

Biotransformation of Cyclodextrine-Complexed Semisynthetic Betulin Derivatives by Plant Cells

Authors

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Key words

Nicotiana tabacum, *Catharanthus roseus*, biotransformation, betulonic acid, Solanaceae, Apocynaceae

received November 15, 2017

revised February 22, 2018

accepted February 27, 2018

Bibliography

DOI <https://doi.org/10.1055/a-0585-6081>

Published online March 8, 2018 | *Planta Med* 2018; 84: 743–748 © Georg Thieme Verlag KG Stuttgart · New York | ISSN 0032-0943

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ABSTRACT

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

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, for example, birch bark, have shown to possess various bioactivities including antiviral, antimalarial, anti-inflammatory and anticancer properties [4–6]. A recent review gives a thorough description of pharmacology of various betulonic acid-derived compounds [7]. Previously, Härmä et al. [4] screened altogether 78 different betulin derivatives for their anti-invasive and antiproliferative 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, positions 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

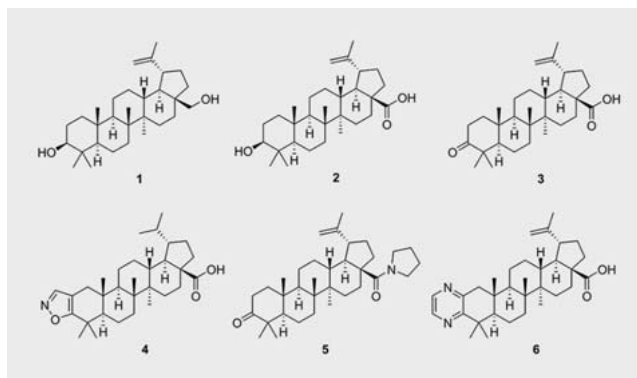
of specific enantiomers is required. Analogous with other platforms exploiting plant cell cultures, several advantages are seen—for example, by utilizing defined culture conditions independent of seasonal fluctuations and pathological constraints [9]. In addition, biotransformation products can acquire improved selectivity, safety, physicochemical properties, and lower toxicity profiles, which can be more suitable for some new therapeutic functions.

Biotechnological processes involve enzyme-catalyzed 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 nonpolar “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 [9–11].

As plant cells have earlier shown to respond to xenobiotics by modifying them to less toxic forms—for example, 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 that 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 semisynthetic 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 semisynthetic 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 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



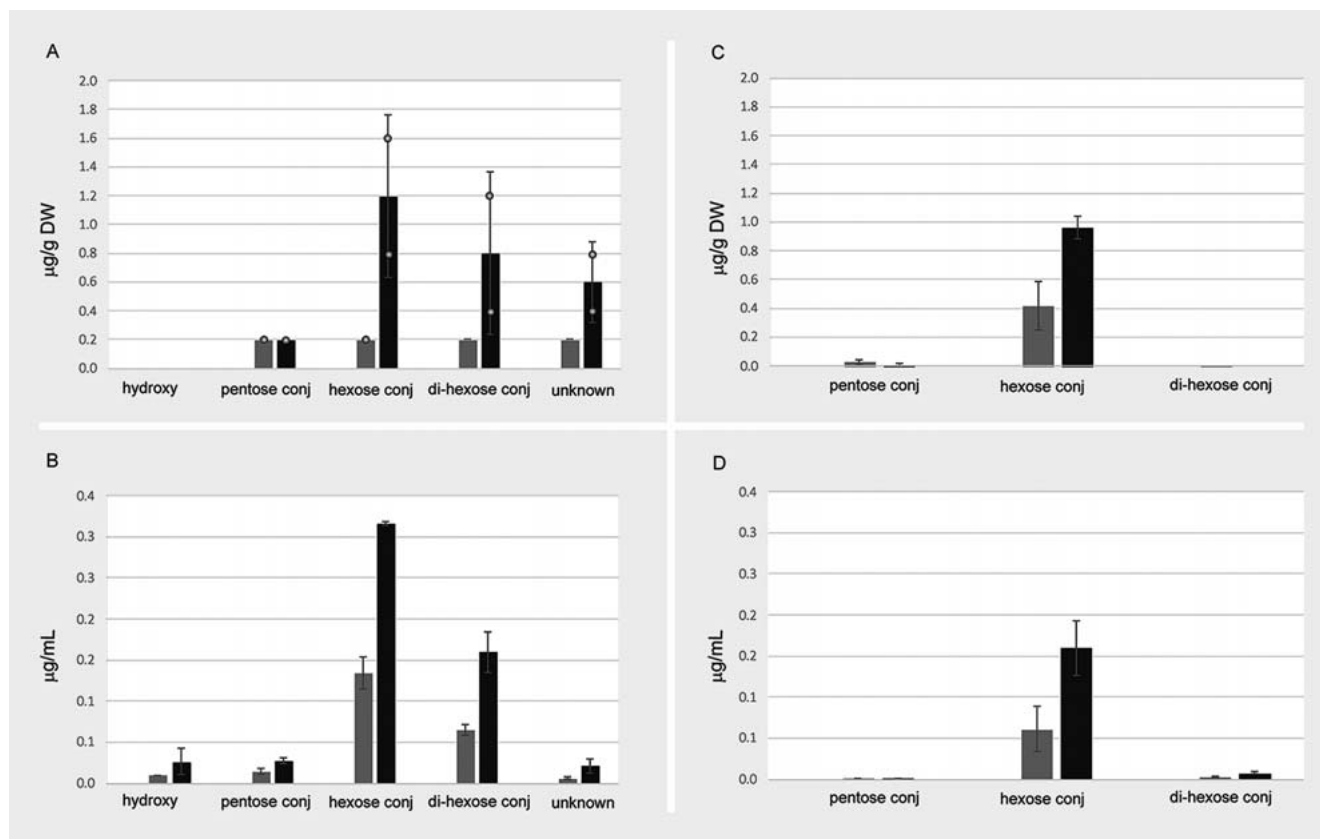
► **Fig. 1** Betulin (1), betulonic acid (2), betulonic acid (3), 20(29)-dihydropupa-2-en[2,3-d]isoxazol-28-oic acid (4), 1-betulonoylpyrrolidine (5), and lupa-2,20(29)-dieno[2,3-b]pyrazin-28-oic acid (6).

