

Summary of Ph.D. Thesis

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

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Introduction

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 which is considered to undergo a tautomer interconversion, forming a 5,7-diene structure. 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. Characteristic OH groups are present in positions 3 β - and 14 α - and further hydroxylation is often observed at C-1, 2, 5, 11, 20, 22, 25, 26 or 27. 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. The structure of the main phytoecdysteroid, 20-hydroxyecdysone (20E) is shown in **Fig. 1**.

Ecdysteroids were discovered as steroid hormones controlling moulting and metamorphosis in insects. The discovery of ecdysteroids from plant sources initiated the systematic and fruitful screening of ecdysteroid-rich plant sources. Plant ecdysteroids are often biosynthesized i.e. 2-5 orders of magnitude higher than their concentration in insects. A 0.1% concentration of 20E is not unusual, and several plant species biosynthesize 20E in 1-3 % of their dry mass. The large number (more than 300) and ready availability of phytoecdysteroids promotes pharmacological studies, which have demonstrated that they influence many physiological functions and are not toxic to mammals. The most pronounced effect of ecdysteroids on mammals is the stimulation of protein synthesis which is not generated via the mammalian androgen, estrogen or glucocorticoid receptors. However, the mechanism of action of ecdysteroids is mainly unknown, their effect on mammalian systems is possibly mediated via the vitamin D non-genomic signalling pathways.

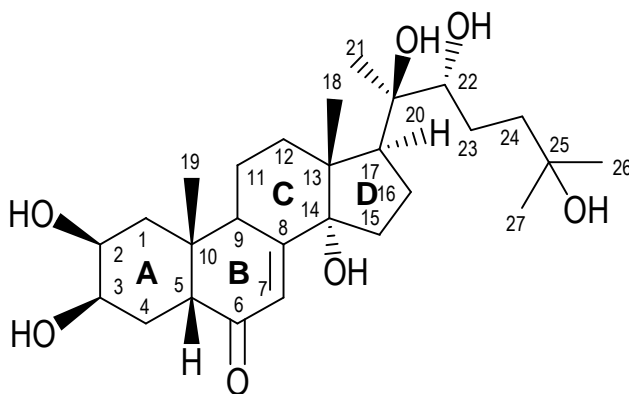


Fig. 1. Structure of 20E

Aims of the study

The increasing number of scientific investigations of the ecdysteroids might reveal new, specific fields, such application in case of muscle atrophies 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, and our preliminary thin layer chromatographic (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. 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, 20E.
- Previously, some ecdysteroid acetonide derivatives were isolated from the plant by our research team¹. 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 20E, 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 20E under physiological conditions.

¹ Presentation held at 53th GA Congress Florence, 21-25 August 2005. Tóth N., Hunyadi A., Máthé I., Báthori M.; New 26-hydroxylated Ecdysteroids from *Silene viridiflora*.

- regenerating muscle (m. soleus, after necrosis induced by a snake venom, notexin) to test, whether 20E is able to accelerate the regenerating process.
- atrophying respiratory muscle (diaphragm), to examine, whether 20E helps to prevent the deleterious effect of glucocorticoids, during co-administration with methylprednisolone.

Experimental

Plant material

The aerial parts of the cultivated *S. viridiflora* L. (Caryophyllaceae) 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).

Reagents and standard ecdysteroid samples

Solvents of high performance liquid chromatography (HPLC) grade were purchased from Merck (Darmstadt, Germany). Solvents of analytical grade were from Reanal (Budapest, Hungary). Reference ecdysteroids were available from earlier isolation work and fully characterized in previous studies of the research group. Their identities and purities were identified by HPLC and nuclear magnetic resonance spectroscopy (NMR).

Procedures for isolation of ecdysteroids

The ecdysteroids were subjected to exhaustive extraction with methanol. The next step involved preliminary purification using solvent-solvent extraction with *n*-hexane and fractionated precipitation with acetone. The isolation of ecdysteroids from the purified plant extract was based on the optimized combination of chromatographic methods: normal- and reversed phase column chromatography (NP-, RP-CC), rotation planar chromatography and HPLC. Each chromatographic step was monitored by conventional TLC.

Structure elucidation

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

In addition to chromatography, all ecdysteroids were characterized by spectroscopic methods: UV spectroscopy, NMR and mass spectrometry (MS) were utilized to identify ecdysteroids.

