Department of Pharmacognosy

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Investigation of plants containing unusual ecdysteroids with ring in their side-chain

Ph.D. Thesis

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ABBREVIATIONS

^{13}C NMR = carbon nuclear magnetic resonance spectroscopy

1D = one-dimensional, 2D = two-dimensional

^{1}H NMR = proton nuclear magnetic resonance

20E = 20-hydroxyecdysone

CC = column chromatography

CID = collision-induced dissociation

CoMFA = comparative molecular field analysis

COSY = correlation spectroscopy

DEPT = distorsionless enhancement by polarization transfer

E = ecdysone

EcR = ecdysteroid receptor

EIMS = electron-impact mass spectroscopy

ESIMS = electrospray ionization mass spectroscopy

FID = free induction decay

HMBC = heteronuclear multiple bond coherence spectroscopy

HMQC = heteronuclear multiple quantum coherence spectroscopy

HPLC = high-performance liquid chromatography

HRESIMS = high resolution electrospray ionization mass spectroscopy

MDR = multiple drug resistance

MS = mass spectroscopy

NOESY = nuclear Overhauser effect spectroscopy

NP = normal-phase

QSAR = quantitative structure-activity relationship

ROESY = rotating-frame Overhauser effect spectroscopy

RP = reversed-phase

RP-CC = reversed-phase column chromatography

RPC = rotation planar chromatography

SPE = solid-phase extraction

TLC = thin-layer chromatography

TOCSY = total correlated spectroscopy

UV = ultraviolet

1. INTRODUCTION

Ecdysteroids are hormones of arthropods and many other invertebrates. The first ecdysteroid, ecdysone (E) was isolated from silkworm pupae (*Bombyx mori*) by German scientists, Butenandt and Karlson, in 1954.[1] Ecdysteroids play essential roles in all insects, as they regulate major transitional events during insect growth: molting, metamorphosis, reproduction and diapause.[2] Many of the morphological, physiological and biochemical processes that occur during molting are induced and modulated by the main molting hormone 20-hydroxyecdysone (20E), although its precursor, E, has a role independent of that of 20E.[3] In the 1960s, phytoecdysteroids, the plant analogs of ecdysteroids, 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, but more than 300 compounds have already been identified. The levels of ecdysteroids in plants are usually between 0.1 and 3% of the dry weight, which is 1000-fold higher than in insects.[6] It should be mentioned that some crop plants, for e.g. spinach (*Spinacia oleracea*) and quinoa (*Chenopodium quinoa*), contain considerable amounts of ecdysteroids (40-300 µg/g fresh weight).[7,8]

Their roles in plants are still unclarified, but it is believed that ecdysteroids offer protection against non-adapted phytophagus invertebrates.[9] The ready availability of ecdysteroids in different plants species has allowed pharmacological studies, which have suggested that they have many positive pharmacological properties. This is in accordance with the use of some ecdysteroid-containing plants in traditional medicine. In spite of the accumulating evidence of their beneficial pharmacological properties, their mode of action and their metabolism in mammals are still unknown.[10] Ecdysteroids are not endogenous participants in the mammalian metabolism, and are not toxic to mammals. They can be toxic to insects by disrupting hormonal processes upon ingestion, which has led to the development of safe insecticides. Nevertheless, the ecdysteroids themselves are not used in the control of pests because of their high polarity and their environmental instability. In contrast, bisacylhydrazines, functional analogs of ecdysteroids, are successful and selective pest control agents.[11,12]

Most of the actions of ecdysteroids in insects are mediated by the intracellular ecdysteroid receptor (EcR) complex, which modifies the activities of specific gene sets. Since neither ecdysteroids nor the EcR proteins are endogenous components of mammalian cells, this system can be used in the regulation of transfected genes. Because of their low mammalian

toxicity, the appropriate ecdysteroid agonists can be used in the generation of ecdysteroid-inducible gene-switch systems.[13,14]

1.1. Structural diversity of ecdysteroids

The basic chemical structure of ecdysteroids is a cyclopentano-perhydrophenanthrene (sterane) skeleton (**Figure 1**); the number of carbon atoms can be 19, 21, 24, 27 or 29. The C/D ring junction is generally *trans*, whereas the A/B ring is normally *cis* (5β-H) and only rarely *trans* (5α-H). Most ecdysteroids possess a 14α-hydroxy group and a 7-en-6-one chromophore in ring B, which results in characteristic ultraviolet (UV) absorption. Other common structural elements of ecdysteroids are the β-side-chain on C-17 and additional hydroxy groups on C-2, C-3, C-20, C-22 and C-25. The great number of possible ecdysteroid structures (more than 1000) can be explained by the variation in number, position and orientation of the hydroxy groups. Some ecdysteroids are conjugated with acids, alcohols or sugars. Additional unsaturation, or the presence of a cyclic ether or a lactone ring (5- or 6-membered) at C-17 can occur. Phytoecdysteroids are biosynthesized from cholesterol or other sterols (lathosterol, stigmasterol or brassicasterol), although the precise biosynthetic pathways involved in the production of phytoecdysteroids are unknown.

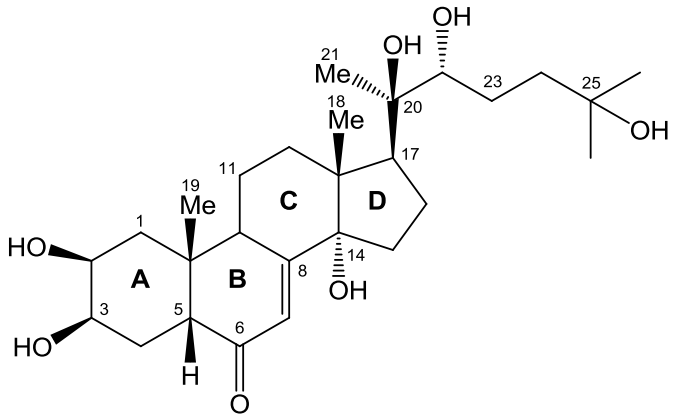


Figure 1. Structure of the most common ecdysteroid, 20E.

The structural diversity of ecdysteroids has provided a good opportunity for investigations of the structure–insect hormone activity relationships by means of comparative molecular field analysis (CoMFA) and 4D-quantitative structure–activity relationship (QSAR) techniques.[15,16]

The data obtained from such studies have led to a pharmacophore hypothesis for the interactions of ligands with the ecdysteroid receptor: it is postulated that the key molecular

features responsible for the activity are not identical to the characteristic structural elements mentioned above. The structure–activity studies have revealed 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.[17]

1.2. Pharmacological effects of ecdysteroids on mammals

The first pharmacological tests on mammals (mice and rats) were performed in the 1960s from the aspect of their potential use as insecticides.[18,19] These tests were followed by many other experiments relating to a wide range of pharmacological effects of ecdysteroids on mammals: anabolic, adaptogenic, wound healing and roborant properties, to mention only a few.

The most important action of ecdysteroids is the stimulation of protein synthesis by increasing the level of mRNA translation in the liver polysomes.[20] Comparison of the physical performance or biochemical parameters of experimental animals treated with ecdysteroids with those of animals which received anabolic vertebrate steroids showed a significant anabolic effect of the ecdysteroids,[21] without the adverse androgenic, antigonadotropic and thymolytic side–effects described after the administration of vertebrate steroid hormones.[22] Study of the structure–anabolic activity relationship revealed that the presence of the 2,3-diol system and the hydroxy groups on C-11 and C-20 are decisive for the appearance of the anabolic effect. Among the ecdysteroids tested for anabolic activity, turkesterone, possessing an 11α-hydroxy group, was found to be the most effective.[23] In rats 20E affects the fiber size in a muscle-specific fashion, and increases the myonuclear number in the fibers of normal and regenerating muscles.[24,25] Gorelick-Feldman *et al*. demonstrated that 20E elicited a rapid elevation in intercellular calcium, in a mouse skeletal muscle cell line C_2C_{12}, followed by sustained Akt activation and enhanced protein synthesis.[26]

The ecdysteroids affect not only protein synthesis, but also the lipid and carbohydrate metabolisms. They have a hypocholesterolemic effect, which is explained by the increased conversion of cholesterol into bile acids, reminiscent of the effect of oxysterols; dietary 20E reduces lipid peroxidation in the membranes.[27,28] Ecdysteroids can reduce the hyperglycaemia induced by the administration of glucagon or alloxan.[29] Ecdysteroid-containing plants are used in traditional medicine for their antidiabetic effect (e.g. *Ajuga iva* and *Morus alba*),[30,31] and some preparations containing ecdysteroids have been proposed for use against elevated blood glucose levels. Ecdysteroids can prevent the hepatotoxic action of

heliotrine and carbon tetrachloride in rats.[32] 20E can restore the normal glomerular filtration rate and suppress albuminuria in rats treated with a nephrotoxic mixture.[33]

Ecdysteroids exert several effects on the central nervous system: the induction of glutamic decarboxylase and acetyl cholinesterase in the rat brain, the production of antiepileptic activity in spontaneously epileptic rats via the $GABA_A$ receptors, and the protection of neurons against the deleterious effects of various drugs.[34-37] Ecdysteroids may accelerate wound healing,[38] and exert beneficial effects on the joint, epiphyseal cartilage tissue and trabecular bone in ovariectomized rats.[39]

The mode of action of ecdysteroids in mammals is not fully understood. There is no specific ecdysteroid receptor in mammals, but there are some indications that the retinoid X receptor (RXR) may be involved in the mediation of the effects of ecdysteroids in mammals.[40] Ecdysteroids do not bind to nuclear receptors in mammals, because their side-chain prevents any binding to the receptors of the steroid hormones. 20E can decrease the cyclic AMP level and increase the synthesis of leukotrienes and prostaglandins.

Ecdysteroids are known to display 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.[41] Many ecdysteroid-containing preparations are frequently used by body builders, and it is known from these users that ecdysteroids have no adverse effects when taken in quite large (~1 g) quantities. Although there have been no clinical trials on the possible effects of ecdysteroids on humans, over 300 different preparations containing ecdysteroids are available, mainly on the internet. Little is known about the metabolism of ecdysteroids in mammals. 20E is rapidly metabolized *in vivo*; the roles of its metabolites in the manifestation of different pharmacological activities are rather unknown. The experiments made by Girault *et al.* revealed that the major steps in the ecdysteroid metabolism are reduction in ring B, epimerization at C-3 and dehydroxylation at C-14.[42] Another important metabolic pathway could be the side-chain cleavage between C-20 and C-22.[43]

Ecdysteroid-inducible gene expression is an area of major interest as concerns biomedical application, e.g. gene therapy. Such gene systems should provide fast and reversible induction or suppression of the target gene, without any interference with the endogenous regulatory networks of the host cells. The ecdysteroids fulfill the above-mentioned requirements, as they are neither toxic nor teratogenic to vertebrates and they can easily penetrate into all tissues. Early ecdysteroid-related gene-switch systems were based on the *Drosophila melanogaster* EcR, in conjunction with co-transfected ultraspiracle or by using endogenous RXR as the heterologous partner.[44,45] In these systems, the most common ecdysteroid, 20E, is ineffective as an elicitor, while the rare phytoecdysteroids muristerone A

and ponasterone A are active.[46] Other gene-switch systems have been developed, including one which is independent of recombinant RXR. Several commercial gene-switch systems are available for experimental use, e.g. those produced by Invitrogen and Rheogene.[47,48]

The resistance of cancer cells to different drugs is a common aspect of anticancer therapy, and multi-drug resistance (MDR) is a major cause of failure in cancer chemotherapy. The most frequent mechanism behind the drug resistance is the increased excretion of the drug from the cell, as a consequence of upregulation of the efflux pumps.[49,50] Intensive research has been carried out to find natural compounds able to counteract MDR. Many natural compounds with potential MDR-reversal effects have been identified, but their application in therapy is very limited.[51] Recent experiments demonstrated that certain ecdysteroids possess a strong MDR-reversal effect, which could be of therapeutic significance.[52,53] Among the ecdysteroids involved in those experiments, those which were more apolar seemed to be the more effective. The strong activity of some ecdysteroids in modulating of multi-drug resistance of a cancer cell line has given a new perspective to ecdysteroid research; further studies are needed to elucidate the mechanisms of the MDR-reversal effects.

1.3. Aims of our studies

The extensive ecdysteroid research at the Department of Pharmacognosy dates back to the late 1970s. During this period a novel, rapid isolation process has been developed by the ecdysteroid research group, based on simplified, improved methodology, which has led to the isolation of many new phytoecdysteroids. The large-scale extraction of ecdysteroids is a key feature of ecdysteroid research, since the synthesis of ecdysteroids is not economically feasible. The investigation of ecdysteroids is a developing area of biomedical chemistry, which involves plant screening, identification of the biologically active compounds and study of the practical application possibilities.

Our most important aims were as follows:

1. To study the ecdysteroid profile of the aerial parts of *Ajuga reptans* var. *reptans*, the rhizome of *Polypodium vulgare* and the roots of *Serratula wolffii*, which involves the isolation, structure determination and identification of new phytoecdysteroids.
2. It was a main objective to find new compounds with unusual structures:
   - The isolation of ecdysteroids with the rare C_{29}-ecdysteroid skeleton
   - The identification of phytoecdysteroids which form a ring in the side-chain
3. To isolate novel compounds with potentially high molting activity

4. To provide a wide range of structurally different ecdysteroids for MDR studies, which would help reveal specific structure–activity relationships and identify compounds with potential MDR-reversal effects

1.4. Botanical characterization and taxonomic classification of *A. reptans*

The common bugle (*Ajuga reptans* L.) is a rhizomatous perennial with long stolons; the stem is 10-40 cm high, pubescent on opposite faces, alternating at each node, and sometimes glabrous at the base. The lower leaves are ovate, entire or crenate. The bracts are ovate, and often tinged with blue; the upper leaves are shorter than the flowers. The verticillasters are crowded, usually 6-flowered. The calyx is 4-6 mm long; the teeth are approximately as long as the tube. The corolla is blue, rarely pink or white; the tube exceeds calyx; the upper lip is entire. The stamens are exserted, and the filaments are hairy. *A. reptans* is native to Europe. It is largely cultivated as an ornamental plant, and has many varieties.[54]

Taxonomic classification:[55]

Division: Magnolyophyta
Class: Magnoliopsida
Subclass: Asteridae
Order: Lamiales
Family: Lamiaceae
Subfamily: Ajugoidae
Genus: *Ajuga* L.
Species: *Ajuga reptans* L.

1.5. Botanical characterization and taxonomic classification of *P. vulgare*

The common polypody (*P. vulgare* L.) is a perennial fern growing to a height of 30 cm. The lanceolate rhizomes are thick and ramifying. New leaves are produced in early summer. The blades are 1-3 times as long as the petiole. The leaves are lanceolate to linear-lanceolate; the obtuse or somewhat acute pinnae are entire, with entire or crenate margins, rarely more deeply serrate. The round, brownish-yellow to rust-brown sori appear in two rows on the underside, one on each side of the midrib, mainly in the upper half of the blade. The secondary veins have 1-3 bifurcations. The spores are yellow, with a warty, folded surface 60-75 μm long. *P. vulgare* is found in shaded, rocky habitats in Eurasia and North America; in a wetter climate, it can grow as an epiphyte on trees.[56]

Taxonomic classification:[57]

Division: Pterydophyta

Class: Polypodiopsida

Order: Polypodiales

Family: Polypodiaceae

Genus: *Polypodium* L.

