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Towards Metabolic Engineering of Podophyllotoxin Production

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Abstract

The pharmaceutically important anticancer drugs etoposide and teniposide are derived from podophyllotoxin, a natural product isolated from roots of *Podophyllum hexandrum* growing in the wild. The overexploitation of this endangered plant has led to the search for alternative sources. Metabolic engineering aimed at constructing the pathway in another host cell is very appealing, but for that approach, an in-depth knowledge of the pathway toward podophyllotoxin is necessary. In this chapter, we give an overview of the lignan pathway leading to podophyllotoxin. Subsequently, we will discuss the engineering possibilities to produce podophyllotoxin in a heterologous host. This will require detailed knowledge on the cellular localization of the enzymes of the lignan biosynthesis pathway. Due to the high number of enzymes involved and the scarce information on compartmentalization, the heterologous production of podophyllotoxin still remains a tremendous challenge. At the moment, research is focusing on the last step(s) in the conversion of deoxypodophyllotoxin to (epi)podophyllotoxin and 4'-demethyldeoxypodophyllotoxin by plant cytochromes.

Keywords: etoposide, podophyllotoxin, *Podophyllum hexandrum*, *Anthriscus sylvestris*, metabolic engineering

1. Introduction

The high demand of podophyllotoxin derivatives for chemotherapy gives a severe pressure on the natural sources, such as *Podophyllum hexandrum* and *Podophyllum peltatum* [1]. The highest concentration of podophyllotoxin is found in *P. hexandrum* roots, with reported

yields up to 6.6% dry weight (d.w.) [2]. The excessive harvesting has resulted in inclusion of *P. hexandrum* in the Convention on International Trade in Endangered Species (CITES) [3]. Chemical synthesis of podophyllotoxin is difficult due to the presence of four contiguous chiral centers and the presence of a base sensitive *trans*-lactone moiety [4]. The shortest synthesis described contains five steps from the commercially available 6-bromopiperonal into (epi) podophyllotoxin [5]. As an alternative, cell suspension cultures have been explored, but these produce only low amounts (max. 0.65% d.w.) of podophyllotoxin [6, 7]. As neither chemical synthesis nor *in vitro* production of podophyllotoxin is economically competitive with the extraction of podophyllotoxin from *P. hexandrum* roots, other alternatives are being searched for. Metabolic engineering aimed at constructing the pathway in a heterologous host is very appealing, but for that approach, an in-depth knowledge of the biosynthetic pathway toward podophyllotoxin is necessary.

2. Lignans and their biological activities

In 1936, Haworth was the first to describe a group of phenylpropanoid dimers (C_6C_3) linked by the central carbon (C8) as lignans [8]. The Haworth's definition of lignan has been adopted by the IUPAC nomenclature recommendations in 2000 [9]. According to this nomenclature, lignans can be divided into eight subgroups based on the oxygen incorporation into the skeleton and the cyclization pattern [10]. In the lignan pathway toward podophyllotoxin, six subgroups of lignans can be defined in the order of occurrence: furofuran, furan, dibenzylbutane, dibenzylbutyrolactol, dibenzylbutyrolactone, and aryltetralin (**Figure 1**). The other two subgroups are aryl-naphthalene and dibenzocyclooctadiene. Dibenzylbutanes are only linked by the 8,8' bond. An additional oxygen bridge is found in furofurans, furans, dibenzylbutyrolactols, and dibenzylbutyrolactones. A second carbon-carbon link is found in aryltetralins, aryl-naphthalenes, and dibenzocyclooctadienes [10, 11]. The majority of the lignans has oxygen at the C9 (C9') carbon; however, some lignans in the dibenzylbutanes, furans, and dibenzocyclooctadiene subgroups are missing this oxygen [10]. Humans metabolize the furofurans pinoresinol and sesamin, the furan lariciresinol, the dibenzylbutane secoisolariciresinol, and the dibenzylbutyrolactone matairesinol. These lignans are phytoestrogens, which can be converted into enterolactone or enterodiol by intestinal bacteria [12, 13]. Enterolactone and enterodiol have antioxidant, estrogenic, and anti-estrogenic activities in humans; furthermore, they may protect against certain chronic diseases [14]. Several lignans have been described to have antiviral properties; however, therapeutic applications are limited due to the toxicity [15]. The extract, podophyllin, of *Podophyllum* roots and rhizome was included in the U.S. Pharmacopeia in 1820. In 1942, it was removed, because of its severe gastrointestinal toxicity [16]. However, Kaplan described in 1944, the successful treatment of venereal warts (*Condylomata acuminata*) in 200 members of the military by topically applied podophyllin [17]. The aryltetralin podophyllotoxin is the active ingredient in podophyllin, which has been commercialized as a treatment for warts caused by the human papilloma virus [18]. Semisynthetic derivatives of podophyllotoxin were designed as chemotherapy compounds for oral administration or for intravenous treatment [19, 20].

