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Published in:
Plant Cell%2C Tissue and Organ Culture

DOI:
[10.1007/BF00037684](https://doi.org/10.1007/BF00037684)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1995

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Uden, W. V., Bouma, A. S., Bracht Waker, J. F., Middel, O., Wichers, H. J., Waard, P. D., Woerdenbag, H. J., Kellogg, R. M., & Pras, N. (1995). The production of podophyllotoxin and its 5-methoxy derivative through bioconversion of cyclodextrin-complexed desoxypodophyllotoxin by plant cell cultures. *Plant Cell%2C Tissue and Organ Culture*, 42(1). <https://doi.org/10.1007/BF00037684>

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The production of podophyllotoxin and its 5-methoxy derivative through bioconversion of cyclodextrin-complexed desoxypodophyllotoxin by plant cell cultures

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Received 5 December 1994; accepted in revised form 1 March 1995

Key words: Glucosides, *Linum flavum*, 5-methoxypodophyllotoxin, podophyllotoxin, *Podophyllum hexandrum*

Abstract

The bioconversion of the lignan desoxypodophyllotoxin by cell suspensions of *Linum flavum* and of *Podophyllum hexandrum* was investigated. The apolar substrate could be easily dissolved in the culture medium at a concentration of 2 mM by complexation with dimethyl-*b*-cyclodextrin. Growth parameters of the cell suspensions were not affected by either the addition of cyclodextrin itself, or when cyclodextrin-complexed desoxypodophyllotoxin was present in the medium. The complexed lignan disappeared from the medium within 7 days for both cell cultures. Cellularly only small amounts of desoxypodophyllotoxin were found. After feeding of desoxypodophyllotoxin, the cell culture of *L. flavum* accumulated 5-methoxypodophyllotoxin and 5-methoxypodophyllotoxin-*b*-D-glucoside. After 7 days a total maximal content of 2.38% on a dry weight basis of 5-methoxypodophyllotoxin was formed, corresponding with 249 mg l⁻¹ suspension. The highest bioconversion percentage of 52.3% was found at day 14. The desoxypodophyllotoxin-fed culture of *P. hexandrum* accumulated podophyllotoxin and its *b*-D-glucoside with a maximal content of 2.87% on a dry weight basis after 9 days, corresponding with 192 mg l⁻¹ suspension. The highest bioconversion percentage of 33.2% was also found at day 9.

Fig. 1. Chemical structures of podophyllotoxin (A), desoxypodophyllotoxin (B), and 5-methoxypodophyllotoxin (C).

Introduction

Podophyllotoxin (Fig. 1A) is a naturally occurring lignan, which is extracted from the rhizomes of *Podophyl-*

lum peltatum and *P. hexandrum* (Berberidaceae) and serves as a starting compound for the preparation of the semi-synthetic cytostatics etoposide (VP-16-213) and teniposide (VM-26) (Clark & Slevin 1987; Holthuis 1988; Stähelin & Von Wartburg 1989). The supply of *P. hexandrum* rhizomes, which contain ca. 4% podophyllotoxin on a dry weight basis, has become limited, due to both intensive collection and lack of cultivation (Gupta & Sethi 1983). Therefore, the production of podophyllotoxin and related lignans by means of biotechnological procedures would be an interesting alternative.

In the biosynthesis pathway for aryltetralin lignans as proposed by Broomhead et al. (1991), the direct precursor of podophyllotoxin is desoxypodophyllotoxin

(Fig. 1B). This proposal is based on the results of feeding experiments with radiolabelled desoxypodophyllotoxin using intact *Podophyllum* plants (Jackson & Dewick 1984; Kamil & Dewick 1986a, 1986b).

Cyclodextrins are cyclic oligosaccharides that are able to form water-soluble inclusion complexes with a whole range of apolar ligands (Duchêne & Wouessidjewe 1990a, 1990b). These clathrating agents have been used to create smooth bioconversion circumstances in plant biotechnology. They combine the advantage of apolar systems (higher solubility of the lipophilic substrate) with that of aqueous systems (compatibility with plant cells with respect to their viability) (Woerdenbag et al. 1990a, 1990b).

In the present study, the poorly water soluble-lignan desoxypodophyllotoxin, was chosen as the substrate and was added as a cyclodextrin complex to cell suspensions of *Podophyllum hexandrum*. In addition, the same experiments were performed with cell suspensions of *Linum flavum* (yellow flax; Linaceae), which normally accumulate 5-methoxypodophyllotoxin (Fig. 1C) as the major lignan (Berlin et al. 1986, 1988; Van Uden et al. 1990, 1991a, 1991b, 1992, 1994; Wichers et al. 1990, 1991).

