Ph.D. Thesis

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Ecdysteroid profile of *Silene viridiflora* and the effect of 20-hydroxyecdysone on rat muscle fibres *in vivo*

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1. Introduction

The steroid hormones can be classified according to their biological relevance. The class of vertebrate steroid hormones: androgens, estrogens, progestogens, corticosteroids and colecalciferols; the class of brassinosteroides, are growth-promoting hormones of plants and the class of ecdysteroids, which were originally discovered in insects, but many are also present in other arthropods, invertebrate phyla and plants as well [1].

1.1 General structure of ecdysteroids

Ecdysteroids comprise a class of polyhydroxylated steroids. They are generally characterized by a basic skeleton containing 27-29 carbon atoms with a long sterol alkyl side-chain on C-17 and by the presence of a 7-en-6-one chromophore group in ring B. In view of these features, the naturally occurring ecdysteroids can be classified among the conformationally flexible steroids, because the side-chain rotates freely around the 5 single C-C bonds. However, ecdysteroids with 19, 21 or 24 carbon atoms may also be formed from C-27 ecdysteroids by cleavage of the sterol side-chain. Moreover, the conjugated oxo function at C-6 has been considered to undergo a tautomer interconversion, forming a 5,7-diene structure [2,3]. Characteristic OH groups are present in positions 3β- and 14α- and further hydroxylation is often observed at C-1, 2, 5, 11, 20, 22, 25, 26 or 27. Other known features include additional double bonds or oxo groups, and glucosylation, esterification or etherification of certain OH groups [4]. The A/B ring junction is mostly *cis* and that of the C/D is *trans*. Moreover, ecdysteroids may occur in nature as acetonides, or esters with organic (acetic, benzoic, coumaric, cinnamic or pyrrole-2-carboxylic) [5,6] or inorganic (phosphoric or sulphuric) acids [7,8]. The structure of the main phytoecdysteroid, 20-hydroxyecdysone (20E, **1**) is shown in **Fig. 1.**

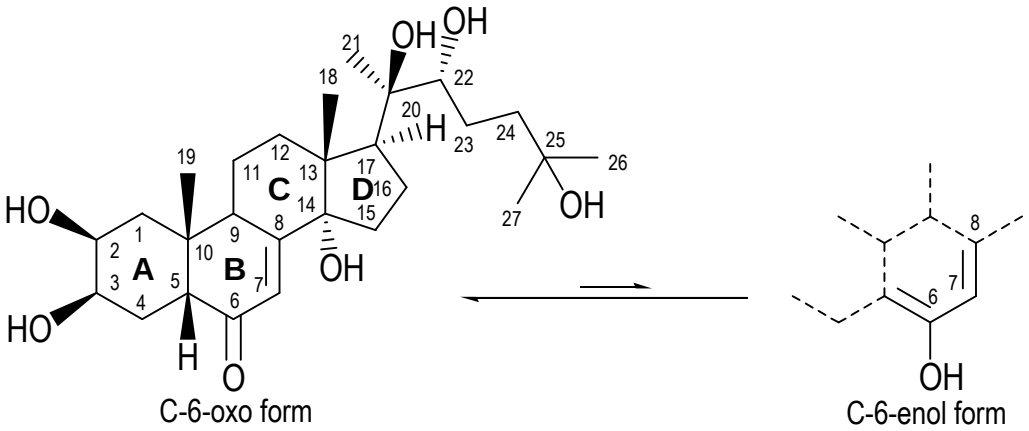

Fig. 1. Structure of 20E (**1**) showing the possible tautomer interconversion of C-6.

1.2 Natural occurrence of ecdysteroids

1.2.1 Presence and role in animals

In insects, ecdysteroids act as moulting hormones, regulating metamorphosis and also several other important life-cycle processes [5]. They may also have role in the reproduction, embryogenesis and diapause of certain other arthropods (insects, crustaceans, arachnids and myriapods). The hormonal effect of ecdysteroids has been proven only in arthropods. The milestones in ecdysteroid research were as follows: It was postulated that the moulting of insects must be under hormonal control [9]. Isolation of the first ecdysteroid, ecdysone, from silkworm pupae [10] proved this assumption. The structure of ecdysone was elucidated 10 years later [11]. In most insect organisms, the main and also the biologically most significant ecdysteroid is 20E (**1**), which was first isolated from crayfish (*Jasus lalandii*) [12]. It has been suggested that other ecdysteroids may play active roles in insects at different stages of their development [13].

1.2.2 Presence and role of ecdysteroids in plants

The fortuitous discovery of ecdysteroids from plant sources initiated the systematic and fruitful screening of ecdysteroid-rich plant sources, leading to the isolation of new ecdysteroids. It is generally accepted that ecdysteroids in plants play an important part in the protection against insect predators and soil nematodes, either as a consequence of their antifeedant activity or by inducing the developmental disruption and even the death of non-adapted phytophagous insects or soil nematodes [5]. Phytoecdysteroids were discovered with rapid success in numerous plant species, and later it emerged that ecdysteroids are widely distributed in the plant kingdom [5]. Ecdysteroids occur in unrelated plant species in great

structural variety and in exceptionally large amounts. Plant ecdysteroids are often biosynthesized i.e. 2-5 orders of magnitude higher than their concentration in insects. A 0.1% concentration of **1** is not unusual, and several plant species biosynthesize **1** in 1-3 % of their dry mass, i.e. a 2-6-fold order of magnitude higher concentration than those of the other ecdysteroid constituents. **1** can be isolated from these plant sources by simple separation procedures [14]. The isolation of minor ecdysteroids requires adequate plant sources, from which these ecdysteroids can be isolated in appropriate quantities by means of sophisticated methods [15]. Such plants are *Ajuga, Serratula, Silene*, *Polypodium* and *Leuzea* species [16]. The large number of phytoecdysteroids (more than 300) promotes continuous research with a view of discovery of unknown pharmacological effects and the expansion of earlier findings.

1.2.3 Silene viridiflora L.- *occurrence, botanical and chemical description*

Fig. 2. Flowers of *S. viridiflora*[1]

The *Silene* genus, one of the largest genera of the word flora, is distributed widely in the Carpathian basin. It comprises approximately 700 predominantly perennial species, about half of which occur in the Mediterranean area. The South Balkan Peninsula and South West Asia are two of the main centers of diversity for the genus [17].

The *Silene* genus belongs to the Angiospermae Division, Dicotyledonae Class, Centrospermae Order, Caryophyllaceae Family and Silenosidae Subtribe.

S. viridiflora is a perennial species, its stem grows up to 40-90 cm. The lower leaves are oblong-spathulate, while the upper ones are ovate-lanceolate or acuminate. The calyx is 15-20 mm long, gradually attenuate at base. The petals are greenish-white with long-exerted claw and linear lobes. The capsule is 12-14 mm, ovoid in shape [18], see in **Fig. 2.**

The *Silene* species were proved to be a rich source of ecdysteroids [19-23]. Up till now almost one hundred *Silene* species have been investigated for the presence of ecdysteroids from which 40% were positive [13,19]. *S. viridiflora* was determined to be an ecdysteroid positive species by our research group [19,24] and was confirmed later by others (reviewed in

[1] Downloaded from http://www.raybrowns.com/perennial-flower-seeds/schizostylis-to-symphytum/SILENE-VIRIDIFLORA-(Caryophlaceae)-HP.html (01. 02. 2010).

[25]). Mamadalieva concluded that the plant is characterized by a relatively high accumulation of ecdysteroid which may be up to 1.1% during budding and flowering [26]. The major ecdysteroids of *S. viridiflora* were proved to be **1**, polypodine B, 2-deoxy-20-hydroxyecdysone, 26-hydroxypolypodine B, integristerone A, silenoside A, D and the 2,22- and 3,22-diacetates of 20,26-dihydroxyecdysone [26-30].

1.3 Extraction, isolation, detection and structure elucidation of ecdysteroids from plant sources

The extraction of dried, milled samples is best performed with a polar solvent such as methanol or ethanol. After the extraction, both conventional (liquid-liquid extraction) and modern (solid-phase extraction) methods are in use for the prepurification [1,31,32]. In the processing of large amounts of sample, further purification is achieved via repeated column chromatography steps on silica, alumina and Sephadex LH_{20} stationary phases [33,34].

Among the chromatographic methods, thin-layer chromatography (TLC) is appropriate to map the ecdysteroid profile of herbal extracts and identify its components, particularly if the analyte is rich in ecdysteroids and a high sensitivity is not necessary. Classical adsorption TLC is the most widely used method [33,35], but chemically bonded stationary phases (primarily octadecyl silica, C_{18}) can also be applied. Ecdysteroids have absorbance at 254 nm and can be visualized with UV light using TLC plates containing fluorescent additives. After spraying the plate with a dehydrating agent (most commonly vanillin-sulfuric acid), colourful spots at daylight and fluorescence at 366 nm can be detected.

Final purification can be carried out with HPLC. If the sample is small, only consecutive steps of NP- and RP-HPLC are applied [36,37]. In NP-HPLC of ecdysteroids both adsorbent and polar, chemically bonded, both apolar, chemically bonded stationary phases are available. Silica, which is the most widely used, yields outstanding resolution in the separation of ecdysteroids, as confirmed by many articles [16,35,38,39]. The most generally applied mobile phases on silica are ternary systems based on CH_2Cl_2 or *c*-hexane, with *i*-PrOH as organic modifier. The strong ecdysteroid adsorption on silica causes the extensive tailing of the ecdysteroid peaks, thus to make them symmetrical, water has to be added to the solvent system [40]. This slows down the equilibrium formation and gradient elution is not possible. Water adsorbs on the silica, slowly deactivating it, and this results in change of the retention times, thereby reducing the reproducibility of the analysis. In RP-

HPLC C_8 and C_{18} modified phases are widely used [41]. MeOH-H_2O and ACN-H_2O solvent systems are generally most appropriate, with somewhat higher resolution in the latter case. The peaks can be made symmetrical by adding a buffer. For the detection of the ecdysteroids during HPLC analysis, mostly UV light is applied. The 7-en-6-one chromophore has sufficient molar absorbance to allow the detection of nanograms of ecdysteroids. With diode array detectors, even more information can be obtained [7,39]. The identification of ecdysteroids can be made more specific if HPLC is connected on-line with MS. HPLC-MS analysis is a very sensitive, selective technique to identify known ecdysteroid compounds [7,38,42,43].

The identification of a pure ecdysteroid is based on physical (melting point, optical rotation) measurements and spectroscopic methods (UV- and IR spectra). The HRMS and NMR spectroscopy are used to elucidate the structures of new compounds. In some cases the final proof of the steric structure is established by using X-ray crystallography [14].

1.4 Effects of the ecdysteroids on mammals

The ecdysteroids influence many physiological functions and have several physiological effects on mammals, including humans; they affect certain major metabolic pathways in mammals: protein synthesis, lipid and carbohydrate metabolisms and ioncurrents. They may act as anabolic, hepatoprotective, immunoprotective, antioxidant and hypoglycemic agents. Their low acute toxicity has been repeatedly demonstrated experimentally [31,44,45], *e.g.* LD_{50} > 6 g/kg and > 9 g/kg for **1** administered intraperitoneal (*i.p.*) or orally (*p.o.*) to mice, respectively. A dose of 0.1 g/kg of **1** administered intravenously (*i.v.*) to rabbits did not cause any toxic reaction, and the subacute treatment of rats with **1** 2 g/kg/day resulted in no toxic symptoms.

1.3.1 Hormonal activity

In consequence of their steroidal structure and anabolic action, ecdysteroids have often been suspected of possessing the hormonal effects of vertebrate steroids (estrogens, androgens and corticoids), especially that of the androgens. The absence of an androgen effect of **1** and some other ecdysteroids has been widely proven in experimental animals. This conclusion was based on measurement of the increase of the prostate and seminal vesicle mass, when no

androgenic effect was observed [46-48]. (The estrogenic effect has been assayed only in the case of **1**). The possibility of estrogenic or antiestrogenic effects **1** was investigated by Prabhu and Nayar [49]. In intravaginal doses of 30- 500 μg, **1** was compared with 17β-estradiol dipropionate in adult female rats. Neither estrogenic nor antiestrogenic effects of **1** were observed. It has also been reported that the effects of anabolic steroids and ecdysteroids are characteristically different on thymocytes. The systemic administration of testosterone and methandrostenolone to male rats in doses of 1-2 mg/100 g for 10 days decreased the mass of thymus and reduced the thymic serum factor content. **1** which does not possess androgenic activity failed to influence the thymus mass and the level of the thymic serum factor [50]. Other work presented similar findings: two anabolic steroids, testosterone and methandrostenolone decreased mass, the quality of DNA and the incorporation of ^{3}H labeled thymidine in thymocytes after daily injection to mice in 5 mg/100 g dose for ten days. In the meantime Ecdysten® (preparation containing mainly **1**) did not influence proliferative activity of thymocytes *in vitro* and thymolytic effect *in vivo*. This indicates that the proliferative process may have a functional significance in thymolitical effect of anabolic steroids [48]. *In vitro* radioligand binding assays using estrogen, glucocorticoid and androgen receptor selective-radioligands were applied to check on the presence or absence of estrogen, glucocorticoid and androgen effects of ecdysteroids, but none of the tested compounds displayed an estrogenic, glucocorticoid or androgenic effect, which reflects that ecdysteroids do not bind to the vertebrate steroid receptors [4]. However, the binding of the ecdysteroids to the vitamin D receptor (VDR) or the activity of the different ecdysteroids on the vitamin D system has not been investigated yet.

1.3.2 Anabolic effect

Ecdysteroids are considered adaptogenic, enhancing physical performance, promoting vitality and increasing the resistance to stress and aging [44,45]. However, their most highlighted effect is increasing muscle size and strength.

The anabolic effect of ecdysteroids on muscle size has been demonstrated in a wide range of animals. Increased body, organ and muscle weight and protein synthesis have been reported in cases of *p.o.* or *i.p.* administration of ecdysteroids in several animal species. The first observed (and classical) pharmacological activity of ecdysteroids is their protein synthesis-stimulatory effect. Okui *et al.* [51] and Otaka *et al.* [52] reported stimulatory effect of ecdysteroids on protein synthesis in the mouse liver. The amino acid incorporation was

determined after oral or *i.p.* administration of ecdysteroids isolated from plants. 4-Chloro-testosterone, an anabolic-androgen, was used as control. Maximal activity was observed for both **1** and 4-chloro-testosterone. An increase in protein synthesis after **1** administration was confirmed by Otaka *et al.* [51] in the microsomal fraction of the mouse liver. The anabolic activities of **1** and cyasterone were likewise determined in mice, where enhanced protein synthesis was detected in the liver and kidney [53]. These early studies were extended to examinations of protein synthesis in other tissues and in other mammalian species (rats, mice, sheep, pigs, quails) [54-61]. For example, improved nitrogen retention and body weight increase with lowered food consumption were observed in pigs and Japanese quails (>12% increase) which received *Leuzea carthamoides* herb, seed and **1** in their diets [57]. Comparing the effect of ecdysteroids and anabolic-androgenic steroids, it has been reported [59] that an androgen dependent development is a prerequisite before the action of ecdysteroids in rat. **1** in 0.5 mg/100 g dose for 7 days resulted in increased weight gain of the whole body, liver, heart, kidneys and musculus tibialis anterior in rats. The accumulation of protein content was also accelerated. These changes were even more pronounced if the animals were still growing (70-80 g). In sexually immature castrated rats, the androgenic action of **1** was not demonstrable in contrast to that of methandrostenolone. Later, an extensive comparative study was presented using a number of purified ecdysteroids including turkesterone, **1** and methandrostenolone [62]. An early report [63] also implied the difference between the mechanisms of actions of ecdysteroids and anabolic steroids. **1**, turkesterone and 2-deoxyecdysone in the same dose (0.5 mg/100 g) were found to stimulate protein synthesis in the liver of laboratory mice. The protein synthesis-increasing ability was associated with polyribosomal activity. The preliminary administration of actinomycin D did not prevent the phytoecdysteroid effect on protein synthesis stimulation. Therefore, it has been concluded that the anabolic effect of ecdysteroids is connected with the acceleration of translocation processes instead of the induction of new RNA synthesis. This shows that ecdysteroids are not likely to act as the classical steroids, via cytoplasmic receptor and regulation of gene transcriptional activity.

The effect of Nerobol® and **1** was tested in insulin-dependent processes and in case of insulin resistance [64]. The insulin resistance was induced by injection of hydrocortisone, whereas the insulin insufficiency by alloxan. The sensitivity of the body to *i.v.* infusion of insulin and the reaction of isolated fat tissue to the hormone increased after administration both Nerobol® and **1**. The above effects of the steroid were more dependent on the nonspecific protein synthesis of the cells than on the increase of insulin secretion. Chermnykh *et al.* [65] compared the anabolic action of ecdysteroids and of methandrostenolone on male

mice, preconditioned with or without a swimming test. Methandrostenolone produced anabolic effects only after constant training, but **1** improved the physical ability of the mice both with and without this preconditioning training. Methandrostenolone stimulated the biosynthesis of myofibrillar proteins in the musculus soleus, but not in the musculus extensor digitorum longus (EDL), while **1** increased the amount of myofibrillar proteins in both muscles. Meanwhile Syrov and co-workers consequently described non-androgenic effects of ecdysteroids [59,62,63], at least one study is at variance with their conclusion. Xu *et al.* [66] have reported that **1**, the effective compound of the extract from *Antherea pernyi* Pas, was able to increase the weight of prostate-semina and levator ani/bulbocavernosus muscles of castrated mice. The extract was also able to accelerate the growth of younger male mice and enhanced the RNA, DNA and protein content in liver. Therefore it has been concluded that **1** has androgen-like anabolic action. These protein synthesis-stimulating effects of ecdysteroids was determined following the *p.o, i.p.* or *i.v.* administration of 0.2-500 mg/kg ecdysteroids from 5 to 150 days.

In a recent study, phytoecdysteroids, applied as various ecdysteroids and extracts of ecdysteroid-containing plants, increased the *in vitro* protein synthesis by 20% in mouse myotubes and human primary myotubes [67]. The gain in protein synthesis was abolished when the PI3K inhibitor was administered to the mouse myotubes together with **1**. This was consistent with the hypothesis that **1** increases protein synthesis via the PI3K pathway, which is a general signalling route for the enhancement of translation on ribosomes [68]. The grip strength of rats was significantly increased by the administration of **1** (50 mg/kg for 28 days) in a dose comparable to that of methandrostenolone, an anabolic steroid (10 mg/kg for 28 days). A similar improvement in grip strength resulted from feeding with a 1 g/kg spinach extract containing 3% of **1** [67].

Considering the above outlined aspects, some ecdysteroids may provide promising alternatives to anabolic-androgenic steroids in therapy. Prospective use of ecdysteroids may extend to treatments of pathological conditions where anabolic steroids are applied, for example in myopathies [69] or to reverse the muscle atrophying effect of glucocorticoids. Because of their potent anti-inflammatory properties, glucocorticoids are often used in the medical practice including the treatment of several respiratory disorders such as asthma and chronic obstructive pulmonary disease, however, one of the most serious side effects of glucocorticoids is the catabolic effect on muscle tissue [70]. While diaphragm is the primary inspiratory muscle; the development of diaphragm muscle atrophy, resulting in loss of force production, might have deleterious effects in patients with lung disease [71]. Anabolic

steroids may have the potential to antagonize glucocorticoid-induced effects on the diaphragm [72], but their hormonal side effects are also well known.

1.4.3 A possible mode of mechanism of action on the muscle

Among the mammalian steroids, calcitriol (1,25D) is the most similar to the ecdysteroids in structure. This raises the hypothetical possibility that the ecdysteroid effects on mammals can be explained by a mechanism similar to the rapid response to 1,25D and its analogues. The connections between 1,25D status and the state of the cardiovascular system [73], insulin resistance and β-cell dysfunction [74-77], autoimmune diseases, including diabetes type 1 [78], cancer, muscle strength and mass [79] has already been described in clinical studies. These effects are most probably linked to non-genomic (NG) action of 1,25D.

The nuclear vitamin D receptor (VDR) belongs to the superfamily of nuclear receptor (NR) proteins, which includes receptors for all steroid hormones and related small-molecule agonists and antagonists [80]. Classically, ligand-occupied steroid hormone receptors have been thought to selectively regulate (activate or diminish) gene transcription [81,82]. In addition, evidence has accumulated to support the participation of the so-called NRs in initiating, at or near the plasma membrane, the rapid (seconds to minutes) activation of signalling cascades that generate NG responses [83,84] that can also modulate genetic activity [85]. In 2004, a VDR ensemble model has been proposed that describes how a classical steroid (nuclear) receptor can accommodate differently shaped ligands to initiate two types of responses: rapid cytoplasmic, membrane or NG responses and slow genomic (G) responses [86,87]. An ensemble model [88] differs from an induced-fit model in that the apo-protein is considered to be highly flexible in absence of ligand; therefore, the apo-protein is capable of sampling one or more active conformations and may contain multiple ligand-binding sites. The VDS-VDR conformational ensemble model posits that two overlapping, functionally distinct ligand-binding pockets (LBPs) exist in the VDR, a genomic pocket (GP, considered thermodynamically favored), responsible for the G effects and an alternative pocket (AP, considered kinetically favored), responsible for the NG effects of vitamin D. **Fig. 3.** illustrates the hypothetical alternative binding site and the VDR ligand binding domain (LBD) as it binds to 1,25D.

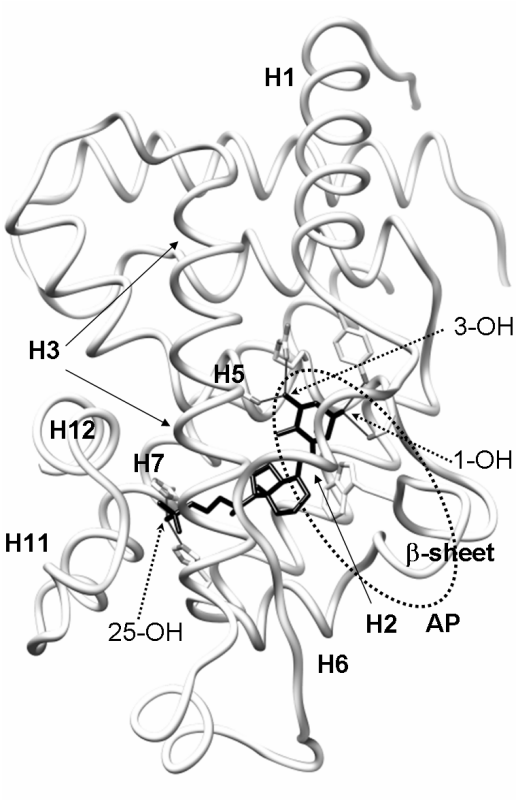

Fig. 3. VDR LBD complexed to 1,25D (based on [89]). α-Helices and the –OH groups of 1,25D are indicated. The dashed oval shows the approximate location of the hypothetical VDR AP (based on [86]).