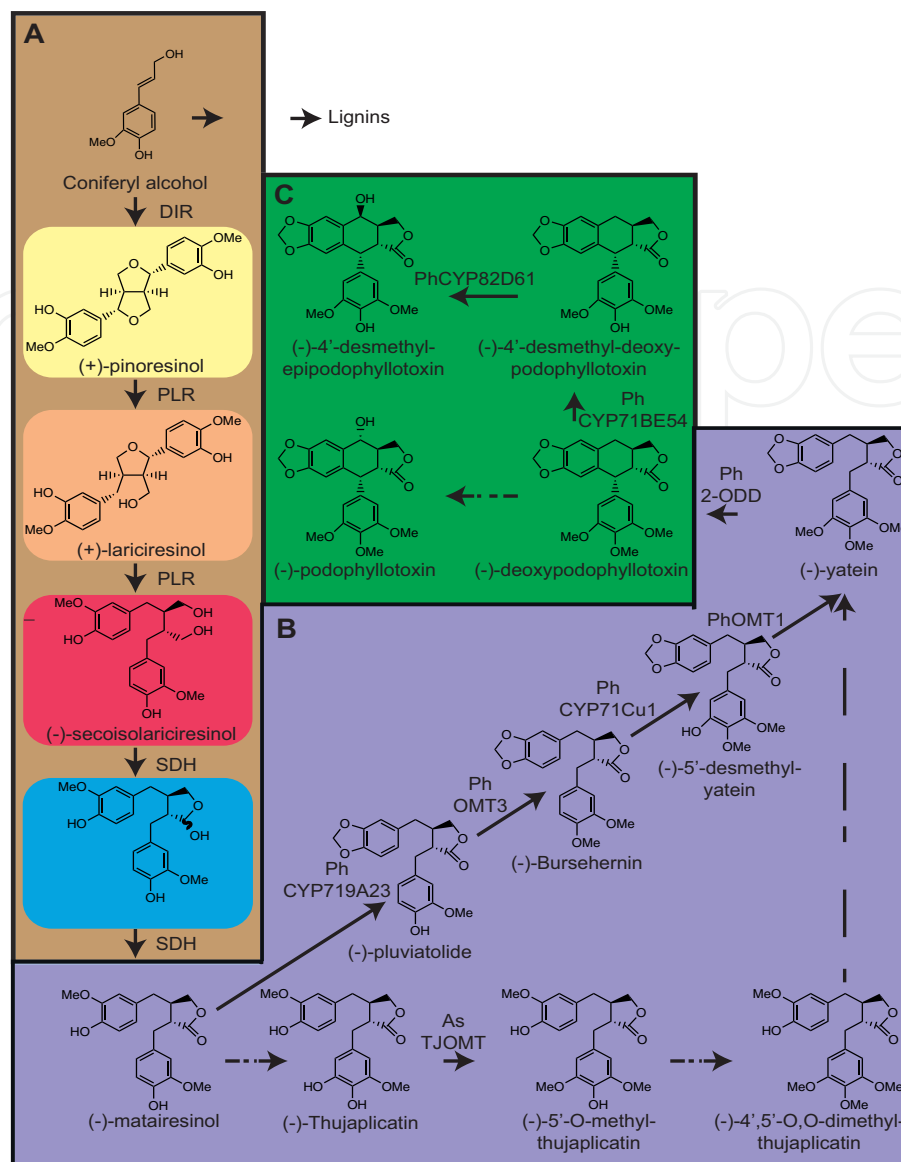


Figure 1. Lignan pathway in *Podophyllum hexandrum* and *Anthriscus sylvestris*. (A) Coniferyl alcohol toward matairesinol (brown box), (B) matairesinol toward deoxypodophyllotoxin (purple box), and (C) deoxypodophyllotoxin toward podophyllotoxin and demethyldeoxypodophyllotoxin (green box). Lignan subgroups are shown by various colors: yellow = furofuran, orange = furan, red = dibenzylbutane, blue = dibenzylbutyrolactol, purple = dibenzylbutyrolactone, and green = aryltetralin.

3. Importance of podophyllotoxin and derivatives for chemotherapy

Podophyllotoxin is a tubulin-interacting agent that inhibits mitotic spindle formation [21]. As podophyllotoxin is severely toxic if applied systemic, a number of less toxic derivatives have been generated and these are now widely used in cancer chemotherapy. Interestingly,

the derivatives currently used in the clinic, etoposide, and teniposide, have a different mode of action than podophyllotoxin. They inhibit topoisomerase II by stabilizing its binding to DNA, which results in double-stranded breaks in the DNA and arrest of the cell cycle in the G2 phase [21]. Etoposide (VP-16, VePesid®) was synthesized in 1966 by Sandoz and was further developed by Bristol-Meyers from 1978 onwards. In 1983, it was approved by the FDA for the treatment of testicular cancer [22]. As etoposide is poorly soluble in water, the etoposide prodrug etoposide phosphate (Etopophos®) was designed by Bristol-Meyers Squibb, which was approved by the FDA in 1996 [23]. The prodrug is converted to etoposide within 30 min presumably by alkaline phosphatases. Furthermore, the pharmacokinetics and toxicity of etoposide phosphate are equal to etoposide [24, 25]. According to the National Cancer Institute and the Dutch government etoposide, phosphate should be used in combination therapy for various cancers (**Table 1**) [26–28]. Teniposide (VM-26, Vumon®) was synthesized in 1967 by

