Bioconversion of deoxypodophyllotoxin into epipodophyllotoxin in *E. coli* using human cytochrome P450 3A4

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Abstract
Biotransformation of deoxypodophyllotoxin to epipodophyllotoxin by three major human hepatic enzymes, CYP1A2, CYP2C9 and CYP3A4, heterologously expressed in *E. coli* DH5α, was investigated. It was shown that CYP3A4 catalysed the hydroxylation of deoxypodophyllotoxin into epipodophyllotoxin in yields up to 90%. The structure of the metabolite was determined using HPLC-MS and HPLC-SPE-NMR techniques. There was no detectable production of epipodophyllotoxin or podophyllotoxin by CYP1A2 and CYP2C9 enzymes. The CYP3A4 enzyme shows a distinctly different reactivity to deoxypodophyllotoxin compared to the semisynthetic derivatives etoposide and teniposide, which are degraded by 3-*O*-demethylation. These findings demonstrate a novel system for the production of 2,7’-cyclolignans, starting from the easily accessible deoxypodophyllotoxin.
Bioconversion of deoxypodophyllotoxin

Introduction

Podophyllotoxin and other 2,7'-cyclolignans are clinically relevant plant compounds. Podophyllotoxin (Fig. 1) is a lignan that possesses potent antimitotic and antiviral activity [201, 202]. The same lignan is also a unique starting compound for the production of two widely used anticancer drugs, etoposide and teniposide. The total synthesis of podophyllotoxin is complicated due to the presence of four chiral centers, a rigid trans-γ-lactone, and an axial 7'-aryl substituent [203]. The isolation of podophyllotoxin from the rhizomes of Podophyllum peltatum and Podophyllum hexandrum (Berberidaceae) plants is also an inefficient means of production. The yield of podophyllotoxin from P. peltatum is low (~0.25% based on dry weight) and the supply of P. hexandrum rhizomes, which contain ~4% of podophyllotoxin by dry weight, is becoming increasingly limited due to both intensive collection and lack of cultivation [204, 205].

An alternative to the synthesis and isolation from natural sources is production by biotechnological techniques starting from deoxypodophyllotoxin (Fig. 1). This drug can be isolated in high amounts from Anthriscus sylvestris (Apiaceae), a common weed in Northwest Europe, whose rhizomes contain considerable amounts of lignans. It has been found that deoxypodophyllotoxin can be converted into podophyllotoxin or 6-methoxypodophyllotoxin and their glucosides by plant cell cultures of Linum flavum [9, 206]. However, the conversion into podophyllotoxin by cell suspension cultures of P. hexandrum is not very efficient [207]. It is known that Aspergillus niger sp. is also able to convert deoxypodophyllotoxin to podophyllotoxin but again in very low yields [208]. Although Penicillium F-0543 and other Penicillium sp. perform the biotransformation of deoxypodophyllotoxin to epipodophyllotoxin in high yields, the overall process was found to be time-consuming [209]. Until now, it has not been clarified which plant or fungal cytochrome is responsible for the hydroxylation of deoxypodophyllotoxin to yield podophyllotoxin or epipodophyllotoxin. It is assumed that in Podophyllum this step is carried out by a cytochrome P450 monooxygenase, as was shown for plant cell cultures of L. flavum [210], but thus far the responsible enzymes have yet to be identified. The discovery of an alternative enzymatic system able to perform the hydroxylation at the 7-position of deoxypodophyllotoxin and related 2,7'-cyclolignans could be an important step forward in the production of epipodophyllotoxin.

