11–12, respectively.

The methanolic washing solution obtained during regeneration of the plate (50 mL) was further purified by RPC in the same way, using solvent systems 3/A, 3/B, and 3/C, with a mobile phase flow rate of 4 mL min^{-1}. Fractions 14–18 contained pure 22-deoxy-integristerone A (3 mg).

RESULTS

Our isolation procedure involved two main steps:

1. Extraction and cleanup of the crude extract using simple non-chromatographic methods.
2. Separation of the ecdysteroids by using preparative-scale chromatographic techniques, such as vacuum RP-CC and preparative RPC on silica.

Figure 1 shows the isolation procedure of ecdysteroids.

The ecdysteroids were subjected to exhaustive extraction with methanol in a percolator at a solvent:plant ratio of 7:1. The extraction resulted in an eight-fold purification. The first step of the cleanup involved fractionated precipitation with acetone, as described earlier.[3] The consecutive precipitation steps removed the overwhelming majority of the polar contaminants. For this, the crude methanolic extract was mixed with acetone in extract:acetone volumetric ratios of 2:1, 1:1, and 1:2. The resulting acetone-methanolic

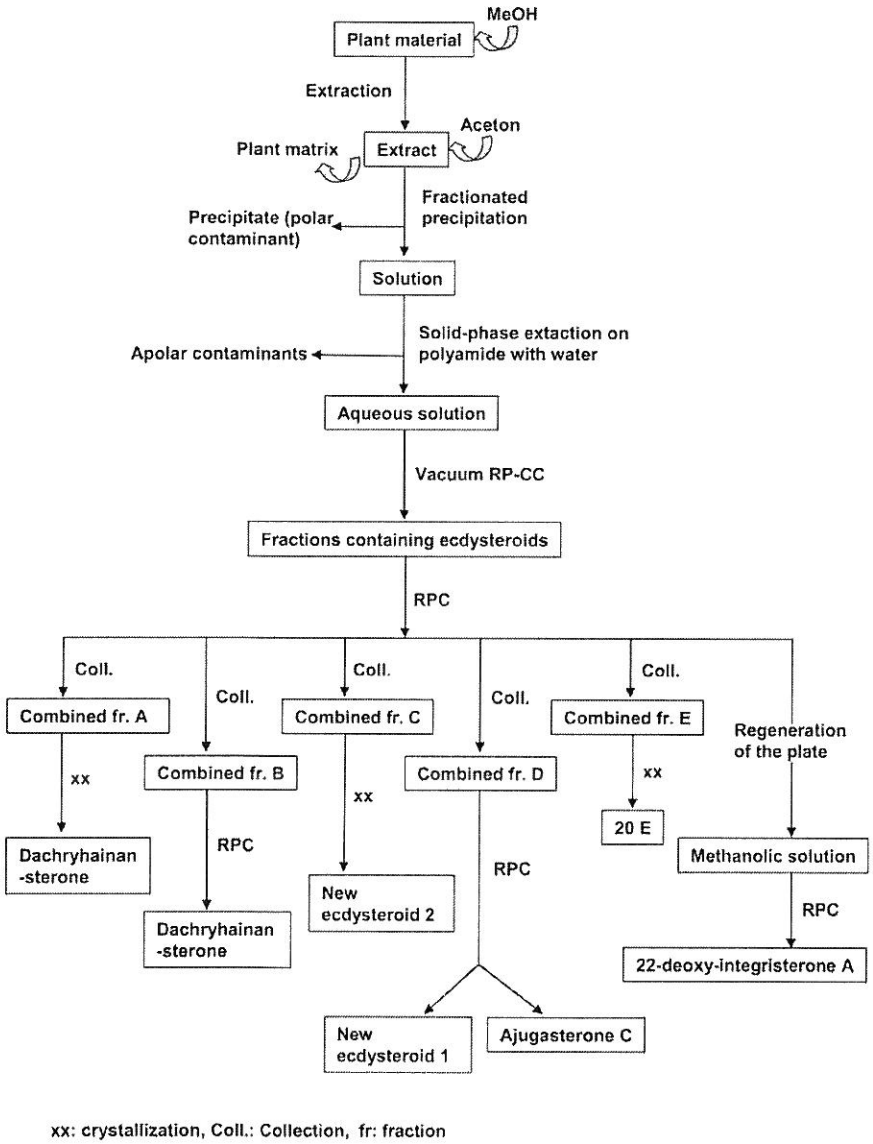

Figure 1. Isolation procedure of ecdysteroids.

solution contained the ecdysteroids, while the precipitate consisted of the impurities. The next cleanup step was by SPE on polyamide, carried out on a home-made set-up, in which 344 g of polyamide was loaded with 137.1 g of sample. The sample was adsorbed onto the polyamide, and the ecdysteroids were eluted from the sorbent with water. The impurities, mainly phenoloids, remained adsorbed on the polyamide. The overall cleanup procedure resulted in a 5-fold purification.

After the cleanup, vacuum RP-CC on octadecyl silica resulted in a crude separation of the prepurified extract. The fractions obtained by RP-CC contained complex mixtures of structurally related ecdysteroids. The ecdysteroids of interest were eluted with 45% and 50% aqueous methanol (Figure 2). These fractions were combined and further purified by preparative RPC on silica.

In the first RPC separation, 42 g of stationary phase was loaded with 390 mg of dry sample, giving an adsorbent-sample ratio of 100:1. Fractionation was carried out with stepwise gradient elution (solvent systems 1/A-1/C) in three steps. The starting mobile phase was chosen by decreasing the solvent strength employed in conventional analytical TLC. The fractionation was controlled by conventional TLC. The fractions containing the same ecdysteroids were combined. TLC analysis has shown that RPC in a single run resulted in three almost pure ecdysteroids: combined fractions A contained dacryhainansterone, combined fractions E 20-hydroxyecdysone, and combined fractions C an earlier unknown new ecdysteroid in almost pure form (Figure 3). To obtain spectroscopically pure ecdysteroids from these fractions, simple crystallization was done.

Combined fractions B contained a mixture of five ecdysteroids (Figure 4A), among which dacrychainanstreone was the main component. These ecdysteroids were further purified by the repeated use of RPC employed with solvent systems with different selectivities (solvent systems 2/A–C). The RPC was carried out as described above. Dacrychainanstreone was eluted at the beginning of the separation with solvent system 2/A (Figure 4B).

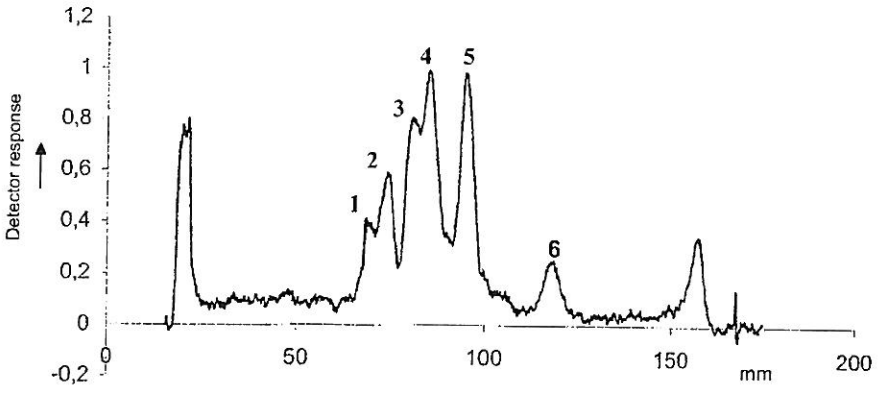

Figure 2. Densitogram of an RP-CC fraction containing the ecdysteroids of interest. Peaks: *1* = 22-deoxy-integristerone A; *2* = 20-hydroxyecdysone; *3* = new ecdysteroid 1; *4* = new ecdysteroid 2; *5* = ajugasterone C; and *6* = dacrychainansterone. Stationary phase: silica gel. Mobile phase: ethyl acetate–96% ethanol - water (80:10:5 v/v/v).

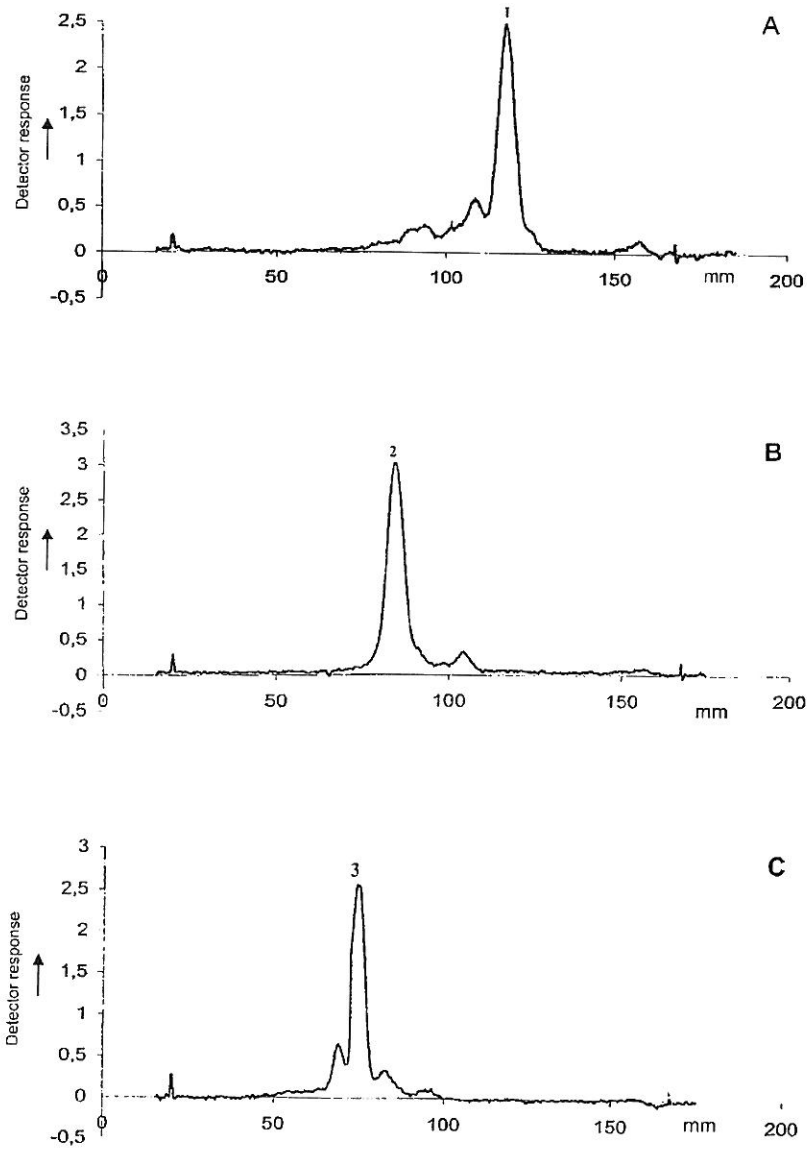

Figure 3. Densitogram of the combined fractions A, C, and E obtained in the first RPC separation. Peaks: *1* = dacrychainansterone (Figure 3A); *2* = new ecdysteroid 2 (Fig. 3B); *3* = 20-hydroxyecdysone (Figure 3C). Stationary phase: silica gel. Mobile phase: ethyl acetate–96% ethanol – water (80:10:5 v/v/v).

