Chapter 11

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′-demethyldesoxypodophyllotoxin 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 arylnaphthalene 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, arylnaphthalenes, 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].

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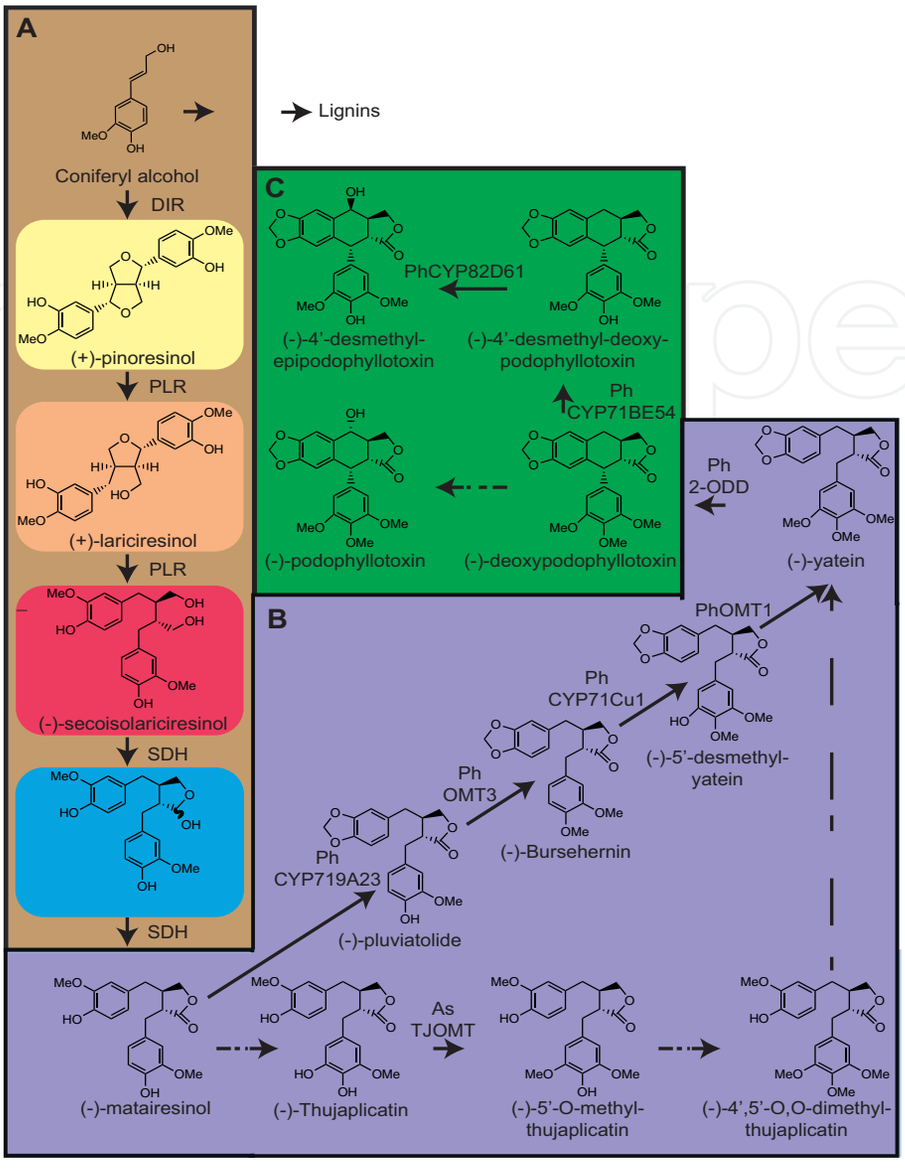

Figure 1. Lignan pathway in *Podophyllum hexandrum* and *Anthriscus sylvestris*. (A) Coniferyl alcohol toward matairesinol (brown box), (B) matairesinol toward deoxypodophyllotoxin (purple box), and (C) deoxypodophyllotoxin toward podophyllotoxin and demethyldesoxypodophyllotoxin (green box). Lignan 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.