CYP1A2, CYP2C9 and CYP3A4 are the major three hepatic P450 enzymes, involved in the metabolism of approximately 65% of human xenobiotics, particularly drugs and carcinogens [181]. CYP3A4 is known for the biotransformation of the podophyllotoxin-related antineoplastic drugs etoposide and teniposide [211-213]. Human CYP3A4 initiates the catabolism of etoposide and teniposide via 3'-O-demethylation. Until now, there are no reports on the effect of this enzyme on podophyllotoxin and other plant derived 2,7'-cyclolignans. Based on the experimental results with etoposide and their derivatives, it may be assumed that 2,7'-cyclolignans containing a 3'-methoxy group will react accordingly. On the other hand, in contrast to the semi-synthetic drugs, the plant 2,7'-cyclolignans do not contain a glucose moiety at C-7 and a hydroxy group at C-4’, which may interfere with the anticipated conversion by CYP3A4. Therefore, the broad specificity of CYP3A4 may have interesting pharmaceutical applications.

This research was initiated to investigate the possible hydroxylation of the 2,7'-cyclolignan deoxypodophyllotoxin by the three main metabolizers, the human liver cytochrome P450 enzymes CYP3A4, CYP2C9, and CYP1A2, using transformed Escherichia coli.
Fig. 1: Chemical structures of the lignans of interest

Materials and Methods

Chemicals
Deoxypodophyllotoxin was isolated by one of us (A. Koulman) from *A. sylvestris* rhizomes, as published elsewhere [9, 214]. Identity and purity of deoxypodophyllotoxin was determined by HPLC and $^1$H and $^{13}$C NMR spectroscopy. All reference compounds (podophyllotoxin, phenacetin, tolbutamide, testosterone, paracetamol, hydroxytolbutamide, and 6β-hydroxytestosterone) were purchased from Sigma (St. Louis, USA). Medium components and glucose were purchased from Duchefa (Haarlem, The Netherlands), and all other chemicals from Merck (Darmstadt, Germany).

Plasmids
The human genes encoding the CYP1A2, CYP2C9, and CYP3A4 were cloned together with a NADPH-P450 reductase gene into a bicistronic pCW vector [182]. The used vector allowed independent expression of the monoxygenase and the reductase gene. Plasmid without a monoxygenase encoding gene was used for control experiments and is referred to as a control plasmid. All described plasmids were a kind gift from F. P. Guengerich (Vanderbilt University School of Medicine, Nashville, USA). Expression was performed in *E. coli* DH5α (Gibco BRL, Gaithersburg, USA).
Expression in E. coli

E. coli DH5α containing the expression plasmid was grown overnight (37°C, 250 rpm) in LB medium (ampicillin 100 µg ml⁻¹). For inoculation (1:100) a culture of 30 ml of TB-medium supplemented with 1% glucose, 100 µg ml⁻¹ of ampicillin and 0.25 ml of a solution of trace elements [195] was additionally grown (37°C, 250 rpm) till an OD₆₀₀ of 0.5. Expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG; 1.0 mM), thiamine (1.0 mM) and δ-aminolevulinic acid (0.5 mM) followed by another 20 h of growth (30°C, 200 rpm). Cells were harvested by centrifugation (10 min, 3000 x g, 4°C) after the determination of the final OD₆₀₀, and washed with potassium phosphate buffer (0.1 M, pH 7.4) by resuspending and centrifugation. Cells were resuspended in 0.1 M potassium phosphate buffer (pH 7.4) containing 20% of glycerol to a final OD₆₀₀ of 15.0. Cells could be used directly or stored in small portions at –80°C until usage.

CYP CO-saturation difference analysis

In order to determine the amount of active CYP enzyme in the bacterial cells, potassium phosphate buffer (0.1 M, pH 7.4) was gently mixed with the cells (1:1) in a 1 ml cuvette. A pinch of sodium hydrosulphite was added and carefully mixed. After 1 min the baseline absorption (400-600 nm) was recorded. Then the mixture was gassed through with carbon monoxide (Hoekloos, Schiedam, The Netherlands) for 30 s, with a gas flow of 1-2 bubbles s⁻¹, and the absorption spectrum at 400-600 nm was recorded again. The amount of enzyme was calculated as described in the literature [196, 215] using the absorbance at 450 nm and 490 nm with 0.091 mM⁻¹ cm⁻¹ as the specific absorptivity. Measurements were performed on a Unicam UV500 Thermo Spectronics spectrometer.