Species: *Polypodium vulgare* L.

1.6. Botanical characterization and taxonomic classification of *S. wolffii*

S. wolffii Andrae is a perennial herb with a stout, erect, subglabrous stem, with a height of 80-150 cm. The basal leaves are irregularly pinnatifid; the segments usually elliptic-lanceolate. The leaflets irregularly serrate with setulae on the margin and veins. The campanulate capitula is 25-30 mm long; there can be up to 15 capitula in a lax, irregular panicle. The outer bracts are acute and velutinous; the inner bracts are rigid and long-attenuate. The florets are purple. *S. wolffii* is native to Eastern Europe.[58]

Taxonomic classification:[59]

Division: Magnolyophyta

Class: Magnoliopsida

Subclass: Asteridae

Order: Asterales

Family: Asteraceae

Subfamily: Tubuliflorae

Tribe: Cynareae

Subtribe: Centureinae

Genus: *Serratula* L.

Species: *Serratula wolffii* Andrae

1.7. Chemical constituents of *A. reptans*

Earlier phytochemical investigations of *A. reptans* revealed the presence of iridoids, diterpenoids, ecdysteroids and anthocyanins, to mention only the most important secondary metabolites. The main iridoid compounds found in this species are 8-acetylharpagide, harpagide, ajureptoside, reptoside and 6-*epi*-acetylharpagide.[60-64] Several diterpenoids with neo-clerodane structure (ajugareptansin, ajureptoside A-D, areptin A-B, etc.) have been isolated from *A. reptans*.[65-69] The phytoecdysteroids are also typical substances of this plant; the occurrence of several ecdysteroids has been documented (**Table 1**).

Table 1. Ecdysteroids isolated from *A. reptans*

Ecdysteroid	Reference
Ajugalactone; cyasterone; polypodine B; 20E, 29-norcyasterone; 29-norsengosterone	Camps *et al.*, 1981 [70]
Sengosterone	Calcagno *et al.*, 1994 [71]
Reptansterone	Calcagno *et al.*, 1996 [72]
Ajugasterone B; polypodine B; 20E; 29-nor-cyasterone; 29-norsengosterone; sengosterone; ajugalactone	Alekseeva *et al.*, 1998 [73]
20E-22-acetate; viticosterone E (20E-25-acetate)	Alekseeva *et al.*, 2000 [74]

1.8. Chemical constituents of *P. vulgare*

P. vulgare is very rich in biologically active compounds, which belong in different classes of plant metabolites. This species has been reported to contain many triterpene hydrocarbons [e.g. hop-22(29)-ene, fern-9(11)-ene and serrate-14-ene], which are not common substances in nature; they are found mainly in different fern species. *P. vulgare* is also known to contain triterpenoid alcohols of the cycloartane group (cycloartanol, cyclolaudenol and their derivatives).[75-77]

Phytoecdysteroids are characteristic plant steroids of common polypody; several have been isolated to date (**Table 2**). The presence of phenolic compounds (polydin) has also been documented.[78,79]

Table 2. Ecdysteroids from *P. vulgare*

Ecdysteroid	Reference
Polypodine B	Jizba *et al.*, 1967 [80]
20E	Jizba *et al.*, 1967 [81]
E	Heinrich *et al.*, 1967 [82]
Polypodosaponine; 26-methylpolypodosaponine	Jizba *et al.*, 1971 [83]
Abutasterone; 5-hydroxy-abutasterone; Inokosterone; 24-hydroxyecdysone; pterosterone	Coll *et al.*, 1994 [84]
Osladine	Jizba *et al.*, 1971,[85] Yamada *et al.*, 1992 [86]

1.9. Chemical constituents of *S. wolffii*

Ecdysteroids have been isolated in spectacular structural diversity from *S. wolffii*. **Table 3** details ecdysteroids isolated from *S. wolffii*. This species has been found by TLC-densitometric determination to contain 0.13-0.85% of 20E.[87]

Table 3. Ecdysteroids isolated from *S. wolffii*

Ecdysteroid	**Reference**
Ajugasterone C; ajugasterone C 20,22-monoacetonide; integristerone A; 20E; 20E 20,22-monoacetonide; 20E-2,3,20,22-diacetonide; polypodine B; pterosterone	Miladera *et al.*, 1992 [88]
Herkesterone; 11α-hydroxypoststerone	Hunyadi *et al.*, 2004 [89]
Ajugasterone C; ajugasterone D; dacryhainansterone; 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	Hunyadi *et al.*, 2007 [90]
20,22-Didehydrotaxisterone; 1-hydroxy-20,22-didehydrotaxisterone	Liktor-Busa *et al.*, 2007 [91]
2β,3β,20*R*,22*R*,2*S*-Pentahydroxy-5β-cholest-6,8(14)-diene, 24-methyleneshidasterone; stachysterone B; 14α,15α-epoxy-14,15-dihydrostachysterone B	Simon *et al.*, 2007 [92]
Serfurosterone A and B	Liktor-Busa *et al.*, 2008 [93]
(11α)-11-Hydroxyshidasterone; (2β,3α,5β,14β,22*R*)-2,3,20,22,25-pentahydroxycholest-7-en-6-one; (2β,3α,5β,22*R*)-2,3,20,22,25-pentahydroxycholest-7-en-6-one; ponasterone A	Simon *et al.*, 2008 [94]
22-Dehydro-20-deoxy-ajugasterone C; 1-hydroxy-22-deoxy-20,21-didehydroecdysone; 22-deoxy-20,21-didehydroecdysone	Takács *et al.*, 2010 [95]

2. MATERIALS AND METHODS

2.1. Plant material

The herb *A. reptans* var. *reptans* was collected in August 2003 from harvested populations at the Experimental Station of the Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary. A voucher specimen (collection number A0308) has been deposited at the Department of Ecotoxicology, Plant Protection Institute, Budapest, Hungary.

Rhizomes of *P. vulgare* L. were collected in October 2008 from three Hungarian locations: the environs of Veszprém, Egerbakta and Kőszeg. A voucher specimen (collection number P71) has been deposited at the Department of Pharmacognosy, University of Szeged, Hungary.

Roots of *S. wolffii* Andrae were collected from cultivated populations 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. Their identities and purities were verified by NMR, MS and HPLC.

2.3. General experimental procedures

2.3.1. General methods and apparatus

Optical rotations were measured with a Perkin-Elmer 341 polarimeter in MeOH. UV spectra were taken 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 Bruker Avance DRX 500, Varian Mercury plus 400, VNMRS 800 and a Varian 800 MHz NMR spectrometer equipped with a 1H{13C/15N} Triple Resonance ^{13}C Enhanced Salt Tolerant Cold Probe operating at 800 MHz for ^{1}H and 201 MHz for ^{13}C NMR. Chemical shifts were given on the δ-scale and were referenced to the solvent (MeOH-d_4: δC = 49.15 and δH = 3.31). In the 1D measurement (^{1}H, ^{13}C and DEPT-135), 64 K data points were used for the FID. The pulse programs of all experiments [gs-COSY, gs-HMQC-TOCSY (mixing time = 80 ms)], gs-HMQC; gs-HMBC, gs-NOESY (mixing times = 350 ms), ROESY

(mixing time = 250-350 ms), and 1D gs-NOESY (mixing time = 300 ms)] were taken from the Bruker and Varian software library.

Mass spectrometric measurements of compounds **1-5** were performed on a Finnigan TSQ 7000 tandem mass spectrometer (Finnigan MAT, San Jose, CA) equipped with a laboratory-built nanoelectrospray ion source. A 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). In the case of compounds **6-21**, MS analyses were performed on LTQ FT Ultra (Thermo Finnigan, San Jose, CA) and Thermo LTQ FT Ultra spectrometers (Thermo Fisher Scientific, Bremen, Germany). The ionization method was ESI and operated in positive ion mode. The ion transfer capillary temperature was set at 280 °C as well as the capillary voltage was 4.7 kV for each measurement. For CID experiment helium was used as the collision gas, and normalized collision energy (expressed in percentage), which is a measure of the amplitude of the resonance excitation RF voltage applied to the endcaps of the linear ion trap, was used to bring about fragmentation. The protonated molecular ion peaks were fragmented by CID at normalized collision energy of 35-40%. Data acquisition and analysis were accomplished with Xcalibur software version 2.0 (Thermo Electron Corp.).

2.3.2. Chromatographic techniques

NP-TLC

NP-TLC was performed on 20×20 cm silica plates (Silicagel 60 F_{254}) (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:

TLC1: dichloromethane–methanol–benzene (25:5:3, v/v/v),

TLC2: ethyl acetate–96% ethanol–water (16:2:1, v/v/v)

TLC3: toluene–acetone–96% ethanol–25% ammonia (100:140:32:9, v/v/v/v).

After development of chromatograms, 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 entire isolation process was controlled by using NP-TLC.

Solid-phase extraction (SPE)

MN-polyamide SC6 (particle size: 0.06-0.16 mm) (Woelm, Eschwege, Germany) was used as stationary phase. The mobile phases were water and aqueous methanol (9:1, 8:2, 7:3 and 1:1, v/v).

NP-CC on alumina

Aluminum oxide (Brockman II neutral, Reanal, Budapest, Hungary) was used as stationary phase. The mobile phases were dichloromethane–ethanol (95:5, 9:1, 85:15 and 8:2, v/v). 40 fractions were collected (100 mL each).

Vacuum reversed-phase column chromatography (RP-CC)

Vacuum RP-CC was carried out on end-capped octadecyl-silica (C_{18}) (0.06-0.2 mm particle size) (Chemie Uetikon-C-gel, C-560, Uetikon, Switzerland) packed into a 400×32 mm glass column.

Elution was performed with a stepwise gradient of 30%, 35%, 40%, 45%, 50%, 55% and 60% aqueous methanol. The pressure was less than 1 atm throughout the entire separation.

Rotation planar chromatography (RPC)

RPC was carried out on a Harrison Model 8924 Chromatotron instrument (Harrison Research, Palo Alto, CA, USA). The stationary phase for RPC was silica gel 60 GF_{254} (Merck, Darmstadt, Germany), manually coated on the rotor as a 1 or 2 mm layer. The mobile phases used for development were as follow:

RPC I: solvent system I/1: chloroform–methanol–benzene (50:3:2, v/v/v)
solvent system I/2: chloroform–methanol–benzene (50:5:3, v/v/v)
solvent system I/3: chloroform–methanol–benzene (50:10:6, v/v/v)

RPC II: solvent system II/1: ethyl acetate–ethanol–water (80:2:1, v/v/v)
solvent system II/2: ethyl acetate–ethanol–water (80:7:3, v/v/v)
solvent system II/3: ethyl acetate–ethanol–water (80:10:5, v/v/v).

The dry stationary phase was completely wetted with the first applied mobile phase (approx. 50 mL). The samples were dissolved in the first elution solvent and were introduced through the inlet. The separation was performed by means of gradient elution in multiple steps. The flow rate was 4 mL/min in the case of the 1 mm layer, and 6 mL/min in the case of the 2 mm layer; 30 fractions (10 mL each) were collected.

HPLC

HPLC analyses were performed with a Jasco Model PU-2080 Pump, Jasco Model UV-2070/2075 Detector (Jasco Inc., Easton, MD, USA).

Stationary phases

Analytical: Zorbax-Sil column (5 µm, 250×4.6 mm i.d.) (DuPont, Paris, France).

Semipreparative: Zorbax-Sil column (5 µm, 250×9.4 mm i.d.) (DuPont, Paris, France).

Mobile phases

NP-HPLC I: dichloromethane–isopropanol–water (125:65:6, v/v/v).

NP-HPLC II: dichloromethane–isopropanol–water (125:50:4, v/v/v).

NP-HPLC III: dichloromethane–isopropanol–water (125:40:4, v/v/v).

NP-HPLC IV: dichloromethane–isopropanol–water (125:40:3, v/v/v).

NP-HPLC V: dichloromethane–isopropanol–water (125:30:2, v/v/v).

NP-HPLC VI: cyclohexane–isopropanol–water (100:65:6, v/v/v).

NP-HPLC VII: cyclohexane–isopropanol–water (100:60:4.5, v/v/v).

NP-HPLC VIII: cyclohexane–isopropanol–water (100:50:4, v/v/v).

Chromatographic separations were monitored at 245 nm; the flow rate was 2.5 mL/min (semipreparative stationary phase) or 1 mL/min (analytical stationary phase).

2.4. Extraction and isolation

2.4.1. Extraction and prepurification of the crude extracts of A. reptans and P. vulgare

The dried plant material was milled, and percolated with methanol at room temperature (see **Table 4** with the data on the extraction and prepurification). The methanolic extract was evaporated to dryness using a Büchi Rotavapor (Büchi Labortechnik AG, Flawil, Switzerland) at 40 °C and 337 mbar. The dry residue was dissolved in methanol, and acetone was added to the solution. The resulting precipitate was separated by decantation and washed three times each with 100 mL of a methanol–acetone (1:1, v/v) mixture. The supernatant and the methanol–acetone solution were combined and evaporated to dryness. This precipitation step was repeated twice, and the final supernatant and the methanol–acetone solution were combined and evaporated to dryness. The dry residue was dissolved in a methanol–water mixture (5:1, v/v), and was further purified by solvent–solvent distribution using *n*-hexane. The aqueous methanolic phases were combined, and evaporated to dryness. The dry residue was dissolved in methanol and absorbed onto a polyamide stationary phase. This was added to the top of a polyamide column; the stationary phase was previously suspended in water. The ecdysteroids were eluted with water and aqueous methanol (9:1, 8:2, 7:3 and 1:1, v/v). The NP-TLC analysis revealed an abundance of ecdysteroids in the fractions eluted with water and with aqueous methanol 9:1 and 8:2. These fractions were further separated by a combination of other chromatographic techniques.

Table 4. Data on the extraction and prepurification of the crude extracts of *A. reptans* and *P. vulgare*

		***A. reptans* herb**	***P. vulgare* rhizome**
Dry weight (kg)		1.24	1.4
Extraction solvent volume (L)		15	15
Dry residue after extraction (g)		159.5	254.1
Methanol volume used for dissolution (mL)		800	500
Fractionated precipitation	Acetone volume I (mL)	800	500
	Acetone volume II (mL)	1600	800
	Acetone volume III (mL)	-	1200
Dry residue after precipitation (g)		113	136.4
Methanol–water (5:1) volume used for dissolution (mL)		600	1000
n-Hexane volume used to solvent–solvent distribution (mL)		5×300	5×400
Solid phase extraction on polyamide	Polyamide (g)	500	500
	Dry residue adsorbed (g)	99.9	107.1
	Fraction volume (L)	2	1.5

2.4.2. Isolation of ecdysteroids from A. reptans

The fraction (62.3 g) eluted with water from the polyamide column was further fractionated by vacuum RP-CC (RP-CC1) on octadecyl-silica (180 g packed into a 400×32 mm glass column). The vacuum CC was performed by using gradient elution with 30%, 35%, 40%, 45%, 50%, 55% and 60% aqueous methanol (1000 mL each) at a flow rate of 5 mL/min; 35 fractions (200 mL each) were collected. The fractions containing the same compounds were combined and evaporated to dryness.