Ajugasterone C was eluted in the first RPC separation with solvent systems 1/B and 1/C, together with another main compound (combined fractions D) (Figure 5A). Repeated RPC resulted in a new ecdysteroid (solvent system 2/A, Figure 5B) and pure ajugasterone C (solvent system 2/B, Figure 5C).

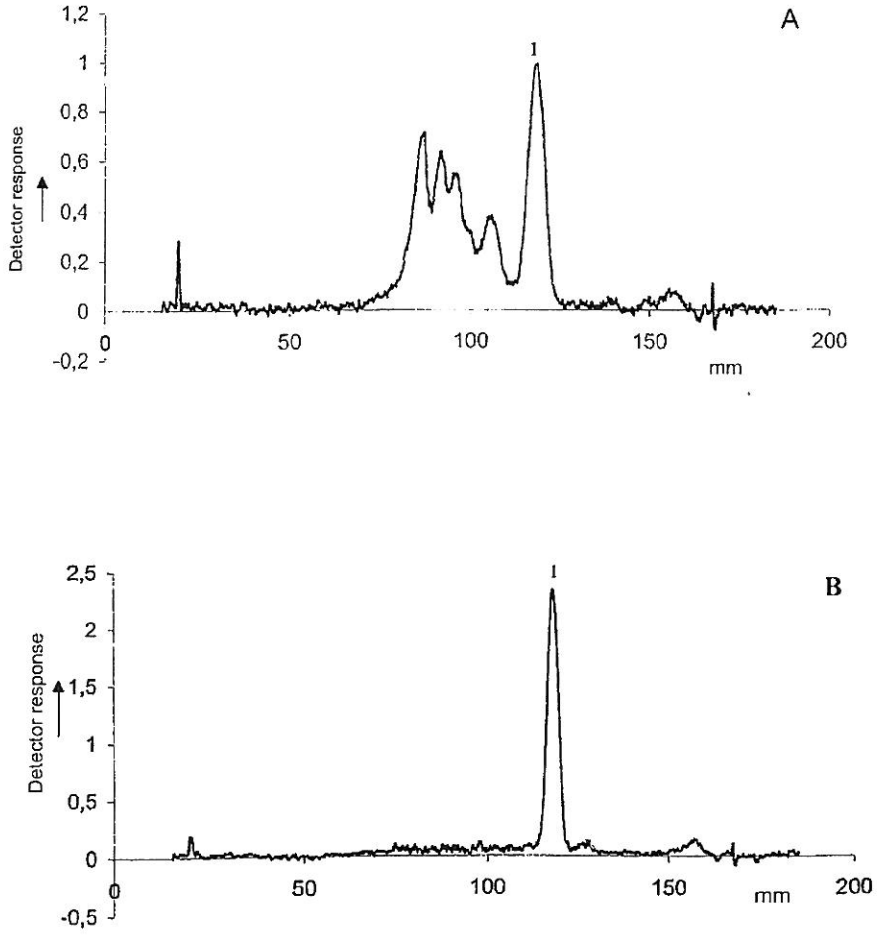

Figure 4. Densitogram of the dacrychainansterone-containing fraction (combined fractions B) obtained in the first RPC separation (Figure 4A) and densitogram of pure dacrychainansterone obtained from this fraction by repeated RPC (Figure 4B). Peak *1* = dacrychainansterone. Stationary phase: silica gel. Mobile phase: ethyl acetate – 96% ethanol–water (80:10:5 v/v/v).

After the fractionation in the first RPC process, a polar ecdysteroid (22-deoxy-integristerone A) remained adsorbed on the stationary phase, and its elution required the use of a polar solvent such as methanol. The use of 50 mL of methanol mm^{-1} of layer thickness was sufficient for the desorption of this ecdysteroid, and 22-deoxy-integristerone A eluted together with some impurities (Figure 6A). The impurities were removed by a repeated RPC process with solvent systems 3/A-3/C to obtain pure 22-deoxy-integristerone A (Figure 6B). The compound was eluted in the middle of this fractionation when solvent system 3/B was in use.

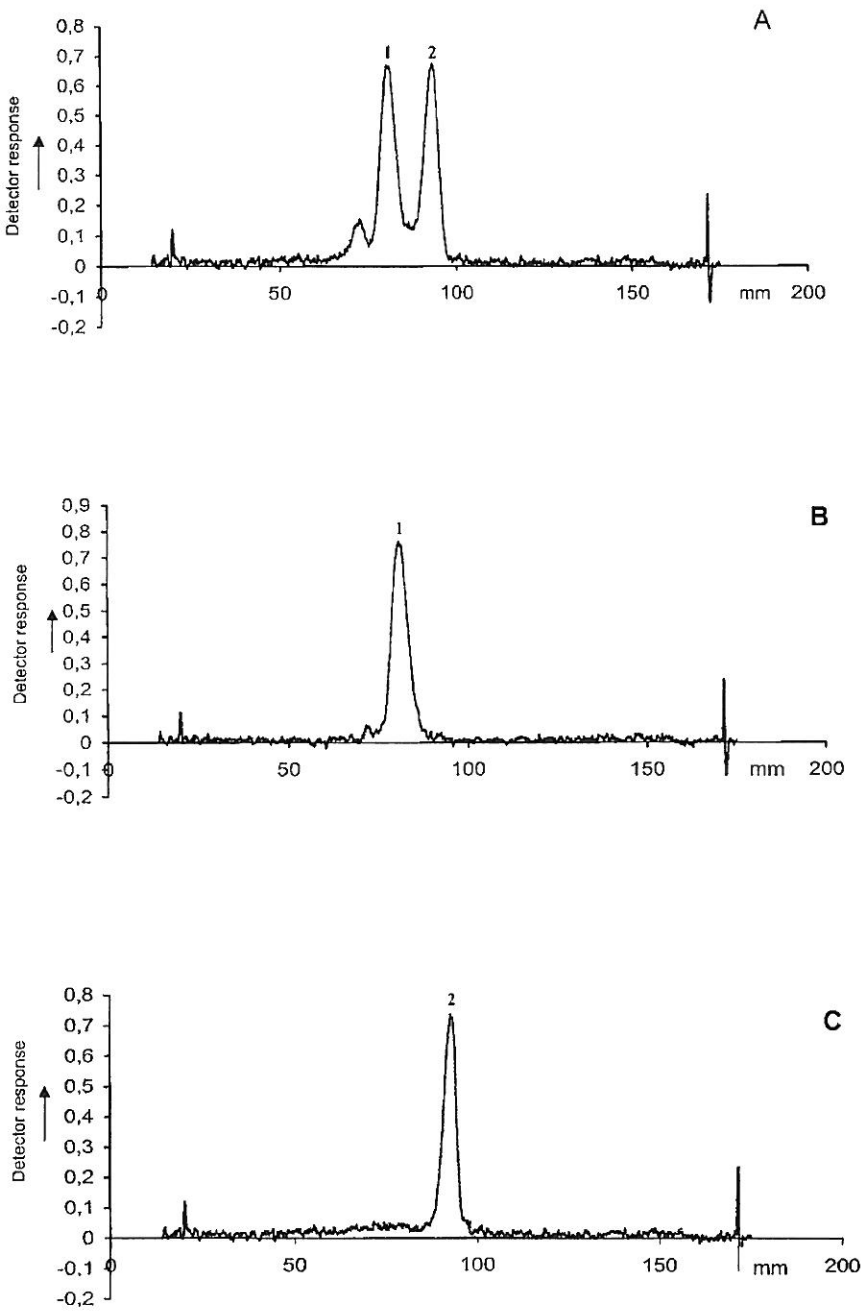

Figure 5. Densitogram of the ajugasterone C-containing fraction (fraction D) obtained in the first RPC separation (Figure 5A) and densitogram of a new ecdysteroid and pure ajugasterone C obtained from this fraction by repeated RPC (Figure 5B and 5C, respectively). Peaks: *1* = new ecdysteroid 1; *2* = ajugasterone C. Stationary phase: silica gel. Mobile phase: ethyl acetate–96% ethanol–water (80:10:5 v/v/v).

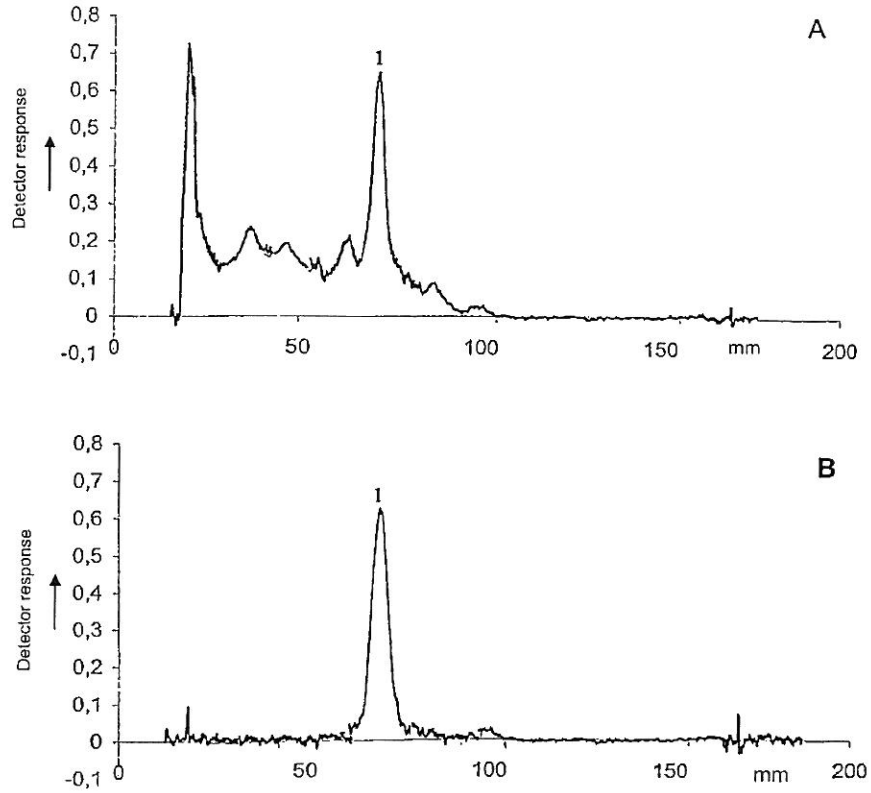

Figure 6. Densitogram of the solution obtained by the regeneration of the rotation plate after the first RPC separation (Figure 6A) and densitogram of pure 22-deoxy-integristerone A obtained from this solution by repeated RPC (Figure 6B). Peak *1* = 22-deoxy-integristerone A. Stationary phase: silica gel. Mobile phase: ethyl acetate – 96% ethanol – water (80:10:5 v/v/v).