Because of the severe side effects of the generally used anti-tumour agents as well as the insensitivity of many neoplastic malignancies for the applied therapeutics the search for new cytostatics must continue (Ayres & Loike 1990; Green 1989; Stringfellow & Schurig 1987). In this respect, the bioconversion products emerging from our experiments may be interesting new starting compounds for the synthesis of improved cytostatics.

Material and methods

Synthesis of the substrate desoxypodophyllotoxin

Podophyllotoxin was isolated from the resin podophyllin, derived from *Podophyllum hexandrum* rhizomes (Merck nr. 7432), based on a method described by Nadkarni et al. (1953). The resin (7 g) was dissolved in 30 ml of the eluents ethanol:toluene (1:1) and was chromatographed on a column (12.5 × 3.0 cm) packed with silica gel (Merck GF₂₅₄). The first yellow-green fraction (ca. 80 ml) was collected and evaporated to dryness. The residue (3.1 g) consisted of 98% podophyllotoxin as determined by NMR.

The isolated podophyllotoxin was reduced to desoxypodophyllotoxin according to Pullockaran and

Kingston (1989). Podophyllotoxin (1.3 g, 3.05 mmol) was dissolved in 70 ml acetic acid and 1.0 g 10% palladium on carbon was added. The reduction was performed under a hydrogen atmosphere at 95 °C during 48 h with stirring. Subsequently, the reaction mixture was filtered and ca. 100 ml diethyl ether was added to the clear solution. The acetic acid was removed by washing the ether layer with a large (1.5 l) quantity of water. The ether fraction was concentrated to ca. 10 ml and submitted to flash chromatography on a column (12.5 × 3.0 cm) packed with silica gel (Merck GF₂₅₄) with diethyl ether as the eluents. After the removal of ether, a light yellow residue (1.0 g, 2.46 mmol), of pure desoxypodophyllotoxin, was obtained.

Polarimetry

Specific optical rotation of desoxypodophyllotoxin was determined using a Perkin-Elmer 241 polarimeter equipped with a 10 cm cuvette.

$$[\alpha]_{\text{D}}^{20} = -116.4^{\circ} \text{ (c = 0.80, CHCl}_3\text{)} \quad (1)$$

Culture methods and growth parameters

Suspension cultures of *Linum flavum* L. (Linaceae) were obtained and grown as described previously (Van Uden et al. 1991a). Cell suspensions of *Podophyllum hexandrum* Royle (Berberidaceae), were routinely grown (Van Uden et al. 1989). In this study subculturing was done after a 3-week growth period, and only dark-grown cultures were used.

Both cell cultures were maintained for more than 5 years in our laboratory. Cells were harvested regularly during the growth cycle. The packed cell volume (PCV) was determined after transferring ca. 10 ml of the suspension to a calibrated conical tube followed by centrifugation for 5 min at 1,500 g. The medium pH and conductance were routinely measured in the resulting supernatant. Cell fresh weight (FW) and dry weight (DW) were determined using a previous method (Van Uden et al. 1991a).

Bioconversion experiments

Desoxypodophyllotoxin, 279 mg (0.7 mmol), was dissolved in 12 ml of a mixture of methanol/butanol (10/2; v/v). To two autoclaved bottles of 500 ml, 6 ml of this solution was transferred aseptically. The methanol/butanol was then evaporated in a laminar

air flow cabinet. Culture media were supplemented with dimethyl-**b**-cyclodextrin at a concentration of 2 mM, and autoclaved during 25 min at 121 °C. These media (175 ml) were added to the bottles containing the desoxypodophyllo-toxin, resulting in a final concentration of 2 mM for this lignan after shaking during 1 day at 140 rpm and 25 °C. The media were transferred to 500 ml Erlenmeyer flasks and subsequently inoculated with 75 ml of cell suspension of *L. flavum* or *P. hexandrum*. The same experiments were performed with media containing the cyclodextrin, but without the substrate. In control experiments, the medium contained neither cyclodextrins nor desoxypodophyllotoxin.