Cancer	Combination of drugs
Hodgkin lymphoma in children	Vincristine sulfate, etoposide phosphate, prednisone, doxorubicin hydrochloride
	Doxorubicin hydrochloride, bleomycin, vincristine sulfate, etoposide phosphate
	Doxorubicin hydrochloride, bleomycin, vincristine sulfate, etoposide phosphate, prednisone, cyclophosphamide
Non-hodgkin lymphoma	
- All	Rituximab, ifosfamide, carboplatin, etoposide phosphate
	Etoposide phosphate, ifosfamide, methotrexate
	Lomustine, etoposide phosphate, chlorambucil, prednisolone
- B-cell	Rituximab, etoposide phosphate, prednisone, vincristine sulfate, cyclophosphamide, doxorubicin hydrochloride
Malignant germ cell tumors	
- Nonbrain	Cisplatin, etoposide phosphate, bleomycin
- Ovarian/testicular	Bleomycin, etoposide phosphate, cisplatin
- Advanced testicular	Etoposide phosphate, ifosfamide, cisplatin
Acute myeloid leukemia	
- Children	Cytarabine, daunorubicin hydrochloride, etoposide phosphate
- Phase II	Cytarabine and amsacrine, etoposide or mitoxantrone
High-risk retinoblastoma in children	Carboplatin, etoposide phosphate, vincristine sulfate
Small cell lung cancer	Etoposide with cisplatin or carboplatin
	Cisplatin, cyclophosphamide, doxorubicin, vincristine, methotrexate
Relapsed Wilms tumor	Ifosfamide, carboplatin, and etoposide

Table 1. Cancer chemotherapy combination treatments with etoposide.

Sandoz and was further developed by Bristol-Meyers from 1978 onwards [22]. It is used in the treatment of acute myeloid leukemia and myelodysplastic syndromes in children and in acute lymphocytic leukemia [29, 30]. Toxicity problems are still an issue with etoposide; therefore, novel derivatives were designed and evaluated in preclinical and clinical studies [31]. The derivatives NK611, GI-311, and TOP-53 were discontinued after phase I or II studies [22, 32, 33]. NK611, which is more water soluble than etoposide, shows similar toxic effects in humans as etoposide. However, only few patients showed efficacy in phase I studies [34–36]. No data of the phase I or II studies were found for GL-311 and TOP-53. Four newer derivatives are tafluposide, F14512, Adva-27a, and QS-ZYX-1-61 [31, 32]. Tafluposide (F-11782), a pentafluorinated epipodophyllotoxin, inhibits topoisomerase I and II activity [37, 38]. In phase I study, stable disease was observed in 7 out of 21 patients with advanced solid tumors, such as choroid and skin melanoma [39]. Increasing the selectivity of anticancer agents is of great interest. As the polyamine transport system is upregulated in cancer cells, F14512 was designed to target the transport system by linking the epipodophyllotoxin core to a spermine chain [40]. Phase I study in adult patients with acute myeloid leukemia showed clinical activity in relapsed patients, but limited activity in refractory patients [41]. F14512 will be tested in combination with cytarabine in a phase II study [41]. The minimal therapeutic effect of etoposide on dogs with relapsing lymphomas has resulted in a phase I study of F14512, which showed a strong therapeutic efficacy [42]. The derivative adva-27a, a GEM-difluorinated C-glycoside derivative of podophyllotoxin, is effective against multidrug resistant cancer cells [43]. Preparations are being made for a phase I study in pancreatic and breast cancer patients in Canada [44]. The derivative QS-ZYX-1-61 induces apoptosis by inhibition of topoisomerase II in human non-small-cell lung cancer [45]. Further investigations are necessary for this compound.

4. Overview of the lignan biosynthetic pathway

Podophyllotoxin is produced in the lignan pathway, which we will discuss in more detail in this section (**Figure 1**). Lignins and lignans are the major metabolic products of the phenylpropanoid pathway in vascular plants. Lignins are derived from coumaryl, coniferyl, and sinapyl alcohol, whereas lignans are derived from coniferyl alcohol [46].

4.1. Coniferyl alcohol toward matairesinol

The pathway toward podophyllotoxin starts with pinoresinol, lariciresinol, secoisolariciresinol, and matairesinol. Pinoresinol and lariciresinol are found in most vascular plants, such as *Arabidopsis thaliana*. Some species follow the lignan pathway toward podophyllotoxin until the branch point matairesinol, such as the *Forsythia* species. Lignans further downstream toward podophyllotoxin are found in more specialized plants. An interesting question is whether the capability of podophyllotoxin production is restricted to a limited number of plants, or that other closely related plants have cryptic pathways as shown in bacteria [47]. To answer this question, an in-depth discussion of the lignan pathway is necessary as we do below. Coniferyl alcohol is converted into matairesinol in five steps by three enzymes: dirigent protein, pinoresinol-lariciresinol reductase, and secoisolariciresinol dehydrogenase (**Figure 1A**).