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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, Gl-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 epipodophylloid, 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 meloid 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 derivate 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 1.95A [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 A, 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 A) 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 constrains, (+)-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. hex``vandrum* 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-demethylyatein in *A. sylvestris*. However, feeding of 5-demethylyatein 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 demethyldesoxypodophyllotoxin

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′-demethyldesoxypodophyllotoxin. In the second step (-)-4′-demethyldesoxypodophyllotoxin is converted to (−)-4′-desmethylepipodophyllotoxin by CYP82D61.

5. Engineering approaches

In this part, we will focus on genetic engineering approach`es 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

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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 helixes. 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.

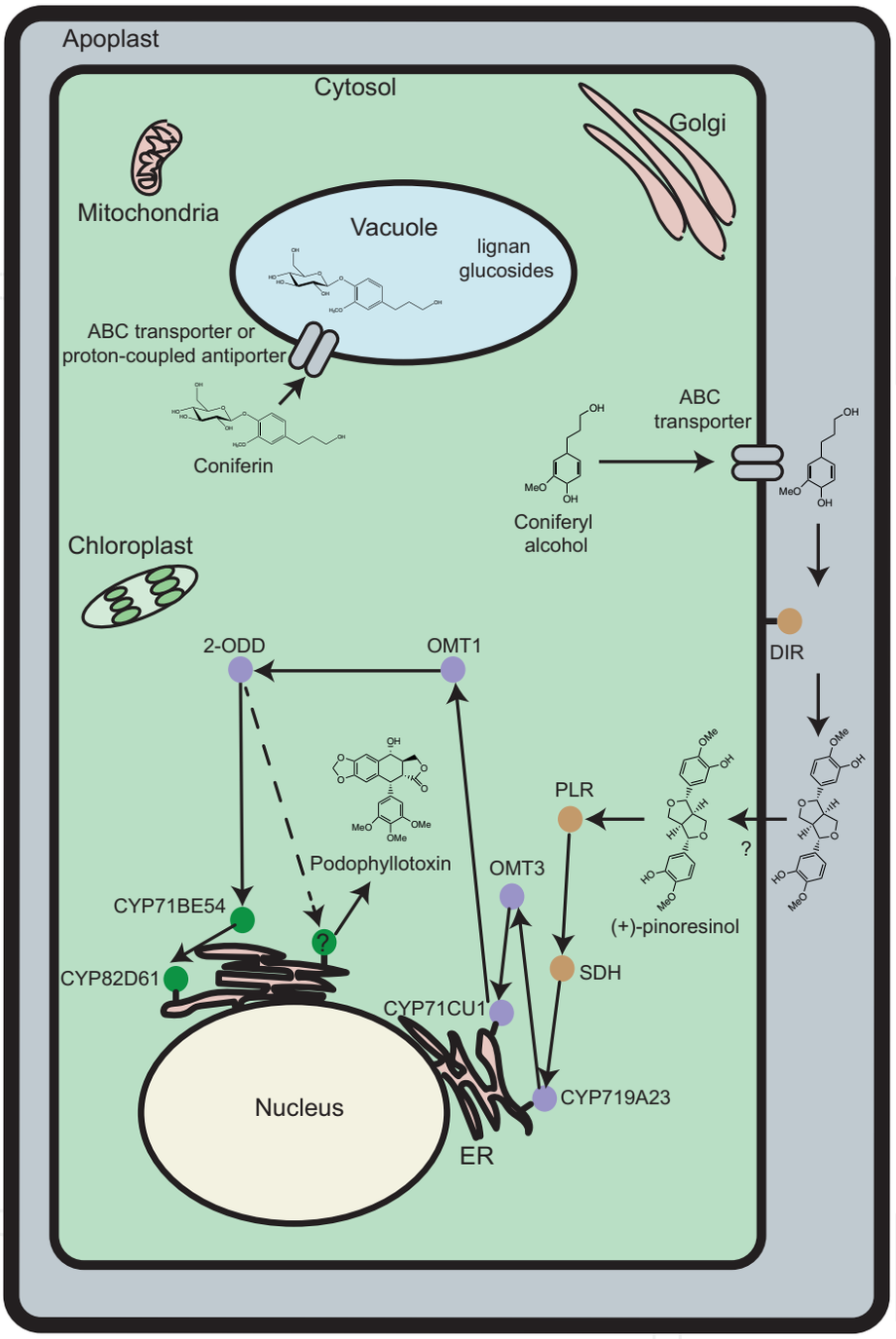

Figure 2. Schematic view of the proposed cellular localization of the enzymes in the lignan pathway in plant cells.