Although a few direct comparisons have been reported on the NG effects of ecdysteroids and vitamin D analogues in mammals, a number of concordant observations can nonetheless be made. For example, both vitamin D analogues [90,91] and **1** activate nuclear p53 in keratinocytes resulting in protection of skin against UV irradiation[2], and in the skeletal muscle both **1** and vitamin D are selectively able to increase the size of the fast glycolytic fibres [92,93]. Moreover, ecdysteroids also seem to act via PI3K/Akt [67,94-96], PKC/MAPK signalling cascades in some *in vitro* systems [96], similarly to vitamin D analogues [97]. These similarities give rise to the suspicion that the "mystic" mechanism of action of ecdysteroids on mammalian systems is mediated by the vitamin D NG signalling pathways, or, in other words, that the ecdysteroids might be able to bind to the VDR AP. Only one attempt is known to have been made in an effort to support this hypothesis, where docking of the 6-enol form of **1** to the VDR AP resulted in a gold score value acceptable for the indication of a strong ligand-protein interaction[2]. This might be explained by (a) the common

[2] Presentation held at XVII[th] International Ecdysone-Workshop 2008, July 20-24, Ulm, Germany. Meybeck, A.; Yang, C.-R.; Zhang, Y.-J.; Salmon, M.; Belot, N.; Toussaint, O.; Wurtz, J.-M.; Moras, D.; Ho, R.; Raharivelomanana, P. Ecdysteroids from plants prevent UV induced premature senescence of human skin fibroblasts. Available on-line:

steroid structure with an entire side-chain of both **1** and vitamin D analogues, (b) the presence of the 3β-OH group in molecule **1**, which –OH is necessary in binding to the AP in the case of vitamin D analogues, (c) the lack of a 1-OH group in **1**, which increases the AP fractional occupancy in case of the vitamin D analogues, (d) the "*cis*-locked" structure of **1**, which also increased the AP fractional occupancy in the vitamin D analogues [98].

If this holds true, then, besides the fields of invertebrate biology, inducible eukaryotic gene expression systems and pesticide research, the ecdysteroids might gain a further importance as agonists of the vitamin D NG signalling pathway, which would provide ecdysteroid research with a significant and completely new direction.

1.5 Objectives

The increasing number of scientific investigations of the ecdysteroids might reveal new, specific fields, such application in case of muscle atrophies [69] or even as vitamin D analogues. The isolation of ecdysteroids from plant sources is the only available way today to obtain them since their synthesis is not economic. The study of ecdysteroid spectrum in *Silene* species has already been in progress by a research group of Institute of Pharmacognosy, University of Szeged in the last decades with the main purpose to find plant species rich in ecdysteroids, which can either be cultivated or grow wild in Hungary. Previous screening and literature data indicated that *S. viridiflora* may contain almost 1% of ecdysteroids [19,24,26], and our preliminary TLC experiments on the extract suggested an interesting ecdysteroid pattern. It is known from literature, that the total phytoecdysteroid cocktail, obtained from the aerial parts of *S. viridiflora* is recommended as an effective adaptogene for use in sports, medicine, reduced functioning, and poor restoration after serious illnesses and heavy physical exertion [30,99]. However, their action on muscle is not well understood ecdysteroids are also promoted to be anabolic agents. Thus, our aims were as follows:

- To determine the ecdysteroid profile of *S. viridiflora* a member of the *Silene* genus. This means first the isolation and elucidation of the structures of new native phytoecdysteroids.
- To improve the efficiency of the earlier isolation procedure, to simplify the methodology and to develop a new, rapid and economical isolation process, principally applicable for the main phytoecdysteroid, **1**.

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http://www.uni-ulm.de/biologie1-ecdysone2008/cms2/files/ecdysone2008_book.pdf2008, pp. 76.

- Previously, some ecdysteroid acetonide derivatives were isolated from the plant by our research team[3]. Because of the probability of artefact formation during the isolation process, our aim was to identify the origin of these ecdysteroid derivatives.
- To examine the effect of **1**, isolated from *S. viridiflora*, *in vivo* in different skeletal muscles and muscle fibres in different rat muscle models, including the size and distribution of the different muscle fibres in:
  - normal skeletal muscles (m. soleus, m. EDL), in order to investigate the effect of **1** under physiological conditions.
  - regenerating muscle (m. soleus, after necrosis induced by a snake venom, notexin) to test, whether **1** is able to accelerate the regenerating process.
  - atrophying respiratory muscle (diaphragm), to examine, whether **1** helps to prevent the deleterious effect of glucocorticoids, during co-administration with methylprednisolone.

2. Experimental

2.1 Plant material

The aerial parts of the cultivated *S. viridiflora* were collected in June 2002 in Vácrátót, Hungary. A voucher specimen was deposited at the Department of Pharmacognosy, University of Szeged, Hungary (specimen number: SV-020612).

2.2 Reagents and standard ecdysteroid samples

Solvents of HPLC grade were purchased from Merk (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 of the research group. Their identities and purities were identified by HPLC and NMR.

2.3 General experimental procedures

2.3.1 Chromatographic techniques

Normal-phase thin-layer chromatography

[3] Presentation held at 53th GA Congress Florence, 21-25 August 2005. Tóth N., Hunyadi A., Máthé I., Báthori M.; New 26-hydroxilated Ecdysteroids from *Silene viridiflora*.

NP-TLC was performed on 20 x 20 cm silica gel plates (Silicagel 60 F_{254}, E. Merck, Darmstadt, Germany). The plates were developed by an ascending technique in a glass chamber (Desaga, Heidelberg, Germany) at room temperature. The following mobile phases were used: **TLC_1**: EtAc-EtOH (96%)-H_2O (80:10:5, v/v/v), **TLC_2**: toluene-acetone-EtOH (96%)-NH_4OH (25%) (100:140:32:9), **TLC_3**: CH_2Cl_2-MeOH-benzene (50:10:6, v/v/v). After development of the TLC the ecdysteroids were detected directly by fluorescence quenching at 254 nm and by the use of vanillin-sulphuric acid spray reagent. After spraying the spots were visualized in daylight and at 366 nm. The whole isolation procedure was controlled by using NP-TLC.

Column chromatography (CC)

Flash-CC (**Flash-CC**) and vacuum reversed-phase column chromatography (**RP-CC**) was carried out on end-capped octadecyl-silica **a**; RP-18 (0.06-0.02 mm), Chemie Ueticon-C-gel, C-560 (Ueticon, Switzerland). Vacuum was produced with a Sue 300E vacuum pump Heto-Holten A/S (Gydevang, Denmark) and the flow rate was 15 ml/min. Normal-phase column chromatography (**NP-CC**) was performed on **b**; Kieselgel 60 (0.063-0.2 mm), Reanal, Budapest, Hungary. Cyano-silica column chromatography (**CN-CC**) was carried out on **c**; CN-phase (0.06-0.2 mm) Chemie Ueticon-C-gel, C-650 (Ueticon, Switzerland) and for polyamide column chromatography (**Po-CC**) **d**; MN-polyamide SC6 (0.06-0.16 mm) Woelm (Eshwege, Germany) was used. During the whole isolation process the fractions containing similar components were combined, taken to dryness on 40 ºC and 337 mbar by a Rotavapor R200, Büchi (Essen, Germany) and redissolved in a small volume of the apparent solvent or solvent system.

Normal-phase rotation planar chromatography (NP-RPC)

NP-RPC was carried out on a Harrison Model 8924 Chromatotron instrument (Harrison Research, Palo Alto, CA). The stationary phase for NP-RPC was silica gel 60 GF_{254} (E. Merk, Darmstadt, Germany), manually coated on the rotor as a 1 mm (**NP-RPC1**, **4**), 2 mm (**NP-RPC3**) or 4 mm (**NP-RPC2**) layer. We used gradient elution with five mobile phases for the development: Solvent system (s. s.): **A**: EtAc-EtOH-H_2O 80:5:2 v/v/v; **B**: EtAc-EtOH-H_2O 80:7:3 v/v/v; **C**: EtAc-EtOH-H_2O 80:10:5 v/v/v; **D**: EtAc-EtOH-H_2O 80:14:7 v/v/v; **E**: EtAc-EtOH-H_2O 80:20:10 v/v/v. As the first step of the procedure the dry stationary phase was completely wetted with the firs applied mobile phase (50-100 ml). The samples were dissolved in the first composition of the elution solvent and were introduced through the inlet. The separation by NP-RPC was achieved with gradient elution in three (**NP-RPC2**: s. s. **C**-**E** and **NP-RPC3**: s. s. **A**-**C**) or four steps (**NP-RPC1**: s. s. **A**, **C**-**E** and **NP-**

RPC4: **A**-**C**, **E**). In case **NP-RPC1-3** ten fractions of 7 ml were collected with each solvent systems, while in case of **NP-RPC4** five fractions of 7 ml were collected. The flow rates were 2-4 ml•min^{-1} for **NP-RPC1**, **4**, 6-8 ml•min^{-1} for **NP-RPC3** and 8-10 ml•min^{-1} for **NP-RPC2**.

High performance liquid chromatography (HPLC)

The HPLC work was performed on an Agilent 1100 Series Isocratic Pump (Agilent Technologies Inc. Palo Alto, United States) coupled with a Jasco UV-2075 Plus detector (Jasco Corporation, Tokyo, Japan). For normal-phase HPLC (**NP-HPLC**) Zorbax RX-Sil column (5 μm, 250 x 4.6 mm i.d., Agilent Technologies Inc. Palo Alto, United States) (for **NP-HPLC1**, **2**, **4**, **5**, **6)**, with a flow rate of 1 ml•min^{-1}, and Zorbax SB-CN column (5 μm, 250 x 4.6 mm i.d., Agilent Technologies Inc. Palo Alto, United States) (for **NP-HPLC3**), with a flow rate of 1 ml•min^{-1}, the following solvent systems were used: **1**; CH_2Cl_2-*i*-PrOH-H_2O, 125:40:3 v/v/v, **2**; CH_2Cl_2-*i*-PrOH-H_2O, 125:40:2 v/v/v, **3**; *c*-hexane-*i*-PrOH, 100:14 v/v, **4**; CH_2Cl_2-EtOH-H_2O, 125:11:2 v/v/v, **5**; CH_2Cl_2-*i*-PrOH-H_2O, 125:25:1 v/v/v, **6**; CH_2Cl_2-*i*-PrOH-H_2O, 125:30:2 v/v/v. For reversed-phase HPLC (**RP-HPLC**) Zorbax ODS C18 column (5 μm, 250 x 9.4 mm i.d., Agilent Technologies Inc. Palo Alto, United States) (for **RP-HPLC1**, **2**, **3**) was used with a flow rate of 2.5 ml•min^{-1}. For **RP-HPLC4**, **5** Zorbax SB C18 column (5 μm, 250 x 4.6 mm i.d., Agilent Technologies Inc. Palo Alto, United States) with a flow rate of 1 ml•min^{-1} was used. Solvent systems were as follows: **1**; H_2O-THF-ACN 85:10:5 v/v/v, **2**; H_2O-ACN 78:22, v/v, **3**; H_2O-ACN 77:23, v/v, **4**; H_2O-ACN 65:35, v/v, **5**; MeOH-H_2O 70:30, v/v.

Chromatographic separations were monitored at 242 nm.

2.3.2 General methods and apparatus for structure elucidation

Optical rotations were measured with a Perkin-Elmer 341 polarimeter. The UV spectra were recorded in MeOH using a Shimadzu UV 2101 PC spectrophotometer. The mass spectrometric measurements were performed on a Finnigan TSQ 7000 tandem mass spectrometer (Finnigan MAT, San Jose, CA) equipped with a laboratory-built nanoelectrospray ion source. A high voltage of about 1000 V was used in the ion source. The instrument was scanned in the normal MS mode over the mass range 10-1500 amu, with a scan time of 2 s. HRESIMS recordings were made on a Finnigan MAT 95SQ tandem mass spectrometer (Finnigan MAT, Bremen, Germany). LC-MS/MS measurements were obtained on a Finnigan TSQ-7000 triple quadrupole mass spectrometer (Finnigan-MAT, San Jose, CA) equipped with a Finnigan Electrospray Ionization (ESI) source. The instrument was operated

in positive ion mode using selective reaction monitoring (SRM). In SRM mode the first quadrupol was set to select 505 Th ($M+H^+$ ions of the steroid investigated) which were fragmented in the collision cell and the two most intense fragments (411 and 429) were measured with the last quadrupol. The ESI needle was adjusted to 5kV and N_2 was used as a nebulizer gas. The collision potential was 25-28 eV. Argon was used as the collision gas and the pressure in the collision cell region was 2 mTorr. Samples were subjected to short HPLC (Applied Biosystems 140C) separation before MS analysis on Phenomenex 100 x 2.1 mm 10 micron 300 Å C4 column (Phenomenex, Yvelines, France). Mobile phases were **6**: H_2O-HCOOH 99.9:0.1, v/v; **7**: MeOH-H_2O-HCOOH 10:90:0.1, v/v/v with a flow rate of 250 µl/min; gradient 10-90 % B 20 min. NMR spectra were recorded in MeOH-d_4 in a Shigemi sample tube at room temperature using a Burker Avance DRX-500 spectrometer. The structures of the products were determined by means of comprehensive 1D and 2D NMR experiments, using widely accepted strategies [100,101]. Chemical shifts are given on the δ-scale and are referenced to the solvent (MeOH-d_4: δ_C 49.15 and δ_H 3.31). In the 1D measurements (^1H, ^{13}C, APT, DEPT-135) 64K data points were used for the FID. The pulse programs of the 2D experiments [gs-COSY, gs-HMQC, HMQC-TOCSY (mixing time = 80 ms), gs-HMBC, 2D NOESY (mixing time = 400 ms)] were taken from the software library.

2.4 Extraction and isolation

2.4.1 Extraction and prepurification

The dried herb (1191 g) was milled and percolated with methanol (12.8 l) at room temperature. The methanolic extract was evaporated to dryness (240.3 g) and redissolved in 400 ml mixture of MeOH-H_2O 1:1, (v/v). The solution was extracted six times (600 ml each) with *n*-hexane. The aqueous-methanolic phase was evaporated to dryness. The residue (206.6 g) was dissolved in 350 ml of MeOH, and 300 ml acetone was added to the solution. The resulting precipitate was separated by decantation, and then rinsed three times with 50 ml of MeOH-acetone 2:1, (v/v). The MeOH-acetone solutions and the supernatant were combined and taken into dryness and precipitation was repeated twice. Supernatants and rinsing liquids were combined and taken to dryness (60.3 g).

2.4.2 Isolation

The dry residue (60.3 g) of the purified extract was redissolved in 150 ml MeOH- H_2O 3:7, v/v then subjected to **Flash-CC** on C_{18} silica and eluted with gradient elution in five steps as follows: MeOH-H_2O 3:7, v/v to 10:0, v/v. Data of the chromatographic separations are detailed in **Table 1**, the scheme of the isolation is shown on **Fig. 4**. From **Flash-CC** five main fractions were collected: MeOH-H_2O 3:7, v/v (37.38 g), 4:6, v/v (6.78 g), 5:5, v/v (2.94 g) and 6:4, v/v (2.62 g) and MeOH (5.18 g). From the fraction eluted with MeOH-H_2O 4:6, v/v compound **1** and **2** co-precipitated and was further purified by repeated crystallization to yield **1** (980 mg) and **2** (63 mg). The mother liquid was separated by repeated vacuum RP-CC (**RP-CC 1**) with MeOH-H_2O 3:7, v/v to 6:4, v/v. Fractions eluted with MeOH-H_2O 4:6, v/v (fr. 41-50, 0.15 g) were subjected to **NP-RPC1** and from fractions eluted with s. s. **E** (fr. 33-36, 20 mg) **3** (2 mg) and **4** (3 mg) were isolated by **RP-HPLC1**. Fractions eluted with MeOH-H_2O 5:5, v/v from **RP-CC 1** (fr. 32-36, 1.07 g) were separated further with **NP-RPC2** and from fractions eluted with s. s. **C**-**D** (fr. 7-12, 10 mg) **5** (4 mg) and **6** (3 mg) were purified by **RP-HPLC2**. Fractions eluted with MeOH-H_2O 6:4, v/v from **RP-CC 1** (fr. 44-48, 3.05 g) were adsorbed to 9 g silica gel, applied onto NP-CC (**NP-CC 2)** and gradient elution was carried out with CH_2Cl_2-EtOH from 10:0, v/v to 8:2, v/v. The fractions eluted with CH_2Cl_2-EtOH 8:2, v/v (fr. 122-137, 0.64 g) were subjected to **NP-RPC3** and from fractions eluted with s. s. **C** (fr. 11-15, 0.14 g) **7** was isolated by **RP-HPLC3**. Fractions containing **8**, **9** and **10** were eluted with s. s. **E** from **NP-RPC3** (fr. 16-20, 0.15 g) and purified by **NP-HPLC1** to yield **8** (7 mg), **9** (2 mg) and **10** (1.8 mg).

The fraction eluted with MeOH-H_2O 5:5, v/v (2.94 g) from **SPE** was subjected to repeated vacuum RP-CC (**RP-CC 3**) and stepwise gradient elution with MeOH-H_2O 3:7, v/v to 5:5, v/v mixtures had been performed. Fractions eluted with MeOH-H_2O 4:6, v/v (fr. 13-17, 0.16 g) was purified further by repeated CN-CC (**CN-CC 4**) with gradient elution of *n*-hexane-acetone 10:0, v/v to 6:4, v/v. Compound **11** (8 mg) was crystallized from the fraction eluted with *n*-hexane-acetone 8:2, v/v (fr. 11-15, 0.5 g), while compound **12** (6 mg) was obtained by **NP-HPLC 2** from fraction eluted with *n*-hexane-acetone 7:3, v/v (fr. 17-18, 0.02 g). The fractions eluted with MeOH-H_2O 5:5, v/v from **RP-CC 3** (fr. 26-29, 1.23 g) were adsorbed to 3.6 g polyamide, applied onto Po-CC (**Po-CC 5**), and the fraction eluted with water (fr. 1, 1.16 g) was purified further by repeated CN-CC (**CN-CC 6**) with gradient elution of *n*-hexane-acetone 9:1, v/v to 6:4, v/v. From the fractions eluted with *n*-hexane-acetone 8:2, v/v compounds **13**-**17** were obtained. Fractions of **CN-CC 6** containing compound **13** (fr. 21-28, 0.23 g) were separated further by **NP-RPC4** and from fractions eluted with s. s. **C** (fr. 23-

26, 0.04 g) **13** (3.4 mg) was isolated by **NP-HPLC1**. Fractions eluted with *n*-hexane-acetone 8:2, v/v from **CN-CC 6** (fr 34-35, 98 mg) were further purified by **NP-HPLC3** to yield **14** (1.2 mg). The fractions eluted with *n*-hexane-acetone 8:2, v/v from **CN-CC 6** and containing compounds **15**-**17** (fr. 37-40, 55 mg) were subjected to vacuum RP-CC (**RP-CC 7**) and gradient elution with MeOH-H_2O 4:6, v/v to 65:35, v/v mixtures had been performed. From the fractions eluted with MeOH-H_2O 6:4, v/v (fr. 21-23, 12 mg) **15** (1.5 mg) and **16** (1 mg) were purified by **NP-HPLC1**. From fractions eluted with MeOH-H_2O 65:35, v/v (fr. 27-28, 15 mg) from **RP-CC 7**, **17** (2 mg) was obtained by **NP-HPLC 4**.

The fraction eluted with MeOH-H_2O 6:4, v/v (2.62 g) from **SPE** was adsorbed to 6.5 g polyamide and applied onto Po-CC (**Po-CC 8**). The fraction eluted with H_2O-MeOH 9:1, v/v (fr. 1, 1.38 g) was separated by CN-CC (**CN-CC 9**) with gradient elution of *n*-hexane-acetone 9:1, v/v to 7:3, v/v, and the fractions eluted with *n*-hexane-acetone 9:1, v/v (fr. 14-23, 0.14 g) were further purified by vacuum RP-CC (**RP-CC 10**) with the increasing gradient of MeOH-H_2O mixtures from 45:55, v/v to 75:25, v/v. Compounds **18** (3.4 mg) and **19** (13.3 mg) were separated from the fractions eluted with MeOH-H_2O 7:3, v/v (fr. 26-30, 76.2 mg) by **NP-HPLC2**. **20** (3.4 mg) was purified from the fractions eluted with MeOH-H_2O 75:25, v/v (fr. 32-34, 34.2 mg) by **NP-HPLC5**.