Extraction of lignans and lignan-b-D-glucosides

To enable measurement of intracellular lignans, the material resulting from the determination of the cell dry weight (DW) was powdered in a mortar. A 100 mg portion of dry mass was extracted by ultra-sonification for 1 h in 2.0 ml of 80% methanol. Four ml of dichloromethane and 4.0 ml of water were added and the mixture was vortexed. After centrifugation during 5 min at 1,500 g, 2.0 ml of the dichloromethane were evaporated to dryness and the residue was redissolved in 1.0 ml methanol and used for TLC or HPLC-analysis.

For the determination of lignan-**b**-D-glucosides, the water phase of the above mentioned extraction was submitted to enzymatic hydrolysis. A 2.5% (w/v) solution of **b**-glucosidase (Sigma G-0395) was prepared in 0.5 M sodium phosphate buffer, pH 5.0. To 4.0 ml of the water phase, 1.0 ml of the enzyme solution was added, followed by an incubation period of 20 h at 37 °C. Then, 4.0 ml of dichloromethane was added, followed by the same treatment as described above for the extraction of dry mass.

For the determination of lignans in the medium, the procedure was as follows: after the determination of the PCV, to 1.0 ml of the supernatant obtained as described under *Culture methods and growth parameters*, 4.0 ml of dichloromethane was added and further treated as described above for the extraction of dry mass. To determine the lignan-**b**-D-glucosides in the spent medium, 1.0 ml of the **b**-glucosidase solution was added to 4.0 ml of the supernatant obtained as described under *Culture methods and growth parameters*. The enzymatic hydrolysis and subsequent extraction of liberated lignan was performed as described above.

Thin layer chromatography (TLC)

Silica gel plates (Merck GF₂₅₄) were used to perform thin layer chromatography of cell extracts. The plates were developed with chloroform/methanol (100/4; v/v) as described by Wichers et al. 1990. Spots were visualized by UV-illumination (254 nm) or after submersion in a mixture of sulphuric acid and methanol (5/100; v/v) followed by heating at 110 °C for 10 min.

High performance liquid chromatography (HPLC)

Podophyllotoxin (Sigma P-4405), 5-methoxypodophyllotoxin (isolated from cell cultures of *L. Flavum*, Van Uden et al. 1992), 5-methoxypodophyllotoxin-**b**-D-glucoside, **b**-peltatin (both kindly provided by TNO-Zeist, The Netherlands) and desoxypodophyllotoxin (prepared at the Laboratory for Organic and Molecular Chemistry, University of Groningen, The Netherlands) were analysed and detected using the HPLC system as reported previously by Van Uden et al. (1989), equipped with a reversed phase Lichrosorb RP-18 (Chrompack, Middelburg, The Netherlands) column (100 × 3 mm i.d.) and a guard column. Methanol/water (4/6; v/v) served as the mobile phase at a flow rate of 1.0 ml min⁻¹. The detection of the lignans was at 290 nm.

Column chromatography

To determine the identity of unknown compounds, cell extracts were prepared from cultures that had grown in the presence of desoxypodophyllotoxin. Ca. 3 g of dry mass was extracted according to the above described procedure. The dichloromethane phases of the extraction were combined and evaporated to dryness. The residue was redissolved in 1 ml chloroform and fractionated on a silica column (40 cm × 2.5 cm) by elution with chloroform containing an increasing concentration of methanol. The collected fractions were analysed by HPLC; fractions containing the unknown compound were submitted to NMR.

Mass spectrometry (MS)

Mass spectrometry (EI) was performed as described previously (Van Uden et al. 1992). In addition, positive ion chemical ionisation (PCI) was performed with NH₃ as the reactant gas.

Desoxypodophyllotoxin, m/z , relative intensities (%): $[M]^+$ 398 (100), 230 (23), 199 (16), 185 (67), 181 (100), 173 (84), 168 (43), 153 (40).

PICI (NH_3): $[M + H]^+$ 399, $[M + NH_4]^+$ 416.

Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were recorded on a Bruker AMX 400 wb or a Varian VXR 300. Samples were dissolved in deuterated chloroform with 0.05% TMS as internal reference.

Results and discussion

Cyclodextrin-complexed desoxypodophyllotoxin as a substrate

To enable feeding of the very apolar desoxypodophyllotoxin to plant cell cultures in a dissolved state, the substrate was first complexed with dimethyl-*b*-cyclodextrin (Van Uden et al. 1993). The complexation of desoxypodophyllotoxin with dimethyl-*b*-cyclodextrin at a ratio 1:1 and at a concentration of 2 mM was rapid and complete, after 3 h.