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

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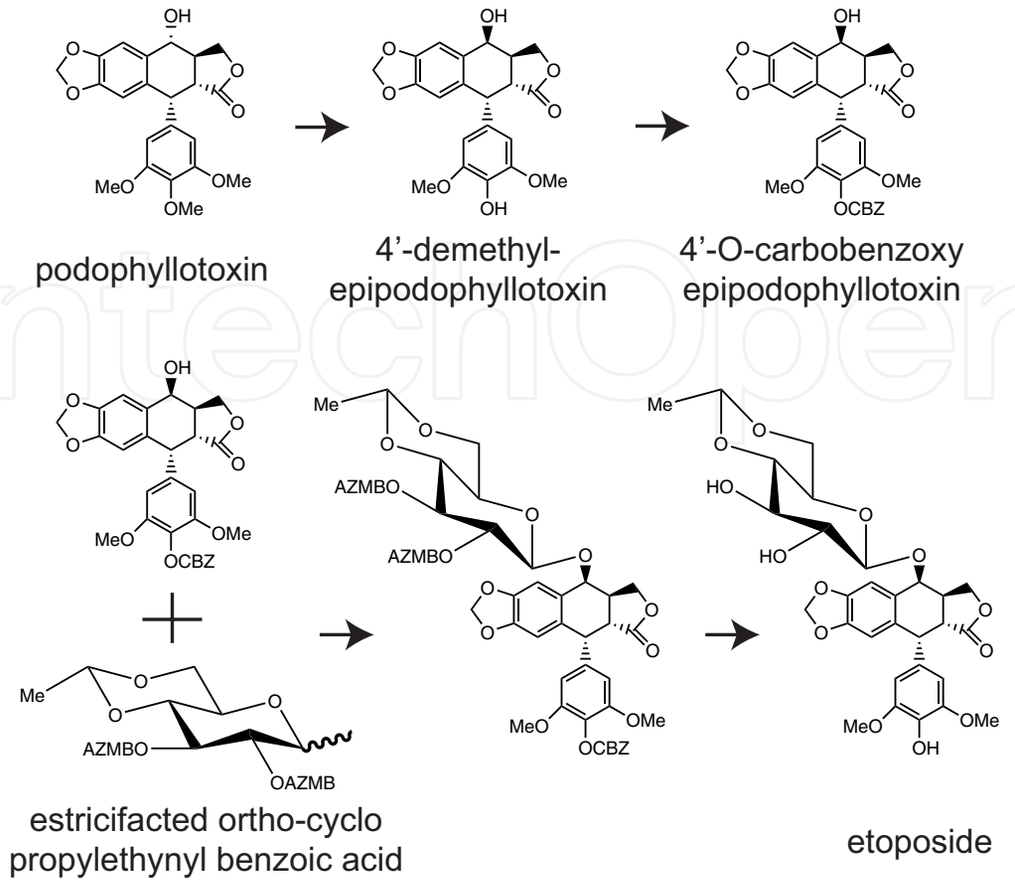

Figure 3. Conversion of podophyllotoxin into etoposide.

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 pentaacetate [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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References

[1] Guerram M, Jiang ZZ, Zhang LY. Podophyllotoxin, a medicinal agent of plant origin: past, present and future. Chin J Nat Med. 2012;10:161-9. doi:10.3724/SP.J.1009.2012.00161.

[2] Alam MA, Naik PK. Impact of soil nutrients and environmental factors on podophyllotoxin content among 28 *podophyllum hexandrum* populations of Northwestern Himalayan region using linear and nonlinear approaches. Commun Soil Sci Plant Anal. 2009;40:2485-504. doi:10.1080/00103620903111368.

[3] Convention of International Trade in Endangered Species of Wild Fauna and Flora. n.d. https://www.cites.org/eng/app/appendices.php#hash2 (accessed October 28, 2015).