The chemical structures of the newly isolated ecdysteroids have been determined by spectroscopic methods (to be published later). The known ecdysteroids were identified by TLC and MS; the compounds were identical to the reference compounds, they migrated together, and they gave the same molecular mass peak and fragments. Figure 7 shows the structures of the isolated known ecdysteroids.

DISCUSSION

As described, a sophisticated strategy was developed for the isolation of ecdysteroids from *S. wolffii*. Their isolation from the partially purified plant extract was based on a combination of vacuum RP-CC and RPC. Our aim in using

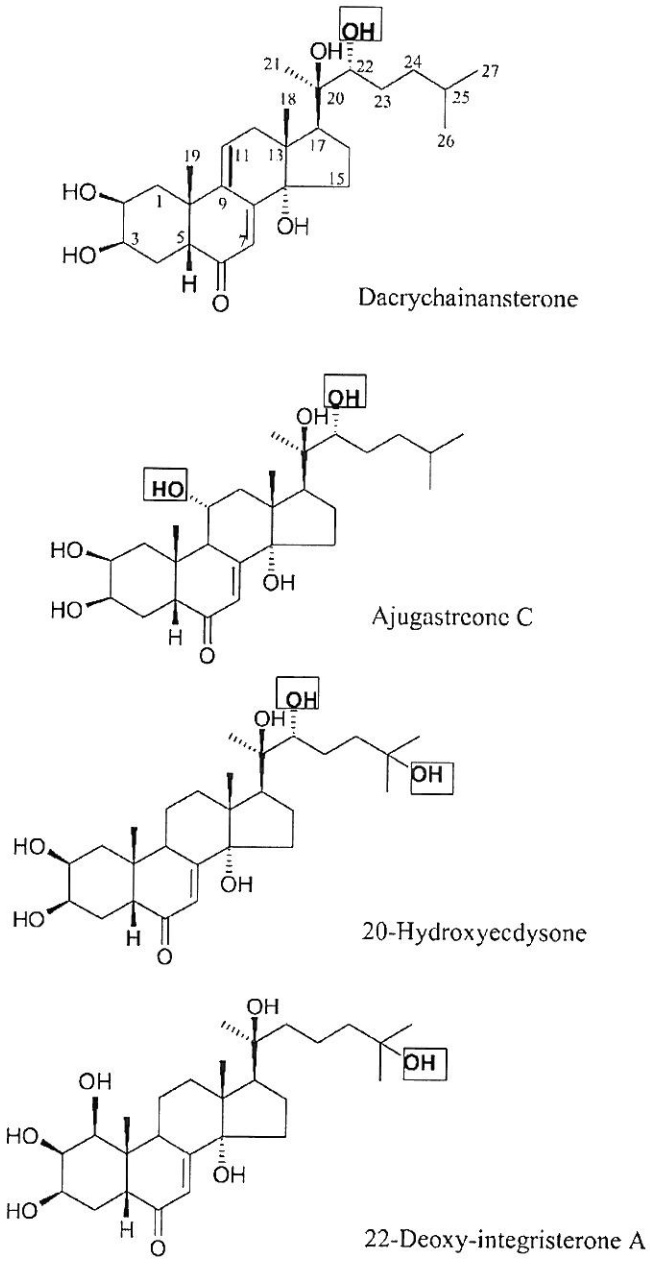

Figure 7. The structures of the isolated known ecdysteroids.

RPC was to develop a new method for the rapid separation of ecdysteroids. Earlier isolation methods consisted of several absorption chromatographic steps, with both column and planar techniques and DCCC, while preparative TLC and/or HPLC was used for final purification. In this new isolation

process, the use of only two chromatographic methods proved sufficient to obtain pure ecdysteroids. Based on the different physical-chemical characteristics of the ecdysteroids, RPC and RP-CC applied in the consecutive steps provided different selectivities and improved resolution. Here, in the final purification step, TLC and HPLC have been replaced by RPC. The earlier separation procedure[2–5] was improved and simplified by the use of RPC on silica, together with RP-CC.

Preparative, centrifugally accelerated, RPC achieved with the Chromatotron instrument ensured good separation of the ecdysteroids from *S. wolffii*. This procedure is generally applicable to other plant sources also.

Several conditions, such as the layer thickness, different mixtures of solvent systems, and the solvent flow rate, which depends on the rotation speed, were adjusted to achieve the best separation.[11–13] It was established that pre-wetting of the silica plate was required for successful separation. A lower flow rate (<4 mLmin^{-1}) did not lead to an improved resolution. RPC gave almost complete separation of three ecdysteroids in a single run from the fractions obtained by prepurification and RP-CC on the crude *S. wolffii* extract. The partial separation in one pass was completed by a repeated pass with another optimized mobile phase, with different selectivity.

The elution order of the ecdysteroids with six hydroxyl groups was unusual when RPC with solvent systems 1/A-1/C were used: ajugasterone C, containing a hydroxyl group at position 11, was eluted earlier than 20-hydroxyecdysone and 22-deoxy-integristerone A. The latter two ecdysteroids are hydroxylated at position 25 (Figure 7). This shows that the chromatographic behavior of the ecdysteroids is strongly affected by the positions of the hydroxy groups.[14,15] Hydroxylation at position 25 has a pronounced effect on the chromatographic characteristics because this hydroxyl group is located in a hydrophobic part of the molecule. It is interesting that 22-deoxy-integristerone A was eluted later than 20-hydroxyecdysone, which has an additional hydroxyl group on the apolar side-chain at position 22.

The use of RPC as a final purification step in ecdysteroid isolation offers some advantages:

- RPC is easier to carry out than the conventional preparative TLC separation.[16]
- The ecdysteroids are in contact with the adsorbent layer for a shorter time than in TLC. Therefore, the problems associated with adsorbent-assisted decomposition are reduced.
- RPC is an on-line preparative chromatographic method, newly introduced for ecdysteroid isolation. It is a simple forced-flow technique driven by centrifugal force. Therefore, this procedure is faster than preparative TLC and provides better separation. RPC is an effective, inexpensive tool for the separation of ecdysteroids in a complex mixture with low solvent usage and less time consumption.
- After one cleaning with methanol, the silica gel layer gave sharper bands.

- RPC permits a larger loading capacity and favorable operating simplicity as compared to HPLC.
- RPC is a very convenient procedure in the final purification steps of ecdysteroid isolation, when 250 mg–1.5 g sample must be separated.

This newly developed purification procedure has led to the isolation of two previously unknown ecdysteroids and several known, biologically important ecdysteroids, such as ajugasterone C, dacrychainansterone, 22-deoxy-integristerone A, and 20-hydroxyecdysone.[6,7] 20-Hydroxyecdysone is the main ecdysteroid of plants and possesses several scientifically proven pharmacological effects. Ajugasterone C is an 11-hydroxylated ecdysteroid, while dacrychainansterone has a 7,9(11)-dienone structure; they attract attention as anabolic and receptor agonists.

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Ecdysteroids from *Serratula wolffii* Roots

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Two new natural ecdysteroids, 20,22-didehydrotaxisterone (**1**) and 1-hydroxy-20,22-didehydrotaxisterone (**2**), were isolated from the roots of *Serratula wolffii*. Their structures were elucidated by 1D and 2D NMR spectroscopy and mass spectrometry. The biological activities of these compounds were determined via oral aphid (*Acyrthosiphon pisum* (Harris)) tests. Compound **1** was inactive and compound **2** exhibited very low toxicity in the oral aphid test. The activities of these two ecdysteroids were in agreement with those of other 22-deoxyecdysteroids.

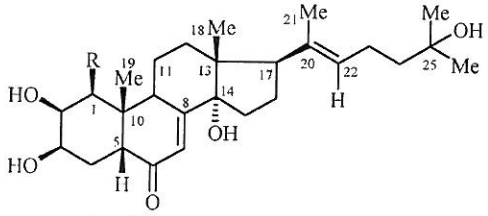
Zooecdysteroids are steroid hormones that control the molting of arthropods.[1] Phytoecdysteroids, compounds structurally related to zooecdysteroids, are widely distributed secondary constituents of plants.[2] Many plant species biosynthesize phytoecdysteroids for protection against insects. Ecdysteroids are lead compounds for the development of selective invertebrate pest control agents.[3] The occurrence of ecdysteroids in relatively large amounts in plant species has made investigation of their pharmacological effects possible. Ecdysteroids exert significant anabolic action without androgenic side effects.[4] Recent research into ecdysteroids has been intensified as a consequence of their application in gene expression systems.[5]

Serratula species are rich sources of ecdysteroids.[6,7] Eighteen ecdysteroids have been reported to occur in *Serratula wolffii* Andrae (Asteraceae).[8] The aerial parts of this species produce not only several known biologically active ecdysteroids containing 11-hydroxy and 7,9(11)-dienone moieties, but also a series of minor new ecdysteroids. These results stimulated our interest in the possible presence of ecdysteroids in the roots of *S. wolffii*. The isolation of known ecdysteroids from this part of this plant has already been published.[9]

We now report the isolation and structure determination of two new 20,22-didehydro derivatives of taxisterone:[10] 20,22-didehydrotaxisterone (**1**) and 1-hydroxyl-20,22-didehydrotaxisterone (**2**). These compounds were tested for toxicity on the L_1−L_2 larvae of *Acyrthosiphon pisum* (Harris) via oral uptake.