Investigation of the genuineness of ecdysteroid acetonides

Investigation of the possibility of ecdysteroid acetonide formation we imitated the conditions of the isolation procedure. To examine the genuine presence of 2-deoxy-20-hydroxyecdysone 20,22-acetonide in the prepurified extracts of *S. viridiflora* herb, NP-, RP-HPLC and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) measurement were performed with independently collected samples.

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 according to the scheme shown on **Fig. 2**.

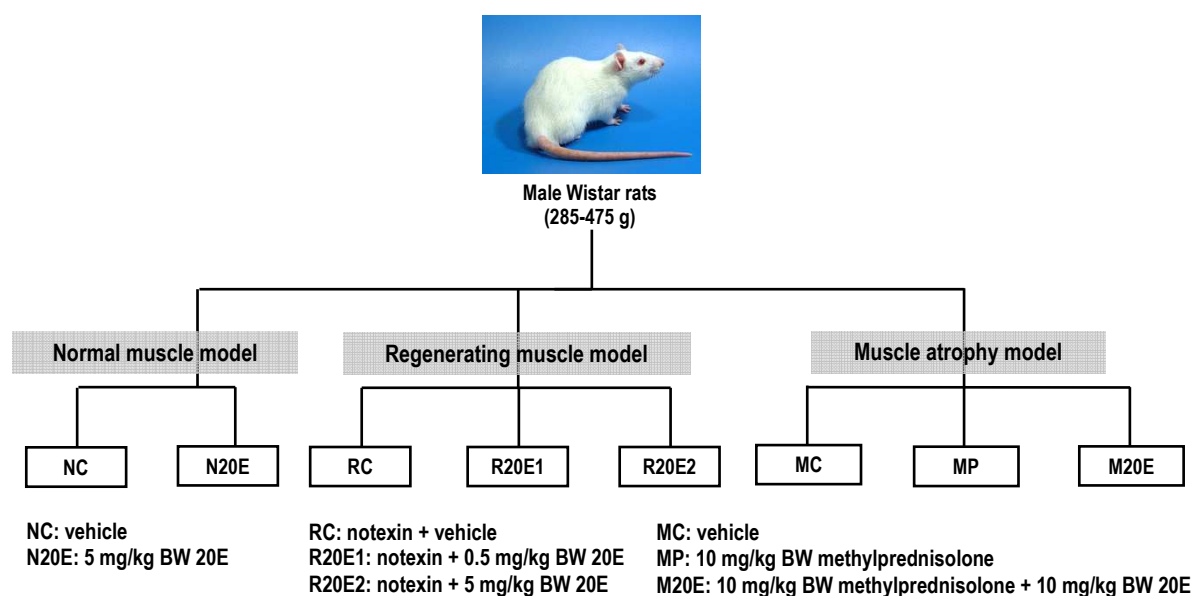


Fig. 2. The scheme of experimental design for the study of 20E effect on muscle fibres.

Collection of muscles, haematoxylin-eosine staining, immunocytochemistry

The dissected regenerating, atrophying and normal muscles were frozen in isopentane cooled with liquid nitrogen and kept at -70 °C. Serial cross sections were cut at 15 µm thickness with a cryostat kept at -20°C and stained with haematoxylin-eosine or peroxidase immunohistochemistry. BA-D5 (mouse, 1:50), SC-71 (mouse, 1:20), BF-F3 (mouse, 1:10) as

primary antibodies were used for MyHC1, 2a, 2b, respectively. BA-D5 (mouse, 1:50), SC-71 (mouse, 1:20); BF-F3 (mouse, 1:10) were combined for MyHC2x staining.

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 hematoxylin-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.

Statistics

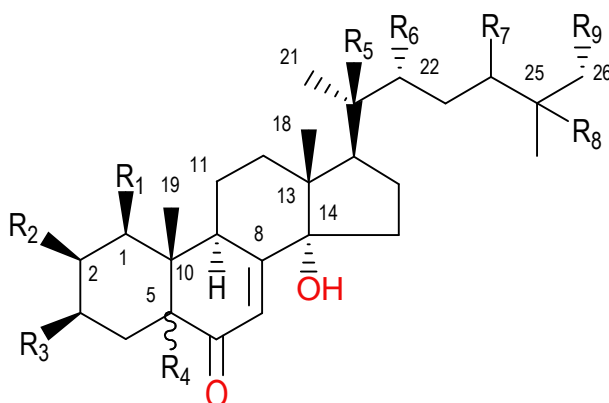
The cumulative data of muscle fibre CSA's obtained from the muscles were compared among groups by using t-test for unpaired samples or one-way analysis of variance followed by Newman-Keuls and Bonferroni post hoc test. The number of myonuclei and the size of myonuclear domain were also compared as cumulative 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 \pm SE.