SDS-PAGE

To detect the presence of heterologous protein in the E. coli cells after the expression, membrane fractions of the cells have been isolated [195] and analyzed using a NuPage system with a 4-12% Bis-Tris gel (Invitrogen, Carlsbad, USA) and Coomassie Blue staining.

Bioconversion assay

Assays were performed in potassium phosphate buffer (0.1 M, pH 7.4) containing glucose (12.5 mM). Cells with expressed CYP were thawed on ice and added to the buffer (20% v/v of total volume). Because the solubility in water for lignans is low (max. 120 mg/l for podophyllotoxin) and good in DMSO and alcohols, all substrates were prepared in 1.0 mM concentration in DMSO and diluted to a final substrate concentration of 10 µM with the buffer solution containing the enzymes. NADP (1 mM), glucose-6-phosphate (5 mM) and glucose-6-phosphate dehydrogenase (1 U ml⁻¹) were added subsequently to the reaction mixture. Finally, MgCl₂ (30 mM) was added, followed by incubation for 2 h (37°C, 250 rpm). Conversions of the CYP1A2 substrate phenacetin, the CYP2C9 substrate tolbutamide and the CYP3A4 substrate testosterone [182, 216] were performed as a control in all experiments. The conversions with a volume of 1.0 ml were performed in Pyrex glass tubes, whereas scaling-up to 50 ml conversions for isolation of the products for HPLC and HPLC-NMR studies were performed in 500 ml glass Erlenmeyer flasks.
Chapter 6

Sample preparation
After incubation of the substrate with the enzyme, the reaction mixture was extracted twice with a double volume of ethyl acetate by vigorous shaking. The layers were separated by centrifugation. The combined organic layers were dried over anhydrous sodium sulphate and evaporated to dryness in a stream of air. Subsequently, the residue was dissolved in 150 µl of methanol and the final solution was analysed by HPLC.

HPLC
HPLC analysis was performed using a Shimadzu-VP system (Shimadzu, ‘s-Hertogenbosch, The Netherlands) consisting of a LC-10AT pump, a Kontron 360 auto sampler, a SPD-M10A DAD detector, a FCV-10AL low pressure gradient mixer, a SCL-10A system controller, a FIAtrom systems CH-30 column heater, operated with CLASS-VP software, version 6.12SP4. The column used was a Luna C18(2), 250 x 4.6 mm, 5µm, together with a Phenomenex guard cartridge C18 (4 x 3 mm) (Phenomenex, Bester, The Netherlands). The injection volume was 20 µl with a flow rate of 1 ml min\(^{-1}\) using a time program of 30 minutes consisting of 5 min of 95% of solvent A [5 mM ammonium formate (0.05% formic acid) : MeCN = 800 : 156 (w/w)] and 5% of solvent B [MeCN : MeOH (0.05% formic acid) : 5 mM ammonium formate (0.05% formic acid) = 585 :40 : 200 (w/w)], followed by a linear gradient with given time endpoints: 24 min: 100% B; 26 min: 100% B; 28 min 5% B ; 30 min 5% B. The capacity factor (k’) was calculated using the formula \(k’ = (R_t - R_{t0}) / R_{t0}\), where \(R_t\) is the retention time of a compound and \(R_{t0}\) is the void time. Concentrations of the metabolites were calculated using a calibration curve established with reference compounds. Concentration of epipodophyllotoxin was calculated as the concentration of podophyllotoxin.