The fractions obtained with 40% aqueous methanol (0.12 g) were dissolved in 5 mL of the first eluent of solvent system RPC I and fractionated with the same solvent system. After TLC analysis, the resulting fractions were combined, and were purified repeatedly with NP-HPLC IV and NP-HPLC VIII to give compound **1** (0.7 mg). Another fraction eluted from the RP column with 45% aqueous methanol (0.17 g) was dissolved in 5 mL of the first eluent of solvent system RPC I, and fractionated with the same solvent system. From the fraction (2.1 mg) eluted with solvent system I/2 by RPC, compound **2** (1.3 mg) was obtained with NP-HPLC V.

The fractions eluted with water–methanol (9:1 and 8:2) (7.8 g) from the polyamide column were combined and further purified by NP-CC on alumina (160 g packed into 400×32 mm glass column). The separation was performed with dichloromethane and dichloromethane–ethanol (96%) mixtures (95:5, 9:1, 85:15, 8:2 and 7:3, v/v) using gradient elution; 50 fractions (10 fractions with each eluent) were collected, each of 100 mL. The combined fraction (0.61 g) obtained by elution from the alumina with dichloromethane–ethanol (9:1) was further separated by RP-CC (RP-CC2) on octadecyl-silica. The fraction eluted with methanol–water (35:65, v/v) (25.2 mg) was subjected to RPC, using solvent system II/1. The residue (2.3 mg) obtained from the elution with methanol was purified by repeated HPLC (NP-HPLC VII and NP-HPLC V) resulting in compound **3** (1.5 mg). The RP-CC2 gave a fraction (0.39 g) eluted with methanol–water, 40-60 and 45:55 v/v, which was purified by RPC using solvent system I to obtain compound **4** (2.7 mg).

Another fraction (0.33 g) eluted from the RP column with methanol–water, 50:50 v/v, was subjected to RPC and fractionated with solvent system I. The combined fraction obtained with solvent system I/3 was finally purified with NP-HPLC IV to give compound **5** (1.6 mg).

2.4.3. Isolation of ecdysteroids from P. vulgare

The fractions eluted with water and with water–methanol 9:1 and 8:2 from the polyamide column were combined (41.7 g) and subjected to RP-CC on octadecyl-silica (150 g packed into a 400×32 mm glass column). Five combined fractions obtained by RP-CC were selected for further purification by RPC on silica.

The fractions (0.94 g) eluted from the reversed-phase column with methanol–water (40%) were dissolved in 5 mL of the first eluent of solvent system RPC I and fractionated with the same solvent system. The fractions eluted with solvent system I/2 were fractionated again with RPC II. The ecdysteroids obtained after RPC II/2 were subjected to normal-phase HPLC (solvent system NP-HPLC II) to obtain **6** (1.5 mg), **7** (2.2 mg) and **8** (1.8 mg).

The fractions obtained by vacuum RP-CC, using 45% aqueous methanol (0.39 g) were separated by repeated RPC, applying solvent systems RPC I and RPC II. The elution with solvent system RPC II/2 resulted in some ecdysteroid-containing fractions, which were further purified by preparative HPLC using solvent system NP-HPLC II to give **9** (1.5 mg).

The RP-CC fractions eluted with 50% aqueous methanol (0.40 g) were applied to an RPC plate and were fractionated by repeated RPC (solvent systems I and II) The fraction obtained with solvent system RPC II/3 was then purified with NP-HPLC II to furnish compounds **10** (1.2 mg) and **11** (1.8 mg). Other RPC fractions obtained with solvent system II/2 were further purified with solvent system NP-HPLC II to give compound **12** (1.6 mg).

Compounds **13** (8.3 mg), **14** (1.2 mg) and **15** (1.6 mg) were isolated from the RP-CC fractions eluted with 55% aqueous methanol (0.44 g) after RPC separation using solvent systems I/1 and II/3. The fractions obtained with solvent system RPC II/2 were subjected to a final purification by repeated HPLC (solvent systems NP-HPLC V and VI).

The RP-CC fractions eluted with 60% aqueous methanol (0.60 g) were separated by RPC (solvent systems I and II). The fractions obtained with RPC II/2 and II/3 were purified by HPLC (NP-HPLC I) to give compounds **16** (0.9 mg), **17** (3.4 mg) and **18** (2.2 mg).

2.4.4. Extraction and isolation of ecdysteroids from S. wolffii

The extraction and prepurification of the roots of *S. wolffii* were carried out by Erika Liktor-Busa, as detailed in her Ph.D. thesis.[96] The fresh roots of *S. wolffii* (4.7 kg) were extracted with methanol at room temperature, and the extract was purified by precipitation with acetone. The supernatant was evaporated to dryness. The dry residue (137.5 g) was dissolved in methanol and was applied to a polyamide column.

The fraction (24.4 g) eluted from the polyamide column with water was subjected to low-pressure RP-CC on octadecyl-silica. The RP-CC fraction (0.39 g) eluted with methanol–water (50:50, v/v) was further fractionated by RPC (solvent systems I and II). The ecdysteroid-containing fractions were purified with NP-HPLC III to obtain compounds **19** (1.2 mg) and **20** (1.1 mg). Another fraction (0.48 g) eluted from the RP column with methanol was purified by repeated RPC (solvent systems I and II), followed by normal-phase HPLC (NP-HPLC IV) which resulted in compound **21** (1.1 mg).

3. RESULTS

3.1. Isolation of ecdysteroids

The entire isolation procedure consisted of two main steps:

– the extraction and the clean-up of the crude extract, followed by

– the isolation of ecdysteroids from the prepurified extract by using a combination of chromatographic methods.

The percolation was carried out with methanol which is the optimal solvent for extraction of the polar ecdysteroids; the plant–methanol ratio was 1:10. The prepurification process comprised three steps: fractionated precipitation, partition and SPE (**Figure 2**).

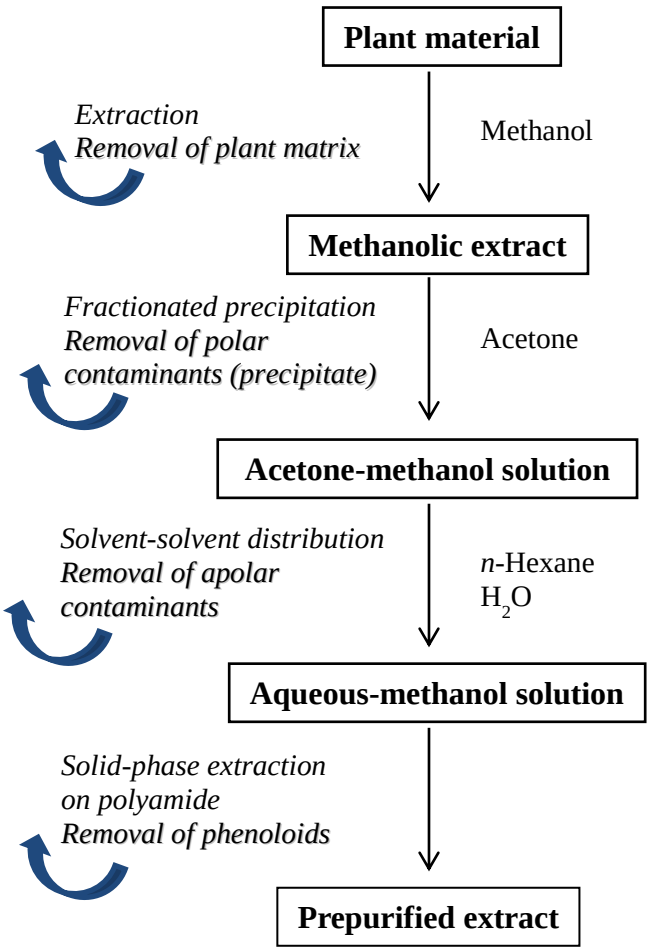


Figure 2. Scheme of the extraction and the prepurification of the crude extracts of *A. reptans* and *P. vulgare*

In the fractionated precipitation carried out in two or three steps, acetone was added to the methanolic extract, which precipitated the polar contaminants, while the ecdysteroids remained dissolved in the methanol–acetone solution. The metanol–acetone volumetric ratios in the three consecutive steps were 2:1, 1:1 and 1:2, with increasing acetone proportion. Solvent–solvent distribution between *n*-hexane and water–methanol (1:5, v/v) allowed purification of the ecdysteroids from apolar contaminants.

The water–methanolic (1:5, v/v) solution was extracted with *n*-hexane, the apolar contaminants passing into the *n*-hexane phase and the ecdysteroids remaining dissolved in the aqueous methanol phase. The residue of the prepurified extract was subjected to CC on polyamide. The ecdysteroids were eluted from the stationary phase with water and with water–methanol 9:1 and 8:2. The phenoloids remained adsorbed on the polyamide.

The prepurified extracts were fractionated by using the combination of NP-CC on alumina, vacuum RP-CC, RPC and preparative HPLC. The separation on the alumina stationary phase was carried out with dichloromethane and dichloromethane–ethanol (96%)

mixtures (95:5, 9:1, 85:15, 8:2 and 7:3, v/v) using gradient elution. The fractions with high ecdysteroid content obtained by elution from the alumina with dichloromethane–ethanol (9:1) were further separated by RP-CC on octadecyl-silica. The sample to stationary phase ratio was 1:3. The fractions eluted with 40, 45, 50, 55 and 60% aqueous methanol by RP-CC contained ecdysteroids, and were further purified by RPC on silica. The RPC separation was performed with stepwise gradient elution in multiple steps (**Figure 3**).

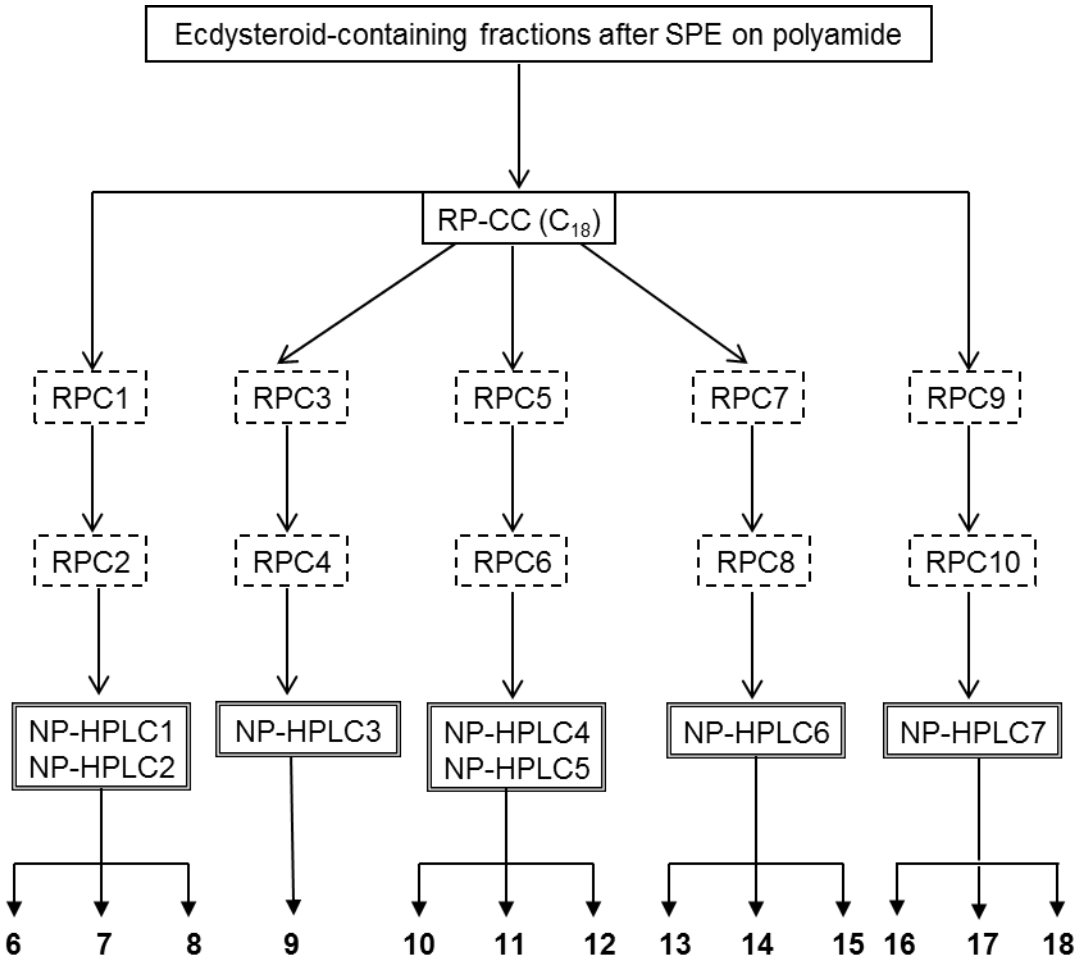


Figure 3. Scheme of the isolation of pure compounds **6-18** from the prepurified extract of *P. vulgare*.

The combination of RPC and HPLC proved to be a very useful chromatographic tool for the separation and purification of structurally related ecdysteroids. **Figure 4** illustrates separation of an ecdysteroid-containing fraction of *P. vulgare* by HPLC.