► **Table 1** Maximum intracellular uptake of betulin derivatives during 10-d incubation in *N. tabacum* and *C. roseus* cell cultures.

Compound	<i>N. tabacum</i>		<i>C. roseus</i>	
	Uptake (%)	time (days)	Uptake (%)	time (days)
15	10	10	44	6
11	20	10	67	10
4	59	6	48	6

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 α -1,4-D-glucopyranosic bonds in α -, β -, or γ -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- γ -cyclodextrin (i.e., 2-(hydroxypropyl)- γ -cyclodextrin complexes).

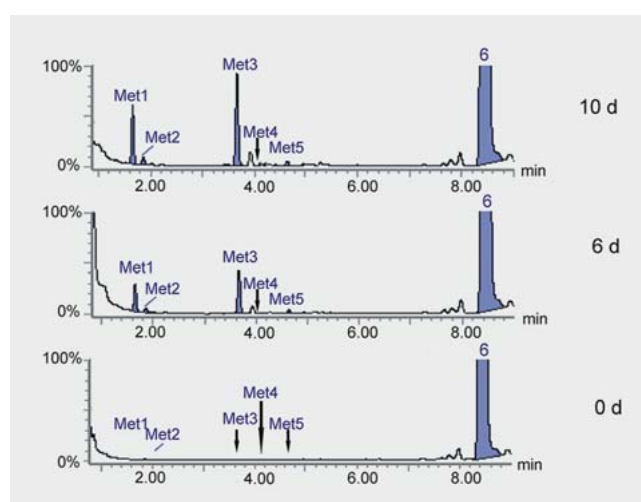
All the tested compounds were taken up from the medium and modified by the cells; however, the majority of fed substrate remained in the extracellular space. Even though trace amounts of oxidation products of compounds 5 and 6 were observed already in the start of the feeding, none of the studied compounds formed derivatives when incubated in cell-free culture medium (i.e., spontaneously during 10 d). 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 d.