[4] Canel C, Moraes RM, Dayan FE, Ferreira D. Podophyllotoxin. Phytochemistry. 2000;54:115-20. doi:10.1016/S0031-9422(00)00094-7.

[5] Ting CP, Maimone TJ. CH bond arylation in the synthesis of aryltetralin lignans: a short total synthesis of podophyllotoxin. Angew Chem Int Ed Engl. 2014;53:3115-9. doi:10.1002/anie.201311112.

[6] Petersen M, Alfermann W. The production of cytotoxic lignans by plant cell cultures. Appl Microbiol Biotechnol. 2001;55:135-42. doi:10.1007/s002530000510.

[7] Ionkova I, Antonova I, Momekov G, Fuss E. Production of podophyllotoxin in Linum linearifolium *in vitro* cultures. Pharmacogn Mag. 2010;6:180-5. doi:10.4103/0973-1296.66932.

[8] Turner EE, Hirst EL, Peat S, Haworth RD, Baker W, Linstead RP, et al. Organic chemistry. Annu Reports Prog Chem. 1936;33:228. doi:10.1039/ar9363300228.

[9] Moss GP. Nomenclature of lignans and neolignans (IUPAC Recommendations 2000). Pure Appl Chem. 2000;72:1493-523. doi:10.1351/pac200072081493.

[10] Umezawa T. Diversity in lignan biosynthesis. Phytochem Rev. 2003;2:371-90. doi:10.1023/b:phyt.0000045487.02836.32.

[11] Whiting DA. Ligans and neolignans. Nat Prod Rep. 1985;2:191. doi:10.1039/np9850200191.

[12] Heinonen S, Nurmi T, Liukkonen K, Poutanen K, Wähälä K, Deyama T, et al. *In vitro* metabolism of plant lignans: new precursors of mammalian lignans enterolactone and enterodiol. J Agric Food Chem. 2001;49:3178-86. doi:10.1021/JF010038A.

[13] Peñalvo JL, Heinonen SM, Aura AM, Adlercreutz H. Dietary sesamin is converted to enterolactone in humans. J Nutr. 2005;135:1056-62.

[14] Landete JM. Plant and mammalian lignans: a review of source, intake, metabolism, intestinal bacteria and health. Food Res Int. 2012;46:410-24. doi:10.1016/j.foodres.2011.12.023.

[15] Charlton JL. Antiviral activity of lignans. J Nat Prod. 1998;61:1447-51. doi:10.1021/NP980136Z.

[16] Ayres DC, Loike JD. Lignans: Chemical, Biological and Clinical Properties. vol. 30. Cambridge, NewYork, Port Chester, Melbourne, Sydney: Cambridge University Press; 1990.

[17] Culp OS, Kaplan IW. Condylomata acuminata: two hundred cases treated with podophyllin. Ann Surg. 1944;120:251-6.

[18] von Krogh G, Lacey CJN, Gross G, Barrasso R, Schneider A. European course on HPV associated pathology: guidelines for primary care physicians for the diagnosis and management of anogenital warts. Sex Transm Infect. 2000;76:162-8. doi:10.1136/sti.76.3.162.

[19] Kelly MG, Hart-Well JL. The biological effects and the chemical composition of podophyllin. a review. J Natl Cancer Inst. 1954;14:967-1010. doi:10.1093/jnci/14.4.967.

[20] Stähelin HF, von Wartburg A. The chemical and biological route from podophyllotoxin glucoside to etoposide: ninth cain memorial award lecture. Cancer Res. 1991;51: 5-15.

[21] Imbert TF. Discovery of podophyllotoxins. Biochimie. 1998;80:207-22. doi:10.1016/S0300-9084(98)80004-7.

[22] Liu YQ, Yang L, Tian X. Podophyllotoxin: current perspectives. Curr Bioact Compd. 2007;3:37-66. doi:10.2174/157340707780126499.

[23] Hande K. Etoposide: four decades of development of a topoisomerase II inhibitor. Eur J Cancer. 1998;34:1514-21. doi:10.1016/S0959-8049(98)00228-7.

