

# **Biotransformation of cyclodextrine-complexed semi-synthetic betulin derivatives by plant cells**

Suvi T. Häkkinen<sup>1\*</sup>, Heli Nygren<sup>1</sup>, Natalia Maiorova<sup>1</sup>, Raisa Haavikko<sup>2</sup>, Sami Alakurtti<sup>1</sup>, Jari Yli-Kauhaluoma<sup>2</sup>, Heiko Rischer<sup>1</sup>, Kirsi-Marja Oksman-Caldentey<sup>1</sup>

## **Affiliations:**

<sup>1</sup>VTT Technical Research Centre of Finland Ltd., Espoo, Finland

<sup>2</sup>University of Helsinki, Drug Research Program, Division of Pharmaceutical Chemistry and Technology, Helsinki, Finland

**\*Corresponding author:** Suvi T. Häkkinen (D. Sc. Tech.); VTT Technical Research Centre of Finland Ltd., Tietotie 2, 02044 Espoo, Finland; tel. +358505610720; fax. +358207227071; [suvi.hakkinen@vtt.fi](mailto:suvi.hakkinen@vtt.fi)

## Abstract

In this study, three semi-synthetic betulonic acid-based compounds, 20(29)-dihydrolup-2-en[2,3-*d*]isoxazol-28-oic acid, 1-betulonoylpyrrolidine and lupane-2,20(29)-dieno[2,3-*b*]pyrazin-28-oic acid were studied in biotransformation experiments using *Nicotiana tabacum* L. and *Catharanthus roseus* (L.) G. Don cell suspension cultures. Biotransformation was performed using cyclodextrin to aid dissolving poorly water soluble substrates. Several new derivatives were found, consisting oxidized and glycosylated (-pentose and -hexose conjugated) products.

**Key words:** *Nicotiana tabacum*, *Catharanthus roseus*, biotransformation, betulonic acid, Solanaceae, Apocynaceae

## Introduction

Triterpenes are a group secondary compounds synthesized by plants via cyclization of squalene [1]. Today, more than 20 000 different triterpenes have been identified in nature, and they are either present as free forms or as glycosides. The pentacyclic triterpenoids, secondary plant metabolites abundantly found in fruit peel, leaves and stem bark, have attracted great interest as therapeutic agents and dietary supplements [1,2]. New functional properties have been obtained by modifying betulin structure, resulting in higher activity and selectivity [3]. In addition, semisynthetic derivatives of the naturally occurring triterpenoids have been studied in search for new anticancer agents, with specific focus on anti-invasiveness properties [4]. Betulin and betulonic acid, lupane-type pentacyclic triterpenes present in *e.g.* birch bark, have shown to possess various bioactivities including antiviral, antimalarial, anti-inflammatory and anti-cancer properties [4,5,6]. A recent review gives a thorough description of pharmacology of various betulonic acid -derived compounds [7]. Previously, Härmä and co-workers (2015) [4] screened altogether 78 different betulin derivatives for their anti-invasive and anti-proliferative effects against castration resistant prostate cancer. The compound library was chemically synthesized by subjecting betulin to Jones oxidation and further by using resulting betulonic acid as a key intermediate for the synthesis of several A-ring fused betulin heterocyclic adducts. Also, position C-28 and C-20 of these compounds were modified. Several betulin derivatives displayed dose-dependent and potent anti-invasive activity at nanomolar concentrations with minimal cytotoxicity.

Biotransformations are chemical reactions catalyzed by cells, organs or enzymes. Plant cell cultures exhibit an enormous biochemical potential with the power to transform cheap and plentiful substances, such as industrial by-products, into rare and expensive products. The biotransformation opens up the possibilities to find improved modifications of already utilized natural compounds and also offers a platform to upgrade the efficacies of known molecules with broader applications through novel of analogs [8]. When it comes to phytochemicals, such procedures carried out by plant cell cultures generate libraries of analog compounds with unique structural modifications and also ensure sustainability of the usage of resources. Also, plant cell-based biotransformations are a potent source of chirality when generation of specific enantiomers is required. Analogous with other platforms exploiting plant cell cultures, several advantages are seen, *e.g.* by utilizing defined culture conditions independent of seasonal fluctuations and pathological constraints [9]. In addition, biotransformation products can acquire improved selectivity, safety, physico-chemical properties and lower toxicity profiles which can be more suitable for some new therapeutic functions.