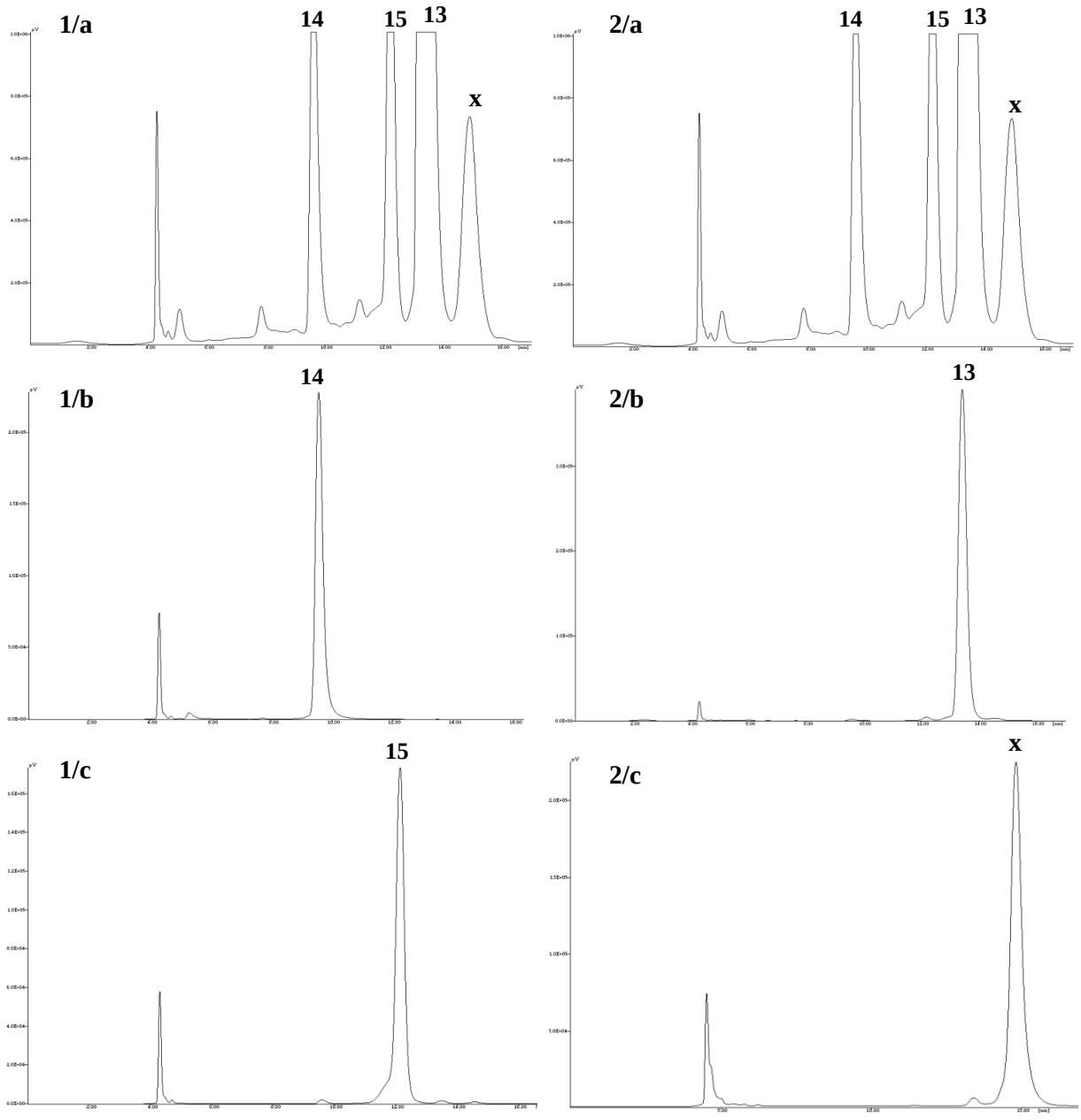


Figure 4. HPLC separation of the fraction containing **13-15** and an unidentified ecdysteroid **x. 1/a** and **2/a** represent the chromatograms of the original fraction, **1/b-c** and **2/b-c** are the chromatograms of pure compounds obtained by HPLC. The separation was carried out on a Zorbax Sil column (5 µm, 250×9.4 mm i.d.), the mobile phase was dichloromethane–isopropanol–water (125:30:2, v/v/v) at 2.5 mL/min.

Repeated RPC using solvent systems with different selectivities was employed for a better separation. The combination of RP-CC and RPC generally resulted in fractions containing a mixture of structurally similar ecdysteroids, which were separated by NP-HPLC with different mobile phases to obtain pure compounds.

3.2. Characterization of the isolated compounds

The chromatographic and the physical behavior of the compounds were examined. Three mobile phases were used for TLC analysis. The stationary phase was silica with a fluorescence indicator. Multiple detections were used to analyze the TLC plates: 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. NP-HPLC systems were also used for the characterization of each compound.

The known ecdysteroids were identified by direct comparison of their physical and spectroscopic characteristics with those available in the literature. In addition to chromatography, all compounds were characterized by different spectroscopic methods, e.g. UV spectroscopy, NMR and MS. The UV spectra provided characteristic information on the 7-en-6-one chromophore. The majority of ecdysteroids have strong UV absorption spectra with a maximum at 240-245 nm. **Table 5** lists some physical and spectroscopic characteristics for the isolated compounds, such as optical rotation and UV spectroscopic data.

The structural elucidation of the compounds involved the evaluation of the MS and NMR spectra data in comparison with those for the well-known ecdysteroid, 20E. The ecdysteroids usually suffer side-chain cleavage, between C-20 and C-22, and between C-17 and C-20. The mass numbers vary, depending on the extent of hydroxylation of the side-chain and the nucleus. The fragmentation results in two major series of fragments, which correspond to the loss of water from the nucleus and from the side-chain. The data on the MS fragmentation of the isolated new ecdysteroids are given in **Table 6**.

The numbers of C, CH, CH_2 and CH_3 fragments in each 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 methyl groups could be used as starting points for structure elucidation, because their signals are singlets and display strong two- and three-bond correlations in the HMBC spectrum. The correlations in the NOESY spectrum provided information about the stereochemistry of the rings and the orientation of the substituents connected to the skeleton. The β-orientation of the H attached to C-14 was justified by the NOESY correlations between H_3-18/H-14. **Tables 6-12** contain the NMR data on the isolated new compounds.

Table 5. Optical rotation and UV spectroscopic data on the isolated ecdysteroids

Comp.	**$[\alpha]_D^{20}$ (*c*, methanol)**	**UV, λ_{max}, nm (logε)**
1	$[\alpha]_D^{25}$ +7° (*c*, 0.1)	243 (4.013)
2	$[\alpha]_D^{25}$ +17° (*c*, 0.1)	239.5 (3.931)
3	$[\alpha]_D^{25}$ +3° (*c*, 0.1)	242 (3.5952)
4	$[\alpha]_D^{25}$ +15° (*c*, 0.1)	242 (3.9459)
5	$[\alpha]_D^{28}$ +22° (*c*, 0.1)	240 (3.62)
6	$[\alpha]_D^{20}$ +7.4° (*c*, 0.1)	243
7	$[\alpha]_D^{20}$ +119°	240
8	–	–
9	$[\alpha]_D^{28}$ +28° (*c*, 0.1)	241 (3.84)
10	–	–
11	–	–
12	$[\alpha]_D^{20}$ +32 ° (*c*, 2.0)	242 (4.14)
13	$[\alpha]_D^{25}$ +65.0° (c 0.18; chloroform)	243 (4.04)
14	–	244-246 (4.07)
15	$[\alpha]_D^{28}$ +12° (*c*, 0.1)	242 (3.72)
16	$[\alpha]_D^{28}$ +13° (*c*, 0.1)	238 (3.83)
17	$[\alpha]_D^{28}$ –37° (*c*, 0.1)	242.2 (2.36)
18	$[\alpha]_D^{28}$ –10° (*c*, 0.1)	245 (2.06)
19	$[\alpha]_D^{28}$ +9° (*c*, 0.1)	242 (2.81)
20	$[\alpha]_D^{28}$ +12° (*c*, 0.1)	242 (2.42)
21	–	245 (3.84)

Table 6. MS fragmentation of the isolated new ecdysteroids

Comp.	M.W.	MS	MS fragments m/z (relative intensity %)
2	520	ESIMS	543 $[M+Na]^+$ (41), 521 $[M+H]^+$ (11), 505 $[M-CH_3]^+$ (11.5), 503 $[M+H-H_2O]^+$ (100), 484 $[M-2H_2O]^+$ (9), 452 $[M+H-3H_2O-CH_3]^+$ (15), 437 (24), 413 (23), 391 (37.5), 365 (17).
	HRESIMS	520.3029 $[M]^+$ (calcd for $C_{29}H_{44}O_8$, 520.3024).
3	536	HRESIMS	536.2961 $[M]^+$ (calcd for $C_{29}H_{44}O_9$, 536.2973), 559.2861 $[M+Na]^+$, (calcd for $C_{29}H_{44}O_9Na$: 559.287).
5	536	ESIMS	559 $[M+Na]^+$ (100), 560 $[M+Na+H]^+$ (27), 537 $[M+H]^+$ (25.5), 521 $[M-CH_3]^+$ (14), 519 $[M+H-H_2O]^+$ (26), 505 (19.5), 482 $[M-3H_2O]^+$ (18), 445 (20.7), 437 (16.5), 413 (27.9), 391 (7.5), 365 (37.9), 356 (14.8)
	HRESIMS	537.3424 $[M+H]^+$ (calcd for $C_{30}H_{49}O_8$, 537.3414).
9	480	ESIMS	481 (0.1), 504 (30) $[M+Na]^+$, 463 (1) $[M+H-H_2O]^+$, 445 (27) $[M+H-2H_2O]^+$, 429 (4) $[M-2H_2O-CH_3]^+$, 427 (15) $[M+H-3H_2O]^+$, 409 (5) $[M+H-4H_2O]^+$, 393 (1), 363 (7) 345 (35), 99 (100), 81 (32)
	HRESIMS	$[M+H]^+$ = 481.3152 (calcd for $C_{27}H_{44}O_7$, 481.3160, delta: -1.6 ppm).
15	446	ESIMS	447 (0.1), 429 (100) $[M+H-H_2O]^+$, 411 (12) $[M+H-2H_2O]^+$, 393 (4) $[M+H-3H_2O]^+$, 375 (1) $[M+H-4H_2O]^+$, 331 (11).
	HRESIMS	$[M+H]^+$ = 447.3105 (calcd for $C_{27}H_{43}O_5$, 447.3105).
16	658	ESIMS	497 (100) $[M+H-C_6H_{10}O_5]^+$, 479 (9) $[M+H-C_6H_{10}O_5-H_2O]^+$, 461 (33) $[M+H-C_6H_{10}O_5-2H_2O]^+$, 443 (17) $[M+H-C_6H_{10}O_5-3H_2O]^+$, 425 (1) $[M+H-C_6H_{10}O_5-4H_2O]^+$, 387 (1), 363 (1).
	HRESIMS	$[M+H]^+$ = 659.3639 (calcd for $C_{33}H_{55}O_{13}$, 659.3637).
17	738	ESIMS	658 (2) $[M-H+D+Na-C_4H_8O_3]^+$, 642 (3) $[M-H+D+Na-C_4H_8O_4]^+$, 616 (100), 598 (3), 331 (2) $[C_{12}H_{20}O_9Na]$.
	HRESIMS	$[M+H]^+$ = 739.4236 (calcd for $C_{39}H_{63}O_{13}$, 739.4263).
18	752	ESIMS	671 (2) $[M+Na-C_4H_8O_3]^+$, 655 (3) $[M+Na-C_4H_8O_4]^+$, 629 (100), 331 (1) $[C_{12}H_{20}O_9Na]^+$.
	HRESIMS	$[M+H]^+$ = 753.4420 (calcd for $C_{40}H_{65}O_{13,}$ 753.4420).
19	596	ESIMS	597 (0.5, $[M+H]^+$), 465 (100, $[M+H-C_5H_8O_4]^+$), 447 (28, $[M+H-C_5H_8O_4-H_2O]^+$), 429 (15, $[M+H-C_5H_8O_4-H_2O-H_2O]^+$), 299 (5, $[C_{19}H_{23}O_3]$).
	HRESIMS	597.36234 ($C_{32}H_{53}O_{10}$; calc. 597.36332).
21	462	ESIMS	ESI-MS: 485 (15, $[M+Na]^+$), 463 (41, $[M+H]^+$), 445 (68, $[M+H-H_2O]^+$), 427 (13, $[M+H-H_2O-H_2O]^+$), 345 (5) 99 [100, C_{22}-$C_{27}]^+$).
	HRESIMS	462.29702 ($C_{27}H_{42}O_6$; calc. 462.29759).

3.3. Structure determination of the isolated ecdysteroids

3.3.1. Structure determination of compounds isolated from A. reptans

A pseudomolecular ion at m/z 519.2939 $[M+H]^+$ in the HRESIMS of compound **1** indicated the molecular formula $C_{29}H_{42}O_8$, in accordance with the ^1H and ^{13}C NMR data. Characteristic fragment ions were formed from the parent compound by the loss of water: m/z 501 $[M+H-H_2O]^+$, 482 $[M-2H_2O]^+$ and 464 $[M-3H_2O]^+$. The molecular formula of **2** was determined as $C_{29}H_{44}O_8$ from the HRESIMS of the protonated molecular ion peak at m/z 520.3029 (calcd. for 520.3024). HRESIMS indicated the pseudomolecular ion for **3** at m/z 559.2861 $[M+Na]^+$, consistent with the molecular formula $C_{29}H_{44}O_9$. On the basis of the molecular ion peak m/z 520.3018 observed by means of HRESIMS, compound **4** was assigned the molecular formula $C_{29}H_{44}O_8$. Characteristic peaks at m/z 503 $[M+H-H_2O]^+$, 487 $[M-H_2O-CH_3]^+$ and 484 $[M-2H_2O]^+$ in the ESIMS of **4** supported its structure. Compound **5** was assigned the molecular formula $C_{30}H_{48}O_8$ through use of HRESIMS. The ESIMS of **5** demonstrated a quasimolecular ion at m/z 559 $[M+Na]^+$.

The alicyclic skeletons of compounds **1-5** are the same, which facilitated the structure elucidation and the determination of the absolute configurations of the stereogenic centers of the ring system. Characteristic HMBC correlations of the methyl ^1H signals over two and three bonds and the olefinic hydrogen (H-7) were utilized in the assignments. The H_3-18/C-14, H-7/C-14, H-7/C-5, H-7/C-9, H_3-19/C-1, H_3-19/C-5 and H_3-19/C-9 HMBC correlations permitted distinctions between the 18-methyl and 19-methyl groups. The H_3-18/C-17 and H_3-21/C-17 cross-peaks identified 21-methyl group. The hydrogen atoms on ring A and on rings B, C and D form common spin systems that were analyzed in $^1H,^1H$-COSY and HMQC-TOCSY experiments.

The HMBC cross peaks of H_3-27 and H_3-29 in compounds **1-4** and of H_3-27, H_3-29 and H_3-30 in compound **5** indicated the connectivity of the side-chain from C-24 to C-29/C-30. The H-22/H_2-23 COSY correlation and H-23/C-24 2J HMBC cross-peak in compounds **1**, **3** and **5** and the H-22/H-23 and H-23/H-24 COSY correlations in compounds **2** and **4** linked the skeleton and side-chain of the compounds. The H-23/C-26 HMBC correlation in compounds **2-4** proved the existence of the five-membered lactone rings. In the HMBC spectra of compounds **1** and **5,** an H-22/C-26 cross-peak was not seen, but the chemical shifts of H-22 and C-22 in compound **1** were in good agreement with those of ajugalactone, 2-dehydroajugalactone and 3-dehydroajugalactone, containing six-membered lactone moieties.[97] The chemical shifts of H-22 and C-22 (^1H: δ 3.29-3.39, ^{13}C: δ 78.4-78.7) in the

corresponding open-chain 22-hydroxy compounds are characteristically highfield-shifted.[89,92] In compound **5,** the H-22/H-26 NOESY correlation verified the presence of a six-membered ring in the side-chain.