[24] Senter PD, Saulnier MG, Schreiber GJ, Hirschberg DL, Brown JP, Hellström I, et al. Anti-tumor effects of antibody-alkaline phosphatase conjugates in combination with etoposide phosphate. Proc Natl Acad Sci U S A. 1988;85:4842-6.

[25] Thompson DS, Greco FA, Miller AA, Srinivas NR, Igwemezie LN, Hainsworth JD, et al. A phase I study of etoposide phosphate administered as a daily 30-minute infusion for 5 days. Clin Pharmacol Ther. 1995;57:499-507. doi:10.1016/0009-9236(95)90034-9.

[26] A to Z List of Cancer Drugs—National Cancer Institute. 2016. https://www.cancer.gov/about-cancer/treatment/drugs.

[27] Cytostatica | Farmacotherapeutisch Kompas. n.d. https://www.farmacotherapeutischkompas.nl/bladeren-volgens-boek/inleidingen/inl-cytostatica.

[28] Kalemkerian GP, Akerley W, Bogner P, Borghaei H, Chow LQ, Downey RJ, et al. Small cell lung cancer. J Natl Compr Canc Netw. 2013;11:78-98.

[29] PDQ Pediatric Treatment Editorial Board PPTE. Childhood Acute Myeloid Leukemia/Other Myeloid Malignancies Treatment (PDQ®): Health Professional Version. Bethesda: National Cancer Institute (US); 2002.

[30] Chemotherapy for acute lymphocytic leukemia. n.d. http://www.cancer.org/cancer/leukemia-acutelymphocyticallinadults/detailedguide/leukemia-acute-lymphocytic-treating-chemo therapy.

[31] Kamal A, Hussaini SMA, Rahim A, Riyaz S. Podophyllotoxin derivatives: a patent review (2012-2014). Expert Opin Ther Pat. 2015;25:1025-34.

[32] Liu YQ, Tian J, Qian K, Zhao XB, Morris-Natschke SL, Yang L, et al. Recent progress on C-4-modified podophyllotoxin analogs as potent antitumor agents. Med Res Rev. 2015;35:1-62. doi:10.1002/med.21319.

[33] Mizugaki H, Yamamoto N, Fujiwara Y, Nokihara H, Yamada Y, Tamura T. Current status of single-agent phase I trials in japan: toward globalization. J Clin Oncol. 2015;33:2051-61. doi:10.1200/JCO.2014.58.4953.

[34] Raβmann I, Schrödel H, Schilling T, Zucchetti M, Kaeser-Fröhlich A, Rastetter J, et al. Clinical and pharmacokinetic phase I trial of oral dimethylaminoetoposide (NK611) administered for 21 days every 35 days. Invest New Drugs. 1996;14:379-86. doi:10.1007/BF00180814.

[35] Raβmann I, Thödtmann R, Thödtmann R, Mross M, Hüttmann A, Berdel WE, et al. Phase I clinical and pharmacokinetic trial of the podophyllotoxin derivative NK611 administered as intravenous short infusion. Invest New Drugs. 1998;16:319-24. doi:10.1023/A:1006293830585.

[36] Pagani O, Zucchetti M, Sessa C, de Jong J, D'Incalci M, Fusco M De, et al. Clinical and pharmacokinetic study of oral NK611, a new podophyllotoxin derivative. Cancer Chemother Pharmacol. 1996;38:541-7. doi:10.1007/s002800050524.

[37] Perrin D, van Hille B, Barret JM, Kruczynski A, Etiévant C, Imbert T, et al. F 11782, a novel epipodophylloid non-intercalating dual catalytic inhibitor of topoisomerases I and II with an original mechanism of action. Biochem Pharmacol. 2000;59:807-19. doi:10.1016/S0006-2952(99)00382-2.

[38] Etiévant C, Kruczynski A, Barret JM, Perrin D, van Hille B, Guminski Y, et al. F 11782, a dual inhibitor of topoisomerases I and II with an original mechanism of action *in vitro*, and markedly superior *in vivo* antitumour activity, relative to three other dual topoisomerase inhibitors, intoplicin, aclarubicin and TAS-103. Cancer Chemother Pharmacol. 2000;46:101-13. doi:10.1007/s002800000133.