4.1.1. Dirigent protein

In 1997, Davin and coworker showed that the dirigent protein (DIR) from *Forsythia suspensa* can couple two coniferyl alcohols stereospecific to (+)-pinoresinol after their oxidation by a nonspecific oxidase or nonenzymatic single-electron oxidant [48]. Davin and coworkers showed that the DIR protein lacks a detectable catalytic active (oxidative) center and that the rate of dimeric lignan formation is similar in the presence or absence of DIR protein; however, the DIR protein is necessary for enantioselectivity [48]. Both (+)- and (-)-pinoresinol-forming proteins were found in plants. The (+)-forming DIR protein is important for the lignan pathway in the direction of podophyllotoxin synthesis. (+)-Forming DIRs are the ScDIR protein from *Schisandra chinensis*, the *psd-Fi1* from *Frullania intermedia*, and PsDRR206 from *Pisum sativum* [49–51]. In *A. thaliana*, 16 DIR homologs were found of which four were characterized as follows: two formed (-)-pinoresinol (AtDIR5 and AtDIR6); the other two showed nonstereoselective coupling of coniferyl alcohols [49, 52]. On the other hand, *Linum usitatissimum* has (+)-forming and (-)-forming DIR proteins [53]. Kim and coworkers solved the crystal structure of the (+)-pinoresinol forming PSDRR206 of *P. sativum* to 1.95 Å [54]. Homology modeling of the (-)-pinoresinol forming AtDIR6 in the PSDRR206 crystal structure showed six additional residues in the longest loop of the (+)-forming DIR, which are present in all (+)-forming DIRs. Site-directed mutagenesis could be used to confirm whether one or more of these residues are responsible for the enantioselectivity of the DIR [54].

4.1.2. Pinoresinol-lariciresinol reductase

In 1996, Dinkova-Kostova and coworkers found the pinoresinol-lariciresinol reductase (PLR) in *F. intermedia*, which could reduce (+)-pinoresinol to (+)-lariciresinol and sequentially to (-)-secoisolariciresinol [55]. The (-)-secoisolariciresinol-forming PLRs are important for podophyllotoxin synthesis. These PLRs were found in *F. intermedia* (PLR-Fi1), *Linum album* (PLR-La1), *L. usitatissimum* (PLR-Lu2) and *Linum corymbulosum* (PLR-Lc1) [56–59]. A PLR with opposite enantioselectivity was found in *L. usitatissimum* (PLR-Lu1) [57, 58]. PLR can have selectivity or preference toward one of the enantiomers. The *Thuja plicata* PLRs accept both enantiomers of pinoresinol; however, they were selective for the lariciresinol substrate, as PLR-TP1 accepts only (-)-lariciresinol and PLR-TP2 only (+)-lariciresinol [60]. In *Linum perenne*, it was found that PLR-Lp1 can convert (±)-pinoresinol to (±)-lariciresinol and (±)-secoisolariciresinol, with a preference for (+)-pinoresinol and (-)-lariciresinol [61]. The *F. intermedia* (PLR-Fi1) and *L. usitatissimum* (PLR-Lu1) PLRs were found to convert (+)-lariciresinol to (-)-secoisolariciresinol before depletion of (-)-pinoresinol [56, 57]. On the other hand, *L. album* (PLR-La1) and *L. perenne* (PLR-LP1) PLRs first seem to convert all (+)-pinoresinol to (+)-lariciresinol before converting (+)-lariciresinol further to (-)-secoisolariciresinol [57, 61]. For *A. thaliana* proteins with strict substrate, specificity toward pinoresinol was found as weak or no activity toward lariciresinol was observed [62]. Therefore, these proteins are annotated as pinoresinol reductases (AtPrRs). AtPrR1 reduces both enantiomers, and AtPrR2 only reduces (-)-pinoresinol [62]. The crystal structures of PLR-Tp1 of *T. plicata* were resolved to 2.5 Å, and a homology model of PLR-Tp2 with opposite enantioselectivity was deduced from the PLR-Tp1 structure [63]. Three residues in the substrate binding site were different, which could explain the enantioselectivity [63].

4.1.3. Secoisolariciresinol dehydrogenase

Secoisolariciresinol dehydrogenase (SDH) from *F. intermedia* and *P. peltatum* convert (–)-secoisolariciresinol into (–)-matairesinol, through the intermediary (–)-lactol. Neither of them was able to convert the opposite enantiomer [64]. Crystallization of *P. peltatum* SDH (1.6 Å) showed that it is a tetramer. The ternary complex was obtained by the addition of cofactors and (–)-matairesinol. Based on the position of (–)-matairesinol, also (–)-secoisolariciresinol could be modeled into the crystal structure. Using the same constraints, (+)-secoisolariciresinol could not be modeled into the crystal structure, which could explain the enantioselectivity [64, 65].