Structure elucidation
HPLC-MS
A Perkin-Elmer SCIEX API-3000 apparatus (Toronto, Canada) with atmospheric pressure electrospray ionisation and triple-quadruple mass spectrometer operating in the positive mode was used for the HPLC-MS analysis. The instrument was equipped with a Perkin-Elmer series 200 HPLC micro-gradient pump and an autosampler. The mass spectrometer was interfaced to a Lichrosphere 100 RP-18 column (250 mm x 4 mm, 5 µm particle size) equipped with a guard column. The mobile phase was 95% H\(_2\)O : 4.9% MeCN (solvent A) and 95% MeCN : 4.9% H\(_2\)O (solvent B), both containing 0.1% HCOOH and 2 mM ammonium formate. The sample was commenced at 70:30 (v/v) of A:B for 5 min, followed by a linear gradient with given time endpoints: 30 min: 90% B; 35 min: 90% B; 37 min: 30% B; 40 min: 30% B. The flow rate was 1 ml min\(^{-1}\) at room temperature. The injection volume was 25 µl. The positive ion mode was employed and spectra were obtained with a spray voltage of 5.2 kV. The source temperature was 400°C. The scan rate was 2 sec scan\(^{-1}\), a full ion scan was applied in the range 100–1200 amu with a step size of 1 amu and 10 V entrance potential. Nitrogen was used both as the nebulizer and curtain gas at a pressure of 0.8 Torr and a flow rate of 20 ml min\(^{-1}\). Data processing was performed using Analyst version 1.4 software (MDS Sciex, Concord, Canada).
HPLC-SPE-NMR

HPLC separations were carried out at 40 °C on a 150 × 4.6 mm i.d. C18(2) Phenomenex Luna column (3 µm, 10 nm) using an Agilent 1100 system consisting of quaternary solvent delivery pump equipped with a degasser (Degases Populaire), an autosampler, and a diode array detector. The chromatograms were monitored at 240 nm, and the sample was separated using mixtures of water (A) and acetonitrile (B) with a flow rate of 0.8 ml min⁻¹. The linear gradient elution program was used starting with 0% B with given time endpoints: 30 min: 100% B; 35 min: 100% B; 40 min: 0% B; 50 min: 0% B. 30 µL of a 40 mg ml⁻¹ sample (in acetonitrile) was injected. A Knauer K100 Wellchrom make-up pump (flow rate 2.0 ml min⁻¹) diluted the post-column eluent flow with water before peak trapping using a Prospekt II SPE unit (Spark Holland, The Nederlands). HySphere GP polymer resin [poly(divinylbenzene)] SPE cartridges (10 x 2 mm i.d.) from Spark Holland were used for trappings. The SPE device was coupled to a Bruker Avance 600 MHz spectrometer equipped with a 30 µL (active volume) ¹H flow probe. For structure determination, a total of eight trappings were performed for each chromatographic peak. The cartridges were dried with a stream of nitrogen for 30 min and the analytes were eluted with 275 µL of acetonitrile-d₃ into the NMR probe for data acquisition. HPLC separations, SPE peak trapping and analyte transfer to the NMR spectrometer were controlled by Bruker HyStar version 2.3 software, while NMR acquisition and processing were performed using Bruker XwinNMR version 3.1 software.

Results

cDNAs for human CYP450 enzyme (1A2, 2C9 or 3A4) and NADPH-P450 reductase were functionally expressed in E. coli DH5α cells, using pCW vector [182]. After expression in E. coli, identification of all cytochromes was performed by SDS-PAGE (Fig. 2) and the CO-saturation difference spectra were measured (Fig. 3).

![Fig. 2: SDS-PAGE analysis of bacterial membranes after expression of the control plasmid (C), CYP1A2, CYP2C9, and CYP3A4 (approximately 56 kDa) and the reductase (approximately 77 kDa). The standards (M) correspond to 97, 64, and 51 kDa.](image)
SDS-PAGE analysis showed bands at approximately 56 kDa (CYP450) and 77 kDa (NADPH-P450 reductase) in bacterial membrane fractions of the expressing strains, but not in membranes derived from cells containing the control plasmid. Expressed but inactive (misfolded) proteins were distinguished from active enzymes by their absorption at 420 nm instead of 450 nm as shown in Fig. 3. Cultures containing expressed CYP1A2, CYP2C9 and CYP3A4 all showed absorbance at 450 nm, whereas no absorption at 450 nm could be detected in cultures containing the control plasmid.