Table 1. Data of column chromatographic separation

Sign of column	S. p.	Size of column (mm x mm)	Weight of s. p. (g)	Weight of app. fr. (g)	Eluent	Proportion of eluent (v/v)	No. of collected fractions	Volume of fr. (ml)
Flash-CC	a	1350 x 62	540	60.03	MeOH-H_2O	3:7, 4:6, 5:5, 6:4, 10:0	1	1000
RP-CC 1	a	1010 x 45	180	6.78	MeOH-H_2O	3:7, 35:65, 4:6, 45:55, 5:5, 55:45, 6:4	6	250
NP-CC 2	b	500 x 35	75	3.12	CH_2Cl_2-EtOH	10:0, 97:3, 95:5, 93:7, 92:8, 85:15, 8:2	20	250
RP-CC 3	a	500 x 35	90	2.94	MeOH-H_2O	3:7, 35:75, 4:6, 45:55, 5:5	6	300
CN-CC 4	c	180 x 20	12	0.63	*n*-hex-Ac	10:0, 9:1, 8:2, 7:3, 6:4	5	50
Po-CC 5	d	180 x 20	12	1.23	H_2O-MeOH	10:0, 9:1, 8:2, 5:5, 0:10	1	150
CN-CC 6	c	180 x 20	12	1.16	*n*-hex-Ac	9:1, 8:2, 7:3, 6:4	20	70
RP-CC 7	a	65 x 30	40	0.055	MeOH-H_2O	4:6, 45:55, 5:5, 55:45, 6:4, 65:35	5	50
Po-CC 8	d	65 x 30	30	2.62	H_2O-MeOH	9:1, 8:2, 7:3, 5:5, 10:0	1	200
CN-CC 9	c	65 x 30	23.5	1.38	*n*-hex-Ac	9:1, 85:15, 8:2, 7:3	25	100
RP-CC 10	a	65 x 30	20	0.14	MeOH-H_2O	45:55, 5:5, 55:45, 6:4, 65:35, 7:3, 75:25	5	50

Abbreviations used in **Table 1** are S. p. = stationary phase, app. = applied, fr. = fractions, No. = number. The stationary phases are detailed in chapter 2.3.1.

2.5 Investigation of the genuineness of ecdysteroid acetonides

2.5.1 Model for acetonide formation

20E (**1**, 1 mg) and polypodine B (**2**, 1 mg) were kept hermetically sealed with acetone (7 ml) and CN-silica (10 mg) for 35 days. Samples (1 ml) were taken on the 1st, 3rd, 7th, 14th, 21st and the 35th days and tested with **$TLC_{1,2,3}$** and **NP-HPLC2**, **4**.

2.6.1 NP-, RP-HPLC and LC-MS / MS analysis

Different air-dried *S. viridiflora* herb samples (5 g) [collected in 05. 2002 (**1**), 05. 2005 (**2**), 06. 2005 (**3**) and 07. 2005 (**4**), respectively] were extracted with 50 ml MeOH, evaporated to dryness and the dried extract was dissolved in 10 ml MeOH. 2 ml H_2O was added to 2 ml methanolic extract and liquid-liquid extraction was performed three times with 5 ml *n*-hexane. The aqueous methanolic phases were dried [0.16 g (**1**), 0.25 g (**2**), 0.31 g (**3**) and 0.30 g (**4**), respectively], absorbed to 0.6 g, 0.85 g, 1 g and 1 g silica gel, respectively and eluted first with 10 ml CH_2Cl_2 and then with 5 ml CH_2Cl_2:MeOH (8:2, v/v). The latter fractions were named **SV-1**, **SV-2**, **SV-3** and **SV-4**. **SV-1** and used for the HPLC experiments (**NP-HPLC6** and **RP-HPLC4**,**5**), while **SV-1-4** were used for HPLC-MS / MS experiments.

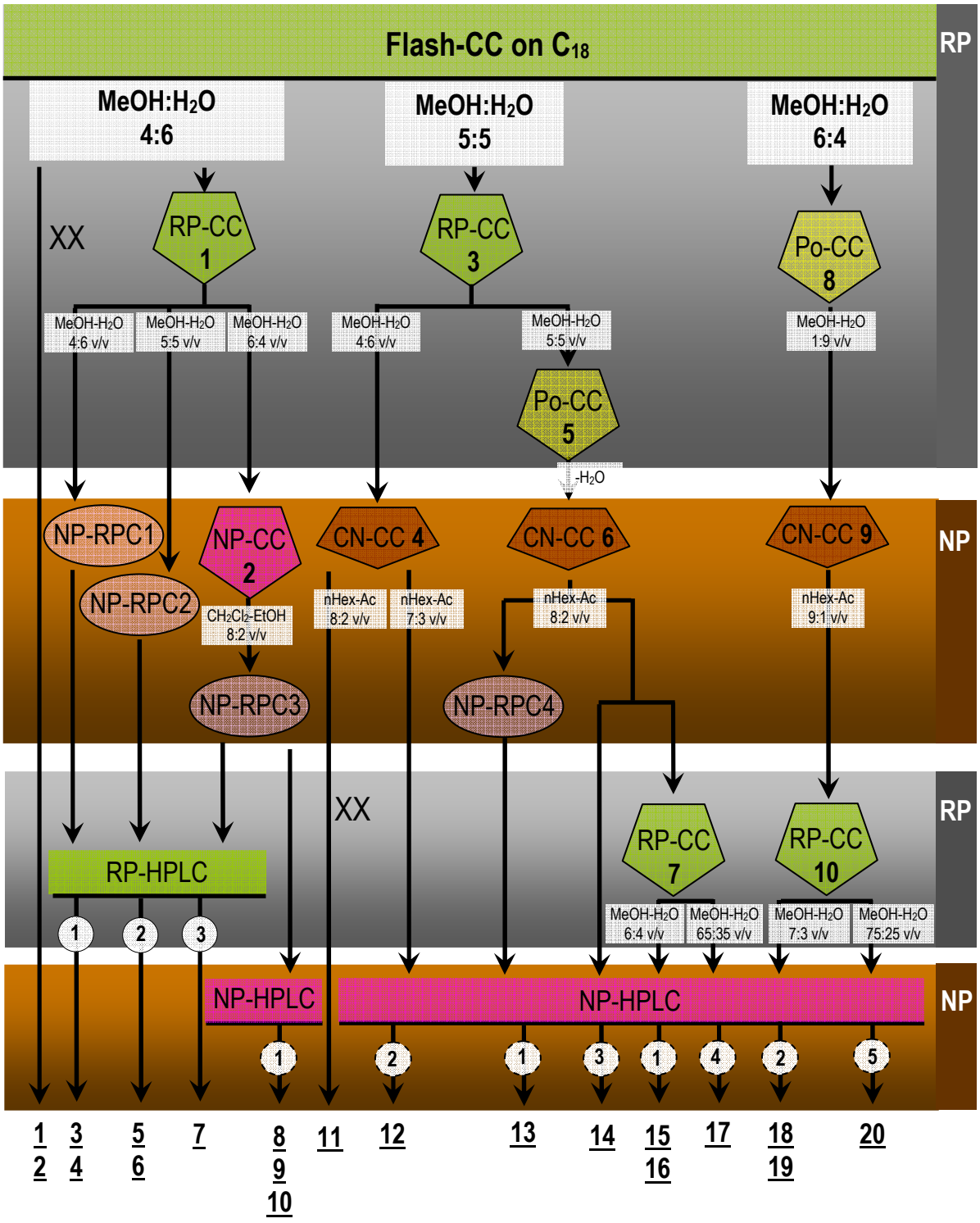


Fig. 4. Scheme of the isolation of ecdysteroids from the prepurified *S. viridiflora* extract.

2.7 In vivo and ex vivo animal experiments

2.7.1 Animals and treatment

Animals were kept and treated according to the regulations of the Ethical Committee of the University of Szeged and Belgian National Guidelines of Animal Care. 38 adult male Wistar rats (304±19 g for the normal and regeneration muscle model, 454±37 g for the muscle atrophy model) were randomized into 8 groups, with 4-6 animals in each, and treated daily in the left thigh according to the scheme shown on **Fig. 5**. **N20E** (5 mg/kg BW/day **1** *s.c.* injection for 7 days); normal control (**NC**; s.c. saline injections according to the protocol of the treated group daily); groups **R20E1**, **R20E2** and **RC** received snake venom (notexin) injection into the left soleus muscle for induction of muscle regeneration as in [102], and treated after five days as follows: **R20E1** (5 mg/kg BW/day **1** *s.c.* injection for 7 days); **R20E2** (0.5 mg/kg BW/day **1** *s.c.* injection for 7 days); regenerating control (**RC**; *s.c.* saline injections according to the protocol of the treated group for 7 days); **MP** (10 mg/kg BW/day methylprednisolone *i.m.* injection for 5 days); **M20E** (10 mg/kg BW/day methylprednisolone *i.m.* injection + 10 mg/kg BW/day **1** *i.m.* injection for 5 days) and methylprednisolone control (**MC**; *i.m.* saline injections according to the protocol of the treated groups daily for 5 days). The animals were housed in individual cages in a temperature-controlled room, fed ad libitum and had free access to water. The animals were injected daily in the left hindlimb after the measurement of their weight.

In case of **N20E** and **NC** on the 8^{th} day the animals were anaesthetized with chloral hydrate and the soleus and EDL muscles on both sides were dissected. In case of **R20E1**, **R20E2** and **RC** on the 12^{th} day after the snake venom injection the animals were anaesthetized with chloral hydrate and the regenerating soleus, the normal soleus and the EDL muscles in the contralateral leg were dissected and the animals were killed with an overdose of chloralhydrate. In case of **M20E**, **MP** and **MC** the animals were anaesthetized with pentobarbital sodium. They were tracheotomized, and a tracheal cannula (polyethylene tubing PE-200) was inserted. The animals were mechanically ventilated with an O_2-enriched gas mixture (tidal volume 5 ml, respiratory frequency 40 breaths/min; Harvard pump respirator, South Natick, MA). The diaphragm was quickly removed through laparotomy and immediately immersed in a cooled oxygenated Krebs solution containing (in mM) 137 NaCl, 4 KCl, 2 $CaC1_2$, 1 $MgC1_2$, 1 KH_2PO_4, 12 $NaHCO_3$, and 6.5 glucose. To avoid regional differences in cross-sectional area (CSA) or fibre type proportions as much as possible, two small rectangular bundles from the middle part of the lateral costal region were obtained by

dissection parallel to the long axis of the fibres. Silk sutures were tied to both ends of the bundle to serve as anchoring points.

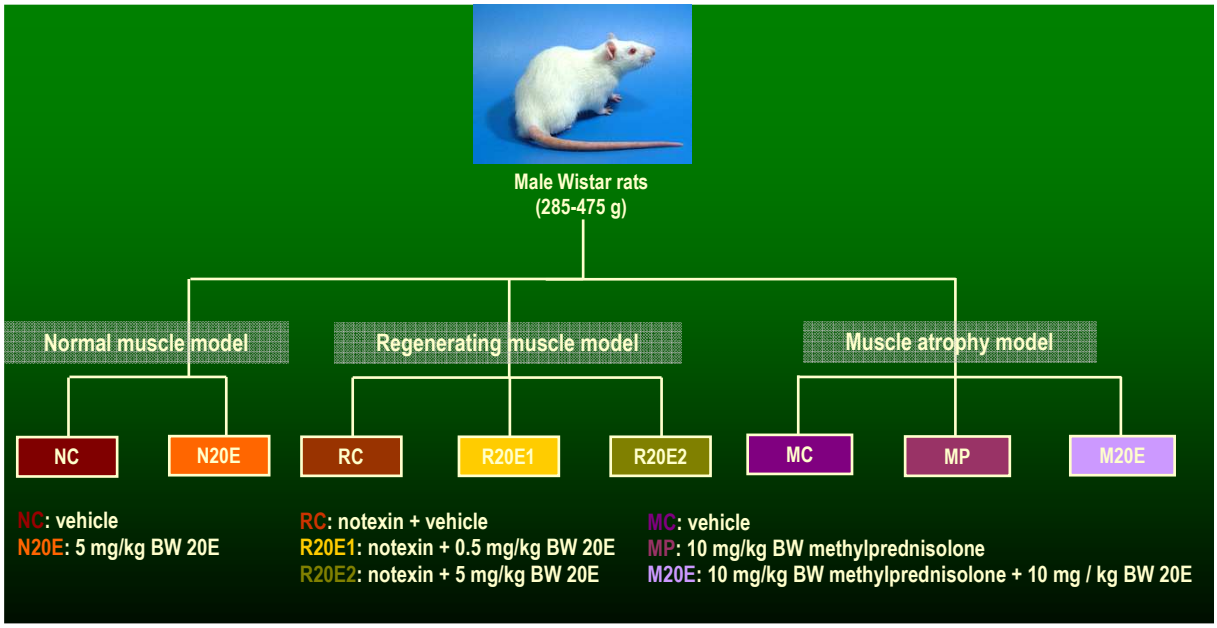


Fig. 5. The scheme of experimental design for the study of 20E (**1**) effect on muscle fibres.

2.7.2 Collection of muscles, haematoxylin-eosine staining, immunocytochemistry

The dissected regenerating and normal muscles were frozen in isopentane cooled with liquid nitrogen and kept at -70 °C. Muscle strips obtained from the costal region of the diaphragm were put into “tissue glue” (Tissue-Tek, Elkhard, IN) on a cork holder with the muscle fibres oriented perpendicularly to the surface of the cork. Proper orientation of the bundles was controlled by using magnifying glasses. Subsequently, these specimens were quickly frozen in isopentane cooled with liquid N_2. Serial cross sections were cut at l5 μm thickness with a cryostat kept at -20°C and stained with peroxidase immunohistochemistry as in [102]. We used BA-D5 (mouse, 1:50), SC-71 (mouse, 1:20), BF-F3 (mouse, 1:10) as primary antibodies for MyHC1, 2a, 2b, respectively [103]. We combined the BA-D5 (mouse, 1:50), SC-71 (mouse, 1:20); BF-F3 (mouse, 1:10) for MyHC2x staining.

2.7.3 Fibre CSA and myonuclear domain

The CSA of 150 fibres of each muscle was measured by Olympus DP-soft, version 3.2 program (Olympus, Hamburg, Germany) on hematoxilin-eosin stained or on immunostained sections. The number of myonuclei in the fibres (in more than 100 fibres of each muscle) was counted on haematoxylin-eosin stained sections in case of groups **NC**, **N20E**, **RC**, **R20E1** and

R20E2 with the 40x objective of the light microscope. The accuracy of this method was controlled by comparing the results of two independent experienced persons. The myonuclear domains in each muscle were calculated from the average myonuclear number per fibre divided by the average fibre CSA. In the EDL the large fibres were counted for myonuclei and divided by the average CSA of the IIx and IIB fibres, as these fibre types were responsible for the larger CSA values and their size increased in response to the **1** treatment. We also counted the number of myonuclei of the small fibres in EDL and divided by the average CSA of the type I and IIA fibres.

2.7.4 Statistics

The cumulative data of muscle fibre CSA's obtained from four muscles were compared among groups by using t-test for unpaired samples or one-way analysis of variance followed by Newman-Keuls and Bonferroni post hoc test. The number of myonuclei and the size of myonuclear domain were also compared as cumulative data of four muscles (from 100 fibres of each). All tests were performed by using the GraphPad Prism version 4.00. Results were considered significant at $p<0.05$. All data are expressed as means ± SE.

3 Results

3.1 Isolation of ecdysteroids from *S. viridiflora*

The isolation procedure consisted of two main steps: extraction and clean-up of the crude extract, followed by fractionation with a combination of chromatographic methods. For the extraction MeOH was used at a solvent-plant 10:1 ratio. The subsequent clean-up methods involved liquid-liquid extraction between hexane and the aqueous-methanolic extract in order to free the crude extract from apolar contaminants (chlorophyll, terpene), while the precipitation with acetone removed the majority of polar contaminants, such as sugars, polysaccharides, or proteins. The residue of the final methanol-acetone solution was subjected to Flash-CC on C_{18} and the

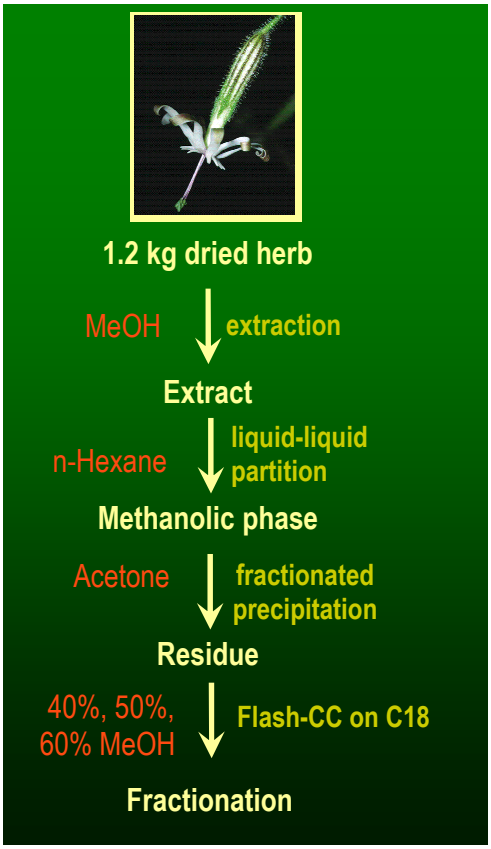


Fig. 6. The scheme of the extraction of *S. viridiflora* and purification of the crude extract

ecdysteroids were removed from the column with 40% (**1**-**10**), 50% (**11**-**17**) and 60% (**18**-**19**) of aqueous methanol as shown in **Fig 6**. These fractions were purified further with different combinations of column chromatographic methods such as NP-CC on silica, repeated vacuum RP-CC on C_{18}, Po-CC, CN-CC or RPC on silica. Fractionations were carried out with stepwise gradient elution with appropriate solvent systems. The final purification was performed either with crystallization [in case of **1**, **2** and **11**], or with preparative or analytical scale NP-HPLC (**8**-**10**, **12**-**20**) or RP-HPLC (**3**-**7**) with good resolution.

3.2 Structure elucidation of the isolated ecdysteroids

Eleven known ecdysteroids were isolated and characterized by direct comparison of their physical and spectroscopic characteristics with those published in the literature [16]. They were also identified by co-chromatography with pure reference ecdysteroids, using NP- and/or RP-HPLC. Nine new ecdysteroids were isolated and characterized by different spectroscopic methods (UV, MS and NMR). MS and NMR provided the basic information on the structures of the components and these spectral data were usually evaluated in comparison with those of **1**.

The UV spectra yielded characteristic information on the 7-en-6-one chromophore. The majority of the ecdysteroids possess strong UV absorption spectra with a maximum at 240-245 nm ($\log \varepsilon \approx 4$). The structural differences between the isolated ecdysteroid components are minor, and in the regions distant from the chomophore they were essentially indistinguishable from each other. Optical rotation and UV spectroscopic data of the new compounds are listed on page 26-29.

The electrospray-ionization mass spectra (ESIMS) are suitable for determination of molecular masses of ecdysteroids. The mass spectra of ecdysteroids may be characterized by the appearance of signals, differing from each other by the loss of water (18 units) from the polyhydroxylated ecdysteroids. Moreover, the majority of ecdysteroids suffer side-chain cleavage; the splitting occurs between C-20, and C-22, and C-17 and C-20. The mass spectra are characterized by mass numbers which depend on the degrees of hydroxylation of the side-chain and nucleus. Fragmentation may result in two major series derived from the nucleus or side-chain, further cleavage may occur between C-22 and C-23, C-23 and C-24 or C-24 and C-25. In ring D either C-13 – C17 or C14 – C15 fragmentation might take place. The MS data for the new components are listed below.

From the ^{13}C, DEPT and HMQC NMR spectra of the new ecdysteroids the number of C, CH, CH_2 and CH_3 fragments of a molecule were identified. From the ^{13}C chemical shifts the number of connecting oxygen atoms was established. The methyl groups could be utilized as starting point for the determination of the structure, because their signals are singlets and display strong two- and three-bond correlations in the HMBC spectrum. With these HMBC correlations and with the knowledge of the ecdysteroid skeleton, methyl groups in positions of C_{18}, C-19, C-21, C-26 and C-27 could be identified. The signals of H-26 and H-27 permitted assignment of the corresponding carbon atoms, while those of H-18 and H-21 led to the identification of C-17. Protons of sp^2 carbon atoms gave correlations to C-5, C-9 and C-14 in the HMBC spectra, which proved the double bonds in all compounds. The NOESY correlations provided information about the stereochemistry of the rings and the orientation of the substituents connected to the skeleton. The anellation of A/B rings were mostly *cis* (NOESY signals were correlating H-9 with H-2 and H-4, and H-19 with H-1 and H-5), except in case of compound **18**, where the anellation of these two rings was determined as *trans* (H-9/H-5, H-19/H-1 and H-19/H-4). In case of ^1H-NMR spectra specific modifications are usually determined in reference to 20E (**1**) molecule which can be utilized for structure assessment. Analysis of the 1D and 2D ^1H-NMR data resulted in the conclusion that the steroid nucleus of the compounds is 'classical' with respect to the presence of 3-βOH, 14-αOH and in some cases 2-βOH (**13**, **15**, **17**, **20**). Special structural elements among the new compounds were as follows: (**a**) 2-deoxyecdysteroids (**5**, **6**, **14**, **16**, **18**) showed the lack of H-2 signal in the hydroxymethin signal zone, broadening of the H-3 signal and correlation of this in the upfield part of the ^1H-^1H COSY sectrum with four signals (H_{ax}-2, H_{eq}-2, H_{ax}-4, H_{eq}-4) could be observed; (**b**) ecdysteroids with 1-βOH (**13**) were characterized by the appearance of a new signal in the hydroxymethyl zone, the H_{ax}-2 signal appearing as a narrow triplet, a downfield shift of the H_{eq}-3 and H-5 signal which resulted from the interaction between 1-OH and H-5, and a strong upfield signal of the C-19 signal with respect to the appropriate signal in **1**; (**c**) ecdysteroids with 5-βOH (**5**, **6**, **14**-**16**) showed the disappearance of the H-5 signal, a modification of the H_{ax}-2, H_{eq}-3 signals and a large downfield shift in the signal of H_{ax}-1, H_{ax}-4 and H_{eq}-4; (**d**) ecdysteroids with 26-OH (**14**-**17**) were characterized by the appearance of a hydroxymethyl signal (two if 25*R* and 25*S* diastereomers are present) as a singlet at δ = 3.35 ppm, the H-26 signal is lost, H-27 signal suffers and upfield shift (-0.05 ppm in MeOH-*d*4); (**e**) C29 ecdysetroids usually contain one or two alkyl groups at C-24 so in case of compound **20** (also a 20,22-acetonide) a 28- and 29-Me signals appeared and the Me-35/C-24 HMBC correlation verified the connection of an ethyl group to the C-24; (**f**) ecdysteroid 2,3-

acetonides (**20**) are present a high chemical shift of C-2 C-3 and C-28, and two extra methyl group could also be seen connected to a typical (O-C-O) sp^3 quaternary carbon atom, (**g**) identification of 20,22-acetonide group in (compounds **13**, **15**-**18**, **20**) was accomplished in the same way using the chemical shifts of C-20 and C-22; (**h**) ecdysteroid glycosides were identified by the presence of a sugar moiety (six additional oxygenated carbon signal, related to hexose moiety) on the certain carbons (22-OH, **5**; 25-OH, **6**). The C-22 (**5**) or the C-25 (**6**) signals were more shielded in respect to **1** and thus suggested the attachment of a sugar unit to C-22, or C-25. From the HMBC correlations H-1´ C-22 (**5**) and H1´ C-25 (**6**) was also observed. The NMR data of the new compounds are listed below and detailed in the Appendix (**Table 7**).