Biotechnological processes involve enzyme-catalysed transformations of substrates in aqueous conditions. As a result, if the substrate is very hydrophobic, such as betulin, the biotransformation process is primarily limited by the substrate solubility. On the other hand, cyclodextrins are cyclic, nonreducing oligosaccharides,

which can form solid inclusion complexes with a range of solid, liquid and gaseous substances by molecular complexation [10]. The lipophilic cavity of cyclodextrins is occupied by enthalpy-rich water molecules, which can be readily substituted by appropriate non-polar “guest” molecules. As a result, a lipophilic substance can be administered in an aqueous solution. Cyclodextrins have been used in various applications such as drug delivery, bioconversion, enzyme mimics and analytical applications [9,10, 11].

As plant cells have earlier shown to respond to xenobiotics by modifying them to less toxic forms *e.g.* by increasing the compound hydrophilicity, the aim of the current study was to investigate whether different forms of betulonic acid derivatives would be obtained by the activity of plant intracellular enzymes. By this way the compounds could possibly offer a broader spectrum for utilization *e.g.* as hit or lead compounds [12]. The selected compounds were two betulin derivatives which were earlier subjected to studies of invasion-specific effects against prostate cancer cells in 2D and 3D cell models [4]. The compounds were selected based on their differential structures deriving from parent compound betulonic acid thus hypothetically resulting in variability in biotransformation products formed. Biotransformation was performed with two cell cultures, *Nicotiana tabacum* L. (Solanaceae) (tobacco) and *Catharanthus roseus* (L.) G.Don (Apocynaceae) (Madagascar periwinkle). Besides tobacco, which is a commonly used model plant in biochemical studies, *C. roseus* was selected for its wide array of biosynthetically active enzymes, especially cytochrome P450s [13]. Both cultures were assayed for their capacity to take up semi-synthetic betulonic acid derivatives and their ability to convert the fed compounds in aqueous environment.

## Results and discussion

The biotransformation of betulin-derived compounds **4**, **5** and **6** (Fig. 1) was accomplished with two plant cell cultures, *i.e.* *N. tabacum* and *C. roseus*. Three semi-synthetic betulonic acid derivatives were fed to these cell cultures using cyclodextrin as carrier. Without cyclodextrin complexation the highly hydrophobic substrates could not be administered to plant cells cultivated in aqueous medium. Often when poorly water soluble substrates are studied, solvents such as dimethyl sulfoxide (DMSO) are considered. However, it is well known that DMSO affects plant cell membrane fluidity already at low concentrations (around 3 % solution) [14] and DMSO is commonly used for this purpose to protect the cell membranes during cryopreservation of plant cells [15]. For this reason, DMSO was not considered. Also, a biphasic system is not promising for biotransformation systems where intracellular enzymes are utilized. The accessibility of the compounds in the intracellular space is crucial and in addition this needs to proceed in aqueous environment. Cyclodextrins are currently extensively used as solubilizers for hydrophobic substances, especially for many steroid compounds [11,, 16, 17]. They are divided in three categories depending whether 6, 7 or 8 glucopyranoses units are linked through  $\alpha$ -1,4-D-glucopyranosic bonds in  $\alpha$ -,  $\beta$ - or  $\gamma$ -cyclodextrin, respectively [18]. In this study, three novel betulin derivatives generated via chemical synthesis were fed to cell cultures of tobacco and Madagascar periwinkle in the form of HP- $\gamma$ -cyclodextrin *i.e.* 2-(hydroxypropyl)- $\gamma$ -cyclodextrin complexes.