[39] Delord J-P, Bennouna J, Dieras V, Campone M, Lefresne F, Aslanis V, et al. First-in-man study of tafluposide, a novel inhibitor of topoisomerase I and II. Mol Cancer Ther. 2007;6:A138.

[40] Barret JM, Kruczynski A, Vispé S, Annereau JP, Brel V, Guminski Y, et al. F14512, a potent antitumor agent targeting topoisomerase II vectored into cancer cells via the polyamine transport system. Cancer Res. 2008;68:9845-53.

[41] Bahleda R, De Botton S, Quesnel B, Soria JC. 12th TAT congress 5-7 march 2014 Washington DC. Tackling Leuk. Phase I study F14512 relapsed or Refract. AML patients, 2014.

[42] Tierny D, Serres F, Segaoula Z, Bemelmans I, Bouchaert E, Pétain A, et al. Phase I clinical pharmacology study of F14512, a new polyamine-vectorized anticancer drug, in naturally occurring canine lymphoma. Clin Cancer Res. 2015;21:5314-23.

[43] Merzouki A, Buschmann MD, Jean M, Young RS, Liao S, Gal S, et al. Adva-27a, a novel podophyllotoxin derivative found to be effective against multidrug resistant human cancer cells. Anticancer Res. 2012;32:4423-32.

[44] Research programme: type II DNA topoisomerase inhibitors—Sunshine Biopharma—AdisInsight. n.d. http://adisinsight.springer.com/drugs/800032587.

[45] Chen MC, Pan SL, Shi Q, Xiao Z, Lee KH, Li TK, et al. QS-ZYX-1-61 induces apoptosis through topoisomerase II in human non-small-cell lung cancer A549 cells. Cancer Sci. 2012;103:80-7. doi:10.1111/j.1349-7006.2011.02103.x.

[46] Lewis NG, Davin LB, Sarkanen S. Lignin and lignan biosynthesis: distinctions and reconciliations. 1998; 697:pp. 1-27. doi:10.1021/bk-1998-0697.ch001.

[47] Rutledge PJ, Challis GL. Discovery of microbial natural products by activation of silent biosynthetic gene clusters. Nat Rev Microbiol. 2015;13:509-23. doi:10.1038/nrmicro3496.

[48] Davin LB, Wang HB, Crowell AL, Bedgar DL, Martin DM, Sarkanen S, et al. Stereoselective bimolecular phenoxy radical coupling by an auxiliary (dirigent) protein without an active center. Science (80-). 1997;275:362-7. doi:10.1126/science.275.5298.362.

[49] Kim KW, Moinuddin SGA, Atwell KM, Costa MA, Davin LB, Lewis NG. Opposite stereoselectivities of dirigent proteins in arabidopsis and schizandra species. J Biol Chem. 2012;287:33957-72. doi:10.1074/jbc.M112.387423.

[50] Gang DR, Costa MA, Fujita M, Dinkova-Kostova AT, Wang HB, Burlat V, et al. Regiochemical control of monolignol radical coupling: a new paradigm for lignin and lignan biosynthesis. Chem Biol. 1999;6:143-51. doi:10.1016/S1074-5521(99)89006-1.

[51] Seneviratne HK, Dalisay DS, Kim KW, Moinuddin SGA, Yang H, Hartshorn CM, et al. Non-host disease resistance response in pea (*Pisum sativum*) pods: biochemical function of DRR206 and phytoalexin pathway localization. Phytochemistry. 2015;113:140-8. doi:10.1016/j.phytochem.2014.10.013.

[52] Pickel B, Constantin MA, Pfannstiel J, Conrad J, Beifuss U, Schaller A. An enantiocomplementary dirigent protein for the enantioselective laccase-catalyzed oxidative coupling of phenols. Angew Chemie Int Ed. 2010;49:202-4. doi:10.1002/anie.200904622.

[53] Dalisay DS, Kim KW, Lee C, Yang H, Rübel O, Bowen BP, et al. Dirigent protein-mediated lignan and cyanogenic glucoside formation in flax seed: integrated omics and MALDI mass spectrometry imaging. J Nat Prod. 2015;78:1231-42. doi:10.1021/acs.jnatprod.5b00023.