Expression of CYP 3A4 in *E. coli* DH5α was investigated over the time course of 24 h after the induction of the cells as depicted in Fig. 4. The protein expression shows a maximum at 20 h. Therefore, the cells were harvested at 20 h in the subsequent experiments with all three CYP enzyme subtypes.
Bioconversion of deoxypodophyllotoxin

The biotransformation capacity of the bacterial cells containing the expressed P450/NADH P450-reductase system was assayed. The metabolites formed in the bioconversion of phenacetin, tolbutamide and testosterone by CYP1A2, CYP2C9 and CYP3A4, respectively, were detected by HPLC-analysis. No metabolite formation was detected in the cells transformed with the control plasmid. Phenacetin (k’ = 5.02) was converted into paracetamol (k’ = 0.60,) by CYP1A2, tolbutamide (k’ = 7.80.) into hydroxytolbutamide (k’ = 4.92) by CYP 2C9, and testosterone (k’ = 8.08) into 6β-hydroxytestosterone (k’ = 5.13). Cells expressing CYP3A4 and incubated with deoxypodophyllotoxin (k’ = 8.80) gave a product peak at k’ = 6.96 under the HPLC-UV conditions. In contrast to CYP3A4 no metabolism was detected for CYP1A2 and CYP2C9 when incubated with deoxypodophyllotoxin.

In a second step, the control and CYP3A4 samples were subjected to HPLC-MS analysis in order to obtain initial information about the nature of the aforementioned HPLC peak arising during the incubation. Deoxypodophyllotoxin was recorded under the applied HPLC-MS conditions at a k’ = 8.86 (Rt = 21.7 min). HPLC-ESIMS analysis led to good responses and produced ion species, typical for electrospray ionisation in positive mode. Specifically, the HPLC-ESIMS spectrum of the extra peak at k’ = 6.86 (Rt = 17.3 min) in CYP3A4 sample (Fig.5) showed abundant ion peaks at m/z 415 ([M+H]+), 432 ([M+NH4]+), 437 ([M+Na]+), and 453 ([M+K+]'), which is consistent with a molecular weight of 414. The ion with m/z 397 in the fragmentation pattern of podophyllotoxin (Fig. 5) showed the highest intensity, which results from successive loss of water [M+H-H2O]+. In contrast to podophyllotoxin, the compound at k’ = 6.86 in CYP3A4 sample under HPLC-NMR conditions has the most abundant ion at m/e 415, which is indicative of a different configuration of the reaction product.

Fig. 4: Levels of CYP3A4 based in CO-saturation spectra and corrected for the amount of cells (OD600).

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Fig. 5: HPLC-MS spectrum of bioconversion of deoxypodophyllotoxin ($R_t = 21.7$ min) to epipodophyllotoxin ($R_t = 17.3$ min) (A) and related mass fragmentation pattern (B). Fragmentation pattern is discussed in detail in the text.
For structural elucidation of the compound accounting for the peak of interest, the HPLC-SPE-NMR technique was employed. One of the main features of this hyphenated technique is the ability to perform multiple trappings of analytes eluted from an HPLC column. The analytes are automatically trapped on solid-phase-extraction cartridges, dried and eluted directly into a NMR flow probe [217]. The concentrations of analytes that are achieved in the flow probe, following analytical scale separation and multiple trappings, are sufficient for the acquisition of high quality 1D and 2D NMR data, thus, enabling structural elucidation of compounds without preparative-scale isolation. HPLC-SPE-NMR analysis of the extract matrix solution of the CYP3A4 sample resulted in the identification of epipodophyllotoxin (peak 3) and deoxypodophyllotoxin (peak 5) (Fig. 6).

**Fig. 6:** HPLC chromatogram (240 nm) of the CYP3A4 matrix solution extract. Gradient elution profile is shown as percent of acetonitrile. The peaks selected for HPLC-SPE-NMR experiments are labeled 1-6 (3; epipodophyllotoxin and 5; deoxypodophyllotoxin).