A methanol extract of the roots of *S. wolffii* was purified by a multistep isolation procedure[9] including precipitation, column chromatography on polyamide and on octadecyl-silica, and rotation planar chromatography. The final chromatographic step using preparative HPLC afforded compounds **1** and **2**.

Compound **1** was assigned the molecular formula $C_{27}H_{42}O_5$ (using HRESIMS). Its UV spectrum revealed absorption at 242 nm (log ϵ = 4.387) characteristic of an α,β-unsaturated ketone. The electrospray mass spectrum demonstrated a quasimolecular ion at *m/z* 485 $[M + K]^+$. The characteristic fragment ions were formed from the intact parent compound by the loss of water: *m/z* 429 $[M + H - H_2O]^+$, *m/z* 411 $[M + H - 2H_2O]^+$, and *m/z* 393 $[M + H - 3H_2O]^+$.



1 R = H
2 R = OH

On the basis of the molecular ion peak observed by HRESIMS, compound **2** was assigned the molecular formula $C_{27}H_{42}O_6$. ESIMS indicated pseudomolecular ions at *m/z* 501 $[M + K]^+$ and 463 $[M + H]^+$. The UV spectrum of compound **2** was consistent with the presence of a 7-en-6-one ecdysteroid chromophore (241 nm (log ϵ = 3.95)).

The structures of **1** and **2** were determined from the ^1H and ^{13}C NMR data. The ^1H and ^{13}C data for compounds **1** and **2** are summarized in the Experimental Section. The singlet methyl signals in the ^1H NMR spectrum aided in their assignments using the characteristic HMBC correlations of these signals over two and three bonds. Identification of the geminal Me-26 and Me-27 groups was unambiguous owing to their mutual HMBC correlation, whereas Me-21 correlated with two olefinic carbon atoms exhibiting strong deshielding (δ 127.7, 134.8). Differentiation between the H_3-19 and H_3-18 atoms of the methyl groups was achieved considering the coupling of the latter with C-17, which also coupled to H_3-21. In accordance with a 6-oxo-$\Delta^{7,8}$-moiety, the H-7 olefinic hydrogens of **1** and **2** (δ 5.81, 5.83) correlated with C-5, C-9, and C-14. The high value of the ^{13}C chemical shift for C-1 in compound **2** (δ 76.6) justifies assignment of an OH group attached to this atom. The hydrogen atoms of ring A form a common spin system, which was analyzed by $^1H,^1H$-COSY and HMQC-TOCSY experiments. The ^1H signal assignments of rings C and D, as well as the side chain attached to C-17, were obtained in an analogous way. Since the amount of compound **2** was limited, signals of C-6 and C-8 remained under noise level. The existence of a conjugated C=O unit was supported by comparison with the ^{13}C chemical shifts of compounds from previous work.[11] The H_α-9/H_α-2 and H-19/H_β-5 correlations in the NOESY spectrum of **1** proved a *cis*-type junction of rings A/B, and the H_β-12/H-18, H_β-12/ H-21, and H_α-12/H_α-17 cross-peaks verify the *trans*-type junction of rings C/D.

In compound **2**, β-orientation of the OH group attached to C-1 was justified by two reasons. First, H_α-2 is axial and its multiplicity

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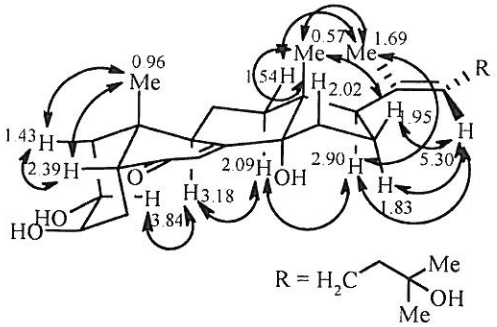

Figure 1. Steric view of compound **1**. Arrows show characteristic proximities obtained by NOESY experiment.

and coupling constant (t; 3.1 Hz) preclude the axial (β) orientation of H-1 because of the absence of an axial/axial coupling constant (9−10 Hz). Second, many ^{1}H and ^{13}C NMR signals of the atoms in ring "A" and methyl-19 are broad, as opposed to the corresponding signals of compound **1**, indicating hindered conformational motion of ring "A". The *trans* arrangement of C-21 and H-22 was proved by the NOESY correlations H-22/H$_\alpha$-16, H-22/H$_\beta$-16, H-22/H$_\alpha$-17, H$_3$-21/H$_\beta$-12, and H$_3$-21/H$_3$-18 (Figure 1).

Compounds **1** and **2** are the first ecdysteroids known to possess an extra double bond in the side chain at position 20(22).[12] These ecdysteroids are also of interest in view of their unusual hydroxylation pattern. The natural ecdysteroids generally contain an OH group in the side chain at position 20 and/or 22. Fourteen ecdysteroids have been isolated previously that do not contain the 20,22-diol structure. Five of these compounds were isolated from plants, mainly from species of the family Cactaceae.[13,14]

Compound **1** (LC_{50} > 100 ppm on day 4) proved inactive, and compound **2** (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). These results verify that the presence of the 20,22-diol is an essential structural requirement for ecdysteroids to attain high mortality in this test. An earlier investigation of the structure−activity relationship indicated that oxygen functions at C-20 and C-22 are important molecular features for activity.[1,15,16]

Experimental Section

General Experimental Procedures. Optical rotations were measured with a Perkin-Elmer 341 polarimeter. The UV spectra were recorded in MeOH with a Shimadzu UV 2101 PC spectrophotometer. NMR spectra were recorded in MeOH-d_4 in a Shigemi sample tube at room temperature, with a Bruker Avance DRX-500 spectrometer. The structures of the products were determined by means of comprehensive 1D and 2D NMR methods, using widely accepted strategies.[17,18] Chemical shifts are given on the δ-scale and were referenced to the solvent (MeOH-d_4: δ_C = 49.15 and δ_H = 3.31). In the 1D measurements (^{1}H, ^{13}C, DEPT-135), 64K data points were used for the FID. The pulse programs of the 2D experiments [gs-COSY, gs-HMQC, HMQC-TOCSY (mixing time = 80 ms), gs-HMBC, 1D NOESY (mixing time = 350 ms), 2D NOESY (mixing time = 400 ms)] were taken from the Bruker software library; the other parameters (pulse length and levels, delays, etc.) were the same as given in our previous work.[17,18] The mass spectrometric measurements were performed on a Finnigan TSQ 7000 tandem mass spectrometer (Finnigan MAT, San Jose, CA) equipped with a laboratory-built nanoelectrospray ion source. A high voltage of about 1000 V was used in the ion source. The instrument was scanned in the normal MS mode over the mass range 10−1500, with a scan time of 2 s. HRESIMS recordings were made on a Finnigan MAT 95SQ tandem mass spectrometer (Finnigan MAT, Bremen, Germany). RPC was carried out on a Harrison Model 8924 Chromatotron instrument (Harrison Research, Palo Alto, CA). The stationary phase for RPC was silica gel 60 GF_{254} (E. Merck). A Zorbax-SIL column (5 μm, DuPont, Paris, France) was used for normal-phase HPLC.

Plant Material. Roots of *Serratula wolffii* 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.

Extraction and Isolation. Fresh roots (4.7 kg) were extracted with MeOH and purified by fractionated precipitation with acetone.[9] The dry residue (137.5 g) of the purified extract was applied to a polyamide column (MN-polyamide SC 6, Woelm, Eshwege, Germany). The fraction eluted with water (24.4 g) was subjected to low-pressure reversed-phase column chromatography on octadecyl-silica (0.06−0.02 μm, Chemie Uetikon, Uetikon, Switzerland). Fractions eluted with 60% MeOH−H_2O (70 mg) were further purified by rotation planar chromatography. From the fractions eluted with CH_2Cl_2−MeOH−C_6H_6 (50:5:3) (1.5 mg), compound **1** (0.5 mg) was obtained and was further purified by normal-phase HPLC [C_6H_{12}−i-PrOH−H_2O (100:40:3)]. Fractions eluted with MeOH were repeatedly separated by RPC. Fractions eluted with EtOAc−EtOH−H_2O (80:5:2) (3 mg) were purified by normal-phase HPLC [C_6H_{12}−i-PrOH−H_2O (100:40:3)] to give compound **2** (2.5 mg).

20,22-Didehydrotaxisterone (1): colorless crystals; mp 231−233 °C; $[\alpha]^{28}_D$ +71 (*c* 0.025, MeOH); UV (MeOH) λ_{max} (log ϵ) 242 (4.387) nm; ^{1}H NMR (CD_3OD, 500 MHz) δ 5.81 (1H, d, *J* = 2.6 Hz, H-7), 5.30 (1H, t, *J* = 7.1 Hz, H-22), 3.96 (1H, m, H-3α), 3.84 (1H, ddd, *J* = 12.0, 4.2, 3.3 Hz, H-2α), 3.18 (1H, ddd, *J* = 11.2, 7.2, 2.6 Hz, H-9α), 2.90 (1H, t, *J* = 9.2 Hz, H-17α), 2.39 (1H, dd, *J* = 12.7, 4.5 Hz, H-5β), 2.13 (2H, m, H-23), 2.09 (1H, td, *J* = 13.0, 8.0 Hz, H-12α), 2.02 (1H, m, H-15β), 1.95 (1H, tdd, *J* = 12.0, 9.2, 2.3 Hz, H-16β), 1.84 (1H, m, H-11α), 1.83 (1H, m, H-16α), 1.79 (1H, m, H-1α), 1.76 (1H, m, H-4α), 1.70 (1H, dt, *J* = 14.2, 4.5 Hz, H-4β), 1.69 (3H, s, H-21), 1.66 (1H, m, H-15α), 1.63 (1H, m, H-11β), 1.54 (1H, ddd, *J* = 13.0, 5.3, 1.7 Hz, H-12β), 1.50 (2H, m, H-24), 1.43 (1H, dd, *J* = 13.4, 12.2 Hz, H-1β), 1.20 (6H, s, H-26, H-27), 0.96 (3H, s, H-19), 0.57 (3H, s, H-18); ^{13}C NMR (CD_3OD, 125 MHz) δ 206.7 (C, C-6), 167.9 (C, C-8), 134.9 (C, C-20), 127.7 (CH, C-22), 121.9 (CH, C-7), 85.0 (C, C-14), 71.5 (CH, C-25), 68.85 (CH, C-2), 68.67 (CH, C-3), 54.3 (CH, C-17), 52.0 (CH, C-5), 48.9 (CH, C-13), 45.0 (CH_2, C-24), 39.5 (C, C-10), 37.6 (CH_2, C-1), 35.4 (CH, C-9), 33.0 (CH_2, C-4), 32.3 (CH_2, C-15), 31.1 (CH_2, C-12), 29.3 (CH_3, C-26, C-27), 24.6 (CH_3, C-19), 24.4 (CH_2, C-23), 24.1 (CH_2, C-16), 21.8 (CH_2, C-11), 18.4 (CH_3, C-21), 17.7 (CH_3, C-18); ESIMS *m/z* 485 $[M + K]^+$ (69), 447 $[M + H]^+$ (93), 429 $[M + H - H_2O]^+$ (100), 411 $[M + H - 2H_2O]^+$ (6), 393 $[M + H - 3H_2O]^+$ (7), 347 (6), 320 (4); HRESIMS *m/z* 447.3025 $[M + H]^+$ (calcd for $C_{27}H_{43}O_5$, 447.3021).