Table 2. contains the structures of all isolated ecdysteroids.

2-deoxypolypodine B 22-*β*-D-glucopyranoside (5): Colourless crystals (4 mg). $[\alpha]^{25}_{D}$ +43˚ (*c* = 0.075, MeOH); UV λ_{max} ^{MeOH} (nm) (log ε): 241 nm (3.83); ^{1}H NMR (CD_3OD, 500 MHz) δ (ppm): 5.85 (1H, d, *J* = 2.4 Hz, H-7), 4.34 (1H, d, *J* = 7.8 Hz, H-1´), 4.08 (1H, s, H-3α), 3.88 (1H, d, *J* = 12.0 Hz, H-6´b), 3.68 (1H, dd, *J* = 12.0, 5.4 Hz, H-6´a), 3.49 (1H, d, *J* = 9.6 Hz, H-22), 3.37 (1H, H-3´), 3.35 (1H, H-4´), 3.33 (1H, H-5´), 3.28 (1H, H-9α), 3.26 (1H, dd, *J* = 9.0, 8.4 Hz, H-2´), 2.36 (1H, dd, *J* = 9, 8.4 Hz, H-17), 2.12 (1H, td, *J* = 12.0, 6.0 Hz, H-12α), 2.03 (3H, H-4α, H-16β, H-24b), 1.98 (1H, H-15β), 1.97 (1H, H-2α), 1.87 (1H, d, *J* = 12.6 Hz, H-12β), 1.77 (1H, H-2β), 1.73 (2H, H-11α, H-11β), 1.74 (1H, H-23b), 1.71 (1H, H-16α), 1.61 (1H, H-15α), 1.60 (1H, H-4β), 1.57 (1H, H-23a), 1.49 (2H, H-1), 1.44 (1H, td, *J* = 12.6, 4.2 Hz, H-24a), 1.24 (3H, s, H-21), 1.20 (3H, s, H-27), 1.19 (3H, s, H-26), 0.91 (3H, s, H-18), 0.89 (3H, s, H-19); ^{13}C NMR (CD_3OD, 125 MHz), *δ* (ppm): 202.9 (C, C-6), 168.2 (C, C-8), 120.7 (CH, C-7), 105.9 (CH, C-1´), 89.9 (CH, C-22), 85.3 (C, C-14), 81.0 (C, C-5), 78.2 (CH, C-5´), 78.1 (CH_2, C-3´), 77.6 (C, C-20), 75.6 (CH_2, C-2´), 71.6 (CH_2, C-4´), 71.5 (C, C-25), 67.2 (CH, C-3), 62.6 (CH_2, C-6´), 51.2 (CH, C-17), 49.1 (C, C-13), 43.4 (C, C-10), 41.1 (CH_2, C-24), 38.1 (CH, C-9), 36.8 (CH_2, C-4), 32.9 (CH_2, C-12), 32.0 (CH_2, C-15), 29.8 (CH_3, C-27), 29.3 (CH_2, C-2), 29.2 (CH_3, C-26), 27.7 (CH_2, C-23), 25.6 (CH_2, C-1), 22.5 (CH_2, C-11), 22.5 (CH_3, C-21), 21.6 (CH_2, C-16), 18.3 (CH_3, C-18), 17.3 (CH_3, C-19); ESIMS *m/z* (rel. int. %): 666 $[M + Na + H]^+$ (36), 665 $[M + Na]^+$ (100), 643 $[M + H]^+$ (56), 462 $[M – sugar]^+$ (9), 393 (43), 391 (45); HRESI-MS *m/z* 665.7744 $[M + Na]^+$ (calcd for $C_{33}H_{54}O_{12}Na$, 665.77436).

2-deoxypolypodine B 25-*β*-D-glucopyranoside (6): Colourless crystals (3 mg). $[\alpha]^{25}_{D}$ +70˚ (*c* = 0.1, MeOH); UV λ_{max} ^{MeOH} (nm) (log ε): 241 nm (3.90); ^{1}H NMR (CD_3OD, 500 MHz) δ (ppm): 5.86 (1H, d, *J* = 2.1 Hz, H-7), 4.456 (1H, d, *J* = 7.7 Hz, H-1´), 4.08 (1H, s, br, H-3α), 3.845 (1H, dd, *J* = 11.9, 1.9 Hz, H-6´b), 3.61 (1H, dd, *J* = 11.9, 5.9 Hz, H-6´a), 3.36 (1H, t, *J* = 8.8 Hz, H-3´), 3.325 (1H, H-22), 3.28 (1H, H-9α), 3.26 (1H, H-5´), 3.22 (1H, H-4´), 3.14 (1H, dd, *J* = 9.1, 8.0 Hz, H-2´), 2.39 (1H,

dd, *J* = 9.3, 8.4 Hz, H-17α), 2.13 (1H, td, *J* = 12.3, 5.9 Hz, H-12α), 2.03 (1H, H-4α), 1.97 (3H, H-2α, H-15β, H-16β), 1.93 (1H, H-24b), 1.87 (1H, H-12β), 1.82 (1H, H-1β), 1.77 (1H, H-2β), 1.75 (1H, H-16α), 1.72 (2H, H-11α, H-11β), 1.70 (1H, H-23b), 1.61 (1H, H-4β), 1.60 (1H, H-15α), 1.49 (1H, H-1α), 1.48 (1H, H-24a), 1.43 (1H, H-23a), 1.28 (3H, s, H-27), 1.27 (3H, s, H-26), 1.19 (1H, s, H-21), 0.896 (3H, s, H-18), 0.892 (3H, s, H-19); ^{13}C NMR (CD_3OD, 125 MHz), δ (ppm): 202.9 (C, C-6), 168.15 (C, C-8), 120.7 (C, C-7), 98.8 (CH, C-1´), 85.4 (C, C-14), 81.0 (CH, C-5), 78.8 (C, C-25), 78.6 (CH, C-22), 78.35 (CH_2, C-3´), 78.11 (C, C-20), 78.05 (CH, C-5´), 75.46 (CH_2, C-2´), 72.0 (CH_2, C-4´), 67.2 (CH, C-3), 63.3 (CH_2, C-6´), 50.5 (CH, C-17), 48.8 (C, C-13), 43.4 (C, C-10), 40.3 (CH_2, C-24), 38.1 (CH, C-9), 36.8 (CH_2, C-4), 32.8 (CH_2, C-12), 31.9 (CH_2, C-15), 29.25 (CH_2, C-2), 27.5 (CH_3, C-26, C-27), 26.9 (CH_2, C-23), 25.6 (CH_2, C-1), 22.44 (CH_2, C-11), 21.6 (CH_2, C-16), 21.2 (CH_3, C-21), 18.2 (CH_3, C-18), 17.3 (CH_3, C-19); ESIMS *m/z* (rel. int. %): 666 $[M + Na + H]^+$ (26), 665 $[M + Na]^+$ (100), 643 $[M + H]^+$ (49), 625 $[M - H_2O]^+$ (21), 421 (55); HRESI-MS *m/z* 665.7744 $[M + Na]^+$ (calcd for $C_{33}H_{54}O_{12}Na$, 665.77436).

Integristerone A 20,22-acetonide (13): Colourless crystals (3.4 mg). $[\alpha]^{25}_D$ +43˚ (*c* = 0.1, MeOH); UV λ_{max}^{MeOH} (nm) (log ε): 242 nm (3.96); ^{1}H NMR (CD_3OD, 500 MHz), δ (ppm): 5.84 (1H, d, *J* = 1.8 Hz, H-7), 4.04 (1H, s, br, H-3α), 3.87 (1H, t, *J* = 3 Hz, H-2α), 3.82 (1H, br, H-1α), 3.68 (1H, dd, *J* = 8.4, 3.6 Hz, H-22), 3.08 (1H, H-9α), 2.61, (1H, dd, *J* = 12.9, 4.5 Hz, H-5), 2.31 (1H, dd, *J* = 9, 8.4 Hz, H-17α), 2.09 (1H, td, *J* = 12.6, 4.8 Hz, H-12α), 2.02 (1H, 16-H β), 1.97 (1H, dd, *J* = 12.6, 6.6 Hz, H-15β), 1.86 (1H, H-16α), 1.84 (1H, H-12β), 1.77, (2H, H-4α, H-4β), 1.73 (1H, H-24b), 1.72 (2H, H-11α, H-11β), 1.62 (1H, dd, *J* = 10.8, 10.2 Hz, H-15α), 1.53 (2H, H-23a, H-23b), 1.48 (1H, H-24a), 1.39 (3H, s, H-30), 1.32 (3H, s, H-29), 1.21 (3H, s, H-27), 1.20 (3H, s, H-26), 1.18 (H_3-21, s), 1.08 (3H, s, H-19), 0.84 (3H, s, H-18); ^{13}C NMR (CD_3OD, 125 MHz), δ (ppm): 205.8 (C, C-6), 167.0 (C, C-8), 122.3 (CH, C-7), 108.2 (C, C-28), 86.0 (C, C-20), 85.5 (C, C-14), 83.5 (CH, C-22), 76.6 (CH, C-1), 71.3 (C, C-25), 71.1 (CH, C-3), 68.6 (CH, C-2), 50.7 (CH, C-17), 48.7 (C, C-13), 46.9 (CH, C-5), 44.0 (C, C-10), 42.4 (CH_2, C-24), 35.8 (CH, C-9), 33.7 (CH_2, C-4), 32.5 (CH_2, C-12), 31.9 (CH_2, C-15), 29.6 (CH_3, C-27), 29.5 (CH_3, C-30), 29.1 (CH_3, C-26), 27.3 (CH_3, C-29), 24.9 (CH_2, C-23), 22.7 (CH_3, C-21), 22.5 (CH_2, C-16), 22.1 (CH_2, C-11), 20.2 (CH_3, C-19), 17.8 (CH_3, C-18); ESIMS *m/z* (rel. int. %): 560 $[M + Na + H]^+$ (22), 559 $[M + Na]^+$ (100), 537 $[M + H]^+$ (20), 519 $[M - H_2O]^+$ (70), 479 $[M + H - acetone]^+$ (25), 461 $[M + H - H_2O - acetone]^+$ (27), 450 (58), 430 (35); HRESI-MS *m/z* 559.6962 $[M + Na]^+$ (calcd for $C_{33}H_{54}O_{12}Na$, 559.69612).

2-deoxy-26-hydroxypolypodine B (14): Colourless crystals (1.2 mg). $[\alpha]^{25}_D$ +41˚ (*c* = 0.05, MeOH); UV λ_{max}^{MeOH} (nm) (log ε): 243 nm (4.02); ^{1}H NMR (CD_3OD, 500 MHz) δ (ppm): 5.85 (1H, d, *J* = 2.2 Hz, H-7), 4.08 (1H, s, br, H-3α), 3.37 (2H, s, H-26a, H-26b), 3.34 (1H, m, H-22), 3.27 (1H, m, H-9α), 2.39 (1H, t, *J* = 8.8 Hz, H-17α), 1.19 (3H, s, br, H-21), 1.14 (3H, s, H-27), 0.90 (3H, s, H-18), 0.89 (3H, s, H-19); ^{13}C NMR (CD_3OD, 125 MHz) δ (ppm): 120.7 (CH, C-7), 85.2 (C, C-14), 81.0 (C, C-5), 78.7 (CH, C-22), 77.9 (C, C-20), 73.6 (C, C-25), 70.8 (CH_2, C-26), 67.2 (CH, C-3), 50.5 (CH, C-17),

48.8 (C, C-13), 43.3 (C, C-10), 37.9 (CH, C-9), 37.2 (CH_2, C-24), 32.6 (CH, C-12), 25.3 (CH_2, C-1), 23.6 (CH_3, C-27), 21.1 (CH_3, C-21), 18.2 (CH_3, C-18), 17.2 (CH_3, C-19); ESIMS *m/z* (rel. int. %): 535 $[M + K]^+$ (100); HRESI-MS *m/z* 497.3108 $[M + H]^+$ (calcd for $C_{27}H_{45}O_8$, 497.3102).

26-hydroxypolypodine B 20,22-acetonide (15): Colourless crystals (1.6 mg). $[\alpha]^{25}_D$ +89˚ (*c* = 0.05, MeOH); UV λ_{max} ^{MeOH} (nm) (log ε): 227 (3.764) nm; ^{1}H NMR (CD_3OD, 500 MHz) δ (ppm): 5.86 (1H, d, *J* = 2.8 Hz, H-7), 3.99 (1H, q, *J* = 3.0 Hz, H-3α), 3.95 (1H, ddd, *J* = 10.0, 7.4, 3.6 Hz, H-2α), 3.695 (1H, t, *J* = 6.0 Hz, H-22), 3.375 (1H, d, *J* = 11.0 Hz, H-26b), 3.355 (1H, d, *J* = 11.0 Hz, H-26a), 3.19 (1H, ddd, *J* = 11.3, 7.0, 2.7 Hz, H-9α), 2.31 (1H, dd, *J* = 9.4, 8.1 Hz, H-17α), 2.12 (1H, td, *J* = 13.1, 5.0 Hz, H-12α), 2.075 (1H, dd, *J* = 14.7, 3.0 Hz, H-4α), 2.03 (1H, m, H-16β), 1.96 (1H, dd, *J* = 12.4, 6.5 Hz, H-15β), 1.87 (1H, m, H-16α), 1.86 (1H, m, H-12β), 1.81 (1H, m, H-11b), 1.77 (1H, dd, *J* = 14.9, 3.0 Hz, H-4β), 1.74 (1H, m, H-11α), 1.73 (2H, m, H-1α, H-1β), 1.71 (1H, m, H-24b), 1.61 (1H, m, H-15α), 1.55 (1H, m, H-23b), 1.53 (1H, m, H-23a), 1.52 (1H, m, H-24a), 1.39 (3H, s, H-30), 1.315 (3H, s, H-29), 1.18 (3H, s, H-21), 1.15 (3H, s, H-27), 0.915 (3H, s, H-19), 0.83 (3H, s, H-18); ^{13}C NMR (CD_3OD, 125 MHz) δ 202.5 (C, C-6), 167.4 (C, C-8), 120.7 (CH, C-7), 108.2 (C, C-28), 86.0 (C, C-20), 85.3 (C, C-14), 83.6 (CH, C-22), 80.4 (C, C-5), 73.6 (C, C-25), 70.7 (CH_2, C-26), 70.4 (CH, C-3), 68.6 (CH, C-2), 50.5 (CH, C-17), 48.7 (C, C-13), 45.5 (C, C-10), 39.2 (CH, C-9), 37.2 (CH_2, C-24), 36.3 (CH_2, C-4), 34.3 (CH_2, C-1), 32.6 (CH_2, C-12), 31.8 (CH_2, C-15), 29.5 (CH_3, C-30), 27.3 (CH_3, C-29), 24.05 (CH_2, C-23), 23.9 (CH_3, C-27), 22.7 (CH_3, C-21), 22.65 (CH_2, C-11), 22.5 (CH_2, C-16), 17.8 (CH_3, C-18), 17.1 (CH_3, C-19); ESIMS *m/z* (rel. int. %): 575 $[M + Na]^+$ (46), 553 $[M + H]^+$ (100), 537 $[M - CH_3]^{+\bullet}$ (5), 535 $[M + H - H_2O]^+$ (2), 520 $[M + H - H_2O - CH_3]^{+\bullet}$ (2), 495 $[M + H - \text{acetone}]^+$ (59), 481 (1), 477 (3), 437 (3), 359 (3), 328 (14); HRESI-MS *m/z* 553.3366 $[M + H]^+$ (calcd for $C_{30}H_{49}O_9$, 553.3363).

2-deoxy-26-hydroxypolypodine B 20,22-acetonide (16): Colourless crystals (1.00 mg). $[\alpha]^{25}_D$ +25˚ (*c* = 0.05, MeOH); UV λ_{max} ^{MeOH} (nm) (log ε): 238 (4.082); ^{1}H NMR (CD_3OD, 500 MHz) δ (ppm): 5.86 (1H, s, br, H-7), 4.08 (1H, s, br, H-3β), 3.70 (1H, m, H-22), 3.37 (1H, d, *J* = 11.0 Hz, H-26b), 3.36 (1H, d, *J* = 11.0 Hz, H-26a), 3.28 (1H, m, H-9α), 2.32 (1H, t, *J* = 8.7 Hz, H-17α), 2.12 (1H, td, *J* = 12.4, 5.7 Hz, H-12α), 2.04 (1H, m, H-16β), 2.035 (1H, m, H-4b), 1.97 (1H, m, H-15β), 1.96 (1H, m, H-2b), 1.88 (1H, m, H-16α), 1.86 (1H, m, H-12β), 1.84 (1H, m, H-1b), 1.77 (1H, m, H-2a), 1.73 (1H, m, H-11a), 1.72 (1H, m, H-24b) 1.61 (2H, m, H-4a, H-15α), 1.55 (2H, m, H-23a, H-23b), 1.53 (1H, m, H-24a), 1.50 (1H, m, H-1a), 1.39 (3H, s, H-30), 1.32 (3H, s, H-29), 1.18 (3H, s, H-21), 1.15 (3H, s, H-27), 0.89 (3H, s, H-19), 0.83 (3H, s, H-18); ^{13}C NMR (CD_3OD, 125 MHz) δ (ppm): 167.9 (C, C-8), 120.7 (CH, C-7), 108.2 (C, C-28), 86.0 (C, C-20), 85.4 (C, C-14), 83.6 (CH, C-22), 81.2 (C, C-5), 73.6 (C, C-25), 70.7 (CH_2, C-26), 67.2 (CH, C-3), 50.6 (CH, C-17), 48.7 (C, C-13), 43.25 (C, C-10), 38.1 (CH, C-9), 37.2 (CH_2, C-24), 36.9 (CH_2, C-4), 32.6 (CH_2, C-12), 31.8 (CH_2, C-15), 29.5 (CH_3, C-30), 29.3 (CH_2, C-2), 27.3 (CH_3, C-29), 25.6 (CH_2, C-1), 24.05 (CH_2, C-23), 23.8 (CH_3, C-27), 22.7 (CH_3, C-21), 22.5 (CH_2, C-11), 22.5 (CH_2, C-16), 17.8 (CH_3, C-18), 17.3 (CH_3, C-19); ESIMS *m/z* (rel. int.

%): 559 $[M + Na]^+$ (100), 537 $[M + H]^+$ (36), 518 $[M – H_2O]^+$ (3), 541 $[M + Na – H_2O]^+$ (12), 501 $[M + H – 2\ H_2O]^+$ (2), 445 (10), 385 $[M + H – H_2O – C_6O_3H_{14}]^+$ (3), 315 (12), 304 (24); HRESI-MS *m/z* 537.3420 $[M + H]^+$ (calcd for $C_{30}H_{49}O_8$, 537.3414).

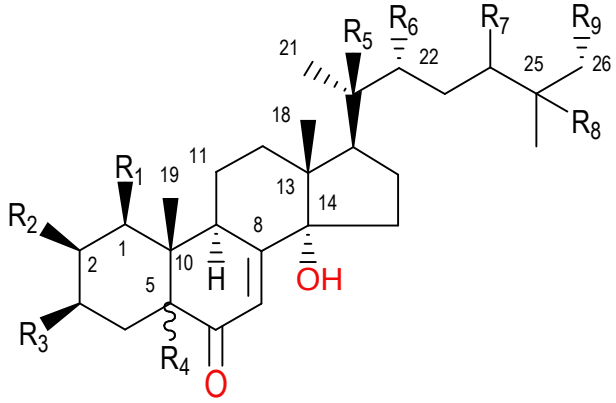
20,26-dihydroxyecdysone 20,22-acetonide (<u>17</u>): Colourless crystals (2.00 mg). $[\alpha]^{25}_D$ +145˚ (*c* = 0.005, MeOH); UV λ_{max} ^{MeOH} (nm) (log ε): 242 (4.011); ^{1}H NMR (CD_3OD, 500 MHz) δ (ppm): 5.81 (1H, d, *J* = 2.6 Hz, H-7), 3.70 (1H, m, H-22), 3.375 (1H, d, *J* = 10.9 Hz, H-26b), 3.36 (1H, d, *J* = 11.0 Hz, H-26°), 2.42 (1H, dd, *J* = 12.6, 4.0 Hz, H-5β), 2.33 (1H, dd, *J* = 9.2, 8.6 Hz, H-17α), 1.39 (3H, s, H-30), 1.32 (3H, s, H-29), 1.18 (3H, s, H-21), 1.15 (3H, s, H-27), 0.96 (3H, s, H-19), 0.83 (3H, s, H-18); ^{13}C NMR (CD_3OD, 125 MHz) δ (ppm): 121.8 (CH, C-7), 85.9 (C, C-20), 85.5 (C, C-14), 83.6 (CH, C-22), 73.2 (C, C-25), 70.7 (CH_2, C-26), 50.5 (CH, C-17), 49.3 (C, C-13), 37.1 (CH_2, C-24), 32.55 (CH_2, C-12), 29.4 (CH_3, C-30), 27.3 (CH_3, C-29), 24.4 (CH_3, C-19), 23.7 (CH_3, C-27), 22.7 (CH_3, C-21), 17.8 (CH_3, C-18); ESIMS *m/z* (rel. int. %): 575 $[M + K]^+$ (14), 560 $[M + H + Na]^+$ (6), 559 $[M + Na]^+$ (5), 542 (100), 521 $[M – CH_3]^{+\bullet}$ (23), 519 $[M + H – H_2O]^+$ (2), 503 $[M – CH_3 – H_2O]^{+\bullet}$ (7), 501 $[M +H – 2H_2O]^+$ (23), 478 $[M – acetone]^+$ (4), 445 (14), 413 (6), 314 (10), 304 (55); HRESI-MS *m/z* 537.3418 $[M + H]^+$ (calcd for $C_{30}H_{48}O_8$, 537.3414).