The *cis* junction of rings A/B was indicated from the H_{α}-9/H_{α}-2 and H_3-19/H_{β}-5 NOESY correlations in compounds **1**-**5.** The H_3-18/H_{β}-12, H_{β}-12/H_3-21, H_{α}-12/H_{α}-17, H_3-18/H_{β}-15 and H_3-18/H_{β}-16 NOESY cross-peaks of these compounds confirmed the *trans* junction of rings C/D. It should be mentioned that, in the case of 14-*epi* steroids, steric interactions resulted in characteristic 9 ppm shifts of C-12 and C-15 (e.g. in 20E and 14-*epi*-20E).[97]

The H_{β}-12/H_3-21, H_3-18/H_3-21, H-22/H_2-16, H_{eq}-23/H_2-16 and H_3-29/H_{α}-16 NOESY correlations in compounds **1** and **5** revealed the absolute configurations of C-20 and C-22 as shown in the chemical structures. In the NOESY spectrum of compound **1,** the H_3-27/H-28 NOESY correlation was very strong and the H_3-27/H_3-29 NOESY cross-peak was weak, proving that H-28 is oriented towards H_3-27 in the preferred conformation. A NOESY cross-peak was observed between H_3-29 and H_{eq}-23, but there was no H_3-29/H_{ax}-23 NOESY proximity. These facts described the positions of 28-hydroxy and 29-methyl in relation to the plane of the lactone ring.

In compound **5**, the $^3J_{\text{H-22,Ha-23}}$ = 12.0 Hz proved the antiperiplanar arrangement of these hydrogens. The H-22/H-26, H-23/H-25 and H-26/H_3-27 NOESY correlations indicated the antiperiplanar arrangement of H-25 and H-26, and also determined the positions of the 27-methyl and 26-methoxy groups. The H_3-29/H_{eq}-23 NOESY cross-peak indicated that the conformation around the C-24 – C-28 bond was similar to that in compound **1**.

Compounds **2** and **4** are diastereomers. In compound **2,** $^3J_{\text{H-22,H-23}}$ = 9.9 Hz and the H_3-18/H_3-21, H_3-21/H-23 and H-22/H_2-16 NOESY correlations proved the antiperiplanar arrangement of H-22 and H-23 and the absolute configuration of C-20. The H-22/H_2-28, H-23/H_3-27 and H-25/H_3-29 NOESY cross-peaks verified the *trans* arrangement of the 27-methyl and 24-ethyl groups. Through a consideration of different configurations of C-22 and C-23, four isomers were taken into account in PM3 semiempirical calculations (Hyperchem 7). In the NOESY spectrum of compound **2** an H-23/H-17 cross-peak was detected, which eliminated two of these isomers because of the distance of 4.3 Å between these hydrogens.

The distinction between the remaining two isomers was achieved by considering the fact that a 24-ethyl group under ring D would result in strong spatial congestion. Moreover, no NOESY steric proximities were detected between the hydrogens on ring D and the lactone ring. Further, the antiperiplanar arrangement of H-22 and H-23 was ruled out on the basis of

the PM3 calculations. The structure of compound **2** was assigned to the isomer with the 20*R*,22*R*,23*R*,24*S*,25*S* configuration.

In the isomeric compound **4**, the H_3-18/H_3-21, H_3-21/H-23, H-22/H_2-16 and H-23/H-17 NOESY correlations determined the absolute configurations of C-20 and C-22. Strong H-23/H_3-29 and H-24/H-25 NOESY cross-peaks and the absence of a H_3-27/H_2-24 correlation verified the *cis* arrangement of H-23, and the 27-methyl and 24-ethyl groups. The $^3J_{\text{H-22,H-23}}$ = 4.3 Hz indicated their gauche arrangement. The very strong H-23/H-17 NOESY correlation indicated their predominance of the conformer in which the C-23–H-23 bond points towards ring D. The strong H_3-21/H-24 NOESY response allowed determination of the absolute configuration in the lactone ring. Castro *et al.* reported breviflorasterone, an ecdysteroid with the same structure as **4**.[98] On their assumption of a common biosynthesis, the relative configuration was considered to be the same as that of cyasterone (24*R*,25*S*). Unfortunately, they determined the ^{1}H and ^{13}C chemical shifts in pyridine-d_5, whereas our data were measured in methanol-d_4, and therefore the identity of brevisterone and compound **4** does not follow from the NMR measurements. The $[\alpha]_D$ values of compound **4** and breviflorasterone are very different, indicating that the configurations in the lactone rings may be different.

In compound **3**, the H_3-29/H-25 NOESY cross-peaks verified the *trans* arrangement of the 24-ethyl and 27-methyl groups. The measured $^3J_{\text{H-22,H-23}}$ = 9.7 Hz and the H-22/H-25 and H-23/H_3-27 NOESY correlations proved the antiperiplanar arrangement of H-22 and H-23. The measured H_3-21/H-23 and H-22/H_2-16 correlations verified the configuration of C-20. The H-23/H-17 NOESY response could not be detected, so the configuration of C-22 remained undetermined. Considering the similarity of the spatial structure and the $^3J_{\text{H-22,H-23}}$ values of compounds **2** and **3,** the configuration of C-22 should be *R** (the change of the configurations of C-23 and C-24 follows from the H→OH substitution), i.e. the possible spatial arrangement is the same as depicted for compound **2** (24-hydroxy instead of H-24).

3.3.2. Structure determination of compounds isolated from P. vulgare

On the basis of the protonated molecular ion peak observed by means of HRESI-MS/MS, the molecular formula of **9** was $C_{27}H_{44}O_7$, that of **15** was $C_{27}H_{42}O_5$, that of **16** was $C_{33}H_{54}O_{13}$, that of **17** was $C_{39}H_{62}O_{13}$, and that of **18** was $C_{40}H_{64}O_{13}$. In most cases, the characteristic fragment ions were formed from the protonated molecular ion through the loss of sugar units, or sugars and water.

The ^{1}H, ^{13}C and HMQC correlations of compound **14** and previous data on polypodine B and shidasterone allowed us to elucidate the structure of compound **14** as 5-hydroxyshidasterone (ajugasterone D). From a consideration of the available NMR data, it

can be concluded that the fused ring skeletons of compounds **9** and **15** are the same as in polypodine B and 20E, respectively. Additionally, the side-chain in compound **16** is identical with that in 20E.

The characteristic HMBC correlations over two and three bonds of the methyl signals and the olefinic hydrogen (H-7) were initially utilized in the assignment. The H_3-18/C-14, H-7/C-14, H-7/C-5, H-7/C-9, H3-19/C-1, H_3-19/C-5 and H_3-19/C-9 HMBC correlations resulted in the assignment of 18- and 19-methyl groups. The H_3-18/C-17 and H_3-21/C-17 cross-peaks identified 21-methyl signals. The H_3-26/C-27 and H_3-27/C-26, and the H_3-26/C-25 H_3-26/C-24, H_3-27/C-25 and H_3-27/C-24 HMBC correlations permitted the identification of the geminal 26-methyl/27-methyl groups in compounds **9**, **14**, **15** and **16**.[92,99] These correlations are characteristic of C_{27}-ecdysteroids. The hydrogen atoms on ring A, rings B, C and D, the side-chain and the sugar units form separated spin systems that were analyzed in z-filtered-TOCSY, ^{1}H,^{1}H-COSY and HMQC-TOCSY experiments.

The characteristic H-26/C-22 cross-peak observed in the HMBC spectrum of compound **17**, the H-26/H-22 responses in the ROESY spectra of compounds **17** and **18**, and the chemical shifts of the C-26 signals of **17** (δ 102.6) and **18** (δ 109.9) furnished evidence of a cyclic hemiacetal between C-22 and C-26 in both compounds.

The HMBC cross-peak of H-1'/C-2 in compound **16** resulted in the position of the sugar moiety. The H-1'/C-3, H-2'/C-1" and H-1"/C-2' correlations in compounds **17** and **18** proved the C-3 connection of the disaccharide units to the aglycone. The z-filtered 1D-TOCSY gave the coupling constants of the hydrogens in the sugar moieties, and in this way we could identify the sugar units as β-*D*-glucose and *L*-rhamnose. H-2" in rhamnose has an equatorial position, and therefore the axial α- or equatorial β-position of H-1" could not be given from the value of the ^3J (H-1"/H-2") coupling constant. To overcome this uncertainty, the ^1J(H-1"/C-1") couplings were utilized, which were determined in the ^{13}C-coupled HMQC experiments. It is well known that, in consequence of the lone-pair effect, an antiperiplanar arrangement of the lone-pair on the ring oxygen atom with respect to the anomeric C-H bond (axial hydrogen), the characteristic value is about 160 Hz, i.e. 10 Hz less than for the anomeric counterpart (equatorial hydrogen).[100,101] For the glucose moiety in compound **17,** the magnitude of ^1J(H-1"/C-1") was measured as 160 Hz, whereas the corresponding value for the rhamnose moiety was 173 Hz. This unequivocally proves the presence of an α-*L*-rhamnose moiety.

The coupling constants of the H-2 signal and the H-2/H-9 NOESY response of compounds **8**, **9**, **15** and **16** supported the *cis* A/B ring junction and the β-orientation of the

OR substituent. The coupling constants of the H-3 signal and the H-3/H-5 cross-peaks in the NOESY and ROESY spectra of compounds **17** and **18** indicated the *trans* A/B ring junction in both compounds. Further support for the *trans* ring junction is gained from the chemical shifts of C-19 (13.6 ppm in both compounds), which characteristically differ from the value measured for 20E (24.4 ppm).[97] We have earlier reported similar C-19/C-2 and C-19/C-4 γ-gauche interactions in analogous *trans* A/B ring derivatives.[100] In compounds **8**, **9**, **15** and **16**, the H_β-12/H_3-18, H_α-12/H_α-17, H_3-18/H_β-15 and H_3-18/H_β-16 NOESY cross-peaks revealed the *trans* C/D ring junction. It is known that the C-12 and C-15 chemical shifts show significant differences between 20E (32.5 and 31.8 ppm) and in the isomeric 14-*epi*-20E (41.7 and 40.8 ppm).[97] For **9**, **15** and **16**, we measured chemical shifts of ~32.2, 31.2 and 32.7 ppm for C-12, whereas the C-15 signals were detected at ~32.2; 32.3 and 31.8 ppm. These chemical shifts provide a further strong evidence of the *trans* junction of rings C/D. We earlier reported the structure elucidation of two analogous H-14 epimers.[94] The chemical shifts of C-14 and C-15 were 59.2 and 34.1 ppm, respectively, for the H_β-14 epimer and 57.2 and 23.6 ppm, respectively, for the H_α-14 epimer. A comparison with the chemical shifts of C-14 (56.5 ppm) and C-15 (23.8 ppm) in **17** and **18** indicated good concordance with those of the H_α-14 epimer.

The configurations of stereogenic centers C-20 and C-22 proposed for **9**, **14** and **16** were deduced on the basis of the close similarity of the ^{13}C chemical shifts measured in these compounds and the reference compounds (E, shidasterone and 20E, respectively).[97,102] For compounds **17** and **18** the configurations of the stereogenic centers of the steroid skeleton including C-20, are known,[97] but the configuration in the pyranose unit has not been determined. The relative configuration of the tetrahydropyran moiety in **17** and **18** was studied in a one-dimensional selective z-filtered TOCSY experiment. The measured values of ^3J(H-26/H-25) ~ 8.5 Hz indicate the axial-axial arrangement of these protons. The ^3J(H-22/H_a-23) ~ ^3J(H_a-23/H_a-24) ~ ^3J(H_a-24/H-25) coupling constants of ~ 11 Hz indicated the chair conformation of the tetrahydropyran ring, and the equatorial position of C-20. The substituents in positions 22, 25 and 26 are equatorial, which can be correlated with the 22*S*,25*R*,26*R* or 22*R*,25*S*,26*S* configurations. It should be mentioned that we failed to observe a highly resolved H-22 signal, but the ^{1}H spectrum and the corresponding cross-peak in the HMQC spectrum indicate only one coupling with a value of ~12 Hz. On this basis, we can conclude that H-20 and H-22 are gauche located in the preferred conformation around the C-20–C-22 bond. Further information on the predominant conformation can be gained from a consideration of the NOE responses between H_3-21/H_β-12, H_3-21/H_3-18 and H_3-21/H_2-23.

This measurement revealed that there is no steric proximity between H_3-21/H_2-16 and H_β-12/ H_2-23. Such a steric arrangement could be explained by 22*S*,25*R*,26*R* but we do not have unambiguous evidence for exclusion of the alternative 22*R*,25*S*,26*S* configurations. A compound similar to **9** and **10** is polypodoside A,[103,104] where instead of R = H, Me the substituent is a rhamnopyranosyl (rha-1→C-26) moiety. The determined 20-S, 22-R, 25-S and 26-R configurations correlate well with our configurations 20*S*,22*R*,25*S*,26*S* in compounds **17** and **18**. It should be mentioned that the introduction of a rhamnopyranosyl moiety resulted in change of the configuration at C-26.

Unfortunately, H-22 is a part of "X" of a higher-ordered spin system of H-17/H-20 and H2-23 in compound **15**, which prevents the utilization of ^{3}J(H/H) couplings to steric analysis. Thus the configurations of C-20 and C-22 must be left unresolved.

3.3.3. Structure determination of compounds isolated from S. wolffii

For **19,** HRESIMS indicated the molecular formula $C_{32}H_{53}O_{10}$ with the molecular ion peak at m/z 597.36234 ($[M+H]^+$). ^{1}H and ^{13}C NMR assignments of the fused ring skeleton were first made from the characteristic two- and three-bond HMBC correlations of the methyl resonances, followed by analysis of the TOCSY and NOESY correlations of H-5, H-17 and H-8. The spectral data were in good agreement with those reported for ponasterone A-22-glycolate[105] and ponasterone A (except those relating to H-22 and C-22).[4] Besides the skeletal resonances, the ^{13}C and HSQC NMR spectra confirmed the presence of two oxymethylene groups, two oxymethine groups and an oxygen-linked tetrasubstituted carbon atom. The TOCSY and HMBC correlations demonstrated that these resonances were those of an apiose unit. In agreement with the downfield-shifted C-22 signal (as compared with that observed in ponasterone A), the HMBC correlations between H-22 and C-1' and between H-1' and C-22 confirmed that the apiose moiety was connected to the oxygen atom linked to C-22. The relative configuration of the sugar moiety was determined from the coupling constant of 3.6 Hz between H-1' and H-2' and the NOE correlations between H-1' and H-2' and between H-2' and H-5'. The coupling constants (10.6 and 1.6 Hz) observed between H-22 and the two H-23 protons were similar to those in ponasterone A[4] and ponasterone A-22 glycolate,[105] indicating that C-22 has the same configuration in these compounds. The absolute configuration of the sugar moiety could not be determined. The proposed structure was in accordance with the fragment ions detected in the HRMS-MS spectrum, indicating loss of the sugar moiety followed by successive losses of water molecules: 465 ($[M+H-C_5H_8O_4]^+$), 447 ($[M+H-C_5H_8O_4-H_2O]^+$), 429 ($[M+H-C_5H_8O_4-H_2O-H_2O]^+$) and 299 ($[C_{19}H_{23}O_3]$).