1-Hydroxy-20,22-didehydrotaxisterone (2): colorless crystals; mp 218−220 °C; $[\alpha]^{28}_D$ +10 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ϵ) 241 (3.95) nm; ^{1}H NMR (CD_3OD, 500 MHz) δ 5.84 (1H, d, *J* = 2.5 Hz, H-7), 5.30 (1H, t, *J* = 7.0 Hz, H-22), 4.04 (1H, br, H-3α), 3.88 (1H, t, *J* = 3.1 Hz, H-2α), 3.82 (1H, br, H-1α), 3.10 (1H, t, *J* = 8.8 Hz, br, H-9α), 2.89 (1H, t, *J* = 9.2 Hz, H-17α), 2.61 (1H, dd, *J* = 12.8, 4.5 Hz, H-5β), 2.13 (2H, m, H-23), 2.06 (1H, m, H-12α), 2.05 (1H, m, H-15β), 1.95 (1H, tdd, *J* = 12.0, 9.2, 2.2 Hz, H-16β), 1.83 (1H, m, H-4β), 1.82 (1H, m, H-16α), 1.78 (1H, m, H-4α), 1.72−1.69 (2H, m, H-11α, H-11β), 1.68 (3H, s, H-21), 1.66 (1H, m, H-15α), 1.52 (1H, m, H-12β), 1.50 (2H, m, H-24), 1.20 (6H, s, H-26, H-27), 1.07 (3H, s, br, H-19), 0.59 (3H, s, H-18); ^{13}C NMR (CD_3OD, 125 MHz) δ 134.8 (C, C-20), 127.7 (CH, C-22), 122.0 (CH, C-7), 84.8 (C, C-14), 76.6 (CH, C-1), 71.5 (CH, C-25), 71.1 (CH, C-3), 68.6 (CH, C-2), 54.3 (CH, C-17), 48.9 (CH, C-13), 47.0 (CH, C-5), 44.9 (CH_2, C-24), 44.0 (C, C-10), 36.0 (CH, C-9), 33.7 (CH_2, C-4), 32.3 (CH_2, C-15), 31.1 (CH_2, C-12), 29.3 (CH_3, C-26, C-27), 24.3 (CH_2, C-23), 24.0 (CH_2, C-16), 22.2 (CH_2, C-11), 20.2 (CH_3, C-19), 18.4 (CH_3, C-21), 17.7 (CH_3, C-18); ESIMS *m/z* 501 $[M + K]^+$ (26), 463 $[M + H]^+$ (4), 445 $[M + H - H_2O]^+$ (100), 427 $[M + H - 2H_2O]^+$ (8), 408 $[M + H - 3H_2O]^+$ (2), 391 $[M + H - 4H_2O]^+$ (14), 374 (4), 363 (5), 336 (4); HRESIMS *m/z* 463.2976 $[M + H]^+$ (calcd for $C_{27}H_{43}O_6$, 463.2970).

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Three new steroids from the roots of *Serratula wolffii*

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14α,15α-epoxy-14,15-dihydrostachysterone
B

ABSTRACT

Investigation of the methanol extract of the roots of *Serratula wolffii* resulted in an ecdysone-related compound, 2β,3β,20R,22R,25-pentahydroxy-5β-cholest-6,8(14)-dien (**1**), a new ecdysteroid, 24-methylene-shidasterone (**2**), the known compound stachysterone B (**3**) and its 14,15-α-epoxide (**4**), a novel natural product. The structures of compounds **1–4** were established by spectral analysis (^{1}H NMR, ^{13}C NMR, COSY, NOESY, HMQC, HMQC-TOCSY and HMBC).

1. Introduction

Ecdysteroids were discovered as insect moulting hormones. However, besides their controlling the processes of metamorphosis in insects, they possess a wide range of biological activities. One of their main pharmacological effects is the stimulation of protein synthesis [1]. Ecdysteroids have recently attracted attention because of their possible use in gene therapy [2].

One of the richest sources of ecdysteroids is *Serratula wolffii* (Andrae) from the family Asteraceae [3]. This species biosynthesizes predominantly 20-hydroxyecdysone, the major ecdysteroid of plants, and a series of minor ecdysteroids. We earlier reported several new compounds from the herbs and the roots of *S. wolffii* [4].

As a continuation of our research on unusual steroids of this species, the present paper describes the isolation and structure elucidation of a new ecdysteroid-related compound,

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2β,3β,20R,22R,25-pentahydroxy-5β-cholest-6,8(14)-dien (1), two new ecdysteroids, 24-methylene-shidasterone (2) and 14α,15α-epoxy-14,15-dihydrostachysterone B (4), and the known stachysterone B (3) from the roots of the plant.

2. Experimental

2.1. General methods

Optical rotations were measured with a Perkin-Elmer 341 polarimeter. UV spectra were recorded in MeOH with a Shimadzu UV 2101 PC spectrophotometer. NMR spectra were recorded in MeOH-d_4 in a Shigemi sample tube at room temperature with a Bruker Avance DRX-500 and Varian Unity Inova-500 and Inova-600 spectrometers. The structures of products were determined by means of comprehensive one- and two-dimensional NMR methods, using widely accepted strategies [5,6]. Chemical shifts are given on the δ-scale and were referenced to the solvent (MeOH-d_4: $\delta_C = 49.15$ and $\delta_H = 3.31$). In the 1D measurements (^{1}H, ^{13}C and DEPT-135), 64K data points were used for the FID. The pulse programs of all experiments [gs-COSY, phase-sensitive DQF-COSY, gs-HMQC, HMQC-TOCSY (mixing time = 80 ms), edited gs-HSQC; gs-HMBC, NOESY (mixing times = 400 ms, 500 ms and 600 ms), and 1D gs-NOESY (mixing time = 300 ms)] were taken from the Bruker and Varian software library. The mass spectrometric measurements were performed on a Finnigan TSQ 7000 tandem mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with a laboratory-built nanoelectrospray ion source. A high voltage of about 1000 V was used in the ion source. The instrument was scanned in the normal MS mode over the mass range 10–1500, with a scan time of 2 s. HRESIMS recordings were made on a Finnigan MAT 95SQ tandem mass spectrometer (Finnigan MAT, Bremen, Germany). HPLC analyses were performed with a Jasco Model PU-2080 Pump, Jasco Model UV-2070/2075 detector. A Zorbax-SIL column (5 μm, 9.4 mm × 250 mm, DuPont, Paris, France) was used for normal-phase HPLC; and a Zorbax SB C18 column (5 μm, 4.6 mm × 250 mm, DuPont, Paris, France) was used for reversed-phase HPLC. Rotation planar chromatography (RPC) was carried out on a Harrison Model 8924 Chromatotron instrument (Harrison Research, Palo Alto, CA). The stationary phase for RPC was silica gel 60 GF_{254} (E. Merck). Column chromatographic support: Chemie Ueticon-C-Gel octadecyl silica (0.06–0.02 μm, Chemie Ueticon, Ueticon, Switzerland).

2.2. 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.

2.3. Extraction and isolation

The fresh roots (4.7 kg) were extracted with MeOH, and the extract was purified by fractionated precipitation. The dry residue (137.5 g) of the purified extract was subjected to polyamide column chromatography (MN-polyamide SC 6, Woelm, Eschwege, Germany). The fraction eluted with water (24.4 g) was separated by low-pressure reversed-phase column chromatography on octadecyl silica. The fraction (120 mg) eluted with 55% MeOH–H_2O was purified by RPC (CH_2Cl_2–MeOH–C_6H_6 (50:5:3 v/v/v)) and normal-phase HPLC on silica (c-C_6H_{12}–i-PrOH–H_2O (100:40:3 v/v/v), 2 ml/min, UV detection 245 nm) to give **1** (0.7 mg). Another fraction (70 mg) was eluted from the reversed-phase column with MeOH–H_2O (60:40 v/v) and was purified by a combination of RPC and reversed-phase HPLC. From the fraction eluted with CH_2Cl_2–MeOH–C_6H_6 (50:3:2 v/v/v), compound **2** (0.5 mg) was obtained by reversed-phase HPLC (ACN-H_2O (35:65 v/v), 0.8 ml/min, UV detection 245 nm). The reversed-phase column chromatography gave a fraction (390 mg) which was separated by repeated RPC (CH_2Cl_2–MeOH–C_6H_6 (50:5:3 v/v/v) in the first step and EtOAc–EtOH–H_2O (80:2:1 v/v/v), in the second one). Further purification by normal-phase HPLC (c-C_6H_{12}–i-PrOH–H_2O (100:40.3 v/v/v), 3 ml/min, UV detection 245 nm) yielded **3** (4 mg) and **4** (3 mg).

2.3.1. 2β,3β,20R,22R,25-Pentahydroxy-5β-cholest-6,8(14)-dien (1)

$[\alpha]_D^{28} - 7°$ (c = 0.05, MeOH); UV λ_{max}^{MeOH} (nm) (log ε): 237 (3.2); ^{1}H and ^{13}C NMR (MeOH-d_4) (see Table 1); ESIMS *m/z* (relative abundance (%)): 487 (30) $[M+K]^+$, 471 (26) $[M+Na]^+$, 449 (29) $[M+H]^+$, 431 (40) $[M+H-H_2O]^+$, 413 (100) $[M+H-2H_2O]^+$, 395 (37) $[M+H-3H_2O]^+$; HRESI-MS: M = 448.3179 (calcd for $C_{27}H_{44}O_5$, 448.3177).