5α-2-Deoxy-20-hydroxyecdysone 20,22-acetonide (<u>18</u>): Colourless crystals (3.4 mg). $[\alpha]^{25}_D$ +57˚ (*c* = 0.05, MeOH); UV λ_{max} ^{MeOH} (nm) (log ε): 238 (3.4); ^{1}H NMR (CD_3OD, 500 MHz) δ (ppm): 5.83 (1H, d, *J* = 2.8 Hz, H-7), 3.685 (1H, m, H-22), 3.55 (1H, tt, *J* = 11.0, 4.4 Hz, H-3α), 2.75 (1H, ddd, *J* = 11.7, 7.2, 2.7 Hz, H-9α), 2.355 (1H, dd, *J* = 12.2, 3.6 Hz, H-5α), 2.30 (1H, dd, *J* = 9.1, 8.6 Hz, H-17α) 2.11 (2H, H-4α, H-12-α), 2.03 (1H, H-16β), 1.95 (1H, H-15β), 1.87 (1H, H-1β), 1.86 (1H, H-16α), 1.815 (1H, H-12β), 1.79 (2H, 2-Hα, 11-Hα), 1.74 (1H, H-24b), 1.635 (1H, H-11β), 1.615 (1H, H-15α), 1.53 (2H, H-23°, H-23b), 1.485 (1H, H-24b), 1.41 (1H, H-1α), 1.39 (1H, H-2β), 1.39 (3H, s, H-30), 1.365 (1H, t, H-4β), 1.32 (3H, s, H-29), 1.205 (3H, s, H-27), 1.196 (3H, s, H-26), 1.175 (3H, s, H-21), 0.854 (3H, s, H-19), 0.826 (3H, s, H-18); ^{13}C NMR (CD_3OD, 125 MHz) δ (ppm): 203.25 (C, C-6), 166.5 (C, C-8), 123.6 (C, C-7), 108.15 (C, C-28), 86.0 (C, C-20), 85.3 (C, C-14), 83.5 (CH, C-22), 71.31 (CH, C-3), 71.27 (C, C-25), 54.7 (CH, C-5), 50.7 (CH, C-17), 48.4 (C, C-13), 47.6 (CH, C-9), 42.4 (CH_2, C-24), 39.7 (C, C-10), 38.0 (CH_2, C-1), 32.4 (CH_2, C-12), 31.9 (CH_2, C-15), 31.5 (CH_2, C-2), 31.0 (CH_2, C-4), 29.6 (CH_3, C-27), 29.5 (CH_3, C-30), 29.1 (CH_3, C-26), 27.3 (CH_3, C-29), 24.9 (CH_2, C-23), 22.7 (CH_3, C-21), 22.5 (CH_2, C-16), 21.7 (CH_2, C-11), 17.8 (CH_3, C-18), 13.4 (CH_3, C-19); ESIMS *m/z* (rel. int. %): 1031 $[2M + Na]^+$ (9), 544 $[M + H + K]^+$ (9), 528 $[M + H+ Na]^+$ (14), 527 $[M + Na]^+$ (41), 504 $[M]^+$ (10), 487 $[M + H – H_2O]^+$ (39), 429 $[M + H – H_2O – acetone]^+$ (100), 413 (23), 391 (60), 333 (21); HRESI-MS *m/z* 527.3336 $[M + Na]^+$ (calcd for $C_{30}H_{48}O_6Na$, 527.3339).

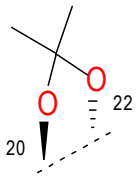
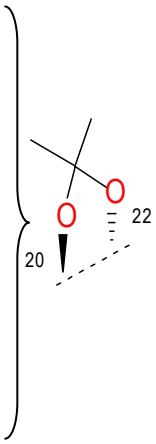
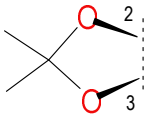
Makisterone C 2,3;20,22-diacetonide (<u>20</u>): Colourless crystals (13.3 mg). $[\alpha]^{25}_D$ +65˚ (*c* = 0.005, MeOH); UV λ_{max} ^{MeOH} (nm) (log ε): 240 (3.4); ^{1}H NMR (CD_3OD, 500 MHz) δ (ppm): 5.80 (1H, d, J = 2.5 Hz, H-7), 4.30 (1H, q, H-3α), 4.27 (1H, dt, *J* = 9.4, 5.0 Hz, H-2α), 3.834 (1H, d, *J* = 10.0 Hz), 2.935 (1H, ddd, *J* = 11.7, 7.0, 2.5 Hz, H-9α), 2.304 (1H, t, *J* = 8.9 Hz), 2.245 (1H, dd, *J* = 9.3, 8.2 Hz,

H-5β), 2.105 (1H, td, *J* = 13.0, 4.9 Hz, H-12α), 2.06 (1H, H-16β), 1.99 (2H, d, H-1α, H-15β), 1.98 (2H, H-4a, H-4b), 1.87 (1H, H-16α), 1.85 (1H, H-12β), 1.77 (1H, H-11α), 1.74 (1H, H-23b), 1.68 (1H, H-28b), 1.67 (1H, H-11β), 1.62 (1H, H-15α), 1.47 (3H, s, H-35), 1.45 (1H, ddd, *J* = 12.5, 6.6, 3.0 Hz, H-24), 1.39 (3H, s, H-32), 1.325 (3H, s, H-31), 1.32 (3H, s, H-34), 1.22 (1H, H-28a), 1.20 (1H, H-23a), 1.18 (3H, s, H-27), 1.173 (3H, s, H-21), 1.16 (3H, s, H-26), 1.006 (3H, t, *J* = 7.5 Hz, H-29), 0.96 (3H, s, H-19), 0.825 (3H, s, H-18); ^{13}C NMR (CD_3OD, 125 MHz) δ (ppm): 205.8 (C, C-6), 167.0 (C, C-8), 122.0 (C, C-7), 109.6 (C, C-33), 108.1 (C, C-30), 86.1 (C, C-20), 85.4 (C, C-14), 81.5 (CH, C-22), 74.4 (C, C-25), 73.8 (CH, C-2), 73.3 (CH, C-3), 52.6 (CH, C-5), 50.4 (CH, C-17), 50.0 (CH, C-24), 49.0 (C, C-13), 39.0 (C, C-10), 38.9 (CH_2, C-1), 35.9 (CH, C-9), 32.5 (CH_2, C-12), 31.8 (CH_2, C-15), 30.9 (CH_2, C-23), 29.5 (CH_3, C-32), 29.0 (CH_3, C-35), 27.9 (CH_3, C-27), 27.8 (CH_2, C-4), 27.2 (CH_3, C-31), 26.8 (CH_3, C-26, C-34), 25.3 (CH_3, C-28), 24.2 (CH_3, C-19), 22.7 (CH_2, C-16), 22.6 (CH_3, C-21), 21.8 (CH_2, C-11), 14.9 (CH_3, C-29); ESIMS *m/z* (rel. int. %): 627 $[M + K]^+$ (42), 612 $[M + H + Na]^+$ (41), 611 $[M + Na]^+$ (100), 588 $[M]^+$ (6.4), 576 $[M + H + Na - 2H_2O]^+$ (55), 563 (41), 545 (55), 531 $[M + H -$ acetone$]^+$ (40), 513 $[M + H - H_2O-$ acetone$]^+$ (42), 495 $[M + H - 2H_2O-$ acetone$]^+$ (20), 473 $[M + H -$ 2 acetone$]^+$ (17), 454 $[M + H - H_2O-$ 2 acetone$]^+$ (24), 436 $[M + H -$ 2 H_2O- 2 acetone$]^+$ (25), 418 $[M + H -$ 3 H_2O- 2 acetone$]^+$ (56), 391 (86), 381 (28), 338 (25), 333 (29); HRESI-MS *m/z* 611.3913 $[M + Na]^+$ (calcd for $C_{35}H_{56}O_7Na$, 611.3909).

Table 2. Structure of the isolated ecdysteroids. The new compounds are indicated with bold characters with asterisk.



No.	Name	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9
1	**20-hydroxyecdysone**	H	OH	OH	βH	OH	OH	H	OH	H
2	**polypodine B**	H	OH	OH	βOH	OH	OH	H	OH	H
3	**2-deoxy-20-hydroxyecdysone 3-*β*-D-glucopyranoside**	H	H	O-β-D-glu	βH	OH	OH	H	OH	H
4	**2-deoxypolypodine B 3-*β*-D-glucopyranoside**	H	H	O-β-D-glu	βOH	OH	OH	H	OH	H
5	***2-deoxypolypodine B 22-β-D-glucopyranoside****	H	H	OH	βOH	OH	O-β-D-glu	H	OH	H
6	***2-deoxypolypodine B25-β-D-glucopyranoside****	H	H	OH	βOH	OH	OH	H	O-β-D-glu	H
7	**2-deoxy-20,26-dihydroxyecdysone**	H	H	OH	βH	OH	OH	H	OH	OH

8	**integristerone A**	OH	OH	OH	βH	OH	OH	H	OH	H
9	**26-hydroxypolypodine B**	H	OH	OH	βOH	OH	OH	H	OH	OH
10	**20,26-dihydroxyecdysone**	H	OH	OH	βH	OH	OH	H	OH	OH
11	**2-deoxy-20-hydroxyecdysone**	H	H	OH	βH	OH	OH	H	OH	H
12	**2-deoxy-integristerone A**	OH	H	OH	βH	OH	OH	H	OH	H
13	***integristerone A 20,22-acetonide****	OH	OH	OH	βH			H	OH	H
14	***2-deoxy-26-hydroxypolypodine B****	H	H	OH	βOH	OH		H	OH	OH
15	***26-hydroxypolypodine B 20,22-acetonide****	H	OH	OH	βOH			H	OH	OH
16	***2-deoxy-26-hydroxypolypodine B 20,22-acetonide****	H	H	OH	βOH			H	OH	OH
17	***20,26-dihydroxyecdysone 20,22-acetonide****	H	OH	OH	βH			H	OH	OH
18	***5α,2-deoxy-20-hydroxyecdysone 20,22-acetonide****	H	H	OH	αH			H	OH	H
19	**2-deoxy-20-hydroxyecdysone 20,22-acetonide**	H	H	OH	βH			H	OH	H
20	***makisterone C 2,3 20,22-diacetonide****	H			βH			CH_2-CH_3	OH	H

3.3 Investigation of the genuineness of ecdysteroid acetonides

Our experiment to model the acetonide formation did not give results in none of the sampling days (1st, 3rd, 7th, 14th, 21st, 35th), we did not found acetonide formation via testing with **TLC$_{1,2,3}$** and **NP-HPLC2,4** systems.

To prove that the dried herb originally contained ecdysteroid acetonides, NP- and RP-HPLC experiments were performed on the methanolic extract of the herb after a two-step pre-purification procedure in which acetone was not used. The pre-purification was carried out with solvent-solvent distribution between aqueous MeOH and *n*-hexane to remove the apolar contaminants followed by coarse column chromatography on silica with two elution steps. The apolar contaminating components remained in the extract were washed from the column after the first purification with CH_2Cl_2, and the ecdysteroids of interest were eluted with CH_2Cl_2-MeOH (8:2, v/v) in the second elution step. The bulk of the polar contaminating components was retained on the adsorbent (silica). The purified extract (**SV-1**) and an ecdysteroid acetonide (compound **19**), most abundantly present in the herb, was subjected to

HPLC. **Fig. 7A-C** depict the HPLC chromatograms of the pre-purified extract and compound **19**.

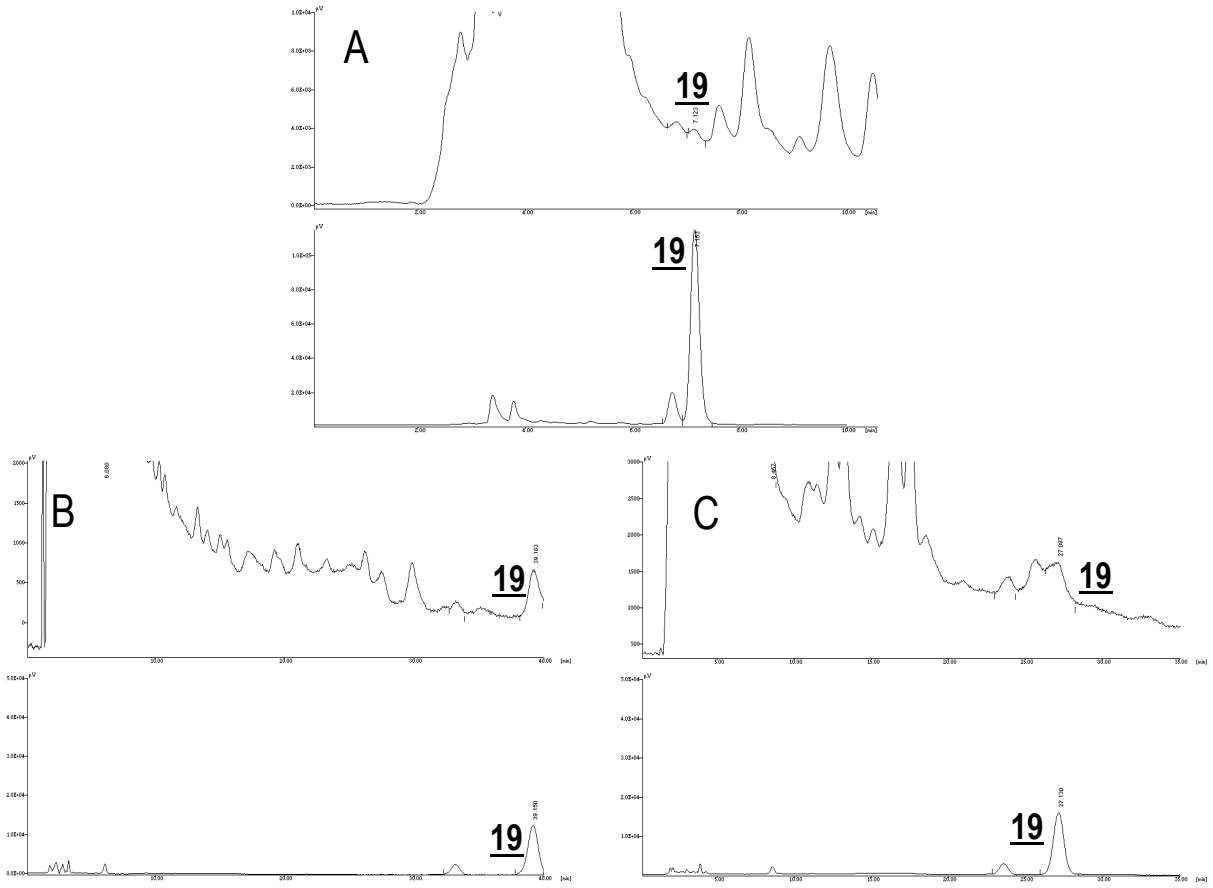


Fig. 7. HPLC chromatograms of **SV-1** and the ecdysteroid acetonide, compound **19**.
A: Chromatographic conditions: **NP-HPLC6**
B: Chromatographic conditions **RP-HPLC4**.
C: Chromatographic conditions: **RP-HPLC5**.

Table 3. Retention times of compound **19** using different HPLC systems

Chromatographic conditions	t_R (min)	
	Compound in SV-1	**19**
RP-HPLC4	39.183	39.150
RP-HPLC5	27.097	27.130
NP-HPLC7	7.123	7.153

The R_t values of the compound **19** coincided with those of the corresponding compounds in the in **SV-1** (shown in **Table 3**).

Mass spectrometric detection was made to verify the presence of compound **19** in four pre-purified extracts of *S. viridiflora*, collected in different time (**SV-1**, **SV-2**, **SV-3** and **SV-4**) with positive ion electrospray ionization and LC–MS/MS analysis. Following the RP-HPLC separation the samples were subjected to mass fragmentation. A triple-quadrupole mass spectrometer was utilized for SRM of MH^+ (*m/z* 505) as major molecular ion of compound **19**. MS/MS experiments were performed on the

two most intense product ion fragments *m/z* 429 and 411, which were eluted from the HPLC column at 5.94 min and 5.87 min, respectively. Presence of these molecular ion fragments at the corresponding retention times were proved in all four extract. **Fig. 8** presents the LC–MS/MS chromatogram of compound **<u>19</u>** and **SV-2**.

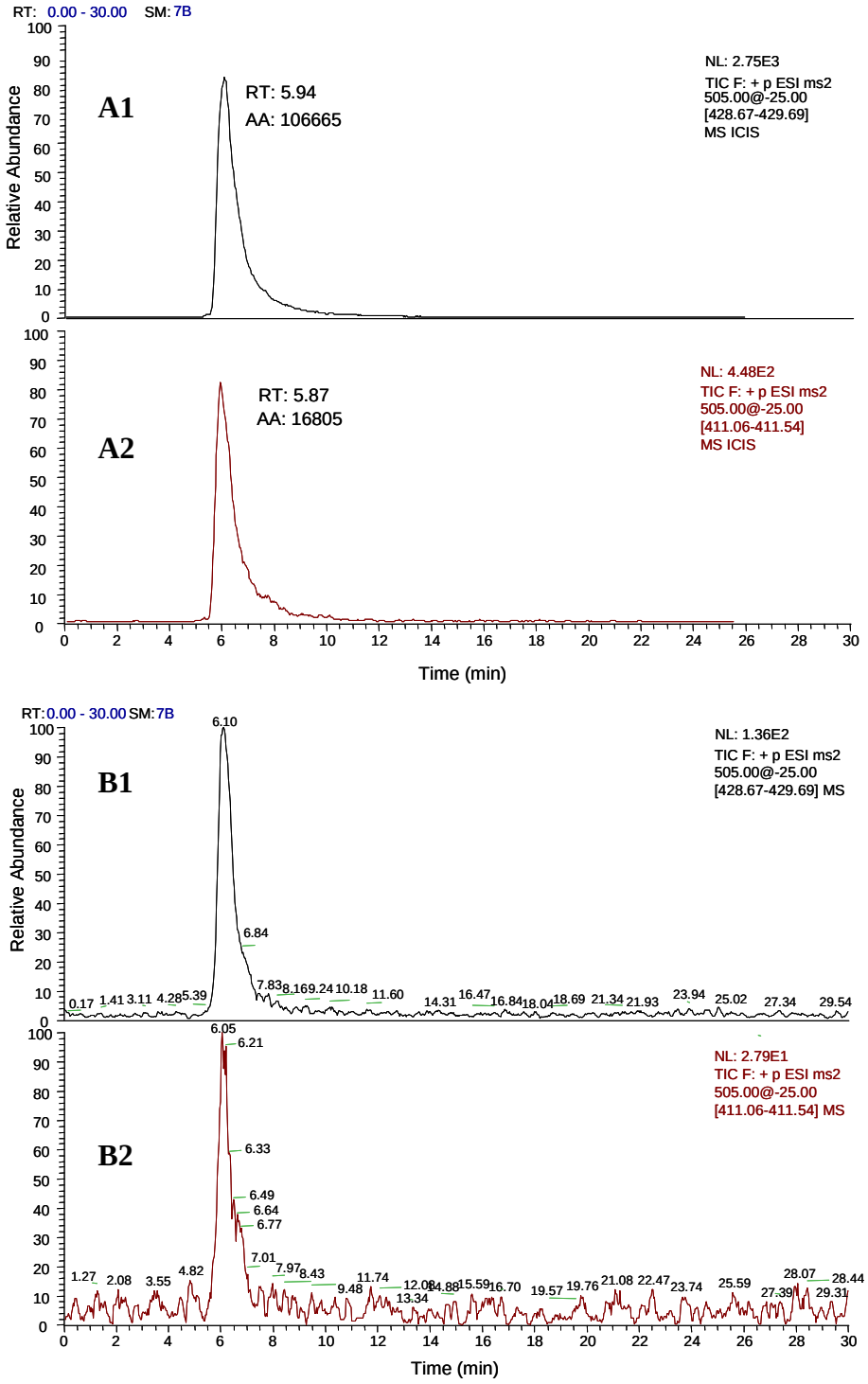


Fig. 8. LC-MS/MS chromatogram of compound **<u>19</u>** (*m/z* 429 **A1**, *m/z* 411 **A2**) and **SV-2** (*m/z* 429 **B1**, *m/z* 411 **B2**).

3.4 Effect of 20E (1) on rat muscle fibres

3.4.1 Body and muscle mass

In the normal and regenerating muscle model, the body and muscle mass increased significantly in group **N20E** compared to group **NC**. However, these two parameters did not change in group **R20E1** and only the muscle mass of the right EDL increased in group **R20E2** compared to group **RC**. In the muscle atrophy model, the body weight was not different between the three groups at the start of the treatment. In the **MC** group the weight increased ~3% during the treatment period, whereas it decreased by ~5% and ~ 3.5% in the **MP** and **M20E** groups respectively which showed a highly significant difference compared to C (p<0.001). Although there was no difference between **MP** and **M20E**, the rate of weight loss tended to decrease slower in **M20E** than in **MP** group. There was no difference in the diaphragm weight between the three groups. The results are shown in **Table 4**.