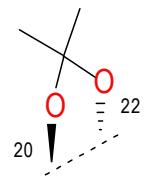
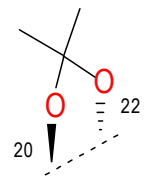
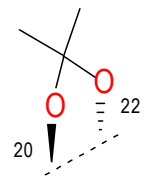
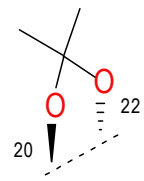
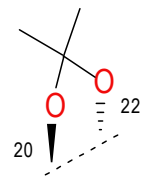
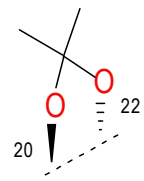
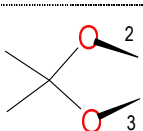
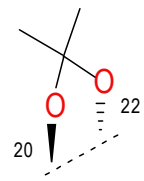
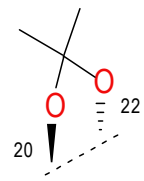
Main results and discussion

Isolation and structure elucidation

With combined chromatographic methods 20 ecdysteroids were isolated and characterized from the herb of *S. viridiflora*. 9 of the compounds have been discovered for the first time in a natural source and 12 of them were reported for the first time from this plant. 20E has been successfully isolated in two steps, involving crystallisation from the *S. viridiflora* herb extract and used for *in vivo* animal experiments. The structure of the isolated compounds is shown in **Table 1**.

Table 1. 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 B 25-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	

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-hydroxypolypodine 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. 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. Other derivatives such as glycosides (**3-6**) and several ecdysteroid acetonides (**13** and **15-20**) were also isolated. The acetonide group decreases the polarity of the compounds and thus changes their chromatographic behaviour and compared to the rather polar ecdysteroid derivatives, makes their isolation easier.

Genuineness of ecdysteroid acetonides

The genuineness of ecdysteroid acetonides has been verified via indirect and direct methods in *S. viridiflora* herb extract. 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 prepurified 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 prepurified extracts of four, independently collected *S. viridiflora* herb samples. 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.

In vivo effect of 20E on rat skeletal muscle

We demonstrated that 20E modified muscle fibre size in normal (m. soleus, m. EDL) and regenerating muscles (m. soleus) even after administration for 7 days.

20E increased fibre CSA in the regenerating soleus muscle, suggesting that 20E exerted a beneficial effect on muscle regeneration. The effect of 20E was different in the two applied doses (5 mg/kg, 0.5 mg/kg), which might indicate a dose-dependent action (**Fig. 3**).

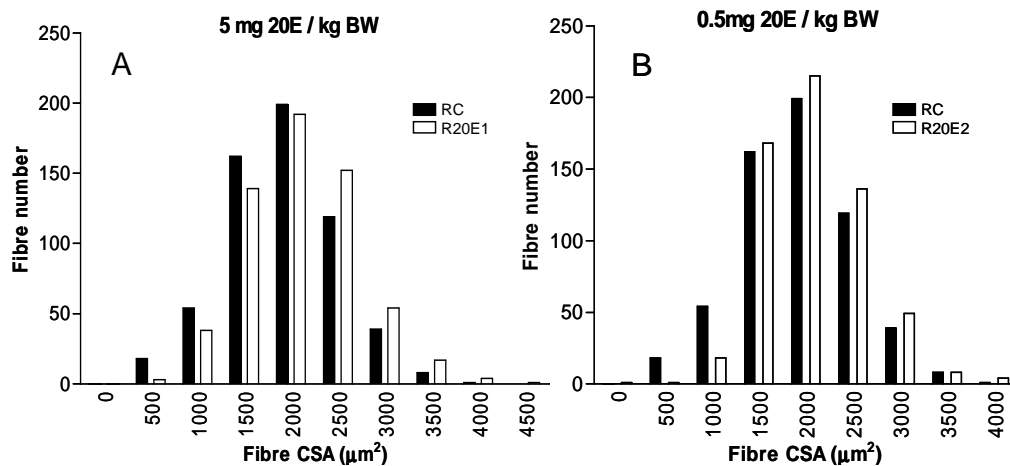


Fig. 3. Fibre size distribution of regenerating m. soleus after administration of 20E in a dose of 5 mg/kg BW (**A**) and 0.5 mg/kg BW (**B**). Mean fibre CSA of group RC is $1714 \pm 28.34 \mu\text{m}^2$. Mean fibre CSAs of groups **R20E1** and **R20E2** are $2077 \pm 24.58 \mu\text{m}^2$ and $1857 \pm 21.40 \mu\text{m}^2$, respectively. Data are means \pm SE. Note that the number of larger muscle fibres is increased by the 20E treatment in a concentration dependent manner in the regenerating muscles.