2.3.2. 24-Methylene-shidasterone (2)

$[\alpha]_D^{28} + 3°$ (c = 0.05, MeOH); UV λ_{max}^{MeOH} (nm) (log ε): 241.8 (3.7); ^{1}H and ^{13}C NMR (MeOH-d_4) (see Table 1); ESIMS *m/z* (relative abundance (%)): 497 (10) $[M+Na]^+$, 475 (100) $[M+H]^+$, 457 (72) $[M+H-H_2O]^+$, 439 (5.7) $[M+H-2H_2O]^+$, 421 (3) $[M+H-3H_2O]^+$, 364 (2); HRESI-MS: M = 474.2975 (calcd for $C_{28}H_{42}O_6$, 474.2970).

2.3.3. Stachysterone B (3)

Table 1 shows the ^{1}H and ^{13}C NMR data. The other spectroscopic data are in accordance with the reported structure [7].

2.3.4. 14α,15α-epoxy-14,15-dihydrostachysterone B

$[\alpha]_D^{28} - 5°$ (c = 0.1, MeOH); UV λ_{max}^{MeOH} (nm) (log ε): 240 (3.8); ^{1}H and ^{13}C NMR (MeOH-d_4) (see Table 1); ESIMS *m/z* (relative abundance (%)): 501 (13) $[M+Na]^+$, 479 (100) $[M+H]^+$, 461 (24) $[M+H-H_2O]^+$, 443 (27.5) $[M+H-2H_2O]^+$, 425 (2.6) $[M+H-3H_2O]^+$, 393 (3), 330 (53); HRESI-MS: M = 478.2924 (calcd for $C_{27}H_{42}O_7$, 478.2919).

3. Results and discussion

The isolation of compounds **1–4** from the methanol extract involves fractionated precipitation and combined chromatographic procedures, including column chromatography on polyamide and octadecyl silica, RPC and preparative HPLC.

The structures of compounds **1–4** (Fig. 1) were elucidated by using NMR, UV and MS measurements. The UV spectrum of compound **1** verified the presence of a conjugated double bond system. The molecular formula of **1**, $C_{27}H_{44}O_5$, was established

Table 1 – The ^1H (500 MHz) and ^{13}C (125 MHz) chemical shifts, multiplicities (m) and couplings constants (*J*) of compounds 1–4 (MeOH-d_4) (δ in ppm, *J* in Hz)

		1			2			3		4		
No.		^{13}C	^1H	m; *J* (Hz)	^{13}C	^1H	m; *J* (Hz)	^{13}C	^1H	^{13}C	^1H	m; *J* (Hz)
1	α	37.4	1.70	dd; 13.3, 4.4	37.5	1.795		37.2	1.81	37.1	1.82	dd; 13.5, 4.3
	β		1.59			1.43	dd; 13.1, 12.5		1.465		1.46	t; 12.8
2	α	69.0	3.66	ddd; 12.5, 4.3, 2.9	68.9	3.84	ddd; 12.1, 4.3, 3.3	68.71	3.80	68.7	3.84	ddd; 12.1, 4.2, 3.3
3	α	70.5	3.90	q; 2.6	68.7	3.95	q; 2.9	68.67	3.94	68.5	3.95	q; 3.0
4	α	36.9	1.48		33.2	1.72[a]		33.0	1.65[a]	32.8	1.66	dd; 7.8, 3.0
	β		1.86	dt; 14.8, 3.9		1.76[a]			1.72[a]		1.66	
5	β	39.7	2.09		51.9	2.38	dt; 8.1, 4.7	51.6	2.385	52.0	2.41	dd; 9.0, 8.4
6		130.7	5.53	dd; 10.0, 5.5	206.6	–	–	205.9	–	205.9	–	–
7		125.6	6.09	d; 9.9	122.3	5.81	d; 2.7	121.2	6.08	124.0	5.89	d; 2.8
8		125.9	–	–	168.1	–	–	158.9	–	159.8	–	–
9	α	36.5	2.39		35.3	3.15	ddd; 11.3, 7.1, 2.7	39.96	2.72	39.1	2.84	ddd; 10.2, 6.9, 2.8
10		37.6	–	–	39.4	–	–	40.06	–	39.7	–	–
11	α	20.8	1.66		21.6	1.80		21.8	1.86	21.7	1.93	
	β		1.60			1.67			1.77		1.81	
12	α	39.5	1.46		32.45	2.165	td; 13.3, 4.8	41.2	1.64	35.6	1.81	
	β		2.22	dt; 12.4, 3.0		1.84			2.31		2.20	dd; 9.0, 2.8
13		45.5	–	–	48.4	–	–	49.1	–	42.4	–	–
14		147.6	–	–	85.4	–	–	150.8	–	73.3	–	–
15	α	25.4	2.41		31.8	1.62		130.4	6.08[#]	61.3	–	–
	β		2.29			1.97					3.97	s
16	α	22.9	1.65		22.0	1.83		32.1	2.25	27.9	1.92	
	β		2.04			2.00			2.625		1.94	
17	α	56.4	1.59		52.0	2.42	dd; 9.5, 8.2	59.1	2.20	48.4	1.71	dd; 10.6, 7.0
18	β	21.5	1.13	s	18.35	0.84	s	20.2	1.14	16.5	1.02	s
19	β	23.7	0.77	s	24.5	0.96	s	24.15	0.97	24.5	1.01	s
20		77.9	–	–	76.8	–	–	77.3	–	77.0	–	–
21		21.0	1.23	s	20.7	1.21	s	20.5	1.25	20.8	1.19	s
22		78.4	3.39	dd; 10.5, 1.3	82.4	3.91	t; 8.1	78.7	3.335	78.4	3.29	dd; 10.5, 1.8
23	a	27.4	1.27		35.6	2.545	dt; 8.2, 2.2	27.4	1.32	27.4	1.28	td; 11.8, 4.4
	b		1.59			2.545	dt; 8.2, 2.2		1.61		1.55	tdd; 11.8, 4.4, 1.8
24	a	42.5	1.42		158.3	–	–	42.4	1.425	42.3	1.44	td; 12.4, 4.2
	b		1.79	td; 12.8, 4.8					1.81		1.79	td; 12.6, 4.6
25		71.4	–	–	83.2	–	–	71.4	–	71.4	–	–
26		28.9	1.17	s	27.9	1.28	s	29.0	1.185	29.0	1.19	s
27		30.0	1.20	s	29.2	1.33	s	30.0	1.21	30.0	1.20	s
28	a				104.1	4.805	t; 2.3					
	b					4.895	t; 2.2					

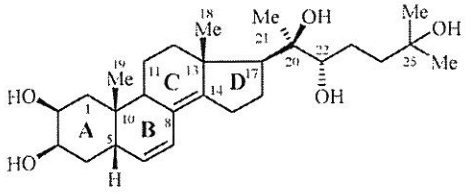
[a] Tentative.
[#] Meaningless remark on the stereochemistry of H-15.

by high-resolution measurement of the protonated molecular ion peak at *m/z* 448.3179 in the ESIMS (calcd 448.3177), which corresponds to the suggested structure. The presence of the characteristic peaks at *m/z* 431 $[M+H-H_2O]^+$ and 413 $[M+H-2H_2O]^+$ in the ESIMS of **1** also supported its structure.

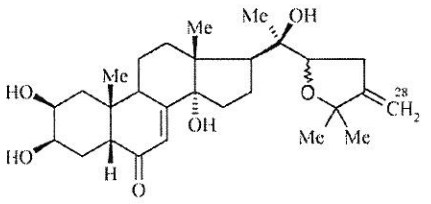
The UV spectrum of compound **2** is in accordance with the presence of the 7-en-6-one chromophore of ecdysteroids. The molecular formula of **2**, $C_{28}H_{42}O_6$, was established via the molecular ion peak (*m/z* 475), and coincided with the ^1H and ^{13}C NMR data (see Table 1).

The UV spectrum of compound **4** indicates the presence of an α,β-unsaturated keto group. The ESIMS of **4** revealed a peak at *m/z* 479 for $[M+H]^+$, which is consistent with the molecular formula $C_{27}H_{42}O_7$. The characteristic fragment ions were formed from the intact parent compound by the loss of water: *m/z* 461 $[M+H-H_2O]^+$, 443 $[M+H-2H_2O]^+$ and 425 $[M+H-3H_2O]^+$.

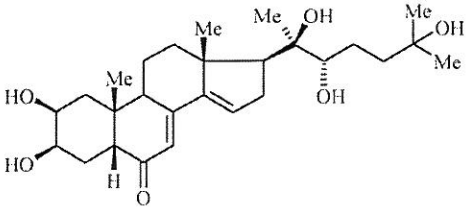
The ^1H and ^{13}C chemical shifts of the compounds **1–4** are summarized in Table 1. For the signal assignment we identified at first the five methyl signals appearing as singlets in the ^1H NMR spectrum. The characteristic HMBC correlations of the methyl groups through two and three bonds were utilised in the assignment. The identification of the geminal Me-26 and Me-27 groups are straightforward owing to their mutual HMBC correlation. In compound **2** these methyl groups marked out a quaternary sp^2 carbon atom at 158.3 ppm proving the attachment of the terminal methylene group to C-24. H_3-21 gave correlation only to three carbon atoms in contrast to the other methyl groups. The differentiation between H_3-19 and H_3-18 atoms of the angular methyl groups was achieved considering the coupling of the latter with C-17. H_3-21/C-17 HMBC responses were also detected in these compounds. In compound **2** the ^{13}C chemical shift values of C-22 (82.4 ppm) and C-25 (83.2 ppm) prove the presence of OR ($R \neq H$) sub-



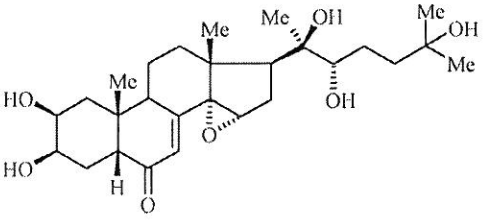
1 2β,3β,20*R*,22*R*,25-pentahydroxy-5β-cholest-6,8(14)-dien



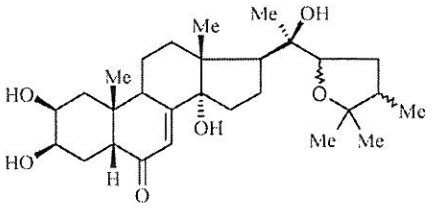
2 24-methylene-shidasterone



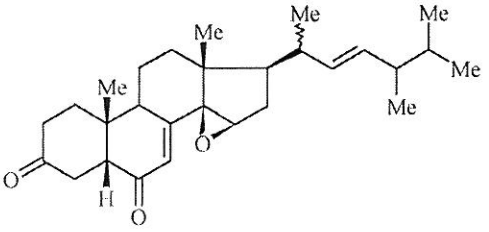
3 stachysterone B



4 14α,15α-epoxy-14,15-dihydrostachysterone B



5 24-methylshidasterone



6 gymnasterone B

Fig. 1 – Structures of compounds 1–6.

stituent. The strong H-22/H-26 NOESY response may indicate the existence of a five-membered ring. Further support of this structure can be gained from the comparison of the chemical shifts of C-22 (81.6 ppm) and C-25 (82.5 ppm) measured for 24-methyl-shidasterone (5) (Fig. 1) [7].