It has been reported previously that cyclodextrins applied at a concentration of 2 mM did not affect the growth characteristics of cell suspensions of *Podophyllum hexandrum* (Woerdenbag et al. 1990b) and of *Linum flavum* (Van Uden et al. 1993). Also in this study the use of dimethyl-*b*-cyclodextrin at a final concentration of 1.4 mM did not affect the growth characteristics. The cell cultures of *L. flavum* exhibited normal growth under all experimental conditions. The PCV increased from ca. 26 to 60%, the FW from 120 to 300 g l⁻¹, the DW from 6 to 16 g l⁻¹, and the conductance decreased from 4.5 to 1.3 mS within one growth cycle.

The cell cultures of *P. hexandrum* grew at a much slower rate, the PCV increased from ca. 16 to 25%, the FW from 80 to 100 g l⁻¹, the DW from 5 to 6.5 g l⁻¹, and the conductance decreased from 4 to 1.7 mS.

All bioconversion experiments were performed at a final desoxypodophyllotoxin concentration of 1.4 mM. The concentration of substrate in the culture medium decreased immediately after inoculation with the cells and had dropped to zero after 7 days for both cell cultures (Fig. 2).

In an earlier study, it was found that dimethyl-*b*-cyclodextrin-complexed podophyllotoxin at a compa-

Fig. 2. Time course of the desoxypodophyllotoxin concentration in the spent medium of the cell culture of *L. flavum* (o) and of *P. hexandrum* (•).

table concentration and fed to the same cell line of *L. flavum*, vanished from the medium within 1 day (Van Uden et al. 1993). Due to the higher bioconversion rate of podophyllotoxin as compared with desoxypodophyllotoxin, the flux of that substrate into the plant cell might have been higher.

Cellularly, desoxypodophyllotoxin was detectable directly after the inoculation although only small amounts were found. Maximal values, 0.14% DW, were found after 5 h, corresponding with 8.8 mg l⁻¹ suspension for *L. flavum* and 0.05% DW, 2.4 mg l⁻¹, for *P. hexandrum* cell cultures. After 7 days, no substrate was detectable cellularly.

Bioconversion of cyclodextrin-complexed desoxypodophyllotoxin

TLC- and HPLC-analysis revealed that the fed desoxypodophyllotoxin was converted into 5-methoxypodophyllotoxin by the cell cultures of *L. flavum*, whereas podophyllotoxin was the main product in the cells of *P. hexandrum* cultures.

In the control cultures none of the above mentioned compounds could be detected, and in the cell free medium neither lignans nor lignan glucosides were found.

The time course of accumulation of 5-methoxypodophyllotoxin and podophyllotoxin in cell cultures of *L. flavum* and *P. hexandrum*, respectively, is depicted in Fig. 3 and Fig. 4, respectively.

The bioconversion of desoxypodophyllotoxin into 5-methoxypodophyllotoxin with the cell cultures of *L. flavum* reached a maximal value of 24.0% at day 14. The biosynthesis of podophyllotoxin in *P. hexandrum* started immediately and a maximal bioconversion per-

Fig. 3. Time course of cellularly accumulated 5-methoxypodophyllotoxin (%DW), as aglucone (o), as **b**-D-glucoside (•) and as **b**-D-glucoside (including the unknown) (Δ), after feeding of dimethyl-**b**-cyclodextrin-complexed desoxypodophyllotoxin to cell cultures of *L. flavum*.

Fig. 4. Time course of cellularly accumulated podophyllotoxin (%DW), as aglucone (o), and as **b**-D-glucoside (•), after feeding of dimethyl-**b**-cyclodextrin-complexed desoxypodophyllotoxin to cell cultures of *P. hexandrum*.

centage of 32.8% was found at day 9. Furthermore, the **b**-D-glucosides of both lignans accumulated, following approximately the same pattern as the corresponding aglucones. *Linum* cultures showed the highest bioconversion percentage of 9.8% at day 7 (Fig. 3), *Podophyllum*, 1.4% bioconversion, at day 5 (Fig. 4). In the cell culture of *P. hexandrum*, at day 9 the highest bioconversion percentage of desoxypodophyllotoxin into podophyllotoxin (aglucone and glucoside) was found, 33.2%. Bioconversion data are summarized in Table 1.