The analyses of the MS and NMR data on **20** and **21** via the above protocol revealed that these two compounds have the same steroid skeleton and differ only in the side-chains. The assignments of the steroid skeleton were in good agreement with the available NMR data on 3-*epi*-20E.[106] For **21**, the COSY and HMQC correlations and the similarity of the ^{1}H and ^{13}C chemical shifts in the side-chain to those reported earlier for 11α-hydroxyshidasterone[94] in accordance with the elemental composition determined from the HR-MS data, indicated that **21** was 3-*epi*-shidasterone. Its molecular formula, $C_{27}H_{42}O_6$, was established via the molecular ion peak observed by HRESI-MS/MS. In accordance with the elemental composition $C_{27}H_{44}O_6$ derived from HR-MS measurements, the HMBC correlations of H_3-21, H_3-26 and H_3-27 suggested that **20** is 3-*epi*-22-deoxy-20-hydroxyecdysone.

Table 7. NMR data on reptanslactone A (**2**)

No.		^{13}C	^{1}H	m; *J* (Hz)	HMBC	NOESY
1	α	37.5	1.79			
	β		1.42	t; 12.8	68.7-8, 39.4, 35.3, 24.5	2.38, 0.96
2	α	68.8	3.85	ddd; 12.1, 4.2, 3.4	-	3.95, 3.16, 1.80, 1.71
3	α	68.7	3.95	td; 3.3, 2.5	52.0	3.85, 1.75, 1.70
4	a	33.05	1.70			
	b		1.75			
5	β	52.0	2.38	dd; 12.7, 4.9	206.7, 35.3, 33.05, 24.5	1.71, 1.73, 0.96
6		206.7	-	-	-	-
7		122.0	5.795	d; 2.5	85.3, 52.0, 35.3	1.97, 1.61
8		168.6	-	-	-	-
9	α	35.3	3.16	ddd; 11.4, 7.1, 2.6	168.6, 39.4, 24.5, 21.65-68	3.85, 2.19, 1.81, 1.79, 1.72
10		39.4	-	-	-	-
11	α	21.65	1.80			
	β		1.69			
12	α	32.5	2.19	td; 13.0, 4.9	48.9, 21.9, 18.6	3.16, 2.75
	β		1.87			
13		48.9	-	-	-	-
14		85.3	-	-	-	-
15	α	31.8	1.60	t, 10.0	85.3, 50.7, 48.8	5.80, 1.32
	β		1.97			
16	α	21.9	1.78			
	β		2.00			
17	α	50.7	2.75	dd; 9.5, 8.4	48.8, 32.5, 21.9, 18.6	4.40, 3.59, 2.19, 1.79, 1.31
18	β	18.6	0.89	s	85.3, 50.7, 32.5, 48.9	2.00, 1.97, 1.89, 1.70, 1.31
19	β	24.5	0.96	s	52.0, 39.4, 37.5, 35.3	
20		77.5	-	-	-	-
21		21.68	1.315	s	77.5, 72.8, 50.7	4.40, 3.59 , 2.75, 1.87, 0.89
22		72.8	3.59	d; 9.8	81.4, 77.5, 50.7, 21.65-68	4.40, 2.75, 1.98, 1.90, 1.80, 1.31, 1.28
23		81.4	4.40	dd; 10.0, 2.5	72.8, 21.4	3.59, 2.75 , 2.11, 1.29-1.31
24		48.9	2.11		-	4.40, 1.29, 1.27
25		41.4	2.48	qd; 7.5, 3.0	182.3, 81.4, 21.65-68, 15.45	2.12, 1.29, 0.98
26		182.3	-	-	-	-
27		15.45	1.29	d; 7.6	182.3, 48.9, 41.4	4.40?, 3.59, 2.49, 2.12, 1.84-1.94, 0.98
28	a	21.4	1.27			
	b		1.90			
29		12.0	0.98	t; 7.3	48.9, 21.4	2.49, 2.12, 1.91

Table 8. NMR data on sendreisterone (**5**)

No.		^{13}C	^{1}H	m; *J* (Hz)	HMBC	NOESY
1	α	37.5	1.80			
	β		1.43	dd; 13.0, 12.6	68.7-9, 39.4, 35.3	3.84, 2.38, 0.96
2	α	68.9	3.84	ddd; 11.7, 3.9, 3.5	-	3.95, 3.16, 1.79, 1.72
3	α	68.7	3.95	q; 2.8	-	3.84, 1.71
4	α	33.0	1.73			
	β		1.70			
5	β	51.9	2.38	dd; 12.2, 4.8	206.6, 35.3, 33.0	1.71, 1.43, 0.96
6		206.6	-	-	-	-
7		122.2	5.80	d; 2.7	85.3, 51.9, 35.3	1.935, 1.575, 0.96, 0.88
8		168.2	-	-	-	-
9	α	35.3	3.16	ddd; 7.0, 2.6	168.2, 24.6, 21.7	3.84, 2.12, 1.79, 1.72
10		39.4	-	-	-	-
11	α	21.7	1.805			
	β		1.70			
12	α	32.6	2.12	td; 13.1, 4.9	18.1	3.16, 2.36
	β		1.875	ddd; 12.8, 4.9, 1.9	85.3, 35.3	1.20, 0.88
13		48.7	-	-	-	-
14		85.3	-	-	-	-
15	α	31.9	1.575	dd; 10.4, 9.4	85.3, 50.5, 48.8	5.80, 1.74
	β		1.945			
16	α	21.5	1.68			
	β		1.94			
17	α	50.5	2.36	t; ~8.0	48.6, 21.5-7, 18.1	3.35, 2.12, 1.69, 1.54, 1.20
18	β	18.1	0.88	s	85.3, 50.5, 48.7, 32.6-7	1.93, 1.89, 1.69, 1.20
19	β	24.6	0.96	s	51.9, 39.4, 37.5, 35.3	2.38, 1.80, 1.70-2, 1.43
20		78.0	-	-	-	-
21		21.2	1.20	s	78.0, 75.2, 50.5	2.36, 1.88, 1.74, 1.54, 0.88
22		75.2	3.35	d; 10.2	78.0, 53.9, 21.2	2.36, 2.24, 1.93, 1.69, 1.54
23	a	32.7	1.54	ddd; 13.6, 11.1, 1.0	-	4.82, 3.605, 3.35, 2.36
	b		1.74	ddd; 13.8, 11.1, 2.9		
24		53.9	2.24	ddd; 11.2, 8.8, 3.2	147.2, 114.3, 20.3	4.82, 3.605, 3.35, 1.75, 1.70, 1.13
25		147.2	-	-	-	-
26	a	114.3	4.815		53.9, 20.3	1.68
	b		4.865		53.9, 20.3	1.68
27		20.3	1.68	s	147.2, 117.3, 53.9	4.87, 3.605, 3.35, 2.24, 1.12
28		70.7	3.605	dq; 8.9, 6.2	33.0	2.24, 1.75, 1.69, 1.54, 1.13
29		22.5	1.13	d; 6.2	70.7, 53.9	3.605, 2.24, 1.68

Table 9. NMR data on 5-hydroxyecdysone (**9**)

No.		^{13}C	^{1}H	m; *J* (Hz)	HMBC	COSY	NOESY
1	α	34.3	1.74		80.4, 70.4, 68.5, 45.6, 39.3	3.99, 3.95	
	β		1.74		80.4, 70.4, 68.5, 45.6, 39.3	3.99, 3.95	
2	α	68.5	3.95	ddd; 16.7, 6.9, 3.2		1.74	3.19, 2.08, 1.68-1.82
3	α	70.4	3.99	q; 3.0		2.08, 1.77, 1.74	2.08, 1.68-1.82
4	α	36.3	2.08	dd; 14.8, 3.0		3.99, 1.77, 1.74	1.68-1.82
	β		1.77		80.4, 70.4, 68.5, 45.6	3.99, 2.08	2.08, 1.41, 1.21, 1.19, 0.95
5		80.4	-				
6		202.6	-				
7		120.6	5.86	d; 2.7	85.0, 80.4, 39.3	3.19	1.97, 1.60
8		167.3	-				
9	α	39.3	3.19		167.3	5.86, 1.74	2.13, 2.08, 1.82
10		45.6	-				
11	a	22.7	1.74		45.6, 39.3	3.19	
	b		1.82				
12	α	32.3/32.2	2.13			1.79	3.19
	β		1.79				
13		48.3	-				
14		85.0	-				
15	α	32.3/32.2	1.60			1.97	5.86, 1.97
	β		1.97			1.60	5.86, 1.60, 1.55
16	α	27.1	1.52				1.21, 1.19, 0.95
	β		1.97			1.60	
17	α	48.9	2.02		27.1		0.95
18	β	16.3	0.74	s	85.0, 48.9, 48.3, 32.3, 32.2		5.86, 1.97, 1.76, 1.52, 0.95, 0.92
19	β	17.1	0.92	s	80.4, 45.6, 39.3, 34.3		2.08, 1.74-1.76, 0.74
20		43.6	1.76			0.95	
21		13.4	0.95	d; 6.7	75.4, 48.9, 43.6,	1.76	2.02, 1.76, 1.55, 1.33, 0.74
22		75.4	3.59			1.32	2.02, 1.97, 1.55, 1.41
23	a	25.5	1.32			3.59, 1.80, 1.76	
	b		1.55			1.80, 1.41	3.59, 1.21, 1.19, 0.95
24	a	42.4	1.41		25.5	1.80, 1.55	3.59
	b		1.80			1.55, 1.41	
25		71.6	-				
26		29.2	1.19	s	71.6, 42.4, 29.8		1.80
27		29.8	1.21	s	71.6, 42.4, 29.2		1.80, 1.55, 1.41, 0.95

Table 10. NMR data on 3-*epi*-shidasterone (**21**)

No.		^{13}C	^{1}H	m; *J* (Hz)	COSY	NOESY
1	α	43.2	2.09	dd; 13.4, 4.2	3.64	3.64, 3.18, 1.81, 0.95
	β		1.08	dd; 13.7, 11.9	3.64	3.35
2	α	72.3	3.64	ddd; 11.7, 8.8, 4.4	3.35, 2.09, 1.08	3.17, 2.09, 1.57
3	β	75.5	3.35	overlapped	3.64, 1.75, 1.57	
4	α	33.8	1.56	td; 13.0, 11.5	3.35, 2.09	3.64, 3.18, 0.85
	β		1.76			
5	β	57.6	2.09	dd; 13.4, 4.2	1.76, 1.56	3.35, 0.95
6		204.8	-			
7		122.1	5.82	d; 2.7	3.17	1.96, 1.62
8		168.2	-			
9	α	36.1	3.17	ddd; 11.7, 7.4, 2.7	5.82, 1.81, 1.69	3.64, 2.14, 1.81, 1.57
10		39.7	-			
11	α	21.7	1.82	overlapped		
	β		1.70	overlapped		
12	α	32.5	2.16	td; 13.0, 4.5		3.18, 2.38, 0.85
	β		1.86	overlapped		
13		48.5	-			
14		85.3	-			
15	α	31.9	1.61	overlapped		
	β		1.97	dd; 12.7, 6.4		5.82, 0.85
16		21.9	1.82	overlapped		
			2.02	dtm; 12.6, 10.0	2.37	0.85
17	α	52.0	2.37	t; 9.2	2.00, 1.83	3.92, 2.17, 1.92, 1.80, 1.22
18	β	18.3	0.85	s		1.97, 1.87, 1.69, 1.22
19	β	24.0	0.95	s		2.10, 1.69
20		77.2	-			
21		20.9	1.22	s		2.38, 1.99, 1.87, 1.76, 0.85
22		85.7	3.92	dd; 8.4, 6.2	1.90, 1.75	2.38, 1.99, 1.88, 1.79, 1.24
23	a	28.6	1.76	overlapped		
	b		1.90	overlapped		
24	a	39.8	1.75	overlapped		
	b		1.75	overlapped		
25		81.9	-			
26		28.5	1.24	s		3.92, 1.89, 1.76
27		29.1	1.25	s		3.92, 1.89, 1.76

Tables 11-12. NMR data on 20-deoxy-shidasterone (**15**) (on the left) and ponasterone A-22-apioside (**19**) (on the right)

No.		^{13}C	^{1}H	m; *J* (Hz)
1	α	37.5	1.80	
	β		1.43	dd; 13.3, 12.5
2	α	68.9	3.84	m
3	α	68.7	3.95	q; 2.9
4	a	33.0	1.70	
	b		1.75	
5	β	51.9	2.385	dd; 12.8, 4.6
6		*	–	–
7		122.2	5.815	d; 2.6
8		167.7	–	–
9	α	35.6	3.15	m (ddd)
10		39.5	–	–
11	α	21.7	1.81	
	β		1.67	
12	α	32.16	2.105	td; 13.0, 4.9
	β		1.79	
13		48.5	–	–
14		85.2	–	–
15	α	32.25	1.60	
	β		1.98	
16	a	27.3	1.52	
	b		1.98	
17	α	49.5	1.935	
18	β	16.3	0.735	s
19	β	24.6	0.97	s
20		39.3	1.85	
21		13.1	0.935	d; 6.5
22		82.2	4.11	ABX
23	a	25.9	1.79	
	b		1.79	
24	a	39.8	1.75	
	b		1.75	
25		81.8	–	–
26		28.4	1.246	s
27		29.0	1.246	s

No.		^{13}C	^{1}H m; *J* (Hz)
1	α	37.5	1.79 m
	β		1.43 dd (13.1, 12.0)
2	α	68.9	3.84 ddd (12.0, 4.1, 3.4)
3	α	68.7	3.95 q (2.5)
4	α	33.0	1.75 m
	β		1.70 m
5	β	52.0	2.38 dd (13.0, 4.1)
6		206.6	
7		122.3	5.81 d (2.6)
8		168.1	
9	α	35.2	3.15 ddd (10.8, 7.4, 2.5)
10		39.4	
11	α	21.7	1.81 m
	β		1.69 m
12	α	32.8	2.11 td (12.9, 4.7)
	β		1.88 ddd (12.9, 4.7, 1.8)
13		48.8	
14		85.4	
15	α	31.9	1.58 m
	β		1.98 m
16	α	21.5	1.67 m
	β		2.01 m
17	α	51.2	2.30 t (9.0)
18		18.3	0.88 s
19		24.5	0.97 s
20		77.3	
21		22.6	1.195 s
22		90.2	3.35 dd (10.6, 1.6)
23	’	30.8	1.37 m
	’’		1.61 m
24	’	36.9	1.25 m
	’’		1.51 m
25		29.4	1.55 quint (6.7)
26		22.8	0.92 d
27		23.5	0.93 d
1’		113.0	4.98 d (3.6)
2’		77.9	3.96 d (3.6)
3’		80.3	
4’	’	74.7	3.79 d (9.6)
	’’		4.10 d (9.6)
5’	’	64.8	3.55 d (11.5)
	’’		3.58 d (11.5)

Table 13. NMR data on polypodine B-2-glucoside (**16**)