Table 4. Body weight and muscle mass of the treated rats

Body weight (g)			m. soleus (mg)		m. EDL (mg)		Reg. m. soleus (mg)	Diaph. (mg)
Start of treatment	End of exp.	Change in weight	Right	Left	Right	Left		
				NC				
324.5±2.50 (318-330)	336.5±6.55 (320-352)	12.0±4.16 (2-22)	140.3±5.69 (128-155)	143.5±4.09 (135-151)	142.8±3.64 (136-152)	146.0±3.03 (137-150)	-	-
				N20E				
309.0±6.00 (304-316)	373.0±5.26 (364-388)	64.0±4.32*** (52-72)	160.0±4.77** (150-170)	175±7.14* (160-194)	166.0±6.38* (156-184)	172.0±9.58* (154-197)	-	-
				RC				
285.3±3.82 (276-292)	353.8±7.96 (340-376)	68.5±10.65 (49-94)	158.0±3 (150-160)	-	155.0±5 (150-170)	-	113.0±6 (100-130)	-
				R20E1				
293.5±2.06 (290-298)	352.5±4.78 (340-360)	59.0±3.11 (50-64)	168.0±7.5 (150-180)	-	150.0±4.08 (140-160)	-	113.0±7 (100-130)	-
				R20E2				
314.0±8.29 (296-336)	387.0±5.80 (376-402)	73.0±4.04 (66-80)	174.0±6.96 (158-189)	-	178.0±4.05* (169-188)	-	116.0±10.5 (90-136)	-
				MC				
441.0±5.52	454.8±7.03	13.83±1.89	-	-	-	-	-	597.8±10.2
				MP				
460.7±8.41	438±8.43	-22.67±1.73***	-	-	-	-	-	566.9±2.4
				M20E				
443.8±5.53	429.0±2.08	-14.83±4.07***	-	-	-	-	-	566.9±12.1

*, **, *** p<0.05, 0.01, 0.001 compared to the correspondent control (0.9% NaCl or notexin + 0.9% NaCl). Abbreviations used: exp. = experiment, reg. = regenerating, diaph. = diaphragm

3.4.2 CSA of MyHC fibre types

Normal muscle model

The size of muscle fibres increased significantly in response to the **1** treatment in the left soleus of group **N20E** (5011±67.44 μm^2) compared to group **NC** (4261±49.68 μm^2). The rat soleus muscle consists of slow-oxidative type I (90%) and fast-oxidative type IIA (9%) fibres expressing MyHC1 and MyHC2a isoforms, respectively. The rate of IIx/d (mentioned as IIx) fibres is only 1%. The CSA of both type I and type IIA fibres in the soleus muscle of the treated left hindlimb increased (p<0.001) in group **N20E** compared to the control group **NC** (**Fig. 9A**, black and empty columns). **1** also increased the size of muscle fibres in the EDL (3137±56.98 μm^2 vs. 2488±48.07 μm^2) and the fibre size distribution of the treated muscle showed a bimodular pattern suggesting a distinct effect on the different fibre types. In the EDL four fibre types (I, IIA, IIx and IIB) are present expressing correspondingly the MyHC1, MyHC2a MyHC2x and MyHC2b isoforms. However in the left EDL the CSA of the type IIx fibres increased (p<0.001), while the type I and type IIB fibres did not change but the CSA of type IIA fibre´s decreased (p<0.001) compared to those of group **NC** (**Fig. 9B,** black and empty columns). This shows that **1** affects fibre size in a muscle specific fashion instead of a fibre type dependent manner.

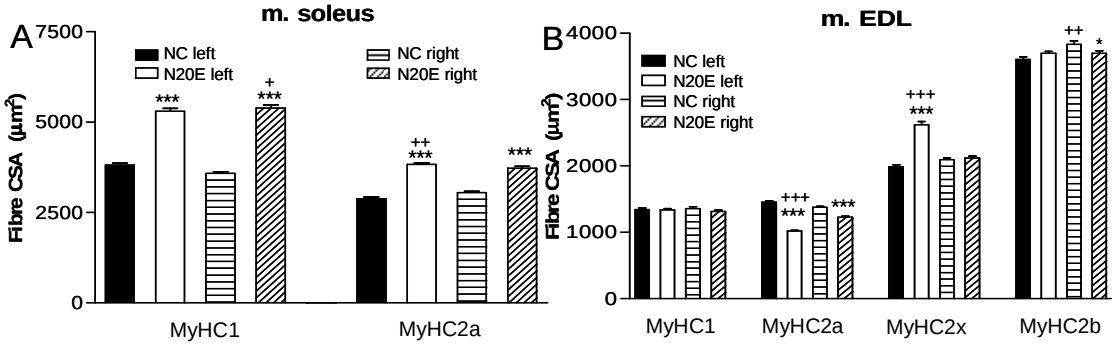


Fig. 9A/B The size of MyHC fibre types in the left and right soleus and EDL muscles of rats in group **N20E** treated with 20E in the left leg. Symbols * p<0.05, *** p<0.001 compared to the control, + p<0.05, ++ p<0.01, +++ p<0.001 compared to the change in fibre size of the corresponding muscle in the other hindlimb.

*The effect of 20E (**1**) on the contralateral hindlimb*

The fibre sizes in the treated (left) and non-treated (right) hindlimbs of group **N20E** were compared with those of the corresponding hindlimbs in the control. Interestingly, the size of the same fibre types was different in the left than in the right hindlimb muscles even in

the control group (**Fig. 9**). In group **N20E**, the type I fibres were larger (p<0.05) in the right than in the left soleus while the IIA fibres became larger in the left than in the right soleus (p<0.01) compared to those in group NC (**Fig. 9A**). In the EDL, the CSA of type I fibres were not different from the control but the IIA fibres became smaller after the **1** treatment in the left than in the right muscle. Moreover the size of IIx fibres increased in the left (p<0.001) but not in the right EDL, while the size of the IIB fibres was decreased in the right but not in the left EDL compared to the controls (**Fig. 9B**). This shows that the distance from the site of the 20E treatment blunted the decrease of IIA fibres and the increase of IIx fibres in the EDL.

Regenerating muscle model

The soleus muscle consisted of entirely new myofibres after five days of the notexin treatment, 98% of them expressed slow myosin [104]. The size of the regenerating fibres has been increased by **1** in group **R20E1** (**Fig. 10A**). Injection of a ten times lower dose of **1** (0.5 mg/kg BW) for 7 days into rats (group **R20E2**) increased the fibre size only in the regenerating soleus, but this increase was less pronounced (p<0.001) than in the animals treated with 5 mg/kg BW **1** (group **R20E1**) (**Fig. 10B**). This showed that the effect of **1** was dose dependent and the 0.5 mg/kg BW was still an effective dose on the regenerating soleus but not on the fibres of normal muscles in the contralateral leg (data not shown).

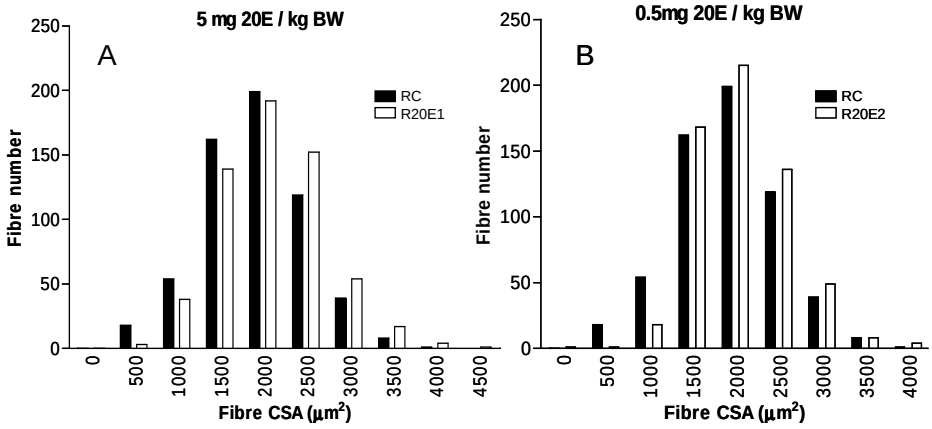


Fig. 10. Fibre size distribution of regenerating m. soleus after administration of **1** in a dose of 5 mg/kg BW (**A**) and 0.5 mg/kg BW (**B**). Mean fibre CSA of group RC is 1714±28.34 µm^2. Mean fibre CSAs of groups **R20E1** and **R20E2** are 2077±24.58 µm^2 and 1857±21.40 µm^2, respectively. Data are means ± SE. Note that the number of larger muscle fibres is increased by the **1** treatment in a concentration dependent manner in the regenerating muscles.

The regenerating soleus modified the effect of **1** on fibre size of normal muscles. The relative changes in CSA of fibre types in soleus and EDL of the right hindlimb of group **R20E1** and **N20E** were different compared to the corresponding controls, **RC** and **NC** (**Fig.**

11A and **B**). The increase of type I fibre sizes in the right soleus was smaller (p<0.001) in the **R20E1** than in the **N20E** group. A smaller but still significant difference was also found between the relative changes of the type IIA fibres of the right soleus muscles of the two groups (p<0.05). In the EDL the type I fibres were not changed compared to controls. The IIA fibres were more decreased in **R20E1** than in the **N20E** group (p<0.001). Interestingly, the size of IIx fibres was increased only in the **R20E1** group while the size of IIB fibres increased in the R20E1 group and decreased in the **N20E** group. This showed that the presence of a regenerating soleus in the rat modifies the effect of **1** on the size of fibre types.

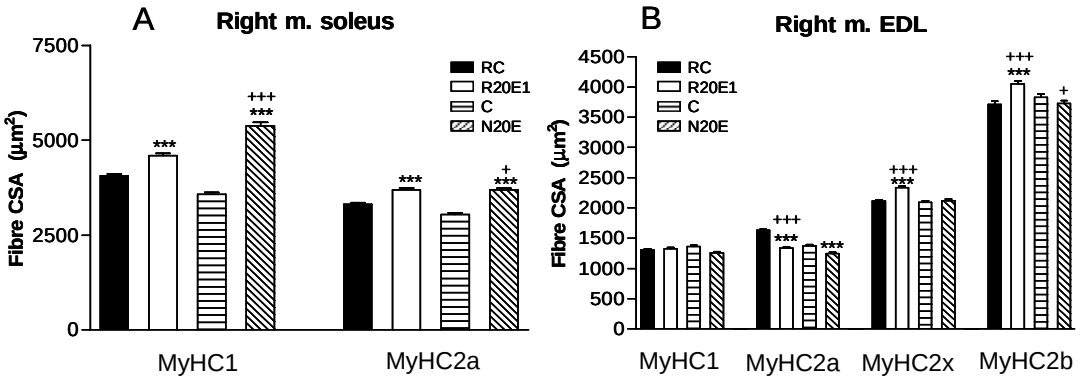


Fig. 11. The presence of a regenerating soleus in the left hindlimb modifies the effect of **1** in the contralateral normal soleus (**A**) and EDL (**B**). *** p<0.001 compared to the corresponding control. + p<0.05, +++ p<0.001 compared to the change in fibre size of the corresponding muscle of the other group of rats with or without notexin treatment.

Muscle atrophy model

Fibre type distribution was not changed by any of the treatments. In general the diaphragm consisted of 40% type I, 39% type IIa and 27% type IIx and 4% IIb fibres. As it was expected the methylprednisolone treatment did not alter the size of the type I (723.8±11.90 μm^2) and type Iia fibres (874.±10.02 μm^2) in the **MP** group compared to the control (734.5±8.54 μm^2; 827.7±12.68 μm^2, respectively) in the **MC** group, while the corticosteroids cause the selective atrophy of the IIb/IIx fibres [105]. **1** and methylprednisolone together also did not cause any changes in the size of these two fibres in the **M20E** group (722.5±5.58 μm^2; 8.42.1±6.75 μm^2, respectively). On the contrary, the CSA of the IIx fibres decreased significantly (p<0.001) in the **MP** group (2480±39.47 μm^2) compared to the **MC** group (2740±36.65 μm^2), whereas there was no difference in the mean CSA between the **ME** and **MC** groups (2845±32.86 μm^2 vs. 2740±36.65 μm^2; p>0.05). A significant decrease (p<0.001) was also observable in the mean CSA of the IIb fibres in the

MP (3514±52.47 μm^2) and slightly less decrease (p<0.01) in the **M20E** group (4061±44.40 μm^2) compared to the **MC** group (4292±57.77 μm^2). Difference in the size of the Iib fibres between the **MP** and the **M20E** group was also highly significant (p<0.001) with a higher mean CSA value in the **M20E** group (**Fig. 12**)

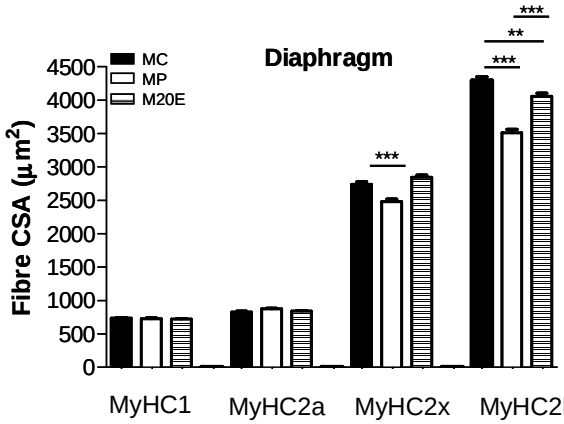


Fig. 12. CSA of the different fibre types of the diaphragm. ** p<0.01, *** p<0.001.

Myonuclear number

The numbers of myonuclei followed the increase of cross sectional areas in most muscles after the **1** treatment. This meant that the size of myonuclear domains did not change, except in group **N20E** when an increase (p<0.01) in the right soleus and decreases in large fibres (IIx and IIB) in EDL on both sides were found (**Table 5**). The positions of myonuclei did not change in response to the **1** treatment, i.e. they remained peripheral in normal and central in regenerating muscles.

Fibre type proportion

The relative fibre type proportion did not change in the muscles of any of the groups. **1** also did not influence the formation of predominantly slow type fibres in the regenerated soleus.

Table 5. Myonuclear numbers and domains in **1** treated muscles.

Myonuclei / fibre						
m. soleus		**m. EDL**				
Right	**Left**	**Large fibres**		**Small fibres**		**Regenerating m. soleus**
		Right	**Left**	**Right**	**Left**	
			NC			
0.88±0.06	0.91±0.03	1.590±0.05	1.563±0.03	0.45±0.017	0.52±0.013	-
			N20E			
0.87±0.01	1.09±0.03*	1.96±0.07*	2.04±0.08**	0.44±0.02	0.45±0.02	-
			RC			
0.92±0.05	-	1.67±0.05	-	0.42±0.018	-	1.57±0.06
			R20E1			
0.96±0.04	-	2.00±0.13	-	0.52±0.01**	-	1.91±0.06**
			R20E2			
-	-	-	-	-	-	1.82±0.03**
Myonuclear domain (μm^2)						
			NC			
4405±81.73	4481±179.9	1808±96.41	1759±55.42	3072±197.6	2713±168.4	-
			N20E			
5532±306.0*	4493±145.1	1447±81.16*	1539±42.02*	2860±81.22	2613±6.4	-
			RC			
4011±167.1	-	1774±221.4	-	3449±260.30	-	1234±55.63
			R20E1			
4378±89.0	-	1596±105.7	-	2590±134.20*	-	1082±32.75
			R20E2			
-	-	-	-	-	-	1028±104.2

*, **, *** p<0.05, 0.01, 0.001 compared to the correspondent control (0.9% NaCl and notexin + 0.9% NaCl)

4. Discussion

The steps of the isolation and the type of separation methods used are dependent on the concentration of the ecdysteroids to be isolated. Our isolation process includes two important steps, such as solid-liquid extraction followed by the clean-up of the crude extract, and chromatographic fractionations. The whole isolation procedure was controlled by NP-

TLC. Since ecdysteroids are semipolar compounds, methanol is the most relevant solvent for their extraction, although it extracts a wide scale of compounds of different type. Apolar constituents such as chlorophyll or less oxidized terpenoids can be eliminated from the extract by using solvent-solvent partition, while polar contaminants, such as proteins or sugars can be precipitated with acetone [15]. Flash-CC on a C_{18} column is capable to extract the ecdysteroids from an aqueous phase. The column was washed with 30 % aqueous methanol to remove the remaining polar impurities of the extract. The ecdysteroids of our interest were eluted with 40%, 50% and 60% aqueous methanol. Flash-CC on C_{18} is an ideal, rapid clean-up method. The separation on the chemically bonded octadecyl silica is based on hydrophobic interaction, so the irreversible adsorption, which is characteristic to adsorption chromatography, could be avoided. Although the capacity of this stationary phase is lower than that of silica gel, but in this initial step overloading of the column did not cause any efficiency problem. The isolation of the main ecdysteroids, 20E (**1**) and polypodine B (**2**), which are present in *S. viridiflora* in relatively high concentration (0.1 % and 0.05% respectively dry weight, respectively) were crystallized with a multi-step procedure from 40% aqueous methanol fraction. The entire isolation process of **1** is economical and can easily be carried out; it requires only a minimum of technical setup and eliminates the need for complicated apparatus. In case of the isolation of minor ecdysteroids, a multi-step isolation protocol was developed. The clean-up was followed by some preparative-scale chromatography, including classical NP-CC on silica stationary phase, polyamide CC which provides a group separation between ecdysteroids and phenoloids, including flavonoids. The abundant amount of ecdysteroids, possessing alcoholic hydroxyl groups, were firstly eluted with water, whereas flavonoids, containing phenolic hydroxyl group remained adsorbed on the stationary phase, since they need a higher solvent force to be eluted. CN-CC permits a quick and convenient purification step because of its simplicity and as an advantage apolar solvent systems can be used such as *n*-hexane-acetone in our case. This successful method for the separation of apolar ecdysteroids, it is perfectly suitable for the ecdysteroid acetonides as well. The isolation procedure was improved by the use of RPC on silica adsorbent. Our aim was to simplify the earlier isolation work with the introduction of RPC and to study the role of RPC in this process. RPC is easy to carry out, and the ecdysteroids are in contact with the adsorbent layer only for a short time. The forced-flow method, driven by a centrifugal force provides faster and better separation. Therefore, the problem associated with adsorbent-assisted decomposition was minimized [106-108]. RPC is an inexpensive, effective tool for the separation of ecdysteroids, with low solvent usage and less time consumption. The final

purification was carried out with preparative scale normal- or reversed-phase HPLC depending on the polarity of the ecdysteroids to be isolated. In case of NP-HPLC dichloromethane and cyclohexane based solvent systems were used. Ecdysteroids bind to the silica stationary phase quite strongly so small amount of water at a maximum of 3% displayed the best selectivity and provided the best peak symmetry [40]. In RP systems C_{18} bounded columns provided efficient separation, where water-miscible organic solvents such as methanol or acetonitrile were used as mobile phases. The consecutive steps of whole separation procedure were based on the different characteristics of the ecdysteroids and the accompanying compounds, such as distribution between two immiscible phases, lipophilicity and adsorption to the stationary phase. After each step a selectivity change was carried out as such a reversed phase mode of separation was followed by normal phase techniques as illustrated in **Fig. 4**.

With combined chromatographic methods 11 known and 9 new ecdysteroids were isolated from the *S. viridiflora*. Among the components there are several 2-deoxyecdysteroids, such as 2-deoxy-20-hydroxyecdysone 3-*β*-D-glucopyranoside (**3**), 2-deoxypolypodine B 3-*β*-D-glucopyranoside (**4**), 2-deoxypolypodine B 22-*β*-D-glucopyranoside (**5**), 2-deoxypolypodine B 25-*β*-D-glucopyranoside (**6**), 2-deoxy-20,26-dihydroxyecdysone (**7**), 2-deoxy-20-hydroxyecdysone (**11**), 2-deoxy-integristerone A (**12**), 2-deoxy-26-hydroxy-polypodine B 20,22-acetonide (**16**), 5*α*,2-deoxy-20-hydroxyecdysone 20,22-acetonide (**18**) and 2-deoxy-20-hydroxyecdysone 20,22-acetonide (**19**), which are highly characteristic to the *Silene* genus. Several isolated components, as such 2-deoxy-20,26-dihydroxyecdysone (**7**), 26-hydroxypolypodine B (**9**), 20,26-dihydroxyecdysone (**10**), 2-deoxy-26-hydroxypolypodine B (**14**), 26-hydroxypolypodine B 20,22-acetonide (**15**), 2-deoxy-26-hydroxypolypodine B 20,22-acetonide (**16**) and 20,26-dihydroxyecdysone 20,22-acetonide (**17**), bears a 26-OH group. The presence of 26-hydroxylated ecdysteroids in nature is not unusual. They have previously been described in both some insects [16] and plant species from the *Silene* [23,109], *Ajuga*, *Leuzea* [110], *Vitex* [111], *Lychnis* [109] genus. Plants synthesize 26-hydroxylated ecdysteroids with greater structural variety than insects. 26-hydroxylated ecdysteroids frequently occur in the *Silene* species, including *S. viridiflora*, in 2-deoxy, 22-deoxy and further hydroxylated forms of ecdysone or as acetate derivatives of the basic molecules [26,28,30,109,112-114]. Other derivatives such as glycosides (**3**-**6**) and several ecdysteroid acetonides (**13** and **15**-**20**) were also isolated.

A comparison of the ecdysteroid profile of *S. viridiflora* cultivated in Hungary, Vácrátót and in the Republic of Uzbekistan, Tashkent is shown in **Table 6**. Regarding to the

ecdysteroid profile, similarity of *S. viridiflora* herbs, derived from the distinct ecological regions, could be ascertained. In most of the cases, either the parent compound or its derivative was detectable in both samples. Interestingly, the glycosylation pattern of the plants is slightly altered and instead of ecdysteroid acetonides, 2,22- and 3,22-diacetate derivatives were described from the Uzbekistanis sample [28].

Table 6. Comparison of the ecdysteroid profile of S. viridiflora grown in Hungary (H) and Uzbekistan (U). Data based on the following publications: [26,28,30,115].