4.2. Matairesinol toward deoxypodophyllotoxin

Plant feeding experiments performed by various groups have revealed the metabolites intermediate between matairesinol and podophyllotoxin, such as yatein and deoxypodophyllotoxin in *P. hexandrum* [66, 67]. This was followed by the identification of the enzymes in *P. hexandrum* (**Figure 1B**). Marques and coworkers found that pluviatolide synthases in *P. hexandrum* (CYP719A23) and *P. peltatum* (CYP719A24) can convert (–)-matairesinol into (–)-pluviatolide by formation of the methylenedioxy bridge [68]. Lau and Sattely used transcriptome mining in *P. hexandrum* to identify four additional biosynthetic enzymes in the lignan pathway, which convert (–)-pluviatolide into deoxypodophyllotoxin [69]. Pluviatolide 4-O-methyltransferase (PhOMT3) converts (–)-pluviatolide into bursehernin by methylation at C4'OH. Bursehernin 5'-hydroxylase (CYP71CU1) incorporates a molecular oxygen at C5' in bursehernin, which results in (–)-5'-desmethyl-yatein. In the following step, 5'-demethyl-yatein O-transferase (OMT1) converts (–)-demethyl-yatein to (–)-yatein by methylation at C5'OH. In the last step, deoxypodophyllotoxin synthase (2-ODD) converts (–)-yatein to (–)-deoxypodophyllotoxin by ring closure between C2 and C7' [69]. Sakakibara and coworkers suggest a different route toward deoxypodophyllotoxin for *Anthriscus sylvestris* (**Figure 1B**) [70]. Feeding experiments showed incorporation of matairesinol, thujaplicatin, 5-methylthujaplicatin, and 4,5-dimethylthujaplicatin into yatein [70]. This was followed by the discovery of the enzyme thujaplicatin O-methyltransferase (AsTJOMT), which methylates thujaplicatin to form 5-O-methylthujaplicatin [71]. Furthermore, they found incorporation of matairesinol and pluviatolide in bursehernin, but no further incorporation into yatein. No literature has been reported in the presence of 5-demethyl-yatein in *A. sylvestris*. However, feeding of 5-demethyl-yatein to *A. sylvestris* results in yatein formation [70]. In the transcriptome of *L. album*, genes related to OMT3 and CYP71CU1 were found; however, no gene related to CYP719A24 was found (**Figure 1B**) [72, 73]. The differences in the lignan pathways in *P. hexandrum*, *A. sylvestris*, and *L. album* indicate the possibility that the later part of the lignan pathway might have convergently evolved in the various species, which decreases the probability of the presence of a cryptic pathway in other species.

4.3. Conversion of deoxypodophyllotoxin into demethyldeoxypodophyllotoxin

The *P. hexandrum* enzyme that converts deoxypodophyllotoxin into podophyllotoxin has not been identified yet. Lau and Sattely, attempted to find this enzyme, presumably a cytochrome, by mining the publicly available RNA-sequencing data set from the Medicinal Plants Consortium.

Furthermore, they analyzed transcriptome data from *P. hexandrum* after upregulating the podophyllotoxin biosynthesis genes by wounding the leaves. Both methods were successful in identifying podophyllotoxin biosynthesis genes as described in the previous session; however, the enzyme converting deoxypodophyllotoxin into podophyllotoxin was not found (**Figure 1C**). They found two P450 cytochromes that can convert deoxypodophyllotoxin into 4'-desmethylepipodophyllotoxin [69]. In the first step, CYP71BE54 converts (-)-deoxypodophyllotoxin to (-)-4'-demethyldeoxypodophyllotoxin. In the second step (-)-4'-demethyldeoxypodophyllotoxin is converted to (-)-4'-desmethylepipodophyllotoxin by CYP82D61.

5. Engineering approaches

In this part, we will focus on genetic engineering approaches to produce podophyllotoxin in a heterologous system. In order to produce podophyllotoxin in *Escherichia coli* or *Saccharomyces cerevisiae*, the pathway from the easily available glucose toward coniferyl alcohol has to be implemented into these organisms.

5.1. Production of coniferyl alcohol in *E. coli* and *S. cerevisiae*

Coniferyl alcohol can be produced in *E. coli* by a co-culture system. Coumaryl alcohol is produced upon insertion of four phenylpropanoid pathway genes [74]. The production can be increased by addition of four key shikimate pathway genes to overproduce tyrosine [75]. Addition of the genes for methyltransferase and HpaBC in another strain resulted in the accumulation of 125 mg/L coniferyl alcohol after 24 h. Co-culturing was necessary as HpaBC converts tyrosine to an unwanted side product [74]. The full biosynthetic pathway toward coniferyl alcohol has not been tested for expression in *S. cerevisiae* yet. However, production of ± 100 mg/L coumaric acid has been shown [76]. To convert coumaric acid to coniferyl alcohol in *S. cerevisiae*, four or five additional genes have to be expressed; therefore, in order to produce coniferyl alcohol levels similar to *E. coli*, further optimization of coumaric acid production is necessary.