No.		^{13}C	^{1}H	m; *J* (Hz)	No.		^{13}C	^{1}H	m; *J* (Hz)
1	a	33.0	1.82		18	β	18.2	0.895	s
	b		1.90		19	β	16.9	0.93	s
2	α	76.4	4.15	ddd; 12.1, 4.8, 3.5	20		78.03	–	–
3	α	68.1	4.21	q; 3.0	21		21.2	1.197	s
4	α	35.8	2.075	dd; 14.9, 3.0	22		78.6	3.32	
	β		1.805		23	a	27.5	1.29	
5	β	80.4	–	–		b		1.66	
6		*	–	–	24	a	42.5	1.435	
7		120.9	5.85	d; 2.7		b		1.80	
8		*	–	–	25		71.4	–	–
9	α	39.1	3.20		26		29.1	1.188	s
10		45.9	–	–	27		29.9	1.202	s
11	α	22.4	1.915		1’		103.4	4.45	d; 7.8
	β		1.72		2’		75.4	3.23	dd; 8.8, 7.9
12	α	32.7	2.125	td; 13.1, 5.0	3’		77.99	3.375	t; 9.0
	β		1.89		4’		71.8	3.315	
13		48.6	–	–	5’		78.2	3.31	
14		85.2	–	–	6’	a	62.9	3.70	dd; 11.8, 5.3
15	α	31.8	1.60			b		3.885	dd; 12.0, 1.8
	β		1.96						
16	α	21.6	1.73						
	β		1.99						
17	α	50.6	2.385	ABX					

Table 14. NMR data on polypodosaponine (**17**)

No.		^{13}C	^{1}H	m; *J* (Hz)	No.		^{13}C	^{1}H	m; *J* (Hz)
1	a	37.9	1.415	t; (HMQC)	20		41.6	1.78	
	b		1.86	d; HMQC	21		14.4	1.01	d; ~6.8
2	a	29.9	1.46		22		79.3-5	3.48	
	b		1.90		23	a	25.1	1.32	
3	α	77.6	3.80	tt; 11.3, 4.3		b		1.43	
4	a	27.3	1.39		24	a	32.5	1.19	
	b		2.33			b		1.82	
5	α	54.36	2.35		25		38.9	1.31	
6		202.6	-	-	26		17.3	0.92	d; 6.5
7		123.6	5.67	t; 2.1	27		102.6	4.237	d; 8.3
8		167.2	-	-	1’		100.1	4.55	d; 7.7
9	α	51.4	2.31		2’		79.3-5	3.37	dd; 9.3, 7.8 (1D-TOCSY)
10		39.6	-	-	3’		79.55	3.48	t; 9.1 (1D-TOCSY)
11	a	23.0	1.69	q; HMQC	4’		72.04	3.26	
	b		1.86		5’		77.9	3.26	
12	a	40.1	1.485	t; HMQC	6’	a	62.95	3.645	dd; (1D-TOCSY)
	b		2.165	d; HMQC		b		3.85	d; (1D-TOCSY)
13		46.1	-	-	1”		102.4	5.19	d; 1.6
14	α	56.5	2.175		2”		72.4	3.935	dd; 3.3, 1.8
15	a	23.8	1.56		3”		72.5	3.725	dd; 9.5, 3.3
	b		1.675		4”		74.2	3.37	
16	a	28.2	1.47		5”		70.0	4.125	
	b		1.89		6”		18.2	1.24	d; 6.3
17	α	54.25	1.425						
18	β	12.8	0.665	s					
19	β	13.6	0.870	s					

3.3.4. Structures of the isolated ecdysteroids

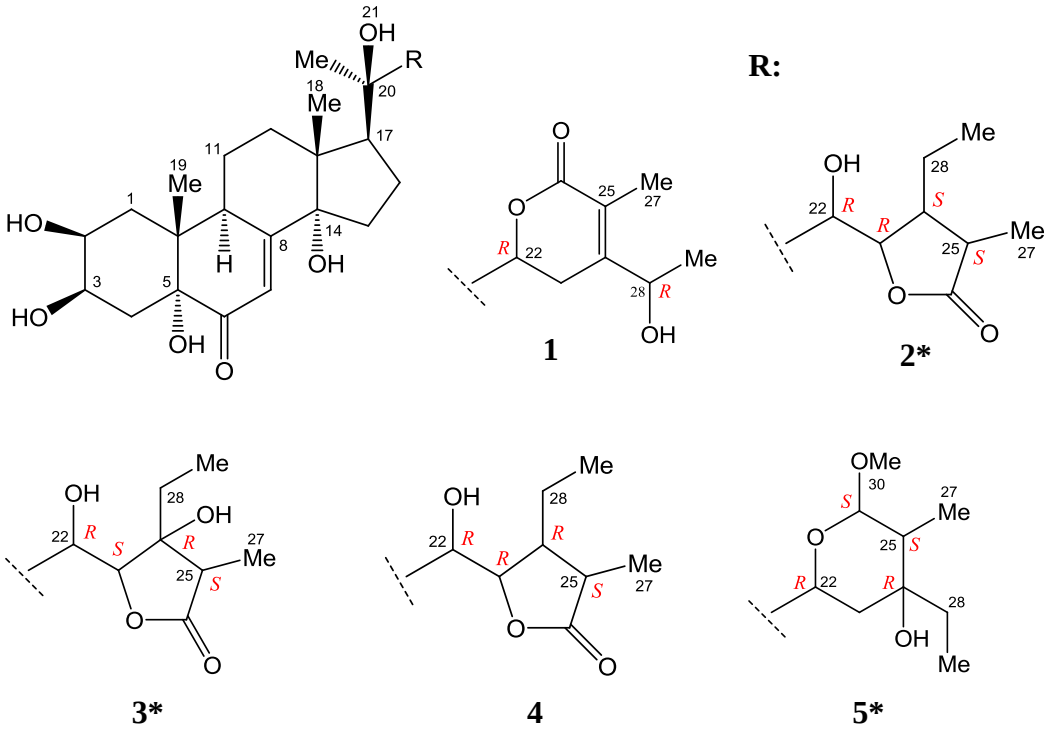


Figure 5. Structures of compounds isolated from *A. reptans*: 24-dehydroprecyasterone (**1**), reptanslactone A (**2**)*, reptanslactone B (**3**)*, breviflorasterone (**4**) and sendreisterone (**5**)*. New compounds are denoted by asterisks (*)

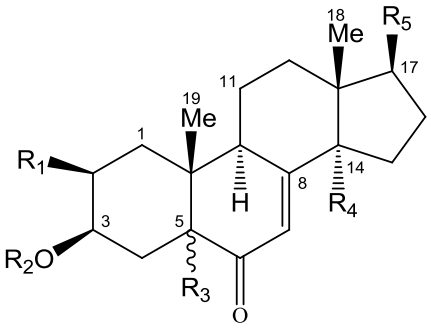
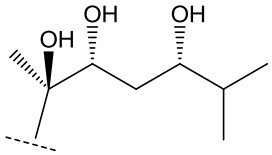
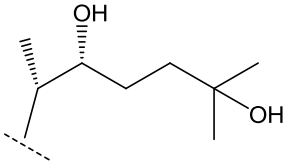
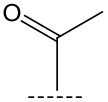
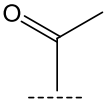
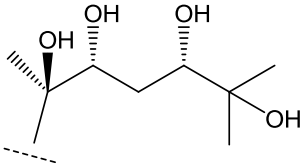
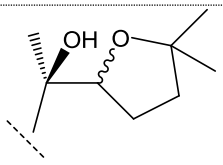
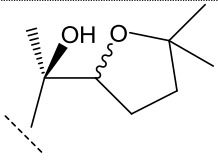
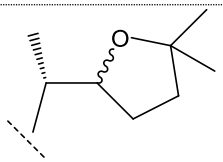
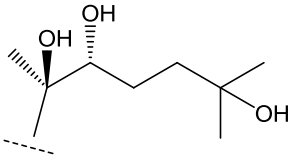
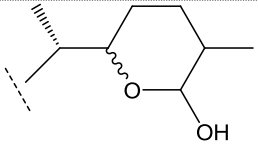
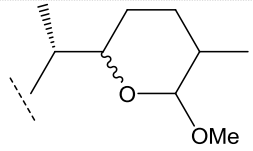
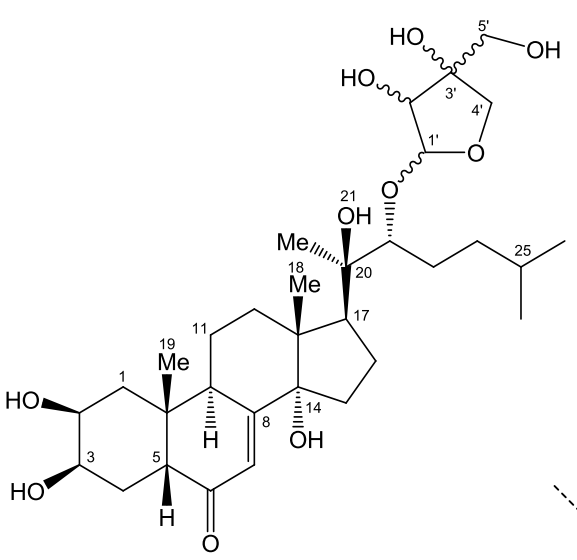


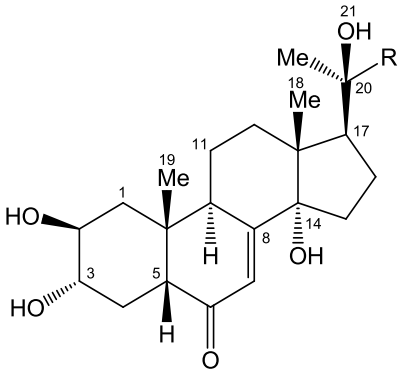
Table 15. Compounds isolated from *P. vulgare*. New compounds are denoted by asterisks (*)

Ecdysteroid	**R_1**	**R_2**	**R_3**	**R_4**	**R_5**
Pterosterone (6)	OH	H	*β*H	OH	
Rubrosterone (7)	OH	H	*β*H	OH	
5-Hydroxyrubrosterone (8)	OH	H	*β*OH	OH	
5-Hydroxyecdysone (9)*	OH	H	*β*OH	OH	
5-Hydroxypoststerone (10)	OH	H	*β*OH	OH	
Poststerone (11)	OH	H	*β*H	OH	
Abutasterone (12)	OH	H	*β*H	OH	
Shidasterone (13)	OH	H	*β*H	OH	
5-Hydroxyshidasterone (14)	OH	H	*β*OH	OH	

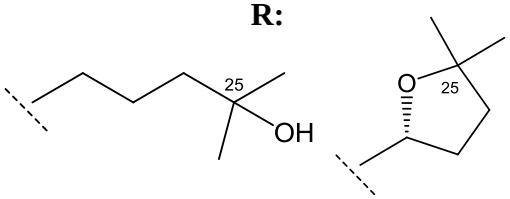
20-Deoxyshidasterone (15)*	OH	H	*β*H	OH	
Polypodine B-2-glucoside (16)*	Glu	OH	*β*OH	OH	
Polypodosaponin (17)	H	Rha-Glu	*α*H	H	
26-Methoxypolypodosaponin (18)	H	Rha-Glu	*α*H	H	



19*



R:



20 **21***

Figure 6. Structures of compounds isolated from *S. wolffii*: ponasterone A-22-apioside (**19**)*, 3-*epi*-22-deoxy-20-hydroxyecdysone (**20**) and 3-*epi*-shidasterone (**21**)*. New compounds are denoted by asterisks (*)

4. DISCUSSION

The ecdysteroids, which contain several hydroxy groups, are rather polar compounds, and can be extracted with a polar solvent such as methanol. However, although methanol is very effective for the extraction of ecdysteroids, it is not selective for them. The crude methanolic extract contains not only the structurally different ecdysteroids, but a series of other compounds. This methanolic extract was subjected to a multiple-step clean-up procedure by using simple, preparative-scale separation techniques. The principal steps were liquid–liquid extraction, precipitation, and SPE on polyamide. The majority of the contaminants, representing 37-58% of the dry residue obtained by extraction, were removed by liquid–liquid extraction between *n*-hexane and the aqueous methanolic extract, and precipitation with acetone from the crude methanolic extract. In this way, the methanolic extract was made free of apolar (chlorophyll and terpenoids) and polar contaminants (sugars and proteins), with a very good recovery of the ecdysteroids. The following prepurification step was solid-phase extraction, which was carried out on polyamide. Ecdysteroids with alcoholic hydroxyl groups were eluted by using water and aqueous methanol (10% and 20%); the flavonoids remained adsorbed on the polyamide, because the polyamide strongly binds the flavonoids, which contain phenolic hydroxy groups. SPE on polyamide permits a group separation between ecdysteroids and flavonoids.

The isolation of different ecdysteroids from the prepurified plant extract needed the utilization of a combination of sophisticated preparative-scale chromatographic methods, which included NP-CC on alumina, RP-CC on octadecyl-silica, RPC on silica, and preparative HPLC. RP-CC was performed according to the conditions of vacuum chromatography. RP-CC on octadecyl-silica and NP-CC on an alumina column were used to separate the remaining apolar and polar impurities from the ecdysteroids. During RP-CC, the apolar impurities have a greater affinity for the stationary phase; these impurities were therefore retained on the sorbent. An important advantage of octadecyl-silica as stationary phase is that it does not adsorb ecdysteroids. Alumina proved to be very efficient in the elimination of polar contaminants, because these compounds remain adsorbed on alumina. It can be used as a cheap stationary phase whenever there is a considerable amount of polar contaminants to be eliminated, but the adsorption on alumina must be considered. The isolation procedure was improved by the use of RPC, which is easy to carry out, and the ecdysteroids are in contact with the adsorbent layer for a short time. The problem with adsorbent-promoted decomposition was therefore reduced. RPC on silica is an inexpensive,

effective tool for the separation of ecdysteroids, which requires low solvent usage and less time.

The final purification was carried out with repeated preparative-scale NP-HPLC with good resolution. Our well-conceived separation method was in fact a combination of different chromatographic methods with varying selectivity. Chromatographic steps based on different interactions were consecutively used to achieve the best separation: alumina (adsorption interactions), octadecyl-silica (hydrophobic interactions), RPC and HPLC (adsorption interactions).

The species selected for our study had to fulfill two essential criteria: high contents of ecdysteroids (*P. vulgare* rhizomes contain 10‰ and *S. wolffii* roots 10-20‰ of ecdysteroids),[107] and the ability to biosynthesize ecdysteroids with a broad range of structures.[97] *A. reptans* and *P. vulgare* are starting materials for the manufacturing of preparations, while *S. wolffii* could be an alternative source to *Leuzea carthamoides*, which is also a frequent starting material for many herbal preparations.[108]

Our selected species had previously been investigated for their ecdysteroid content; *S. wolffii* was examined among others by our research group, too. Research carried out in the last decades revealed that these species contain ecdysteroids with uncommon structures: *A. reptans* produces C_{29}-ecdysteroids with a lactone ring, *P. vulgare* produces compounds with multiple sugar molecules, and *S. wolffii* produces ecdysteroids with furan ring and multiple double bonds. Our isolated ecdysteroids deserve attention for their unusual structures: all three species biosynthesize compounds which possess a five- or six-membered ring in their side-chain. Such ecdysteroids with a cyclic ether, acetal or lactone ring are very useful for structure–activity relationship studies, and can reveal essential features of biological activity.