Investigating the effect of 20E on rat muscles we could demonstrate that 20E 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 (**Fig. 4**).

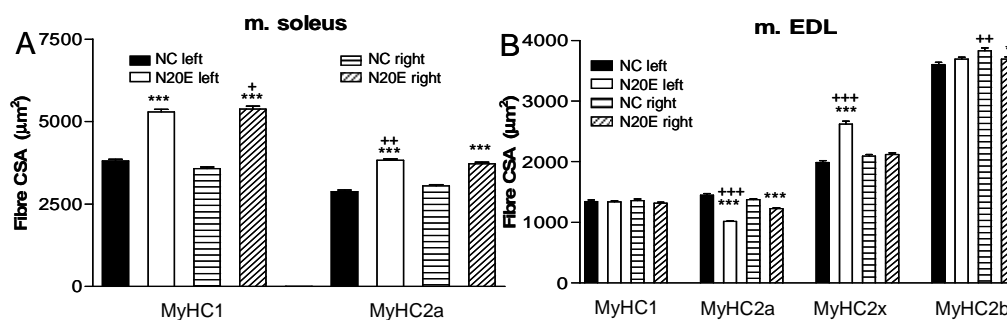


Fig. 4. The size of MyHC fibre types in the left and right soleus and EDL muscles of rats in group **N20E** treated with 20E in the left leg. Symbols * $p < 0.05$, *** $p < 0.001$ compared to the control, + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$ compared to the change in fibre size of the corresponding muscle in the other hindlimb.

In our experiments, the regenerating soleus muscle in the left hindlimb influenced the effect of 20E 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, 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 20E on the right EDL (**Fig. 5**).

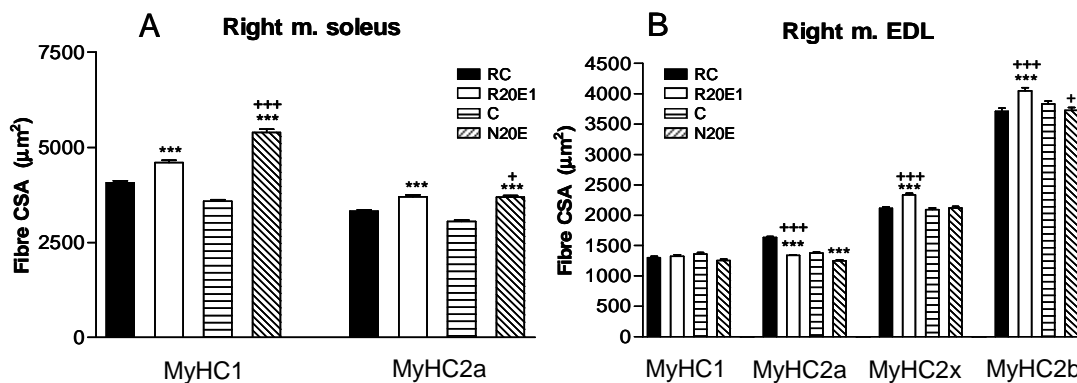


Fig. 5. The presence of a regenerating soleus in the left hindlimb modifies the effect of 20E in the contralateral normal soleus (A) and EDL (B). *** $p < 0.001$ compared to the corresponding control. + $p < 0.05$, +++ $p < 0.001$ compared to the change in fibre size of the corresponding muscle of the other group of rats with or without notexin treatment.

20E 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 20E involves the activation of satellite cells.

After five days treatment, 20E was able to prevent the IIB and IIX fibres of the diaphragm from the atrophying effect of the methylprednisolone, even if it was administered in the same concentration as the applied glucocorticoid (10 mg/kg BW). This effect was obvious considering the size of the CSAs of the diaphragm fibres (**Fig. 6**).