All the tested compounds were taken up from the medium and modified by the cells; however, the majority of fed substrate remained in the extracellular space. Even though trace amounts of oxidation products of compounds **5** and **6** were observed already in the start of the feeding, none of the studied compounds formed derivatives when incubated in cell-free culture medium, *i.e.* spontaneously during 10 days. The uptake of fed compounds varied depending on the compound and cell culture (**Table 1**). Uptake was better in *C. roseus* than in *N. tabacum* cell cultures. The highest intracellular level, 67 %, was observed for compound **5**, while total conversion rates of the fed compounds remained low, between 0.3 % to 1.8 %, during the study period of 10 days.

Biotransformation of compound **6** by tobacco cell cultures resulted in five derivatives (hydroxyl conjugate, pentose conjugate, hexose conjugate, di-hexose conjugate and one unidentified derivative) (**Fig. 2A-B**). The standard deviation represents the biological variation, which is in some instances rather high. However, it should not be considered exceptionally high, as 50 % biological variation has been reported being rather typical for plant systems [19]. The characteristic accumulation of the derivatives within a period of 10 days in culture medium is shown in **Fig. 3**. Chromatograms and mass spectra of the derivatives resulting from the biotransformation of compound **6** are presented in **Fig. 1S**.

The MS<sup>2</sup> spectra of the precursor ion at  $m/z$  623.41  $[M + H]^+$  show a product ion at  $m/z$  491.3  $[(M + H) - 132]^+$ , corresponding to the loss of a pentose group. Further MS<sup>3</sup> fragmentation of the product ion at  $m/z$  491.3 produced a spectra equal to the parent compound  $([M + H]^+)$ . Similarly, the MS<sup>2</sup> spectra of the precursor ion at  $m/z$  653.42  $[M + H]^+$  reveal a product ion at  $m/z$  491.3  $[(M + H) - 162]^+$ , corresponding to the loss of a hexose group from the parent compound (**Fig. 4**). Based on the mass fragmentation pattern, the pentose derivative ( $m/z = 623$ ) releases the 132 unit fragment from compound **6**, while hexose derivative ( $m/z = 653$ ) releases a 162 unit fragment. In mass fragmentation pattern of the di-hexose derivative, a 324 unit moiety is released from the precursor ion at  $m/z$  815.47. Additionally, an unknown biotransformation product at  $m/z$  901.45 producing the same product ion at  $m/z$  491.3 was detected in *N. tabacum* (**Fig. 2A-B**). The position of the mono-pentose or mono-hexose in **6** cannot be deduced from MS spectra but most probably, the moiety is bound to the carboxyl group. It seems that glycosylation is rather a plant specific process, while hydroxylations are commonly achieved by fungal metabolism, too [20].

In *C. roseus* cells compound **6** was transformed to three derivatives (pentose, hexose and di-hexose conjugate) (**Fig. 2C-D**). While the accumulation of the hexose conjugate occurred similarly in both species, di-hexose was more abundant in *N. tabacum*. In addition, the unidentified derivative detected in the tobacco system was not present in *C. roseus*.

Biotransformation of compound **5** resulted in minor amounts of several oxidation products in both *N. tabacum* and *C. roseus*, however further conjugates were not detected. Unlike compounds **6** and **4**, compound **5** does not possess a carboxyl moiety which is the typical position for glycosylation. Uptake of compound **5** was high in *C. roseus* cells (**Table 1**).

Compound **4** showed interesting differences in modification patterns between tobacco and *C. roseus* cells (**Fig. 5A-D**). Five derivatives (mono-hydroxy, pentose conjugate, hexose conjugate, di-hexose conjugate and one unidentified derivative) appeared in tobacco cells while in *C. roseus* cells only one derivative was found (**Fig 5A-B**). Interestingly, intracellular levels of compound **4** in *C. roseus* cells were remarkably high (**Fig. 5C**). Compound **4** has a structurally interesting heterocycle attached to the A ring, which might act as a substrate for endogenous terpene indole alkaloid modifying enzymes, or abundant cytochrome P450 responsible in various oxidative reactions in plants.