5.2. Cellular localization of enzymes from the lignan pathway

In order to engineer the lignan pathway for podophyllotoxin production in a heterologous cell, knowledge about the localization of lignans and their corresponding enzymes is necessary. Localization to the wrong organelle might abolish or lower production, as was shown for penicillin production [77]. The monolignol coniferyl alcohol is synthesized in the cytosol and transported over the plasma membrane for incorporation into lignin or lignan by an ABC membrane transporter, whereas the glucosylated form (coniferin) for storage could only be transported over the vacuolar membrane possibly by another ABC membrane transporter or proton-coupled antiporter [78, 79]. Analyses of transmembrane helices by the TMHMM predictor [80] indicated that DIR has one transmembrane helix. Furthermore, the DIR protein is a glycoprotein with a secretory signal peptide [50]. This indicates that the DIR protein is membrane attached, which is consistent with the findings in *F. suspense* stems. Only the insoluble fraction was

capable of stereoselective conversion of coniferyl alcohol to (+)-pinoresinol, whereas soluble enzyme preparations only form racemic pinoresinol [81, 82]. As the DIR protein was found primarily localized within the plant cell wall [83], it might be difficult to target DIR to its natural compartment in bacteria and yeast. However, there is strong indication that monolignol dimerization also occurs intracellular as shown by protoplast experiments in *A. thaliana* and the racemic pinoresinol formation in crude cell-free enzyme preparation of *F. suspense* stems [81, 84]. The disadvantage is the absence of stereoselectivity in the coupling of the two coniferyl alcohols. However, this should not be a problem, if the influx of coniferyl alcohol is large enough. The following proteins lack a transmembrane helix or signal peptide according to the TMHMM predictor and SignalP [85]: PLR, SDH, OMT3, OMT1, and 2-ODD. PLR and 2-ODD are localized to the cytoplasm, and SDH, OMT3, and OMT1 to the chloroplast according to the plant specific localization tool Plant-mPloc [86]. However, the specific chloroplast localization tools ChloroP and PCLR suggest no chloroplast localization, which was confirmed by the localization tools MultiLoc2-LowRes and LocTree3 [87–90]. Therefore, we think that the proteins PLR, SDH, OMT3, OMT1, and 2-ODD are all localized in the cytoplasm. The four cytochromes CYP719A23, CYP71CU1, CYP71BE54, and CYP82D61 contain a targeting peptide and one or two transmembrane helices. They are probably located in the endoplasmic reticulum (ER) membrane (according to an analysis by Plant-mPloc and MultiLoc2) as most plant cytochromes are anchored in the ER membrane and face the cytosolic side [91]. Our hypothesis is that deoxypodophyllotoxin is converted to podophyllotoxin by a cytochrome that is ER bound (**Figure 2**). Production of podophyllotoxin in *E. coli* would be feasible assuming that PLR, SDH, OMT3, OMT1, and 2-ODD can be actively expressed in the cytosol. As coniferyl alcohol has been produced before in this organism and cytochrome P450 enzymes with modified N-terminus have also been expressed successfully [92], some of the major steps toward podophyllotoxin might be performed in *E. coli*. The disadvantage of *E. coli* is the lack of NAD(P)H P450 reductase, the redox partner of cytochromes necessary for the supply of electrons from the cofactor NAD(P)H [92]. The establishment of a renewable supply has been proven difficult in *E. coli*.

5.3. Conversion of deoxypodophyllotoxin to (epi)podophyllotoxin by engineering

In 2006, Vasilev and coworkers showed that the human liver cytochrome P450 3A4 (CYP3A4) together with human NADPH P450 reductase can convert deoxypodophyllotoxin stereoselectivity into epipodophyllotoxin [93]. The disadvantage of this system is the usage of frozen cells and therefore the need to supply a regenerative system, such as glucose-6-phosphate dehydrogenase and NADP. Changing the system to a resting cell assay or cell-free assay with the usage of a cheaper cofactor and increasing the electron transfer between cytochrome and reductase would greatly increase the usability of this system. As CYP3A4 is quite unspecific, an approach to find a dedicated cytochrome converting deoxypodophyllotoxin into podophyllotoxin could be provided by the systematic analysis of cytochrome encoding genes found by Kumari and coworkers, who analyzed the transcriptome of *P. hexandrum* cultivated at two temperatures. The expression of DIR protein, PLR and SDH were upregulated by at least a factor two at 15°C compared to 25°C [94], accompanied by an increase of podophyllotoxin accumulation at 15°C. Fifteen cytochrome transcripts were upregulated by at least a factor two at 15°C compared to 25°C. These fifteen upregulated cytochrome transcripts would be interesting candidates for future investigation.