[54] Kim KW, Smith CA, Daily MD, Cort JR, Davin LB, Lewis NG. Trimeric structure of (+)-pinoresinol-forming dirigent protein at 1.95 Å resolution with three isolated active sites. J Biol Chem. 2015;290:1308-18. doi:10.1074/jbc.M114.611780.

[55] Dinkova-Kostova AT, Gang DR, Davin LB, Bedgar DL, Chu A, Lewis NG. (+)-Pinoresinol/(+)-Lariciresinol Reductase from Forsythia intermedia: protein purrification, cDNA cloning, heterologous expression and comparision to isoflavone reductase. J Biol Chem. 1996;271:29473-82. doi:10.1074/jbc.271.46.29473.

[56] Katayama T, Davin LB, Chu A, Lewis NG. Novel benzylic ether reductions in lignan biogenesis in Forsythia intermedia. Phytochemistry. 1993;33:581-91. doi:10.1016/0031-9422(93)85452-W.

[57] von Heimendahl CBI, Schäfer KM, Eklund P, Sjöholm R, Schmidt TJ, Fuss E. Pinoresinol–lariciresinol reductases with different stereospecificity from Linum album and Linum usitatissimum. Phytochemistry. 2005;66:1254-63. doi:10.1016/j.phytochem.2005.04.026.

[58] Hemmati S, Heimendahl CBI von, Klaes M, Alfermann AW, Schmidt TJ, Fuss E, et al. Pinoresinol-Lariciresinol reductases with opposite enantiospecificity determine the enantiomeric composition of lignans in the different organs of *Linum usitatissimum* L. Planta Med. 2010;76:928-34. doi:10.1055/s-0030-1250036.

[59] Bayindir Ü, Alfermann AW, Fuss E. Hinokinin biosynthesis in Linum corymbulosum Reichenb. Plant J. 2008;55:810-20. doi:10.1111/j.1365-313X.2008.03558.x.

[60] Fujita M, Gang DR, Davin LB, Lewis NG. Recombinant pinoresinol-lariciresinol reductases from western Red Cedar (*Thuja plicata*) catalyze opposite enantiospecific conversions. J Biol Chem. 1999;274:618-27. doi:10.1074/jbc.274.2.618.

[61] Hemmati S, Schmidt TJ, Fuss E. (+)-Pinoresinol/(−)-lariciresinol reductase from Linum perenne Himmelszelt involved in the biosynthesis of justicidin B. FEBS Lett. 2007;581:603-10. doi:10.1016/j.febslet.2007.01.018.

[62] Nakatsubo T, Mizutani M, Suzuki S, Hattori T, Umezawa T. Characterization of *Arabidopsis thaliana* pinoresinol reductase, a new type of enzyme involved in lignan biosynthesis. J Biol Chem. 2008;283:15550-7. doi:10.1074/jbc.M801131200.

[63] Min T, Kasahara H, Bedgar DL, Youn B, Lawrence PK, Gang DR, et al. Crystal structures of pinoresinol-lariciresinol and phenylcoumaran benzylic ether reductases and their relationship to isoflavone reductases. J Biol Chem. 2003;278:50714-23. doi:10.1074/jbc.M308493200.

[64] Xia ZQ, Costa M a, Pelissier HC, Davin LB, Lewis NG. Secoisolariciresinol dehydrogenase purification, cloning, and functional expression. Implications for human health protection. J Biol Chem. 2001;276:12614-23. doi:10.1074/jbc.M008622200.

[65] Youn B, Moinuddin SGA, Davin LB, Lewis NG, Kang C. Crystal structures of apo-form and binary/ternary complexes of podophyllum secoisolariciresinol dehydrogenase, an enzyme involved in formation of health-protecting and plant defense lignans. J Biol Chem. 2005;280:12917-26. doi:10.1074/jbc.M413266200.

[66] Kamil WM, Dewick PM. Biosynthetic relationship of aryltetralin lactone lignans to dibenzylbutyrolactone lignans. Phytochemistry. 1986;25:2093-102. doi: 10.1016/0031-9422(86)80072-3.