Plant species respond to the exposure of xenobiotics by differential substrate conversion [21] and conjugation of a glucose moiety is a typical detoxification reaction mediated by the abundant glycosyltransferases in plants. Glycosylation takes place in -OH, -SH, -NH or carboxyl groups [22]. Among the studied compounds, compounds **6** and **4** were glycosylated, but compound **5** formed only hydroxy-derivatives. This is expected since the former two possess carbonyl moieties, while the latter does not. Compound **6** displays only one -OH group for glycosylation and therefore the di-hexose is probably bound to this position of the molecule. MS/MS analysis, however cannot distinguish between 1 →2 or 1 →6 linkage. Interestingly, in their studies with betulinic acid derivatives, Baratto and co-workers [23] showed variability

of biotransformation products resulting from fungal and plant-based metabolism. While fungal conversion products consisted of oxidation and esterification products, *Daucus carota* cell suspension resulted in aldehyde product. Glycosylated products were not reported in this case.

In conclusion, using biotransformation, we could produce new, more hydrophilic betulin-derivatives which would be difficult to obtain via chemical synthesis. Due to their efficient metabolic machinery, *e.g.* related to xenobiotic detoxification processes, plant cells have proven to be an interesting system for biotransformation. Possibilities to produce high-value chemicals from natural sources has become in recent years an exciting research topic. During the past decades, more than half of all new drug entities were originally found in nature [24]. Vast amounts of bark raw material is obtained as by-products from the forest industry. Birch bark contains betulin up to 35% of its dry weight. Many of the betulinic acid derivatives are known for their bioactive properties, however the poor water solubility limits their use in various applications. As an example, recently, Haavikko and co-workers [25] reported anti-leishmanial activities of various synthetic heterocyclic betulin derivatives. Leishmaniasis is a spectrum of diseases which affect 12 million people, with 1-2 million new cases annually and new treatments are searched to treat this disease. Earlier, betulin glycosides which were introduced into lecithin liposomes were shown to possess cholesterol lowering effects in blood in experimental hypercholesterolemia [26]. The glycosylated biotransformation products observed in this study constitute promising, more hydrophilic candidates for bioactivity testing due to their increased water solubility.

## Materials and Methods

### *Plant cell cultures*

*N. tabacum* SR1 'Petit Havana' cell suspension (VTTC P-120003) was a gift from Fraunhofer Institute for Molecular Biology and Applied Ecology, IME (Aachen, Germany) and it was maintained in modified Gamborg B5 medium (G0210, Duchefa) according to [27], with 1.0 ppm naphthaleneacetic acid, 0.1 ppm kinetin and 20 g/L sucrose. Culture was subcultured once a week with 30% (v/v) inoculum. *C. roseus* PC-1140 (DSMZ, PC-1140) cell suspension culture was cultured in a similar manner as described for *N. tabacum* SR1. All cells were cultivated at +26 °C under a light 16 h /dark 8 h regime on a rotary shaker at 120 rpm.

### *Synthetic modifications of betulin*

Compounds **4** (20(29)-dihydrolup-2-en[2,3-*d*]isoxazol-28-oic acid), **5** (1-betulonoylpyrrolidine) and **6** (lupa-2,20(29)-dieno[2,3-*b*]pyrazin-28-oic acid) (**Fig. 1**) were synthesized as described [4,21].

### *Biotransformation procedure*

Cyclodextrin was used to facilitate dissolving the poorly water soluble substrates. Biotransformation was performed with both suspensions in late exponential growth phase. Approximately 5 g (FW) of 7 day-old-cell suspension was weighed in crystallising dishes and 5 mL of fresh modified Gamborg B5 growth medium was added. After 3 days adaptation timesubstrate was added by first dissolving it in methanol and then mixing with aqueous cyclodextrin *i.e.* (2-hydroxypropyl)- $\gamma$ -cyclodextrin (Sigma-Aldrich 779229) solution. Final concentrations in the feeding were 88 mg (0.05 mmol) for cyclodextrin and 1.56 mg (0.003 mmol) for the substrate per sample. Sampling was performed after incubating 0 h, 6 d and 10 d. Comparable amount of methanol without the substrate diluted in cyclodextrin solution was used as control. Studied compounds

were also incubated 10 d in sole culture medium without cells. The samples were vacuum-filtered to separate the cells and medium and frozen in liquid nitrogen. All the samples were freeze-dried prior to the extraction and analysis by UPLC-PDA/MS-MS.