These results raise questions about the formation of 5-methoxypodophyllotoxin. When podophyllotoxin was fed to cell cultures of *L. flavum*, podophyllotoxin-**b**-D-glucoside was the only bioconversion product (Van Uden et al. 1993). In the present study, with desoxypodophyllotoxin as the substrate using the same cell cultures, neither podophyllotoxin nor its **b**-D-

Table 1. The bioconversion products formed after feeding of **b**-cyclodextrin-complexed desoxypodophyllotoxin to cell cultures of *L. flavum* and *P. hexandrum*, at the day that the total amount (aglucone + glucoside) was at its maximum.

Bioconversion product	%DW	mg l ⁻¹	t (days)
5-methoxypodophyllotoxin ^a	0.69	72.5	7
5-methoxypodophyllotoxin- b -D-glucoside ^a	1.69	177.0	7
podophyllotoxin ^b	2.84	190	9
podophyllotoxin- b -D-glucoside ^b	0.03	2.0	9

^a *L. flavum*.

^b *P. hexandrum*.

glucoside, but 5-methoxypodophyllotoxin and its **b**-D-glucoside were the bioconversion products. This suggests that 5-methoxypodophyllotoxin is not directly formed from podophyllotoxin but possibly from already hydroxylated desoxypodophyllotoxin (at the 5 position; **b**-peltatin) or already methoxylated desoxypodophyllotoxin (at the 5 position; **b**-peltatin-A-methylether).

Some bioconversions of desoxypodophyllotoxin are known to occur in plants and plant cell cultures. In studies of Jackson and Dewick (1984) and Kamil and Dewick (1986a, 1986b), labelled desoxypodophyllotoxin fed to plants of *P. hexandrum* and *P. peltatum* was converted into podophyllotoxin which, in turn, was oxidized to podophyllotoxone. This latter step appeared to be reversible. In addition, **b**-peltatin was found as a bioconversion product.

Cell suspension cultures of *Forsythia intermedia* have been found to convert desoxypodophyllotoxin into podophyllotoxone, probably via a hydroxylation prior to an oxidation (Broomhead and Dewick 1991).

Microorganisms have been reported to be able to bioconvert desoxypodophyllotoxin into epipodophyllotoxin (Kondo et al. 1988, 1989). The fungus *Penicillium* showed a high bioconversion rate of 0.18 mmol l⁻¹ day⁻¹.

Identification of other bioconversion products

The TLC- and HPLC-analysis of apolar fractions of the extraction revealed that in the cell culture of *L. flavum* a bioconversion product other than 5-methoxypodophyllotoxin was accumulated. During treatment of the polar water phase of the extraction

with **b**-glucosidase, an aglucone was formed. After this enzymatic treatment the unknown bioconversion product was also present, indicating the presence of the corresponding glucoside in the waterphase as well. The unknown compound was found to possess an R_f -value (TLC) of 0.34 and an retention time (HPLC) of 10 min. For comparison. podophyllotoxin had an R_f -value of 0.65 and a retention time of 12 min, 5-methoxypodophyllotoxin an R_f -value of 0.74 and a retention time of 18 min, desoxypodophyllotoxin 0.78 and 26 min, 5-methoxypodophyllotoxin-**b**-D-glucoside 0.19 and 9.5 min, and **b**-peltatin 0.64 and 11.5 min.

Fractions resulting from the column chromatography that contained the unknown component were further analysed by NMR. By method of ^1H -NMR, the unknown compound in the desoxypodophyllotoxin fed cultures of *L. flavum* was identified as 5-methoxypodophyllotoxin-**b**-D-glucoside. The values obtained, corresponded completely with those reported previously by Wichers et al. (1991). Since large amounts of this glucoside were present, only part of it was cleaved by the enzymatic treatment of the water phase, while the remaining glucoside was partitioned between the dichloromethane and water phase, indicating a considerable solubility of this glucoside in dichloromethane.

In conclusion, the substrate desoxypodophyllotoxin was converted by the *L. flavum* cells partly into 5-methoxypodophyllotoxin, but mostly (54-100%) into 5-methoxypodophyllotoxin-**b**-D-glucoside. The amounts of cellularly present 5-methoxypodophyllotoxin, aglucone and glucoside, including the previously unknown compound, are depicted in Fig. 3. At day 14 the highest bioconversion percentage (into aglucone and glucoside) was found, namely 52.3%. Bioconversion data are summarized in Table 1.

In this study, it has been shown that cell cultures of *L. flavum* and *P. hexandrum* are able to bioconvert desoxypodophyllotoxin into several lignans. Optimization experiments as well as isolation and characterization of the enzymes involved are currently being carried out.

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