The remaining compounds eluted in peaks 1, 2, 4 and 6 were not related to the metabolites of interest and full structural elucidation was not conducted for these compounds. Comparison of the retention time and UV spectrum of the compound eluted as peak 3 with that of the pure podophyllotoxin reference might suggest that this compound was podophyllotoxin. However, analysis of the acquired 1D and 2D NMR data clearly showed that this compound is the stereoisomer, epipodophyllotoxin. The $^1$H NMR data of epipodophyllotoxin differ from that of podophyllotoxin by chemical shift values of H-2', H-6, H-6', H-7, H-8, H-8', H-9a and H-9b (Fig. 7). These differences, as well as the multiplicity and coupling constants for H-7 (epipodophyllotoxin H-7, $dd$, $J$= 5.1, 3.5 Hz; podophyllotoxin H-7, $t$, $J$= 8.5 Hz), indicate an $\alpha$-orientation of H-7 in the bioconversion
Fig. 7: Diagnostic signals in the 1D $^1$H NMR spectrum (acetonitrile-$d_3$) of peak 5 (A) (deoxypodophyllotoxin) and peak 3 (B) (epipodophyllotoxin) obtained in HPLC-SPE-NMR mode, and (C) podophyllotoxin after direct injection to the NMR probe. Results are discussed in detail in the text.
product. Coupling patterns in the COSY spectrum confirmed assignments of the H-7, H-7’, H-8, H-8’, H-9a and H-9b protons. The $^1$H NMR chemical shifts determined for the bioconversion product were also in good agreement with those reported for epipodophyllotoxin [218, 219].

**Discussion**

The conversion of deoxypodophyllotoxin into epipodophyllotoxin shows that a recombinant human cytochrome can perform a specific hydroxylation at the C-7 –position. With the bioconversion of deoxypodophyllotoxin, we demonstrated that an alternative for the production of specific lignans is now feasible. Previous studies towards production systems other than *Podophyllum* plants have shown limited success. Although the bioconversion of deoxypodophyllotoxin into epipodophyllotoxin by fungi has been accomplished, the conversion rates were very low and the process took a long period of time [208, 209, 220]. An alternative method for the large-scale production of lignans could be plant cell suspension cultures. Several lignans, including podophyllotoxin β-D-glucopyranoside, have been obtained by this method [221, 222]. However, until now little progress has been reported with respect to optimization of the podophyllotoxin yield. It should also be noted that plant cell cultures are more expensive than the present bacterial cultures.

The application of a heterologous expression system for specific enzymatic modifications of lignans, as shown in our experiments, can be a strong tool for the manufacturing of pharmaceutically important derivatives. Firstly, it shows that non-plant enzymes can perform specific biosynthetic steps in plant metabolism, which largely increases the arsenal of possible bioconversion tools. Secondly, since it forms an inducible system, the disturbing toxicity that various products exhibit towards fungi or bacteria can be bypassed. This will allow the (semi-)synthesis of lignans using microorganisms. The yield of epipodophyllotoxin from conversion of deoxypodophyllotoxin by the CYP3A4 system was at best approx. 90% in the present, non-optimised system (Table 1). The remaining 10% was present as non-converted deoxypodophyllotoxin.

**Table 1:** The percentages conversion of 10 μM testosterone and 10 μM deoxypodophyllotoxin (DOP) by CYP 3A4 after t = 0.33, 1, 2, 8 and 30 hours (% ± sd, n=3).