### *Extraction*

Samples were extracted according to a previously described method [28]. Briefly, lyophilized samples were weighed (50 mg dry weight) and 1 mL chloroform:methanol (2:1) was added together with 200  $\mu$ L ultrapure water. Samples were grinded with a Retsch mill (5 min, 20 Hz), samples were extracted for 30 min and then centrifuged (10 620 *g*, 10 min). Sample aliquots were concentrated under nitrogen flow and the dried residue was dissolved in 200  $\mu$ L methanol before analysis.

### *UPLC-PDA/MS-MS analyses*

The extracts were analyzed with a Waters Q-ToF Premier mass spectrometer (Waters) and a photodiode array (PDA) detector (Waters Inc.) combined with an Acquity UPLC (Waters, Inc.). Chromatography was performed using a Waters Acquity BEH C18 (2.1 x 100 mm, 1.7  $\mu$ m) column, kept at 30 °C. Injection volume was 2  $\mu$ L. Separation was achieved using gradient elution with 0.1 % formic acid in water (v/v) (A) and acetonitrile (B) at a flow rate of 0.43 mL/min. Gradient program was 0 min 50 % B, 0.1 min 50 %, 10 - 13 min 90 % B. PDA detector was scanning from 210 – 600 nm (1.2 nm resolution, 20 points/second). Mass spectrometry was performed in positive polarity using the capillary voltage of 3.0 kV. Data were collected at a mass range of *m/z* 100-1000 with a scan duration of 0.2 s. Desolvation temperature was 350 °C, and source temperature was 125 °C. The desolvation gas flow was 1100 L/h. Calibration curves of the studied compounds were prepared from 2.5 to 1000 ppm. Analytes were quantified using external calibration curves and the biotransformation products were quantified by using the calibration curve of the parent compound.

For further identification of the biotransformation products, fractions collected from UPLC run were infused to a LTQ-Orbitrap (Thermo Scientific) mass spectrometer by a TriVersa Nanomate (Advion Biosciences) using chip-based nanoelectrospray in positive ionisation mode. Identifications were based on the exact mass and MS<sup>n</sup> spectra. MS<sup>2</sup> and MS<sup>3</sup> were acquired using either low resolution (LTQ) or high resolution up to target mass resolution R = 60 000 at *m/z* 400. Alternatively, the higher resolution MS and MS/MS spectra were run using Synapt G2-S (Waters). The UPLC and the chromatographic conditions were same as described above.

### **Supporting information**

Data of the chromatograms and mass spectra of the derivatives resulting from the biotransformation of compound **6** are available as Supporting information.

### **Acknowledgements**

We thank the Academy of Finland (Grant 252308) for financial support. Technical assistance of Mari Lämsä, Siv Matomaa and Ulla Lahtinen is gratefully acknowledged.

The authors declare no conflict of interests.