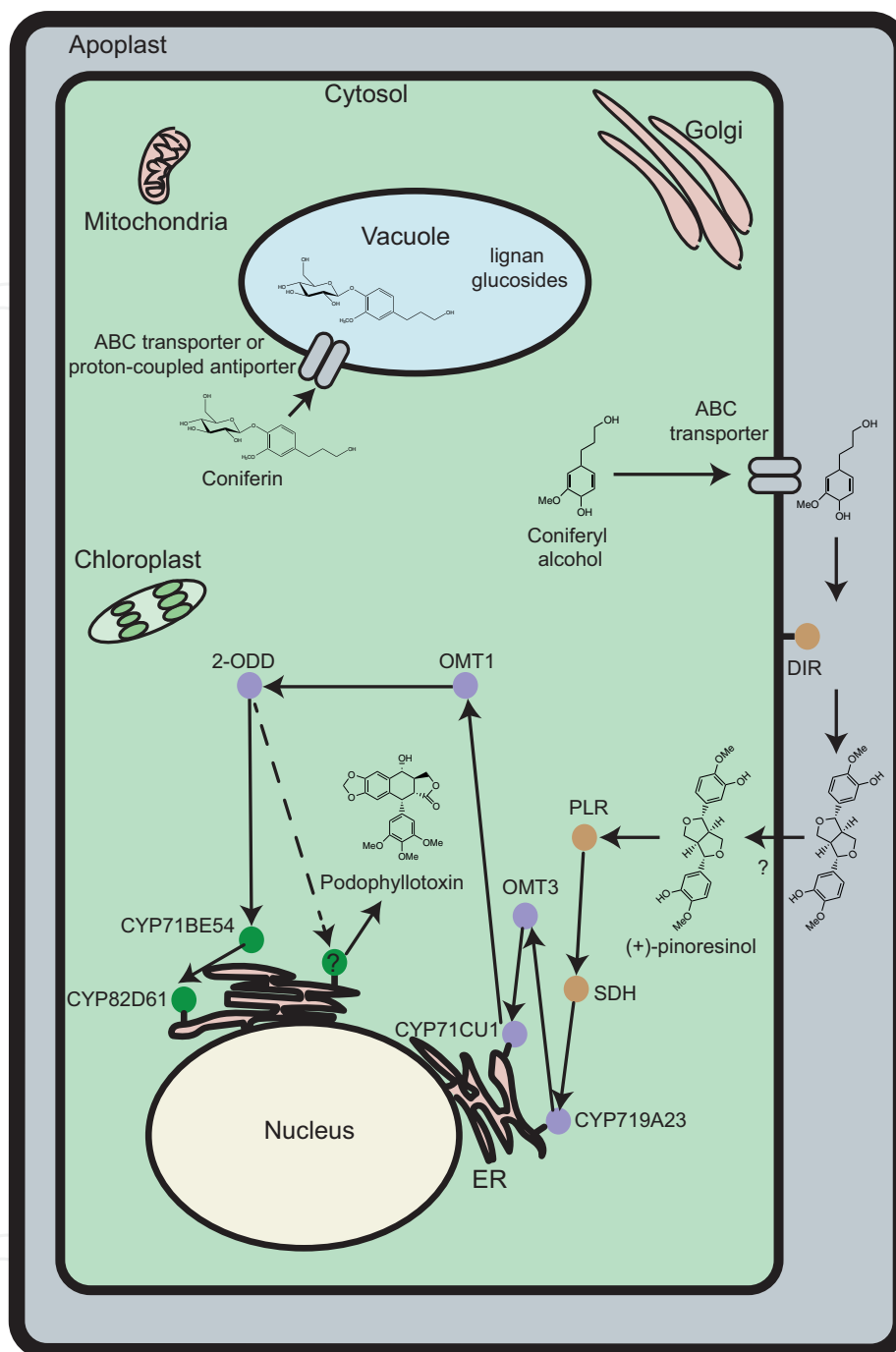


Figure 2. Schematic view of the proposed cellular localization of the enzymes in the lignan pathway in plant cells.

A cytochrome p450 system with high activity toward deoxypodophyllotoxin can form a very interesting production platform in conjunction with a sustainable source of this lignan, as is *A. sylvestris*, a common wild plant in Europe and temperate Asia, that can be cultivated easily [95, 96].

5.4. Production of etoposide

Industrially, podophyllotoxin is chemically converted to etoposide (**Figure 3**). Podophyllotoxin is converted to 4'-demethyl-epipodophyllotoxin by demethylation and epimerization in two steps with a yield of 52% followed by the protection of the phenolic group by conversion to