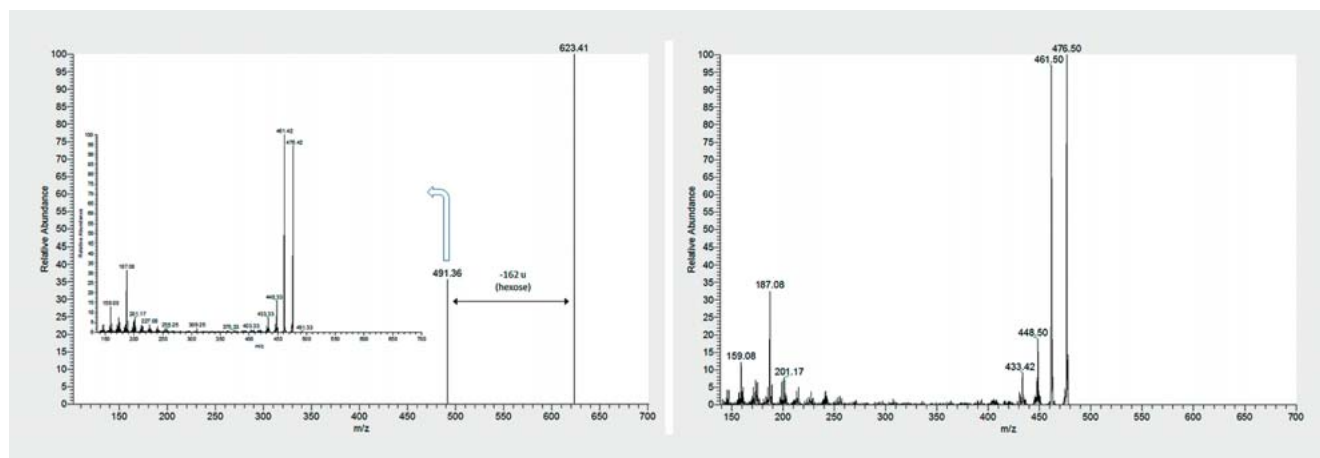
► **Fig. 2** Accumulation of biotransformation products of compound 6. Accumulation in *N. tabacum* cell cultures, intracellular (A) and extracellular (B) levels, and in *C. roseus* cell cultures, intracellular (C) and extracellular (D) levels. Error bars present standard deviations of two biological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

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 d 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**, Supporting Information.

The MS² 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 MS³ fragmentation of the product ion at m/z 491.3 produced a spectra equal to the parent compound ($[M + H]^+$). Similarly, the MS² 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 pre-



► **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 MSⁿ spectra as presented here for hexose conjugate derived from compound **6**. MS² fragmentation of [M + H]⁺ precursor ion at *m/z* 653.42 (A) showed the neutral loss of a hexose moiety, 162 u. Further MS³ fragmentation of the product ion [(M + H) - 162]⁺ 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%.

cursor 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. 2 A, 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. 2 C, 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*.

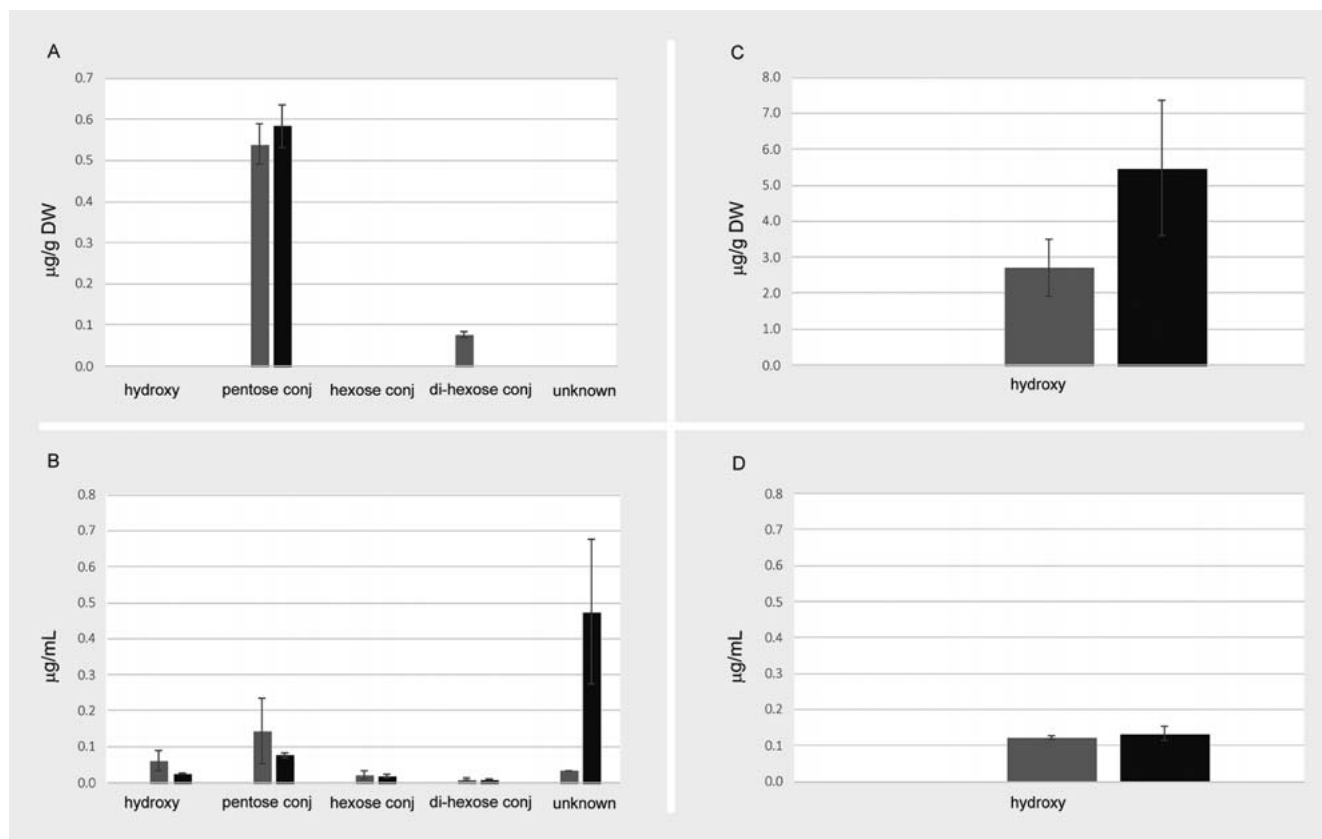
Biotransformation of compound **5** resulted in minor amounts of several oxidation products in both *N. tabacum* and *C. roseus*; however, further conjugates were not detected. Unlike compounds **6** and **4**, compound **5** does not possess a carboxyl moiety, which is the typical position for glycosylation. 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. 5 A–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. 5 A, B**). Interestingly, intracellular levels of compound **4** in *C. roseus* cells were remarkably high (► **Fig. 5 C**). 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 et al. [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* L. (Apiaceae) cell suspension resulted in aldehyde product. Glycosylated products were not reported in this case. Data of the chromatograms and mass spectra of the derivatives resulting from the biotransformation of compound **6** are available as Supporting Information.