In compounds 1 and 3 the HMBC correlations of H_3-18 identicated C-12, a sp^3 and an olefinic sp^2 quaternary carbon atom (147.6, 150.8 ppm) in positions 13 and 14. In compounds 2 and 4 the chemical shift of the quaternary C-14 (85.4, 73.3 ppm) justified the attachment of an oxygen atom. The chemical shift 85.4 ppm for C-14 in compound 2 proves an OH substitution.

H-7 olefinic hydrogen atoms of compounds 1–4 correlated with C-5, C-9 and C-14 carbon atoms in HMBC spectra. In compound 1 H-7 gave a COSY correlation with an olefinic hydrogen in position 6. H-6 marked out a quaternary olefinic carbon atom in the HMBC spectrum proving the existence of an conjugated $\Delta^{6,7;8,14}$-diene-moiety.

The overlapping H-7 and H-15 signals signed out in the HMBC spectrum of the compound 3 C-8, C-13, C-14 and C-16 verified the presence of a conjugated $\Delta^{7,8;14,15}$-diene-moiety in the molecule.

The hydrogen atoms of ring-A form a common spin system which was analysed by ^{1}H, ^{1}H-COSY and HMQC-TOCSY experiments. The assignments of ring-C and -D, as well as the side-chain attached to C-17 were obtained in an analogous way. Surprisingly, we founded a CH group (instead of a CH_2 unit) at position 15 in compound 4. The chemical shift of C-15 (61.3 ppm) indicated the connection of an oxygen atom in this position. At this point there are two alternative structures: a 14,15-dihydroxy- or an 14,15-epoxy-derivative. The $^1J_{C\text{-}15,H\text{-}15}$ coupling constant was measured 188 Hz by ^{1}H-coupled HMQC and proved the existence of an 14,15-epoxy group [8]. The presence of the 14,15-epoxy group was also supported by the chemical shifts of C-14 and C-15 (71.9, 69.0 ppm) of gymnasterone B (6) (Fig. 1) in the literature [9].

The H_α-9/H_α-2 and H-19/H_β-5 correlations in NOESY spectrum of compounds 1–4 established *cis* type junction of rings-A/B. In compounds 2 and 4 the H_β-12/H-18, H_β-12/H_3-21, H_α-12/H_α-17 cross-peaks and the absence of the H_α-9/H_α-15 correlation verified the *trans* type junction of rings-C/D and proved by the correlations of H_β-16/H-15, H_β-16/H_3-18 in 4 the α arrangement of the 14,15-epoxy group. The ^{1}H, ^{13}C and

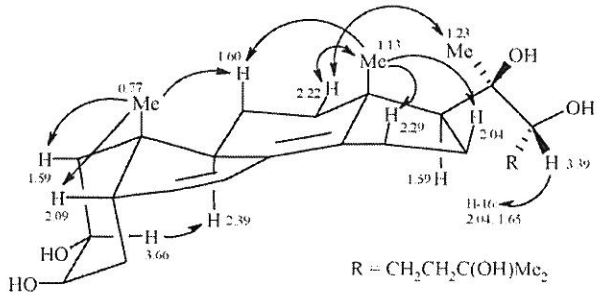


Fig. 2 – Spatial arrangement of compound 1, arrows indicate the steric proximities detected by NOESY experiment.

NOESY correlation of H_3-21 and H-22 for compounds **1**, **3** and **4** were in accordance with the literature data and our results on 20-hydroxyecdysone. These assignations revealed the configuration of C-20 and C-22 which is depicted on Fig. 2.

In the NOESY spectrum of compound **2** the detected H_3-21/H_β-16, H-22/H_β-16, H-22/H_3-21 and H-22/H_3-18 cross-peaks indicate the high mobility of the side-chain contrast with compounds **1** and **3** and this prohibit the determination of the absolute configuration at C-20 and C-22. In compound **5** the configuration of C-20 is known [7]. The differences of ^{13}C chemical shifts from C-13 to C-23 between the compounds **2** and **5** are small despite the diversities of solvents (pyridine-d_5, methanol-d_4) and it gives a hint for the configuration of C-20.

From mechanistic consideration and NOE studies, Roussel et al. [10] revealed, that the C-22 configuration of shidasterone, the basic compound of 24-methylene-shidasterone (**3**), is (22R). According to these experiments and from biogenetic considerations the stereochemistry of C-22 in 24-methylene-shidasterone (**3**) must also be (22R). The intramolecular closure of the furanyl ring from the known precursor, 24(28)-dehydromakisterone A, must proceed in the same way as in the case of shidasterone from 20-hydroxyecdysone.

The NOESY correlations of H_3-26/H-28a, H_3-27/H-28a and H_2-23/H-28b rendered possible the spatial differentiation of H-28a and H-28b in compound **2**.

Compound **1** is of interest from a biosynthetic aspect. It can be explained as a metabolic product of the main plant ecdysteroid, 20-hydroxyecdysone, formed from it by reduction, dehydration and double-bond isomerization during the biosynthesis. Compound **2**, 24-methylene-shidasterone, is the intramolecular ether of 24(28)-dehydromakisterone A. Compound **4** is a five-ringed ecdysteroid containing one epoxide ring. A few ecdysteroids containing 14,15- or 22,23-epoxide moieties have previously been isolated from marine microorganisms and fungi [7]. An ecdysteroid possessing a 14,15-epoxide ring exerts significant cytotoxic activity [9]. Stereoselective synthesis of this antitumour steroid (gymnasterone B) has already been achieved [11].

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The first two ecdysteroids containing a furan ring from *Serratula wolffii*

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Abstract

Two new ecdysteroids, named serfurosterone A and serfurosterone B, were isolated from a methanol extract of the roots of *Serratula wolffii*. Spectroscopic methods revealed that these compounds had previously unknown ecdysteroid structures with acetal functions in the side-chains.

Keywords: *Serratula wolffii*; Asteraceae; Ecdysteroids; NMR; Serfurosterone A; Serfurosterone B

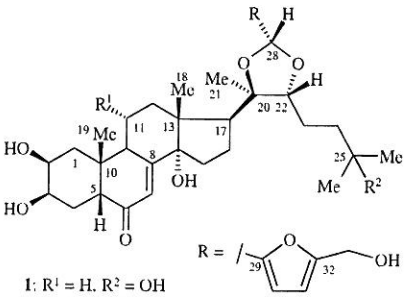
The cascade of morphological changes in insects is triggered by a group of steroid hormones known as ecdysteroids.[1] The phytoecdysteroids, compounds related to insect hormones, also occur in high concentration with diverse structures in several plant species.[2] *Serratula* species have proven to be rich sources of ecdysteroids.[3] *S. wolffii* Andrae (Asteraceae), which is native to the continental climate, is one of the most promising such species, which is cultivated in Hungary.[4]

Ecdysteroids are of great interest for their biological activities. Besides their beneficial pharmacological effects (e.g., anabolic action without androgenic side-effects, and also hypoglycemic and hypocholesterolaemic effects), phytoecdysteroids are inducers of the gene regulation system.[5]

We report here the isolation and structure elucidation of two ecdysteroids, serfurosterone A (**1**) and serfurosterone B (**2**), the first two ecdysteroids found to contain furan ring substituents.

The *S. wolffii* sample examined was collected from Herencsény, Hungary, in 2003. Its roots were extracted with methanol and the extract (208.9 g) was purified by fractional precipitation and column chromatography on polyamide. The fraction eluted with water (24.4 g) from the polyamide was subjected to low-pressure reversed-phase column chromatography on octadecyl silica. Further separation of the fractions containing the ecdysteroids (390 mg and 70 mg) was achieved by rotation planar chromatography on silica, using CH_2Cl_2–MeOH–C_6H_6 (50:10:6, v/v/v) and EtOAc–EtOH–H_2O (80:2:1, v/v/v) as mobile phases and by reversed-phase-HPLC. These separation steps furnished compounds **1** (0.5 mg, which represents 0.00024% of the extract) and **2** (0.5 mg, which represents 0.00024% of the extract).[6]

The structures of **1** and **2** (Fig. 1) were elucidated by using NMR, UV and MS measurements.[7] The UV spectra (DMSO) of **1** and **2** verified the presence of the 7-en-6-one



1: R^1 = H, R^2 = OH
2: R^1 = OH, R^2 = H

Fig. 1. Structures of compounds **1** and **2**.