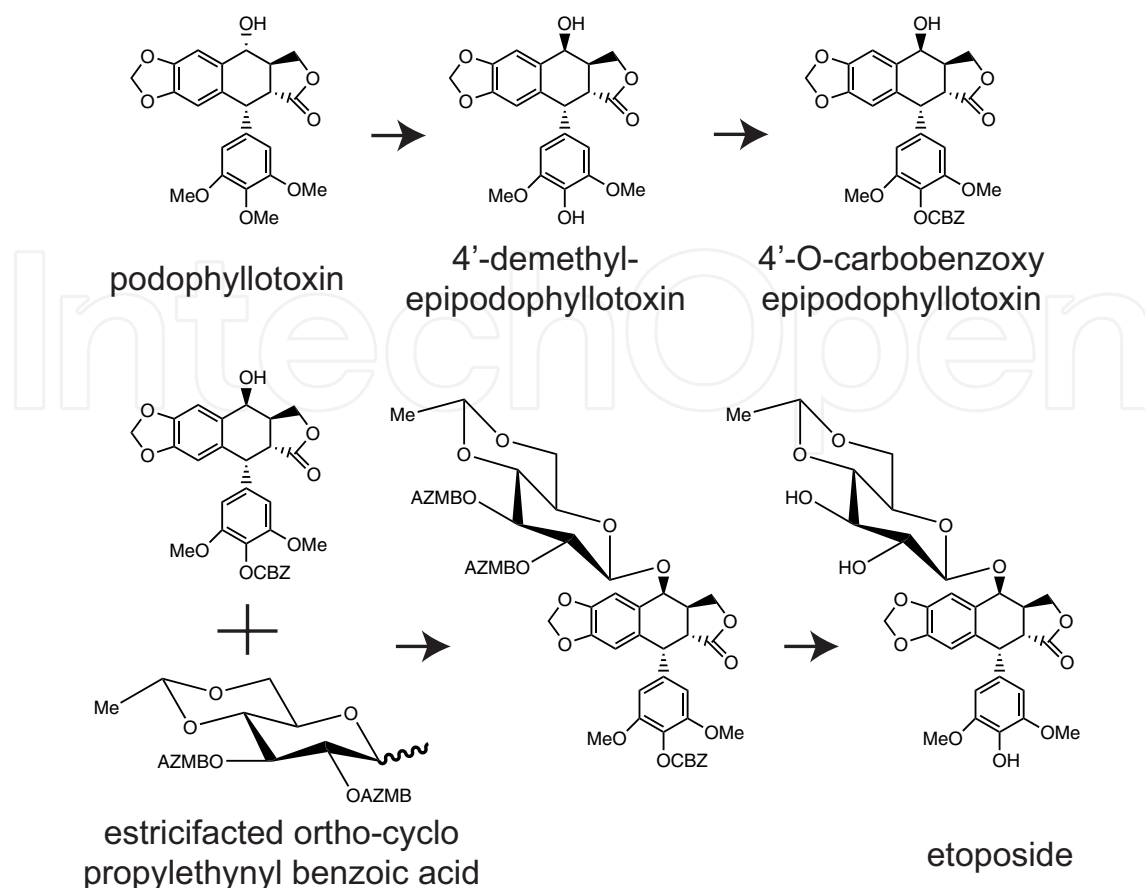


Figure 3. Conversion of podophyllotoxin into etoposide.

4'-O-carbobenzoxy-epipodophyllotoxin in one step with 89% yield [97]. 4'-O-carbobenzoxy-epipodophyllotoxin is then glycosylated to the esterification of *ortho*-cyclopropylethynylbenzoic acid, which is obtained in six steps from β -D-Glucose pentacetate [98, 99]. After glycosylation, the protective groups are removed in one step with 90% yield [98]. As podophyllotoxin production from deoxypodophyllotoxin is not yet applicable on industrial scale, the chemical conversion of deoxypodophyllotoxin into epipodophyllotoxin is of interest, which can be performed in one step with a yield of 53% [100]. Epipodophyllotoxin can be converted chemically to etoposide in the same manner as podophyllotoxin. The chemical synthesis of etoposide from deoxypodophyllotoxin can be shortened by production of 4'-demethyl-epipodophyllotoxin from deoxypodophyllotoxin by CYP71BE54 and CYP82D61 from *P. hexandrum* (see Section 4.3). As only proof of concept has been shown, optimization is required to make this enzymatic conversion suitable for industrial application. Whether deoxypodophyllotoxin can be converted chemically directly to 4'-demethyl-epipodophyllotoxin still needs to be investigated.

6. Future perspectives

Recent insights in the lignan biosynthetic pathway by Lau and Sattely [69] have progressed the research in the lignan pathway enormously. Engineering of the lignan pathway in a heterologous host will become feasible, if the localization of the enzymes in the pathway has been

determined. Depending on this localization, either *E. coli* or *S. cerevisiae* could be a suitable host for production of podophyllotoxin from glucose. The only missing step is the conversion of deoxypodophyllotoxin to podophyllotoxin. Finding this enzyme or replacing this step by the epipodophyllotoxin producing CYP82D61 (with or without CYP71BE54) will advance the development even more. Alternatively, deoxypodophyllotoxin can be chemically converted to etoposide. Considering the huge number of enzymes necessary for conversion of glucose to podophyllotoxin in *E. coli* or *S. cerevisiae*, commercial production in microbial hosts still has a long way to go. Until that time, an alternative approach can be the extraction of deoxypodophyllotoxin from the easy to cultivate *A. sylvestris* and converting this to (epi)podophyllotoxin. Enzymatic conversion needs to be optimized in order to obtain a system that can be used by the industry. Improvement should focus on engineering a cheap system, by usage of a resting cell assay or the usage of a cheap cofactor in a cell-free system. Furthermore, the deoxypodophyllotoxin conversion should be scaled up to industrial production.

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