	H	**U**	**H**	**U**	**H**	**U**	**H**	**U**
Ecdysteroids	***Parent compound***		***Derivative***					
			Glycoside		***Acetate***		***Acetonide***	
20-hydroxyecdysone	+	+	3-*β*-D-glu 22-*β*-D-glu					
polypodine B	+	+						
integristerone A	+	+					+	
2-deoxy-20-hydroxyecdysone	+	+	3-*β*-D-glu				5α 5*β*	
2-deoxypolypodine B			3-*β*-D-glu 22-*β*-D-glu 25-*β*-D-glu	3-*β*-D-glu				
2-deoxy-integristerone A	+							
22-deoxy-20-hydroxyecdysone		+						
2-deoxy-20,26-dihydroxyecdysone	+							
2-deoxy-26-hydroxypolypodine B							+	
26-hydroxypolypodine B	+	+					+	
20,26-dihydroxyecdysone	+	+			2,22-diacetate 3,22-diacetate		+	
makisterone C							+	

The number of the known ecdysteroid acetonides is continuously increasing. They have been reported from *Leuzea carthamoides* [116-118], *Vitex* [119], *Serratula* [120], *Rhaponticum* [121,122], and *Cyathula* [123] species and additionally from *S. brachuica* [124] but this is their first identification in *S. viridiflora*. Although ecdysteroid acetonides are widely reported as natural compounds from different sources, the question may arise whether these molecules are formed during the isolation process. Since we use acetone, as a convenient and common solvent, in the fractionated precipitation during the pre-purification process of the extracts, and in the separation on CN-silica, the natural origin of the ecdysteroid acetonides involves some uncertainty. According to the literature, ecdysteroid acetonide derivatives can not be formed only in the presence of acetone, the process needs a catalyst to promote the synthesis (phosphomolibdenic acid [125], toluene sulfonic acid [126] or $CuSO_4$ [127]). Taking these into consideration, since the lack of catalyst, ecdysteroid-acetonides were not able form in the early phase of our purification method. We also used

acetone during a latter isolation step in CC as well. CN-bounded silica was used as stationary phase and gradient elution was performed with *n*-hexane-acetone solvent systems. If these ecdysteroid acetonides are artificial products, they might have been formed during this process because the CN-phase (hypothetically) might have acted as a catalyst. To exclude this assumption, we performed an experiment to imitate the conditions of the column chromatographic procedure (see in chapter **2.5.1**). Acetonide formation was not found in any of the samples after 35 days by TLC or HPLC.

The genuine presence of the most abundantly isolated ecdysteroid acetonide, 2-deoxy-20-hydroxyecdysone 20,22-acetonide (**19**), was investigated by one NP- and two RP-HPLC chromatographic systems in the extract of *S. viridiflora*. The chromatograms were indicating with high probability, that the isolated acetonide were originally present in the methanolic plant extract. To provide definitive evidence, LC MS/MS measurements were performed to detect **19** in the extracts of four, independently collected *S. viridiflora* herb samples. The base peak observed for compound **19**, when analyzed by ESI with the triple-quadrupole mass spectrometer corresponded to the protonated molecule (*m/z* 505). In further analyses two different SRM transitions, *m/z* 505 → 429 and 505 → 411, could be monitored indicating the loss of the acetonide group and one or two water molecule, respectively. The major fragment ions of compound **19** were identified in all four plant extracts allowing the conclusion that the dried herbs of *S. viridiflora* did originally contain ecdysteroid acetonide. Thus compound **19** is not an artefact formed during the isolation process. This might also indicate, that the other ecdysteroid acetonides isolated from *S. viridiflora* (**13**, **15**-**18** and **20**) are, most probably, also genuine compounds.

Investigating the effect of **1** on rat muscles we could demonstrate that **1** affects the size of fibre types in a different manner in the soleus than in the EDL muscles. This suggests that this compound affects fibre type size in a muscle-specific fashion. A muscle specific effect on fibre size has also been reported in case of anabolic–androgenic steroids (AAS). Treatment with a low (1.5 mg/kg BW/week) and a high (7.5 mg/kg BW/ week) therapeutic dose of nandrolone decanoate over a 5 weeks period increased the dimension of type IIx and IIB fibres in the diaphragm and of type IIa fibres in the gastrocnemius, while the other fibre types remained unchanged [128]. In humans, where the MyHC2b is hardly expressed in type II fibres, a longterm AAS administration (power lifters taking anabolic supplements for nearly 10 years) increased the CSA of type I and type II fibres both in m. vastus lateralis and m. trapezius, while no alteration in the fibre type proportions was found [129-131]. Similar observations were made in healthy young men when the diameter of both type I and II fibres

were increased after 20 weeks supplementation of gonadotrop-releasing hormone agonist (to suppress endogenous testosterone release) and of exogene testosterone [132]. Although the effect of **1** on these muscles is to be determined, it appears that both anabolic steroids and ecdysteroids are acting on the size of fibres in a multiple, muscle-specific fashion. However, it should be noted that AAS are effective in a lower dose than **1**. It is another difference that **1** did not alter the fibre type proportion in the studied muscles as the anabolic steroids [133].

Comparing our findings with the effect of vitamin D on skeletal muscle fibres, we can conclude that vitamin D supplementation is able to increase the CSA, but also the proportion of type II fibres in humans and its deprivation acts conversely (reviewed in [134]). In case of 12 vitamin D–deficient patients, atrophy of type II muscle fibres in the quadriceps muscle was found before vitamin D treatment and a significant improvement after treatment [135]. Muscle biopsies from m. vastus lateralis of older patients with osteoporosis, before and after administration of a vitamin D analog together with 1000 mg of calcium for 3–6 months, showed significant increases in both the percentage and area of type IIA and IIB fibres, despite the lack of any physical training [136]. Two years of treatment with even a low dose of vitamin D (1000 IU of 1,25D / day) significantly increased muscle strength, the mean diameter, and the percentage of type II muscle fibres in nonparetic limbs of 48 severely vitamin D–deficient elderly hemiplegic women [137]. The placebo control group suffered declines in muscle strength and in the size and percentage of type II muscle fibres.

The fast anabolic effect of AAS and vitamin D in skeletal muscle is exerted through signal transduction pathways and not via intracellular steroid receptors [138,139] as reviewed in [4] and [98]. The overexpression of calcineurin, a central player in signalling in muscle, influences fibre phenotype and differentiation in a muscle-specific fashion [140]. This is in line with the muscle-specific effects of AAS and ecdysteroids on fibre types and it implies that ecdysteroids might act on skeletal muscle via signal pathways. In accordance with this, ecdysteroids have been shown to act on signal mechanisms of mammalian hematopoietic [94], human colon carcinoma [96] and cultured mouse myoblast cells [67]. **1** showed a more pronounced effect in the treated left hindlimb (on IIx and IIB fibres of the EDL) than in the untreated right hindlimb, probably because of the distance from the site of administration. This shows that **1** acts locally and via the circulation. **1** increased fibre CSA in the regenerating soleus muscle, suggesting that **1** exerted a beneficial effect on muscle regeneration. A similar effect of AAS (nandrolone) has been reported on the regenerating soleus but not on the EDL [141]. However, in our experiments, the regenerating soleus muscle in the left hindlimb influenced the effect of **1** in the contralateral hindlimb compared

to the animals without regenerating soleus. This cannot be explained by the overload of the right hindlimb (caused by the retained use of the left hindlimb), since overload increases the size and the number of type I fibres [142], unlike it happened in the right hindlimb. It is more likely that the regenerating soleus influences the growth factor environment which interacts with the effect of **1** on the right EDL. In support of this, one example is known when ecdysteroids altered the signalling by interleukin-3 in mammalian hematopoetic cells [94]. A 10 times lower dose (0.5 mg/kg BW) of **1** induced a lower increase of the fibre CSA in the regenerating soleus but had no effect on the fibres in the contralateral normal muscles. However the low dose of **1** increased the weight of the EDL, suggesting dose dependence and a difference in the mechanism of action of a low and high dose of **1**. 20E (**1**) increased the myonuclear number in most of the affected muscles. Muscle fibres also grow in response to AAS with increasing myonuclear number [130]. The myonuclei derive from the accretion of myoblasts produced by the activated satellite cells (muscle resident mesenchymal stem cells), and help to maintain gene expression within the fibre. **1** increased the myonuclear number in proportion to the fibre growth, therefore maintained the size of the myonuclear domains (the sarcoplasm volume around the myonuclei). This suggests that stimulation of fibres size by **1** involves the activation of satellite cells similar to AAS (reviewed by [143]).

As it is already known, that muscle atrophy, caused by corticosteroids, shows fibre-type specification in the rat diaphragm, causing the selective atrophy of type IIB and IIx fibres, but not the type I or IIA fibres [105]. Our study demonstrated that **1** is able to prevent the diaphragm from the atrophying effect of the methylprednisolone. The co-administration of **1** with methylprednisolone in group **M20E** did not decrease the CSA of the IIx fibres, and the decrease of the size of type IIB fibres were remarkably less, than in the **MP** group where the methylprednisolone was administered alone. **1** did not alter the size of the type I and IIA fibres of the diaphragm. It seems that **1** neutralized the atrophying effect methylprednisolone on the IIx and attenuated it in case of the IIB fibres. Muscle specific effect of the anabolic-androgenic steroids on fibre size of the diaphragm has also been reported. Interestingly, the administration of low (1.5 mg/kg BW/week) or high dose (7.5 mg/kg BW/week) of nandrolone decanoate for a 5 weeks period affected only type IIx and IIB fibres in case of rats [128], similar to the case observed after treatment with **1**. Another study could also show that the administration of nandrolon decanoate in low dose (1 mg/kg BW/week) in the last 3 months of a 9 months long, low dose methylprednisolone (0.2 mg/kg BW/day) treatment in rats could reverse the size atrophy of the IIx fibres size in the rat diaphragm [72]. While type IIx and IIB fibres are responsible for the maximal force production in the rat diaphragm, the

atrophy of these fibre types results in the reduction of force generation [72,144], but this was completely abolished by the administration of the anabolic-androgenic steroid [72]. While the above mentioned experiments were long in duration and mostly focused on the effect of AAS on normal diaphragm, or on the reversal of the evolved morphological changes in the diaphragm in consequence of the glucocorticoid treatment, a shorter study was designed by Eason *et* al. They co-administered a higher glucocorticoid (5 mg/kg BW/day) and testosterone dose (0.5 mg/kg BW/day) in order to examine the atrophy preventing and not the reversing ability of the AAS [71]. They could conclude that the simultaneous administration of testosterone with glucocorticoids partially prevents the body and diaphragm weight loss, which also resulted in the complete reversal of the glucocorticoid induced loss in the total force generation [71]. However, it has to be noted that in most of the studies, the AAS were effective in much lower dose than **1**. In contrast, in the present study we administered the methylprednisolone and **1** in a higher but in equal doses (both 10 mg/kg BW/day) only for 5 days. Similar to [71], who also administered testosterone and prednisolone in the same dose (both 5 mg/kg BW/day for 10 days), we also could achieve partial prevention of the deleterious effect of the glucocorticoid treatment such as the complete inhibition of the type IIx and partial inhibition of type IIB fibre atrophy. Based on the results of Eason *et* al. [71] this might mean that **1** is also able to prevent the decrease in the total force production of the diaphragm caused by the glucocorticoid treatment. Unfortunately, our contractile property measurement on the diaphragm such as force-frequency curve and fatigue run, did not gave significant differences between the control and treated groups **C** vs. **MP** or **M20E**, **MP** vs. **M20E** (see method and results in the Appendix). There might have been a problem with treatment duration or dosage since the diaphragm did not show any alteration in the contractile properties after the treatment with methylprednisolone alone (**MP** vs. **C**). A definitive final conclusion in this matter can not been drawn. Further experiments with longer duration or with higher dose are needed to fully establish the effect of **1** on diaphragm force in order to connect the hystochemical findings with functional outcomes.

5. Summary

Our results may be summarized as follows:

1. Isolation

- Twenty ecdysteroids were isolated and characterized from the herb of *S. viridiflora*, twelve of them was reported for the first time from this plant.
- Nine of the compounds have been discovered for the first time in a natural source.
- **1** has been successfully isolated in two steps, involving crystallisation from the *S. viridiflora* herb extract and used for *in vivo* animal experiments.

2. Structure characteristics of the isolated ecdysteroids

- Several of the isolated compounds are 2-deoxyecdysteroids and/or 26-hydroxy derivatives which is characteristic to the *Silene* genus.
- Eight of the ecdysteroids bear acetonide group(s), which decreases the polarity and thus changes their chromatographic behaviour and compared to the rather polar ecdysteroid derivatives, makes their isolation easier.

3. The genuineness of ecdysteroid acetonides has been verified via indirect and direct methods in *S. viridiflora* herb extract:

- The presence of the most abundantly present acetonide derivative, 2-deoxy-20-hydroxyecdysone 20,22-acetonide (**19**) was proved with NP- and RP-HPLC and LC–MS/MS in extracts of four, independently collected *S. viridiflora* herb.

4. *In vivo* effect of **1** on rat skeletal muscle

- **1** modifies muscle fibre size in normal and regenerating muscles even after 7 days administration in a slightly higher dose than the anabolic steroids.
- In case of the regenerating muscle, the effect of **1** was different in the two applied doses, which might indicate a dose-dependent action.
- Similarly to that of anabolic steroids and vitamin D, **1** influenced the size of fibre types in a muscle-specific fashion.
- **1** probably acts in concert with other growth factors because its effect on normal muscles is modified by the presence of a regenerating soleus.
- After five days treatment, **1** was able to prevent the IIB and IIx fibres of the diaphragm, from the atrophying effect of the methylprednisolone, even if they were administered in the same concentration as the applied glucocorticoid. This effect was obvious considering the size of the CSAs of the diaphragm fibres, but was not apparent in the diaphragm contractile properties.

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Abbreviations

1,25D = 1α,25dihydroxyvitamin D_3, calcitriol
1D = one dimensional
20E = 20-hydroxyecdysone
2D = two dimensional
AAS = anabolic-androgenic steroid
ACN = acetonitrile
Akt = protein kinase B
AP = alternative ligand binding pocket
APT = attached proton test
BW = body weight
CC = column chromatography
CH_2Cl_2 = dichloromethane
CN = cyano silica
COSY = correlation spectroscopy
CSA = cross-sectional area
DEPT = distorsionless enhancement by polarization transfer
diaph. = diaphragm
DNA = deoxyribonucleic acid
EDL = extensor digitorum longus
ESI = electrospray ionisation
ESIMS = electrospray ionisation mass spectrometry
EtAC = ethyl acetate
EtOH = ethanol
FID = free induction decay
fr. = fraction
G = genomic
GP = genomic pocket
H_2O = water
HCOOH = formic acid
HMBC = heteronuclear multiple bond coherence spectroscopy
HMQC = heteronuclear multiple quantum coherence spectroscopy
HPLC = high performace liquid chromatography
HRMS = high resolution mass spectrometry
i.m. = intra muscular
i.p. = intra peritoneal
i.v. = intra venous
i-PrOH = isopropanol
IR = infrared spectroscopy
LBD = ligand binding domain
LC-MS/MS = liquid chromatography coupled with tandem mass spectrometry
m. = musculus / muscle
MAPK = mitogen-activated protein kinase
MeOH = methanol
MS = mass spectrometry
MyHC = myosine heavy chain
NG = non-genomic
NH_4OH = ammonium hydroxide
NMR = nuclear magnetic resonance spectroscopy
NOESY = nuclear Overhauser-effect enhancement spectroscopy
NP = normal phase
NR = nuclear receptor
p.o. = per os / oral
PI3K = phosphoinositide 3-kinase
PKC = protein kinase C
Po = polyamide
RNA = deoxyribonucleic acid
RP = reversed phase
RPC = rotation planar chromatography
S. p. = stationary phase
s.c. = sub cutan
s. s. = solvent system
SRM = selected reaction monitoring
THF = tetrahydrofuran
TLC = thin layer chromatography
TOCSY = total correlated spectroscopy
UV = ultraviolet
VDR = vitamin D receptor
VDS = vitamin D sterol
XX = repeated crystallization

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Appendix

Measurement of *ex vivo* contractile properties of the diaphragm

Experimental

Contractile properties

From the properly dissected diaphragm (see in 2.7.1), two small rectangular bundles from the middle part of the lateral costal region were obtained by dissection parallel to the long axis of the fibers. Silk sutures were tied to both ends of the bundle to serve as anchoring points. Each bundle was then placed within the external chamber of a jacketed tissue bath containing Krebs solution, was maintained at 37ºC, and was perfused with a 95% O_2-5% CO_2 mixture. The Krebs solution was changed with each new bundle. One end of the bundle was tied to a rigid support, and the other end was fastened to an isometric force transducer mounted to a micrometer. The muscle was placed between two large platinum stimulating electrodes. The bundles were placed at their optimal length (L_o), defined as the length at which the peak twitch force was obtained. This was followed by a 15 min thermoequilibration period. Stimulations were delivered through a Harvard 50-5016 stimulator (Edenbridge, Kent, UK) connected in series to a power amplifier from power one model HS24-4.8 (R. J. Evans, University of Virginia). Stimuli were applied with a pulse duration of 0.2 ms and a train duration of 250 ms. When the maximum twitch force (P_t) was achieved, the voltage was then increased to 120% (80-100 V) to ensure supramaximal stimulation. This voltage was subsequently used during all stimulations. Isometric force was measured by means of a force transducer (Maywood, Hampshire, UK). The signal was amplified and recorded on a computer via analog-to-digital conversion (DT2802-A) with Labdat software (Labdat/Anadat, RHT-InfoDat, Montreal, Quebec, Canada). Signal analysis was done with Anadat.

The following four measurements were performed.

Twitch characteristics. Two twitches were recorded at L_o to determine P_t, contraction time (CT), and half-relaxation time ($RT_{1/2}$). Average values were used for further analysis.

Maximal tetanic force (P_o)

Bundles were stimulated twice tetanically at 160 Hz for 250 ms to obtain a clear plateau in force generation. The values were expressed corrected for muscle CSA (see below).

Force-frequency curve

Bundles were stimulated at the following frequencies: 25, 160, 50, 160, 80, 160, 120, and 160 Hz. Each stimulus was separated by a 2 minutes interval. Stimulation at 160 Hz was

interposed between each stimulus frequency during the force-frequency curve to relate force output at a certain stimulus frequency to the maximal achievable force output at that moment because some decline in maximal force generation during the force-frequency protocol always occurs.

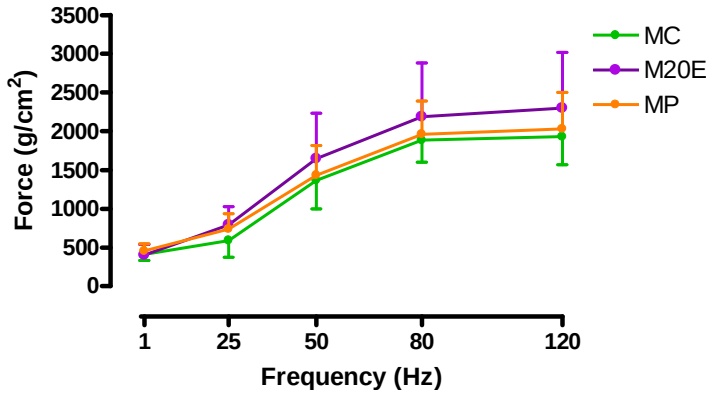
Fatigue properties

Fatigability was assessed as the following. The force output at 160 Hz after each stimulus frequency during the force-frequency curve was measured. After these measurements, each muscle bundle was removed from the bath while its L_o was kept similar to the experimental condition. Subsequently, its L_o, thickness, and width were measured. The bundle was blotted dry and weighed. CSA was calculated by dividing weight by specific density (1.056) and muscle L_o. P_t and P_o, were expressed per unit CSA. The twitch-to-tetanic ratio ($Pt/P_{o)}$ was calculated for each muscle bundle. Finally, the remaining diaphragm tissue was trimmed, blotted, and weighed.

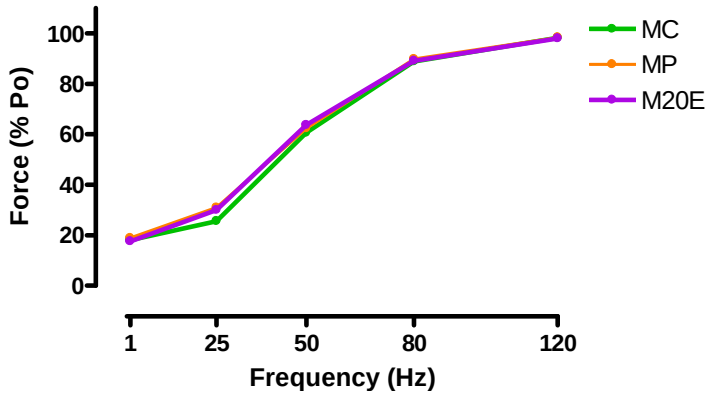
Results

Diaphragm force-frequency curve: not affected by any treatment

a) in absolute values

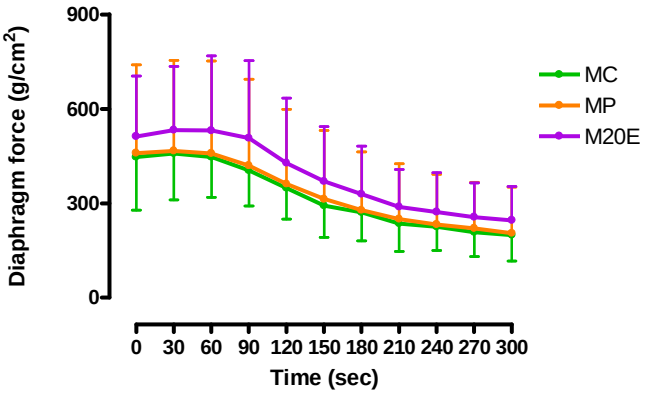


b) as a percentage of tetanic tension

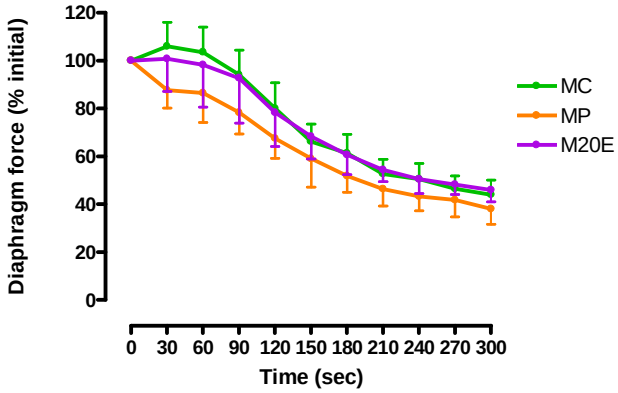


Fatigue run: not affected by any treatment

a) in absolute values



b) as a percentage of initial values



NMR data of the new compounds

Table 7. NMR data of new compounds.