Four of the isolated *Ajuga* ecdysteroids (**1**-**4**) possess a lactone ring, formed by the 26-carboxyl group and a 22- or 23-hydroxy group in the side-chain. The six-membered γ-lactone ring-containing 24-dehydroprecyasterone (**1**), in which the 22-hydroxy group forms a lactone ring with the 26-carboxyl group, was earlier isolated from *Ajuga iva*.[109] Reptanslactone A (**2**) and reptanslactone B (**3**) contain five-membered γ-lactone rings involving a lactone bridge between the 23-hydroxy and 26-carboxyl groups. Compound **3** is the 24-hydroxy derivative of compound **2**, but the 23*R**,24*S** configuration was assigned to compound **2** and the 23*S**,24*R** configuration to compound **3**. Compound **4** (breviflorasterone), previously isolated from *Ajuga macrosperma* var. *breviflora*,[98] is a diastereomer of compound **2**; they differ from each other in the configuration at C-24. Ecdysteroids containing a five- or six-membered lactone ring in their side-chain were first considered specific to *Ajuga* species, but

they have recently been found in other species, too. It is interesting that they are characteristic constituents of the taxonomically very distant *Ajuga* and *Cyathula* species. Apart from these two genera, only *Eriophyton wallichii*, *Leuzea carthamoides*, *Rhaponticum uniflorum*, and two *Silene* species each synthesize one lactone ring-containing ecdysteroid.[97]

The free 23-hydroxy group is also rare in ecdysteroids: only three such compounds are known in plants, in *Ajuga iva*, *Leuzea carthamoides* and *Serratula tinctoria*.[97] The 23-hydroxy group is mainly bound in lactone ring form in the ecdysteroid derivatives in plants. Compounds **1**-**5** are stigmastane-type C_{29}-steroids. Only 15% of the known ecdysteroids isolated from plants have a C_{29} skeleton containing an ethyl group at C-24 in their side-chain.[97] These compounds are also typical ecdysteroids of *Ajuga* spp.

The *Ajuga* spp. contain considerable amounts of ecdysteroids and neo-clerodane diterpenes; they exert both toxic and developmental and reproduction disrupter activity against insects.[110,111] The ecdysteroid profile of *Ajuga* spp. varies strongly in both amount and composition not only between plant species, but also according to plant organs and season, which has an influence on their insecticidal activity.[112] At a molecular level, this activity is closely related to several structural elements. Previous ecdysteroid structure–activity concepts were based on essential or nonessential functional groups,[12] but recent studies have revealed that no single feature is essential for activity, and not all functional groups are equally important for the display of biological activity (**Figure 7**).[15,113] Both activities measured in the BII assay and predictions for hypothetical steroids indicate that a *cis*-A/B-ring junction is not essential for activity. Although the 7-en-6-one moiety is not essential, the absence of both the double bond and the keto group seems to be accompanied by a decreased activity.[15]

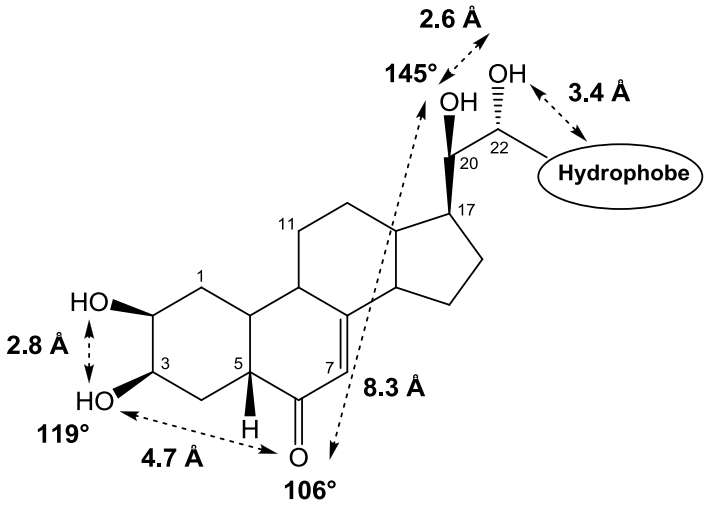


Figure 7. Pharmacophore hypothesis of ecdysteroid activity.[15]

An important functional group on the ring A, is the 2- and/or 3-hydroxy group. The hydroxy groups in positions C-5, C-14 and C-22 play a role in the binding behavior; hydroxy substitution on C-25 rather decreases the activity. A 14-hydroxy group could facilitate the intermolecular connection between the side-chain and ring A.[113] The research carried out by Suksamrarn *et al.*[114] demonstrated that a free 22-hydroxy group is not an essential feature for an ecdysteroid to exhibit high molting activity. The activities of the 22-*O*-substituted ecdysteroids may be higher than those of the parent compounds, providing that the substituent constitutes a functional group that can enhance biological activity. From this aspect, compounds **1**-**5**, which either contain a free 22-hydroxy group or a lactone or acetal ring, may be considered highly active ecdysteroids. The ligand, a steroid molecule with plane restriction will not need more than two main points to reach sufficient associated bond energy, once in contact with the receptor. Ferro *et al.*[113] therefore consider that the 22-hydroxy group should be involved in a direct reaction to induce intermolecular forces, while the other functional groups of the side-chain are contributors to the molecular properties, and modulate the biological activity.

The biological activities of some compounds with heterocyclic ring in the side-chain from *A. reptans* were determined via the oral aphid [(*Acyrtosiphon pisum*(Harris)] test by our research group. Breviflorasterone (**4**) proved to be most active (LC_{50} = 3.65), while reptanslactone B (**3**) (LC_{50} = 78.81) and sendreisterone (**5**) (LC_{50} = 95.14) exhibited only low oral activity against aphid larvae. The activity of **4** was comparable to that of ajugalactone (LC_{50} = 5.37), which is considered one of the most effective antagonist ecdysteroids.[97,115] The activities of these compounds can be explained on the basis of the polarities and the characteristic structural elements of the side-chain. Compound **4** is more balanced in term of polarity as compared to **3**, which contains more hydroxy groups than **4**. Although compounds **4** and **5** have the same number of hydroxy groups, the latter contains a six-membered ring with several relatively bulky substituents (methoxy and ethyl), which probably decreases the activity, and the receptor-binding capacity. *A. reptans*, as a good source of compounds with high molting activity, should be regarded as a leading plant for the development of environmentally friendly compounds, important for integrated pest management programs.

Ecdysteroids possessing an ether or acetal ring in their side-chain are characteristic of fungi, have also been found in species of the Lamiaceae family (*Ajuga macrosperma*, *Ajuga nipponenesis* and *Vitex canescens*), in *Polypodium* spp. and by our research group in *S. wolffii*. In the examinations of the ecdysteroid profiles of our selected species (*A. reptans* var. *reptans*, *P. vulgare* and *S. wolffii*), we were looking in particular for new compounds with an

ether or acetal ring in the side-chain. Our research revealed that all three plants contain such compounds (**Figure 8**). Compounds **5**, **17** and **18** have unusual structures which are closely related to that of ajugacetalsterone A,[116] but the 26-hydroxy group at **5** and **18** is methylated.

The characteristic ecdysteroids of *P. vulgare* have a side-chain in their molecule, which forms an intramolecular ether ring. In three ecdysteroids, shidasterone (**13**) and shidasterone derivatives (**14-15**), the 22-hydroxy group forms an intramolecular ether linkage with the 25-hydroxy group. Shidasterone is fairly common in plants (*Ajuga*, *Leuzea*, *Polypodium*, *Blechnum*, *Stachyurus* and *Vitex* species),[117] whereas its derivatives are rare. The 20-deoxy derivative of shidasterone (**15**), which was previously synthetized from 20E by Roussel *et al.*, is a new natural compound.[118] Other shidasterone derivatives, 11α-hydroxyshidasterone and 24-methyleneshidasterone were earlier obtained from *S. wolffii*, and a new member of this group, compound **21,** has now been identified from the same species.[91,94]

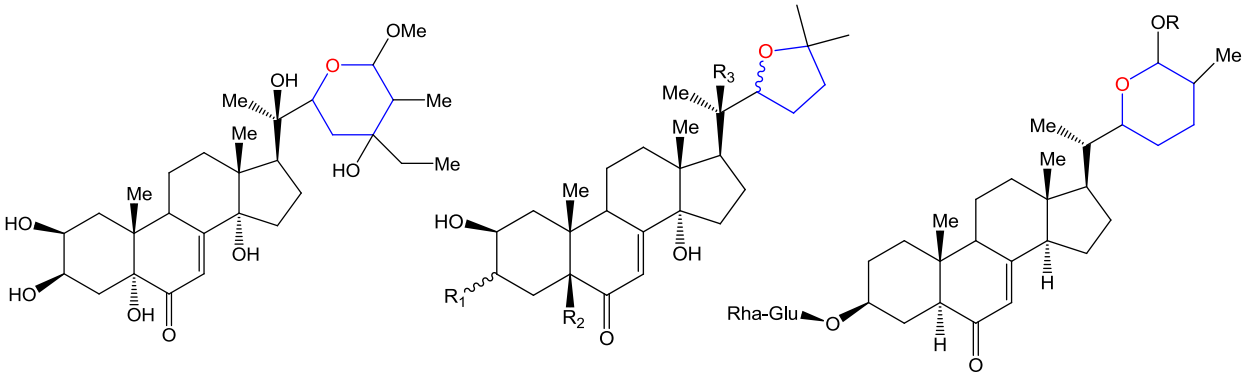
The ecdysteroids **7-8**, **10-11** and **13-15** are reported for the first time from *P. vulgare*. C-5 is the characteristic hydroxylation position in ecdysteroids from the common polypody: several of the isolated ecdysteroids possess a 5-hydroxy group (**Table 16**).[97] The common occurrence of basic ecdysteroid compounds together with their corresponding 5-hydroxy compounds proves that the final hydroxylation step occurs at C-5, in accordance with an earlier hypothesis.[107] Compound **9** is a new, 5-hydroxylated ecdysteroid which is present in the plant together with the parent compound, ecdysone.

Table 16. Parent ecdysteroids and their corresponding 5-hydroxy compounds from *P. vulgare*

Parent ecdysteroid	**5-Hydroxylated compound**
Abutasterone	*5-Hydroxy*abutasterone
Ecdysone	*5-Hydroxy*ecdysone
20-Hydroxyecdysone	*5*,20-Di*hydroxy*ecdysone (polypodine B)
Poststerone	*5-Hydroxy*poststerone
Rubrosterone	*5-Hydroxy*rubrosterone
Shidasterone	*5-Hydroxy*shidasterone

The glycosidic forms of ecdysteroids are known to occur in *Polypodium* species, and exhibit a sweetness intensity much higher than that of sucrose. Such a well-known compound is osladin, which (together with other structures) can be regarded as a potential alternative to sucrose.[85,86] These low-hydroxylated ecdysteroids are glycosylated at C-3 and/or C-26 and

have the rare *trans* A/B ring junction. Compounds **17** and **18**, isolated earlier from *P. vulgare* by Jizba *et al.*, possess such a structure.[83]



(**5**)

	R_1	R_2	R_3
(**13**)	*β*OH	H	OH
(**14**)	*β*OH	OH	OH
(**15**)	*β*OH	H	H
(**21**)	*α*OH	H	OH

	R
(**17**)	H
(**18**)	CH_3

Figure 8. Ecdysteroids with an ether ring from *A. reptans* (**5**), *P. vulgare* (**13**-**15**, **17**-**18**) and *S. wolffii* (**21**)

Besides these glycosides, other compounds containing sugar molecules have been identified and isolated from *P. vulgare* and *S. wolffii*. Ponasterone-22-apioside (**19**) is the first known ecdysteroid glycoside in which apiose is attached as a sugar unit to the aglycone. The 2-glucopyranoside of polypodine B (**16**) is the third example of a C-2 glucosyl derivative among the ecdysteroids.

3-*Epi*-22-deoxy-20-hydroxyecdysone (**20**) and 3-*epi*-shidasterone (**21**) belong among the ecdysteroids that contain a 3α-hydroxy group. The number of such reported 3-*epi*-ecdysteroids is 21, whereas there are around 350 known ecdysteroids. The presence of these 3-*ep*imers in plants is rather unusual; they are mainly biosynthesized in insects. In contrast with the 3-*epi*-ecdysteroids, the other *epi*-ecdysteroids, such as 22-, 14- and 25-*epi*-ecdysteroids, occur only in plant species. The isolation of such kind of 3-epimers from *S. wolffii* has been reported earlier: 3-*epi*-20E and two structurally related 3-*epi*-ecdysteroids, these latter lacking the characteristic 14-hydroxy group.[94]

The Asteraceae family includes two main genera with ecdysteroid-containing species: the genera *Leuzea* and *Serratula*. *Leuzea carthamoides* DC [syn. *Rhaponticum carthamoides* (Willd.) Iljin], a rich source of ecdysteroids, is cultivated on a large scale, especially in Eastern Europe, for the manufacturing of various preparations containing ecdysteroids. *S. wolffii* also has a variegated ecdysteroid content, which is quite similar to that of *L. carthamoides*.[119-121] Considering the similarity of the ecdysteroid profiles of the two plants,

and the high biomass production potential of *S. wolffii*, this could be an alternative source to *L. carthamoides*.

5. SUMMARY

Our main results may be summarized as follows:

1. *Ajuga reptans*
   - Five ecdysteroids were isolated and characterized from *A. reptans*
   - Three of these compounds are new natural compounds
2. *Polypodium vulgare*
   - Thirteen compounds were isolated and characterized from the rhizome of *P. vulgare*
   - Three of these compounds were discovered for the first time in a natural source
3. *Serratula wolffii*
   - Three ecdysteroids were obtained and characterized from *S. wolffii*
   - Two of these compounds are new natural substances
4. Structural characteristics of the isolated ecdysteroids
   - Five compounds contain the rare stigmastane-type C_{29}-steroid skeleton
   - Four ecdysteroids possess a lactone ring, where the 26-carboxyl group forms a lactone ring with a 22- or 23-hydroxy group in the side-chain
   - Four ecdysteroids (shidasterone and its derivatives) have a cyclic ether function in the side-chain
   - Two compounds are 3-epimers; their presence in plants is rather unusual
   - C-5 is the characteristic hydroxylation position of ecdysteroids from the common polypody: five of the isolated ecdysteroids contain a 5-hydroxy group
   - Compound **19** is the first known ecdysteroid glycoside in which apiose is attached as a sugar unit to the aglycone
5. *A. reptans*, as a good source of compounds with high molting activity, could be an important plant for the development of environmentally friendly insecticides
6. *P. vulgare*, evaluated by the *European Medicines Agency*, and generally considered safe, could be a suitable plant for pharmacological studies from the perspective of manufacturing preparations
7. Considering the similarity of the ecdysteroid profiles of the two plants, *S. wolffii* could be an alternative source to *L. carthamoides*.

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