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chromophore in both the structures.[8] The molecular formula, established by high-resolution measurements of the protonated molecular ion peaks in the HRESIMS, was $C_{33}H_{48}O_9$ in each case. The HRESIMS indicated pseudomolecular ions at *m/z* 589.3393 $[M+H]^+$ for **1** and at *m/z* 589.3389 $[M+H]^+$ for **2** (calcd: 589.3363). The peaks at *m/z* 519 $[M+K-R']^+$, 463 $[M+H-H_2O-R']^+$ and 445 $[M+H-2H_2O-R']^+$ in the ESIMS spectra of **1** and **2** supported the presence of a furan ring-containing substituent ($R' = C_6H_4O_2$) in the two molecules. The ^1H and ^{13}C NMR chemical shifts of **1** and **2** are presented in Table 1. Due to the low quantities of the samples of **1** and **2**, some quaternary signals remained under the noise level in the APT and ^{13}C NMR spectra. Their chemical shifts were determined from HMBC spectra. The characteristic HMBC correlations of the methyl signals over two and three bonds were utilized in their assignments. Their mutual HMBC correlations made the identification of the geminal Me-26 and Me-27 groups unambiguous. The singlet multiplicity of the signals of H_3-26 and H_3-27 and the high value of the ^{13}C chemical shift of C-25 (71.4 ppm) verified the existence of the 2-hydroxyisopropyl moiety in **1**. Differentiation between H_3-19 and H_3-18 was achieved by considering the coupling of the latter with C-17, which is also coupled to H_3-21. In both **1** and **2**, the high chemical shifts of C-20 and C-22 (83–86 ppm) proved the oxygen substitution. The H-22/H-28 NOESY correlation in **2** and the chemical shift of C-28 (96.5 ppm) verified the existence of an acetal-type five-membered ring. Moreover, the H-28/C-29, H-33/C-31 and H-33/C-32 HMBC cross-peaks and the H-28/H-30 NOESY correlations revealed a 5-hydroxymethyl-furfurylidene substituent on C-28. The characteristic ^{13}C chemical shifts and the low coupling value $^3J_{H\text{-}30,H\text{-}31} = 3.0$–$3.2$ Hz furnished further support for the structure.[9] The similar chemical shifts and signal multiplicity of H-28, H-30, H-31, H-33 and C-22 likewise indicated the presence of the 5-hydroxymethyl-furfurylidene unit in **1**.

Table 1
The ^1H (500 MHz) and ^{13}C (125 MHz) chemical shifts, multiplicities (m) and couplings constants (*J*) of compound **1** (MeOH-d_4) and compound **2** (DMSO-d_6) (δ in ppm, *J* in Hz)

No.		1			2		
		^{13}C	^1H	m; *J* (Hz)	^{13}C	^1H	m; *J* (Hz)
1	α	37.3	1.79	dt; 13.2, 4.2	38.2	2.46	dd; 12.5, 4.1
	β		1.42	dd; 13.2, 12.0		1.15	t; 11.9
2	α	68.5	3.84	dt; 12.0, 3.4	66.9	3.77	
3	α	68.3	3.95	s; br	66.5	3.76	
4	α	32.7	[a]1.75		32.0	1.62	
	β		[a]1.71			1.46	
5	β	52.1	2.39	dd; 12.6, 4.2	51.1	2.14	dd; 13.1, 3.8
6			—	—		—	—
7		122.0	5.82	d; 2.6	121.0	5.63	d; 2.6
8			—	—	162.6	—	—
9	α	35.0	3.15	ddd; 8.9, 2.6, 10.8	41.2	2.98	dd; 8.9, 2.6
10		39.1	—	—	40.5	—	—
11	α	31.7	1.62		67.2	—	—
	β		1.62			3.87	ddd; 10.9, 9.1, 5.7
12	α	32.2	2.11	td; 12.6, 4.2	42.0	2.08	dd; 12.1, 10.9
	β		1.85			1.93	dd; 12.1, 5.7
13		48.0	—	—	46.6	—	—
14		85.4	—	—	82.7	—	—
15	α	n.d.	n.d.		30.2	1.535	n.d.
	β		n.d.			1.85	n.d.
16	α	n.d.	n.d.		21.3	1.84	n.d.
	β		n.d.			1.84	n.d.
17	α	51.1	2.41	t; 9.1	49.2	2.30	t; 8.6
18	β	17.5	0.86	s	17.5	0.69	s
19	β	24.1	0.96	s	24.08	0.90	s
20		85.6	—	—	83.9	—	—
21		23.6	1.30	s	21.2	1.20	s
22		85.7	3.79	dd; 9.4, 2.3	83.4	3.69	dd; 9.4, 2.6
23	a	n.d.	n.d.		26.0	1.44	
	b		n.d.			1.51	
24	a	41.7	1.53	td; 13.2, 4.2	35.8	1.25	
	b		1.53	td; 13.2, 4.2		1.40	
25		71.4	—	—	27.5	1.58	dt; 13.3, 6.6
26		28.7	1.20	s	22.33	0.88	d; 7.0
27		29.2	1.21	s	22.44	0.89	d; 6.6
28		98.2	5.79	s	96.5	5.74	s
29			—	—	150.3	—	—
30			6.42	d; 3.0	109.9	6.45	d, 3.2
31			6.28	d; 3.0	107.5	6.25	d, 3.2
32			—	—	156.1	—	—
33	a,b	57.2	4.51	s	55.7	4.37	s

[a] Tentative, n.d. = no data observed.

In accordance with a 6-oxo-7-en-6-one moiety, H-7 correlates over $^3J_{C,H}$ couplings with C-5, C-9 and C-14. The hydrogen atoms of ring A form a common spin system analyzed by ^1H, ^1H-COSY and HMQC-TOCSY experiments. The signals of rings C and D, and of the side-chain on C-17, were assigned in an analogous way.

From the H_α-9/H_α-2 and H_3-19/H_β-5 NOESY correlations in **2**, the cis junction of rings A/B is clear. The H_β-12/H_3-18, H_β-12/H_3-21 and H_α-12/H_α-17 cross-peaks confirmed the trans junction of rings C/D. The H_3-18/H-11 NOESY cross-peak and the multiplicity of the H_α-9 signal verified the β-position of H-11.

In **2**, the H_β-12/H_3-21, H_3-18/H_3-21, H_3-18/H-30, H-22/H_2-16 and H-22/H-28 NOESY correlations revealed the steric arrangement of the side-chain and the absolute configurations of C-20 and C-22 as shown in Figure 2.

Compounds **1** and **2** are the first two ecdysteroids known to contain a furan ring: they are acetals of

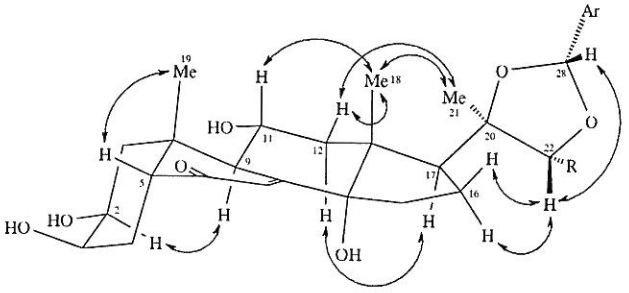

Fig. 2. Steric structure of compound **2**. Ar = 5′-hydroxymethyl-furfuryl-idene, R = $CH_2CH_2CHMe_2$.

5-hydroxymethyl-furfural and 20-hydroxyecdysone in the case of **1** and ajugasterone C in the case of **2**. Structurally related ecdysteroids with an acetal function in the side-chain, 20-hydroxyecdysone 20,22-ethylidene and ajugasterone 20,22-ethylidene, were isolated earlier from *Serratula coronata*.[10]

The plants often biosynthesize C-22 conjugated ecdysteroids to produce defensive constituents against insects. The two isolated compounds are a new type of C-22 conjugated ecdysteroid. These ecdysteroid derivatives are not detectable by the taste receptors of insects and might be hydrolyzed in the guts of insects to the active parent ecdysteroids.

Compound **2** contains an 11α-hydroxy group. Structure/activity experiments have confirmed that the 11α-hydroxy group in ecdysteroids is important for the manifestation of anabolic activity. The protein synthesis-enhancing the effect of turkesterone, an 11α-hydroxylated ecdysteroid, is comparable to that of Nerobol.[11]

Acknowledgements

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6. *Isolation of* **1** *and* **2**: The roots of *S. wolffii* (4.7 kg) were extracted with MeOH at room temperature, and the extract was subjected to fractional precipitation with acetone. The dry residue of the purified extract was chromatographed on a column of MN-polyamide SC 6 (Woelm, Eshwege, Germany). The fraction eluted with water (24.4 g) was separated by low-pressure reversed-phase column chromatography on octadecyl silica. The fraction eluted with 50% MeOH–H_2O (390 mg) was fractionated by repeated rotation planar chromatography (Harrison Model 8924 Chromatotron) on silica. In the second chromatographic step, the fraction eluted with EtOAc–EtOH–H_2O (80:2:1, v/v/v) was purified by reversed-phase HPLC (Zorbax SB C_{18} 250 × 4.6 mm i.d.; ACN–H_2O, 35:65, v/v, 0.8 ml/min) to obtain **1** (0.5 mg). The fraction (70 mg) eluted from the reversed-phase column with 60% MeOH–H_2O was separated by RPC (rotation planar chromatography). The fraction eluted with CH_2Cl_2–MeOH–C_6H_6 (50:10:6, v/v/v) was purified by reversed-phase HPLC (Zorax SB C_{18}, 5 μm, 250 × 4.6 mm i.d.; ACN–H_2O, 35:65, v/v, 1 ml/min) to yield **2** (0.5 mg).
7. *General procedure for NMR measurements*. NMR spectra were recorded in MeOH-d_4 (**1**) or in DMSO-d_6 (**2**) in Shigemi sample tubes at room temperature, using a Varian Inova-600 (**1**) or a Bruker Avance DRX-500 (**2**) spectrometer. The structures of the products were determined by comprehensive one- (1D) and two-dimensional (2D) NMR methods, using widely accepted strategies (Pretsch, E.; Tóth, G.; Munk, M. E.; Badertscher, M. In *Spectra Interpretation and Structure Generation*; Wiley-VCH: Weinheim, 2002; Duddeck, H.; Dietrich, W.; Tóth, G. Structure Elucidation by Modern NMR. In *A Workbook*; Springer-Steinkopff: Darmstadt, 1998).
8. *The physical properties of serfurosterone A [20-hydroxyecdysone 20,22-(5′-hydroxymethyl)-furfurylidene; (20R,22R)-20,22-O-(5′-hydroxymethyl-furfurylidene)-2β,3β,14α,25-tetrahydroxy-5β-cholest-7-en-6-one]* (**1**). $[\alpha]_D^{25.5}$ +56 (*c* 0.0025, DMSO); UV (DMSO) λ_{max} (log ε) 258.7 (3.925) nm. *Serfurosterone B [ajugasterone C 20,22-(5′-hydroxymethyl)-furfurylidene; (20R,22R)-20,22-O-(5′-hydroxymethyl-furfurylidene)-2β,3β,11α,14α-tetrahydroxy-5β-cholest-7-en-6-one]* (**2**). $[\alpha]_D^{25.5}$ +80 (*c* 0.0025, DMSO); UV (DMSO) λ_{max} (log ε) 255.7 (3.874) nm.
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