2-deoxypolypodine B 22-*β*-D-glucopyranoside (5)

No.		^{13}C	^{1}H	m	*J* (Hz)	HMBC	NOESY
1	**α**	25.6	1.49			81.0, 67.2, 38.1	
1	**β**		1.97				4.08
2	**α**	29.3	1.77			67.2, 43.4	
2	**β**	67.2	4.08	s		-	1.97, 2.03
3	**α**	36.8	2.03			81.0, 29.3	3.28, 4.08
4	**α**		1.60			81.0, 67.2, 43.4	
4	**β**	81.0	-	-	-	-	-
5		202.9	-	-	-	-	-
6		120.7	5.85	d	2.4	85.3, 81.0, 38.1	-
7		168.2	-	-	-	-	-
8		38.1	3.28				2.03
9	**α**	43.4	-	-	-	-	-
10		22.5	1.73			49.1	
11	**α**						0.89
11	**β**		2.12	td	12.0, 6	48.7, 18.2	2.36
12	**α**	32.9	1.87	d	12.6	86.9, 48.7, 34.2, 21.7	0.89
12	**β**	49.1	-	-	-	-	-
13		85.3	-	-	-	-	-
14		32.0	1.61			85.3, 49.1	1.98
15	**α**		1.98			85.3, 51.2	1.61
15	**β**	21.6	1.71			49.1, 32.0	1.949, 2.36
16	**α**		2.03			85.3, 77.6, 51.2	1.71, 0.91
16	**β**	51.2	2.36	dd	9, 8.4	49.1, 21.6, 18.3	1.71, 1.74, 2.12, 1.24
17	**α**	18.3	0.91	s	-	85.3, 51.2,49.1, 32.9	1.24, 1.87, 1.73,
18		17.3	0.89	s	-	81.0, 43.4, 38.1, 25.6	1.73, 1.49
19		77.6	-	-	-	-	-
20		22.5	1.24	s	-	105.9, 77.6, 51.2, 41.1	3.49, 0.89, 2.36
21		89.9	3.49	d	9.6	105.9, 77.6, 51.2, 41.1, 27.7, 22.5	2.03, 1.74, 1.57, 1.44, 1.24
22		27.7	1.57			41.1	3.49
23	**a**		1.74			41.1	3.49, 2.36
23	**b**		1.44	td	12.6, 4.2	71.5, 89.9, 27.7, 29.2, 29.8	3.49
24	**a**	41.1	2.03			71.5, 89.9, 27.7, 29.2, 29.8	3.49
24	**b**	71.5	-	-	-	-	-
25		29.2	1.19	s	-	71.5, 41.1, 29.8	
26		29.8	1.20	s	-	71.5, 41.1, 29.2	
27		105.9	4.34	d	7.8	89.9, 67.2	3.33
1'		75.6	3.26	dd	9.0, 8.4	102.9, 78.6	3.35
2'		78.1	3.37			75.4, 71.8,	
3'		71.6	3.35			78.0, 62.9	3.26
4'		78.2	3.33				
5'		62.6	3.68	dd	12.0, 5.4	71.8, 78.0	3.88
6'	**a**		3.88	d	12.0	71.8	3.68, 3.33

2-deoxypolypodine B 25-*β*-D-glucopyranoside (6)

No.		^{13}C	^{1}H	m	*J* (Hz)	HMBC	NOESY
1	**α**	25.6	1.49				
	β		1.82				
2	**α**	29.25	1.97				
	β		1.77				
3	**α**	67.2	4.08	s	broad	81.0	2.03, 1.97, 1.77, 1.61
4	**α**	36.8	2.03				
	β		1.61				
5	**β**	81.0	-	-		-	-
6		202.9	-	-		-	-
7		120.7	5.86	d	2.1	85.4, 81.0, 39.2	1.97, 1.61, 0.895
8		168.15	-	-		-	-
9	**α**	38.1	3.28			168.2, 43.4, 22.4, 17.3	2.03, 1.97, 1.72
10		43.4	-	-		-	-
11	**α**	22.44	1.72				
	β		1.72				
12	**α**	32.8	2.13	td	12.3, 5.9	-	2.39
	β		1.87				
13		48.8	-	-		-	-
14		85.4	-	-		-	-
15	**α**	31.9	1.60			85.4	
	β		1.97			85.4	
16	**α**	21.6	1.75				
	β		1.97				
17	**α**	50.5	2.39	dd	9.3, 8.4	48.8, 21.6, 18.2	2.13, 1.73, 1.19
18	**β**	18.2	0.896	s		85.4, 48.8, 50.5, 32.8	1.98, 1.87, 1.73?
19	**β**	17.3	0.892	s		81.0, 43.4, 38.1, 25.6	1.73, 1.50
20		78.11	-	-		-	-
21		21.2	1.19	s		78.6, 78.05/78.11, 50.5	2.39, 1.88, 1.43
22		78.6	3.325	overlap		40.3, 26.9, 21.2	
23	**a**	26.9	1.43				
	b		1.70				
24	**a**	40.3	1.48				
	b		1.93				
25		78.8	-	-		-	-
26		27.5	1.27	s		78.8, 40.3, 27.5	4.46, 1.93, 1.71, 1.48
27		27.5	1.28	s		78.8, 40.3, 27.5	4.46, 1.93, 1.71, 1.48
1´		98.8	4.456	d	7.7	78.8	
2´		75.46	3.14	dd	9.1, 8.0	98.8, 78.35	
3´		78.35	3.36	t	8.8	75.5, 72.0	
4´		72.0	3.22				
5´		78.05	3.26				
6´	**a**	63.3	3.61	dd	11.9, 5.9	78.05/78.11	
	b		3.845	dd	11.9, 1.9	72.0	

Integristerone A 20,22-acetonide (13)

No.		^{13}C	^{1}H	m	*J* (Hz)	HMBC	NOESY
1	**α**	76.6	3.82		broad		3.08, 1.72, 1.08
2	**α**	68.6	3.87	t	3		4.04, 3.08
3	**α**	71.1	4.04	s	broad		3.87, 1.77
4	**α**	33.7	1.77			46.9	4.04
	β						
5		46.9	2.61	dd	12.9, 4.5	205.8, 122.3, 44.4, 35.8, 20.2	1.77, 1.08
6		205.8	-	-	-	-	-
7		122.3	5.84	d	1.8	85.5, 46.9, 35.8	1.97, 1.62
8		167.0	-	-	-	-	-
9	**α**	35.8	3.08				3.87, 3.82
10		44.0	-	-	-	-	-
11	**α**	22.1	1.72				
11	**β**						
12	**α**	32.5	2.09	td	12.6, 4.8	48.7, 22.5, 17.8	2.31, 1.84
	β		1.84			85.5, 48.7, 35.8, 22.1	2.09
13		48.7	-	-	-	-	-
14		85.5	-	-	-	-	-
15	**α**	31.9	1.62	dd	10.8, 10.2	85.5, 48.7	5.84, 1.97
	β		1.97	dd	12.6, 6.6	85.5, 22.5	5.84, 1.62, 0.84
16	**α**	22.5	1.86			48.7	3.68, 2.02
	β		2.02			86.0, 85.5, 50.7, 31.9	3.68, 1.86, 0.84
17	**α**	50.7	2.31	dd	9, 8.4	83.5, 48.7, 32.5, 22.5, 22.7, 17.8	3.68, 2.09, 1.86, 1.18, 1.53
18		17.8	0.84	s	-	85.5, 50.7, 48.7, 32.5	2.02, 1.97, 1.84, 1.72,1.18
19		20.2	1.08	s	-	76.6, 46.9, 44.4, 35.8	3.82, 2.61, 1.72
20		86.0	-	-	-	-	-
21		22.7	1.18	s	-	86.0, 83.5, 50.7	2.31, 1.86, 1.53, 1.39, 0.84
22		83.5	3.68	dd	8.4, 3.6	86.0, 50.7, 42.4, 24.9, 23.7	2.31, 2.02, 1.86, 1.53, 1.32
23	**a**	24.9	1.53			83.5, 71.3, 42.4	1.18
	b						
24	**a**	42.4	1.48			83.5, 71.3, 29.6, 29.6, 24.9	1.73, 1.53, 1.20, 1.21
	b		1.73			83.5, 71.3, 29.1, 29.6, 24.9	
25		71.3	-	-	-	-	-
26		29.1	1.20	s	-	71.3, 42.4, 29.6	
27		29.6	1.21	s	-	71.3, 42.4, 29.1	
28		108.2	-	-	-	-	
29		27.3	1.32	s	-	108.2, 29.5	3.68, 1.39
30		29.5	1.39	s	-	108.2, 27.3	1.18, 1.32

2-deoxy-26-hydroxypolypodine B (14)

No.		^{13}C	^{1}H	m	J (Hz)	COSY	HMBC
1	**α**	25.3	-	-	-		
	β		-	-	-		
2	**α**		-	-	-		
	β		-	-	-		
3	**α**	67.2	4.08	s	broad	2.05, 1.62	–
4	**α**		-	-	-		
	β		-	-	-		
5	**β**	81.0	-	-	-		
6			-	-	-		
7		120.7	5.85	d	2.2	3.27 (w)	–
8			-	-	-		
9		37.9	3.27	overlap			
10		43.3	-	-	-		
11	**α**		-	-	-		
	β		-	-	-		
12	**α**	32.6	-	-	-		
	β		-	-	-		
13		48.8	-	-	-		
14		85.2	-	-	-		
15	**α**		-	-	-		
	β		-	-	-		
16	**α**		-	-	-		
	β		-	-	-		
17	**α**	50.5	2.39	t	8.8	1.99, 1.75	
18	**β**	18.2	0.90	s		–	85.2, 50.5, 48.8, 32.6
19	**β**	17.2	0.89	s		–	81.0, 43.3, 37.9, 25.3
20		77.9	-	-	-		
21		21.1	1.19	s		–	78.8, 77.9, 50.5
22		78.7	3.34	overlap		1.32, 1.285, 1.24	
23	**a**		-	-	-		
	b		-	-	-		
24	**a**	37.2	-	-	-		
	b		-	-	-		
25		73.6	-	-	-		
26		70.8	3.37	s		–	73.7, 37.2, 23.6
27		23.6	1.14	s		–	73.6, 70.7, 37.2

26-hydroxypolypodine B 20,22-acetonide (15)

No.		^{13}C	^{1}H	m	J (Hz)	HMBC	NOESY
1	a	34.3	1.73				
	b		1.73				
2	α	68.6	3.95	ddd	10.0, 7.4, 3.6	-	3.19, 1.74
3	α	70.4	3.99	q	3.0	161.0	2.07, 1.77
4	α	36.3	2.075	dd	14.7, 3.0	-	3.99, 3.95 (gy), 3.19
	β		1.77	dd	14.9, 3.0	80.4, 70.4, 68.6, 45.5	3.99, 0.92, 0.83
5	β	80.4	-	-	-	-	-
6		202.5	-	-	-	-	-
7		120.7	5.86	d;	2.8	85.3, 80.4, 39.2	1.95, 1.61
8		167.4	-	-	-	-	-
9	α	39.2	3.19	ddd	11.3, 7.0, 2.7	167.4, 120.7, 70.4-7, 45.5, 22.5-7, 17.1	3.95, 2.08, 1.80, 1.73
10		45.5	-	-	-	-	-
11	a	22.65	1.74				
	b		1.81			167.4, 48.6	
12	α	32.6	2.12	td	13.1, 5.0	48.6, 17.8	2.31
	β		1.86				
13		48.7	-	-	-	-	-
14		85.3	-	-	-	-	-
15	α	31.8	1.61			85.3, 48.7	5.86
	β		1.96	dd	12.4, 6.5	22.5-7	5.86, 0.83
16	α	22.5	1.87				
	β		2.03			85.3	3.69, 2.31, 1.31, 0.83
17	α	50.5	2.31	dd	9.4, 8.1	83.6, 48.6, 32.6, 22.5-7, 17.8	3.70, 2.12, 2.02, 1.87, 1.53, 1.19
18	β	17.8	0.83	s		85.3, 50.5, 48.7, 32.6	2.04, 1.95, 1.87, 1.75, 1.18
19	β	17.1	0.915	s		80.4, 68.6, 45.5, 39.2, 34.3	2.13, 1.73
20		86.0	-	-	-	-	-
21		22.7	1.18	s		86.0, 83.6, 50.5	2.32, 1.86, 1.54, 1.39, 0.84
22		83.6	3.695	t	6.0	86.0, 50.7, 37.2, 22.7	2.32, 2.04, 1.89, 1.54, 1.32
23	a	24.05	1.53			83.6,	
	b		1.55			73.6,37.2, 29.5, 23.8-24.0	
24	a	37.2	1.52				
	b		1.71				
25		73.6	-	-	-	-	-
26	a	70.7	3.355	d	11.0	73.6, 37.2, 23.8	1.72, 1.53, 1.15
	b		3.375	d	11.0	73.6, 37.2, 23.8	
27		23.9	1.15	s		73.6, 70.7, 37.2	3.38, 3.35, 1.53
28		108.2	–	–		-	-
29		27.3	1.315	s		108.2, 29.5	3.70, 2.04, 0.83
30		29.5	1.39	s		108.2, 27.3	1.18

2-deoxy-26-hydroxypolypodine B 20,22-acetonide(16)

No.		^{13}C	^{1}H	m	J (Hz)	COSY	HMQC-TOCSY (only TOCSY)	HMBC	NOESY
1	a	25.6	1.50				1.98, 1.77		
	b		1.84						
2	a	29.3	1.77			-	4.08, 1.83, 1.52	-	-
	b		1.96						
3	β	67.2	4.08			2.02, 1.64	1.35?		2.05, 1.63
4	a	36.9	1.61			-	4.08, 2.33?, 2.23?	-	-
	b		2.035						
5	β	81.2	-	-	-	-	-	-	-
6			-	-	-	-	-	-	-
7		120.7	5.86			3.27	3.27	85.45, 81.2, 38.1	1.98, 1.62
8		167.9	-	-	-	-	-	-	-
9	α	38.1	3.28			1.72		167.9	
10		43.2	5-	-	-	-	-	-	-
11	a	22.5	1.73				3.28		
	b		1.73						
12	α	32.6	2.12	dd	12.5, 5.7				
	β		1.86						
13		48.7	-	-	-	-	-	-	-
14		85.4	-	-	-	-	-	-	-
15	α	31.8	1.61				2.32, 1.70?		
	β		1.97						
16	α	22.5	1.88				2.33		
	β		2.04						
17	α	50.6	2.32	t	8.7		2.04, 1.97		
18	β	17.8	0.834	s		-	-	85.4, 50.8, 48.7, 32.6	
19	β	17.3	0.887	s		-	-	81.0, 43.16-35, 38.1, 25.6	
20		86.0	-	-	-		-	-	-
21		22.7	1.18	s		-	-	86.0, 83.6, 50.8	
22		83.6	3.70			1.53	1.72, 1.54	-	1.88, 1.54, 1.29
23	a	24.05	1.55				3.70		
	b		1.55						
24	a	37.2	1.53				3.70		
	b		1.72						
25		73.6	-	-	-	-	-	-	-
26	a	70.7	3.36	d	11.0	-	-	73.6, 37.2, 23.8	
	b		3.37	d	11.0	-	-	73.6, 37.2, 23.8	
27		23.8	1.15	s		-	-	73.6, 70.7, 37.2	
28		108.2	-			-	-	-	-
29		27.3	1.32	s		-	-	108.2	
30		29.5	1.39	s		-	-	108.2	

20,26-dihydroxyecdysone 20,22-acetonide (17)

No.		^{13}C	^{1}H	m	J (Hz)	HMBC
1	**α**	37.5				
	β		-	-	-	
2	**α**		-	-	-	
3	**α**		-	-	-	
4	**a**		-	-	-	
	b		-	-	-	
5	**β**		2.42	dd	12.6, 4.0	-
6			-	-	-	-
7		121.8	5.81	d	2.6	
8			-	-	-	-
9	**α**		-	-	-	
10			-	-	-	-
11	**α**		-	-	-	
	β		-	-	-	
12	**α**	32.55	-	-	-	
	β		-	-	-	
13		49.3	-	-	-	-
14		85.5	-	-	-	-
15	**α**		-	-	-	
	β		-	-	-	
16	**α**		-	-	-	
	β		-	-	-	
17	**α**	50.5	2.33	dd	9.2, 8.6	–
18	**β**	17.8	0.826	s		85.5, 50.5, 49.3, 32.55
19	**β**	24.4	0.96	s		37.5
20		85.9	-	-	-	-
21		22.7	1.18	s		85.9, 83.5, 50.6
22		83.6	3.70	m		-
23	**a**		-	-	-	
	b		-	-	-	
24	**a**	37.1	-	-	-	
	b		-	-	-	
25		73.2	-	-	-	-
26	**a**	70.7	3.36	d	11.0	
	b		3.375	d	10.9	
27		23.7	1.15	s		73.2, 70.7, 37.1
28		108.0	-	-	-	-
29		27.3	1.32	s		108.1, 29.4
30		29.4	1.39	s		107.9, 35.25, 27.3

5α-2-Deoxy-20-hydroxyecdysone 20,22-acetonide (18)

No.		^{13}C	^{1}H	m	*J* (Hz)	HMBC	ROESY
1	**α**	38.0	1.41				
	β		1.87			71.3, 54.7	
2	**α**	31.5	1.79				
	β		1.39				
3	**α**	71.31	3.55	tt;	11.0, 4.4	-	2.36, 2.11, 1.79, 1.40
4	**α**	31.0	2.11				
	β		1.365	t			
5	**α**	54.7	2.355	dd	12.2, 3.6	203.25, 39.7, 13.4	3.55, 2.75, 2.11, 1.41, 1.36
6		203.25	-	-	-	-	-
7		123.6	5.83	d	2.8	85.3, 54.7, 47.6	1.94, 1.61, 0.84
8		166.5	-	-	-	-	-
9	**α**	47.6	2.75	ddd	11.7, 7.2, 2.7	166.5, 123.6, 39.7, 21.7	2.36, 2.11, 1.78, 1.41
10		39.7	-	-	-	-	-
11	**α**	21.7	1.79				
	β		1.635				
12	**α**	32.4	2.11			48.4, 21.7, 17.8	
	β		1.815				
13		48.4	-	-	-	-	-
14		85.3	-	-	-	-	-
15	**α**	31.9	1.615				
	β		1.95			22.5-7	5.84, 0.83
16	**α**	22.5	1.86			-	
	β		2.03			31.9, 22.7	3.69, 1.32, 0.83
17	**α**	50.7	2.30	dd	9.1, 8.6	85.3, 48.4, 32.4, 22.5, 17.8	3.69, 2.11, 1.84, 1.53, 1.178
18	**β**	17.8	0.826	s		85.3, 50.7, 48.4, 32.3	2.02, 1.94, 1.82, 1.63, 1.17
19	**β**	13.4	0.854	s		54.7, 47.6, 39.7, 38.0	1.87, 1.64, 1.39, 1.34
20		86.0	-	-	-	-	-
21		22.7	1.175	s		86.0, 83.5, 50.7	2.30, 2.11, 1.81, 0.83
22		83.5	3.685	m		42.4, 22.7	2.30, 2.03, 1.86, 1.50, 1.32
23	**a**	24.9	1.53				
	b		1.53				
24	**a**	42.4	1.485				
	b		1.74				
25		71.27	-	-	-	-	-
26		29.1	1.196	s		71.3, 42.4, 29.6	2.06, 1.74
27		29.6	1.205	s		71.3, 42.4, 29.1	2.06, 1.74
28		108.15	-	-	-	-	-
29		27.3	1.32	s		108.15, 29.5	3.69, 2.03
30		29.5	1.39	s		108.15, 27.3	1.20, 1.178

Makisterone C 2,3;20,22-diacetonide (20)

No.		^{13}C	^{1}H	m	*J* (Hz)	HMBC	NOESY
1	**α**	38.9	1.99	d			
	β		1.23	t			
2	**α**	73.8	4.27	dt	9.4, 5.0	109.6	2.935, 1.99
3	**α**	73.3	4.30	q		-	1.99, 1.33
4	**a**	27.8	1.98				
	b		1.98				
5	**β**	52.6	2.245	dd	9.3, 8.2	205.8, 122.0, 73.8, 73.3, 39.0, 38.9, 35.9, 28.0, 24.2	1.97, 1.23, 0.96
6		205.8	-	-	-	-	-
7		122.0	5.80	d	2.5		
8		167.0	-	-		-	-
9	**α**	35.9	2.935	ddd	11.7, 7.0, 2.5	167.0, 122.0, 24.2, 39.0, 38.9, 21.8	4.27, 1.99, 1.78
10		39.0	-	-	-	-	-
11	**α**	21.8	1.77				
	β		1.67				
12	**α**	32.5	2.105	td	13.0, 4.9	49.0, 17.8	2.304
	β		1.85				
13		49.0	-	-	-	-	-
14		85.4	-	-	-	-	-
15	**α**	31.8	1.62				
	β		1.99				
16	**α**	22.7	1.87				
	β		2.06				
17	**α**	50.4	2.304	t	8.9	81.5, 49.0, 22.7, 22.6, 17.8	2.05, 1.86, 1.19
18	**β**	17.8	0.825	s		85.4, 50.4, 49.0, 32.5	2.07, 2.00, 1.96, 1.86, 1.67, 1.16-8
19	**β**	24.2	0.96	s		52.6, 39.0, 38.9, 35.9	2.24, 1.99, 1.67, 1.22
20		86.1	-	-	-	-	-
21		22.6	1.173	s		86.1, 81.5, 50.0	3.83, 2.304, 1.86, 0.83
22		81.5	3.834	d	10.0	86.1, 22.6	2.06, 1.88, 1.33, 1.20
23	**a**	30.9	1.20				
	b		1.74				
24		50.0	1.45	ddd	12.5, 6.6, 3.0	-	3.83, 1.17-9, 1.006
25		74.4	-	-	-	-	-
26		26.8	1.16	s		74.0, 50.0	3.83, 2.304, 1.86, 0.83
27		27.9	1.18	s		74.4, 50.0	3.83, 2.304, 1.86, 0.83
28	**a**	25.3	1.22				
	b		1.68				
29		14.9	1.006	t	7.5	50.0, 25.3	3.83, 1.67, 1.21
30		108.1	-	-	-	-	-
31	**β**	27.2	1.325	s		108.1	3.83, 2.05
32	**α**	29.5	1.39	s		108.1, 27.2	1.17-9, 1.20
33		109.6	-	-	-	-	-
34	**α**	26.8	1.32	s		109.6	4.30, 2.05
35	**β**	29.0	1.47	s		109.6, 26.8	1.32, 1.21