In conclusion, using biotransformation, we could produce new, more hydrophilic betulin derivatives that 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 are 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 et al. [25] reported antileishmanial activities of various synthetic heterocyclic betulin derivatives. Leishmaniasis is a spectrum of diseases that 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



► **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; gray bar: 6 d; black bar: 10 d after incubation start.

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 (VTTCC 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-d-old cell suspension was weighed in crystallizing dishes and 5 mL of fresh modified Gamborg B5 growth medium was added. After 3 d adaptation time substrate was added by first dissolving it in MeOH and then mixing with aqueous cyclodextrin—that is, (2-hydroxypropyl)- γ -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 MeOH 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:MeOH (2:1) was added together with 200 μ 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 μ L MeOH before analysis.

UPLC-PDA-MS/MS-MS analyses

The extracts were analyzed with a Waters Q-ToF Premier mass spectrometer (Waters) and a photodiode array (PDA) detector (Waters) combined with an Acquity UPLC (Waters). Chromatography was performed using a Waters Acquity BEH C18 (2.1 \times 100 mm, 1.7 μ m) column, kept at 30 °C. Injection volume was 2 μ 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 to 600 nm (1.2 nm resolution, 20 points/second). MS 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 ionization mode. Identifications were based on the exact mass and MSⁿ spectra. MS² and MS³ 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.

Conflict of Interest

The authors declare no conflicts of interest.

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: Salvador JAR, ed. *Pentacyclic Triterpenes as promising Agents in Cancer*. New York: Nova Science Publishers; 2011: 49–87
- [3] Haavikko R. *Synthesis of Betulin Derivatives with new Bioactivities* [Dissertation]. Helsinki: University of Helsinki; 2015
- [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: e0126111
- [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, Máñez 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: Arora R, ed. *Medicinal Plant Biotechnology*. Oxfordshire: CAB International; 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: 1–7
- [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 CD, Parteni O, Ochiuz L. Applications of cyclodextrins in medical textiles – review. *J Control Release* 2016; 224: 146–157
- [19] Broeckling CD, Huhman DV, 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. *J Exp Bot* 2005; 56: 323–336

- [20] 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
- [21] 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
- [22] Vaistij FE, Lim EK, Edwards R, Bowles DJ. Glycosylation of secondary Metabolites and Xenobiotics. In: Osbourn AE, Lanzotti V, eds. *Plant-derived natural Products: Synthesis, Function, and Application*. New York: Springer; 2009: 209–228
- [23] Baratto LC, Porsani MV, 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
- [24] Newman DJ, Cragg GM. Natural products as sources of new drugs from 1981 to 2014. *J Nat Prod* 2016; 79: 629–661
- [25] 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
- [26] Ivanov AS, Zakharova TS, Odinkova LE, Uvarova NI. Hypolipidemic properties of betulin glycosides. *Pharm Chem J* 1987; 21: 659–662
- [27] Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* 1962; 15: 473–497
- [28] Zhao G, Yan W, Cao D. Simultaneous determination of betulin and betulonic acid in white birch bark using RP-HPLC. *J Pharm Biomed Anal* 2007; 43: 959–962