### **References**

- 1 Sheng H, Sun H. Synthesis, biology and clinical significance of pentacyclic triterpenes: a multi-target approach to prevention and treatment of metabolic and vascular diseases. *Nat Prod Rep* 2011; 28: 543–593
- 2 Bhui K, Srivastava AK, Shukla Y. Cytotoxic action of natural pentacyclic triterpenes on cancer cells: Lupane-type compounds. In: *Pentacyclic Triterpenes as Promising Agents in Cancer*. 2011: 49–87 Im Internet: <http://www.scopus.com/inward/record.url?eid=2-s2.0-84892090676&partnerID=tZOtx3y1>
- 3 Haavikko R. Synthesis of betulin derivatives with new bioactivities. 2015; Im Internet: <http://urn.fi/URN:ISBN:978-951-51-1618-5>
- 4 Härmä V, Haavikko R, Virtanen J, Ahonen I, Schukov HP, Alakurtti S, Purev E, Rischer H, Yli-Kauhaluoma J, Moreira VM, Nees M, Oksman-Caldentey KM. Optimization of invasion-specific effects of betulin derivatives on prostate cancer cells through lead development. *PLoS One* 2015; 10
- 5 Alakurtti S, Mäkelä T, Koskimies S, Yli-Kauhaluoma J. Pharmacological properties of the ubiquitous natural product betulin. *Eur J Pharm Sci* 2006; 29: 1–13
- 6 Rzeski W, Stepulak A, Szymański M, Juszcak M, Grabarska A, Sifringer M, Kaczor J, Kandefer-Szerszeń M. Betulin Elicits Anti-Cancer Effects in Tumour Primary Cultures and Cell Lines in Vitro. *Basic Clin Pharmacol Toxicol* 2009; 105: 425–432
- 7 Ríos JL, Mániz S. New Pharmacological Opportunities for Betulinic Acid. *Planta Med* 2018; 84: 8–19
- 8 Banerjee S, Singh S, Rahman LU. Biotransformation studies using hairy root cultures - A review. *Biotechnol Adv* 2012; 30: 461–468
- 9 Häkkinen ST, Ritala A. Medicinal compounds produced in plant cell factories. In: R A (Hrsg.). *Medicinal plant biotechnology*. United Kingdom: CAB International Oxfordshire, 2010: 13–35
- 10 Del Valle EMM. Cyclodextrins and their uses: A review. *Process Biochem* 2004; 39: 1033–1046
- 11 Singh M, Sharma R, Banerjee UC. Biotechnological applications of cyclodextrins. *Biotechnol Adv* 2002; 20: 341–359
- 12 Thorson JS, Hosted TJ, Jiang JQ, Biggins JB, Ahlert J. Nature's carbohydrate chemists: The enzymatic glycosylation of bioactive bacterial metabolites. *Curr Org Chem* 2001; 5: 139–167
- 13 Morant M, Bak S, Møller BL, Werck-Reichhart D. Plant cytochromes P450: Tools for pharmacology, plant protection and phytoremediation. *Curr Opin Biotechnol* 2003; 14: 151–162
- 14 Orvar BL, Sangwan V, Omann F, Dhindsa RS. Early steps in cold sensing by plant cells: the role of actin cytoskeleton and membrane fluidity. *Plant J* 2000; 23: 785–794
- 15 Menges M, Murray JAH. Cryopreservation of transformed and wild-type Arabidopsis and tobacco cell suspension cultures. *Plant J* 2004; 37: 635–644
- 16 Shen Y, Yu Z, Yang X, Wang F, Luo J, Wang M. A new technique for promoting cyclic utilization of cyclodextrins in biotransformation. *J Ind Microbiol Biotechnol* 2017; 44
- 17 Woerdenbag HJ, Pras N, Frijlink HW, Lerk CF, Malingre TM. Cyclodextrin-facilitated bioconversion of 17 beta-estradiol by a phenoloxidase from *Mucuna pruriens* cell cultures. *Phytochemistry* 1990; 29: 1551–1554
- 18 Radu C-D, Parteni O, Ochiuz L. Applications of cyclodextrins in medical textiles — review. *J Control Release* 2016; 224: 146–157