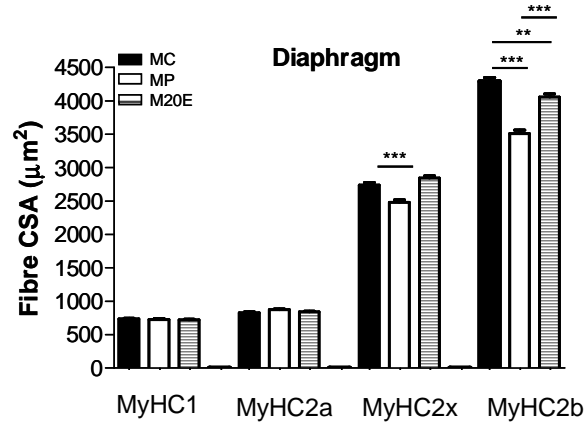


Fig. 6. CSA of the different fibre types of the diaphragm. ** $p < 0.01$, *** $p < 0.001$.

The contractile property measurement on the diaphragm such as force-frequency curve and fatigue run, did not give significant differences between the control and treated groups **C** vs. **MP** or **M20E**, **MP** vs. **M20E** (data not shown). There might have been a problem with treatment duration or dosage since the diaphragm also 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 be drawn. Further experiments with longer duration or with higher dose are needed to fully establish the effect of 20E on diaphragm force in order to connect the histochemical findings with functional outcomes.

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List of publications

The thesis is based on the following publications

- I. **Tóth N.**, Báthori M.
Preparative scale-chromatography of ecdysteroids, a class of biologically active steroids
Journal of Chromatographic Science, **2008**, 46, 111-116. IF: **1.135**
- II. Báthori M., **Tóth N.**, Hunyadi A., Márki Á., Zádor E.
Phytoecdysteroids and Anabolic-Androgenic Steroids – Structure and Effects on Humans
Current Medicinal Chemistry, **2008**, 15, 75-91. IF: **4.823**
- III. **Tóth N.**, Szabó A., Kacsala P., Héger J., Zádor E.
20-Hydroxyecdysone increases fibre size in a muscle-specific fashion in rat
Phytomedicine, **2008**, 15, 691-698. IF: **2.33**
- IV. **Tóth N.**, Simon A., Tóth G., Kele Z., Hunyadi A., Báthori M.
26-Hydroxylated ecdysteroids from *Silene viridiflora*.
Journal of Natural Products, **2008**, 71, 1461-1463. IF: **2.843**
- V. Simon A., **Tóth N.**, Tóth G., Kele Z., Groska J., Báthori M.
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- VI. **Tóth N.**, Hunyadi A., Báthori M., Zádor E.
Phytoecdysteroids and Vitamin D Analogues – Similarities in Structure and Mode of Action
Current Medicinal Chemistry, **2010**, 17, 1974-1994. IF: **4.823 (2008)**

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- I. Báthori M., **Tóth N.**
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Magyar Kémikusok Lapja, **2007**, 62(8-9), 258-260.
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TLC Determination of Hydrophilicity Parameter of Some Pyridinium Aldoximes
Journal of Liquid Chromatography & Related Technologies, **2007**, 30, 13-16. IF: **0.977**

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Kalász, H., Báthori, M., **Tóth N.**

New ecdysteroids from *Silene viridiflora*.

ICOB-5 and ISCND-25 IUPAC International Conference on Biodiversity and Natural Products Kyoto, Japan, 23-28 July 2006.

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Silene viridiflora, mint új ekdiszteroidok forrása

Tudományos Diákköri Konferencia, Szeged, 2006. április 5-7.

Tóth, N.

A 20-hidroxiöldizón hatása patkány vázizomban

Tudományos Diákköri Konferencia, Szeged, 2007. február 8-10.

Tóth, N.

A 20-hidroxiöldizón izolálása és hatása patkány vázizomban

VIII. Clauder Ottó Emlékverseny, Budapest, 2007. április 12-13.

Tóth N., Simon A., Tóth G., Miklóssy Vári V., Máthé I., Báthory M.

Új 26-hidroxiöldizón ekdiszteroidok izolálása a *Silene viridiflora*-ból

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A 20-hidroxiöldizón anabolikus hatása patkány vázizomban

Tavaszi Szél Konferencia, Budapest, 2007. május 17-20.

Bátori M., Liktör-Busa, E., **Tóth, N.**, Hunyadi, A., Máthé, I., Simon, A., Tóth, G.

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Tóth N.

20E and skeletal muscle: Is it a new anabolic agent?

Cell signalling controlling muscle plasticity workshop in the frame of the Flemish-Hungarian Bilateral Cooperation BIL 04/33. Leuven, Belgium, 6 December 2007.

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