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<th>0.33 h</th>
<th>1 h</th>
<th>2 h</th>
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<tr>
<td>Testosterone</td>
<td>19.7 ± 1.08</td>
<td>35.3 ± 4.85</td>
<td>51.6 ± 3.64</td>
<td>70.6 ± 1.48</td>
<td>74.3 ± 3.05</td>
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<tr>
<td>DOP</td>
<td>53.3 ±0.35</td>
<td>86.7 ± 0.69</td>
<td>96.3 ± 2.80</td>
<td>97.2 ± 1.19</td>
<td>98.8 ± 0.31</td>
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It has been reported that etoposide and teniposide are degraded by human CYP 3A4 by 3'-O-demethylation [211, 212]. Deoxypodophyllotoxin possesses methoxy groups at the 3' and 5' positions and therefore a possible O-demethylation of this compound was anticipated. However, no O-demethylation of deoxypodophyllotoxin was observed. The different behaviour can be explained by two major differences in the molecular structure. Firstly, deoxypodophyllotoxin has a 4'-methoxy group instead of a 4'-hydroxy group, as in etoposide and teniposide. This affects the size and polarity of the aromatic ring involved in the reaction. Secondly, etoposide and teniposide already contain an oxygen atom at the C-7 position, and therefore further hydroxylation at this position is highly unlikely. Since the benzylic C-7 position in deoxypodophyllotoxin is highly reactive, its hydroxylation can be anticipated. Fortunately, the hydroxy group introduced in the bioconversion has the same configuration as in etoposide and teniposide. Therefore, epipodophyllotoxin can be converted to these drugs without inversion of the configuration at C-7 that takes place during their commercial synthesis from podophyllotoxin. Derivatization of the hydroxy group at C-7 in epipodophyllotoxin with the retention of configuration is expected to be achievable via an enzymatic glucosylation. This opens a novel biosynthetic strategy for the production of these two potent anticancer drugs.

The HPLC-SPE-NMR experiments allowed the unambiguous identification of the metabolite epipodophyllotoxin in the extract matrix solution. This was achieved on-line prior to any purification or isolation of the different constituents in the extract matrix solution. Therefore, the technique was demonstrated to speed up the analysis of the bioconversion reaction performed on a small scale. For large scale production of epipodophyllotoxin the described bioconversion process must be improved. Firstly, the reconstitution of correctly functioning CYP3A4 enzyme and electron transport must be improved by metabolic engineering or by using another host cell. As mentioned, a major fraction of the recombinant enzyme is misfolded and not active in E. coli [182]. Secondly, the generation of more active enzymes with a higher metabolism rate may be achieved through protein engineering of CYP3A4 enzymes using directed evolution techniques [223].

A major advantage of the described bioprocess is the use of deoxypodophyllotoxin from easily accessible sources, without using endangered Podophyllum species from wild collections. Due to the fact that deoxypodophyllotoxin is accumulated in rhizoma of the common weed A. sylvestris [9], isolation of the natural product is easy and economic. Therefore, the production of epipodophyllotoxin from deoxypodophyllotoxin by heterologous expression of an improved CYP 3A4 may be a good alternative for the P. hexandrum rhizomes in the near future.

**Conclusion**

Hydroxylation at the C-7 position of 2,7'-cyclolignans can be accomplished by CYP3A4. Deoxypodophyllotoxin was converted to epipodophyllotoxin, which contains the same configuration at C-7 as the clinically important drugs etoposide and teniposide. No 3-O-demethylation, which takes place upon the action of CYP3A4 on these drugs, was observed. The bioconversion of deoxypodophyllotoxin by the heterologous expression system shows that a plant specific biosynthetic step can be mimicked by an unrelated human enzyme with high efficiency. Fast growing bacterial cells, high level of enzyme expression, ease of manipulation, high product yield and relatively low costs are the main
advantages of the described system for production of lignans with high cytotoxicity. Hepatotoxicity is well known for podophyllotoxin and other lignans. An *in vitro* system as discussed here, using bacterial cells, can avoid problems caused by toxicity for growth and maintenance of mammalian production cell lines. Furthermore, process engineering for production at a higher scale needs a high supply of NADP to avoid high costs. This problem has to be solved in the future to make this bioconversion a valuable strategy for an industrial production of epipodophyllotoxin. Therefore, heterologous expression of human CYP3A4 in bacteria and subsequent optimization can be used as an alternative strategy for production of epipodophyllotoxin, which is a valuable precursor in the pharmaceutical industry.

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