- <sup>19</sup> Broeckling CD, Huhman D V., Farag MA, Smith JT, May GD, Mendes P, Dixon RA, Sumner LW. Metabolic profiling of *Medicago truncatula* cell cultures reveals the effects of biotic and abiotic elicitors on metabolism. In: *Journal of Experimental Botany*. 2005: 323–336
- <sup>20</sup> Goswami A, Guo Z, Tully TP, Rinaldi FA, Huang XS, Swidorski JJ, Regueiro-Ren A. Microbial transformations of betulinic and betulonic acids. *J Mol Catal B Enzym* 2015; 117: 45–53
- <sup>21</sup> Häkkinen ST, Lackman P, Nygrén H, Oksman-Caldentey KM, Maaheimo H, Rischer H. Differential patterns of dehydroabietic acid biotransformation by *Nicotiana tabacum* and *Catharanthus roseus* cells. *J Biotechnol* 2012; 157: 287–294
- <sup>22</sup> Vaistij FE, Lim EK, Edwards R, Bowles DJ. Glycosylation of secondary metabolites and xenobiotics. In: *Plant-derived Natural Products: Synthesis, Function, and Application*. 2009: 209–228
- <sup>23</sup> Baratto LC, Porsani M V., Pimentel IC, Pereira Netto AB, Paschke R, Oliveira BH. Preparation of betulinic acid derivatives by chemical and biotransformation methods and determination of cytotoxicity against selected cancer cell lines. *Eur J Med Chem* 2013; 68: 121–131
- <sup>24</sup> Newman DJ, Cragg GM. Natural Products as Sources of New Drugs from 1981 to 2014. *J Nat Prod* 2016; 79: 629–661
- <sup>25</sup> Haavikko R, Nasereddin A, Sacerdoti-Sierra N, Kopelyanskiy D, Alakurtti S, Tikka M, Jaffe CL, Yli-Kauhaluoma J. Heterocycle-fused lupane triterpenoids inhibit *Leishmania donovani* amastigotes. *Med Chem Commun* 2014; 5: 445–451
- <sup>26</sup> Ivanov, A.S., Zakharova, T.S., Odinokova, L.E. and Uvarova NI. Hypolipidemic properties of betulin glycosides. *Pharm Chem J* 1987; 21: 659–662
- <sup>27</sup> Murashige T, Skoog F. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiol Plant* 1962; 15: 473–497
- <sup>28</sup> Zhao G, Yan W, Cao D. Simultaneous determination of betulin and betulinic acid in white birch bark using RP-HPLC. *J Pharm Biomed Anal* 2007; 43: 959–962



## Figure legends

**Fig. 1.** Betulin (**1**), betulinic acid (**2**), betulonic acid (**3**), 20(29)-dihydrolup-2-en[2,3-*d*]isoxazol-28-oic acid (**4**), 1-betulonoylpyrrolidine (**5**) and lupa-2,20(29)-dieno[2,3-*b*]pyrazin-28-oic acid (**6**).

**Fig. 2.** Accumulation of biotransformation products of compound **6**. Accumulation in *N. tabacum* cell cultures, intracellular (**A**) and extracellular (**B**) levels, and in *C. roseus* cell cultures, intracellular (**C**) and extracellular (**D**) levels. Error bars present standard deviations of two biological replicates. White bar: 0 h; grey bar: 6 days; black bar: 10 days after incubation start. Due to high standard deviations in Fig 2A, the individual sample points are indicated with dots.

**Fig. 3.** Time-course accumulation of compound **6** derivatives in culture medium. Met1 = di-hexose conjugate; Met2 = unidentified derivative; Met3 = hexose conjugate; Met4 = hydroxyl conjugate; Met5 = pentose conjugate; 6 = compound **6**.

**Fig. 4.** Detected biotransformation products were characterized with a LTQ-Orbitrap mass spectrometer and the assignments were based on the exact mass and MS<sup>n</sup> spectra as presented here for hexose conjugate derived from compound **6**. MS<sup>2</sup> fragmentation of [M + H]<sup>+</sup> precursor ion at *m/z* 653.42 (**A**) showed the neutral loss of a hexose moiety, 162 u. Further MS<sup>3</sup> fragmentation of the product ion [(M + H)-162]<sup>+</sup> at *m/z* 491.3 produced a spectrum identical with the parent compound spectrum (**B**). Spectra were obtained with nanospray using normalized collision energy of 35 % and 30%.

**Fig. 5.** Accumulation of biotransformation products of compound **4**. Accumulation in *N. tabacum* cell culture, intracellular (**A**) and extracellular (**B**) levels, and in *C. roseus* cell culture, intracellular (**C**) and extracellular (**D**) levels. Error bars present standard deviations of two biological replicates. White bar: 0 h; grey bar: 6 days; black bar: 10 days after incubation start.