[67] Jackson DE, Dewick PM. Biosynthesis of *Podophyllum lignans*—II. Interconversions of aryltetralin lignans in *Podophyllum hexandrum*. Phytochemistry. 1984;23:1037-42. doi:10.1016/S0031-9422(00)82604-7.

[68] Marques JV, Kim KW, Lee C, Costa M a, May GD, Crow JA, et al. Next generation sequencing in predicting gene function in podophyllotoxin biosynthesis. J Biol Chem. 2013;288:466-79. doi:10.1074/jbc.M112.400689.

[69] Lau W, Sattely ES. Six enzymes from mayapple that complete the biosynthetic pathway to the etoposide aglycone. Science (80-). 2015;349:1224-8. doi:10.1126/science.aac7202.

[70] Sakakibara N, Suzuki S, Umezawa T, Shimada M. Biosynthesis of yatein in *Anthriscus sylvestris*. Org Biomol Chem. 2003;1:2474-85.

[71] Ragamustari SK, Nakatsubo T, Hattori T, Ono E, Kitamura Y, Suzuki S, et al. A novel O-methyltransferase involved in the first methylation step of yatein biosynthesis from

matairesinol in *Anthriscus sylvestris*. Plant Biotechnol. 2013;30:375-84. doi:10.5511/plantbiotechnology.13.0527b.

[72] Weiss SG, Tin-Wa M, Perdue RE, Farnsworth NR. Potential anticancer agents II: antitumor and cytotoxic lignans from Linum album (Linaceae). J Pharm Sci. 1975;64:95-8. doi:10.1002/jps.2600640119.

[73] Shiraishi A, Murata J, Matsumoto E, Matsubara S, Ono E, Satake H, et al. De novo transcriptomes of *Forsythia koreana* using a novel assembly method: insight into tissue- and species-specific expression of lignan biosynthesis-related gene. PLoS One. 2016;11:e0164805. doi:10.1371/journal.pone.0164805.

[74] Chen Z, Sun X, Li Y, Yan Y, Yuan Q. Metabolic engineering of *Escherichia coli* for microbial synthesis of monolignols. Metab Eng. 2016;39:102-9. doi:10.1016/j.ymben.2016.10.021.

[75] Huang Q, Lin Y, Yan Y. Caffeic acid production enhancement by engineering a phenylalanine over-producing *Escherichia coli* strain. Biotechnol Bioeng. 2013;110:3188-96. doi:10.1002/bit.24988.

[76] Eichenberger M, Lehka BJ, Folly C, Fischer D, Martens S, Simón E, et al. Metabolic engineering of *Saccharomyces cerevisiae* for de novo production of dihydrochalcones with known antioxidant, antidiabetic, and sweet tasting properties. Metab Eng. 2016;39:80-89. doi:10.1016/j.ymben.2016.10.019.

[77] Gidijala L, Kiel JAKW, Douma RD, Seifar RM, van Gulik WM, Bovenberg RAL, et al. An engineered yeast efficiently secreting penicillin. PLoS One. 2009;4:e8317. doi:10.1371/journal.pone.0008317.

[78] Miao YC, Liu CJ. ATP-binding cassette-like transporters are involved in the transport of lignin precursors across plasma and vacuolar membranes. Proc Natl Acad Sci U S A. 2010;107:22728-33. doi:10.1073/pnas.1007747108.

[79] Tsuyama T, Kawai R, Shitan N, Matoh T, Sugiyama J, Yoshinaga A, et al. Proton-dependent coniferin transport, a common major transport event in differentiating xylem tissue of woody plants. Plant Physiol. 2013;162:918-26. doi:10.1104/pp.113.214957.

[80] Krogh A, Larsson B, von Heijne G, Sonnhammer EL. Predicting transmembrane protein topology with a hidden markov model: application to complete genomes. J Mol Biol. 2001;305:567-80. doi:10.1006/jmbi.2000.4315.

[81] Umezawa T, Davin LB, Yamamoto E, Kingston DGI, Lewis NG, Lewis NG, et al. Lignan biosynthesis in forsythia species. J Chem Soc Chem Commun. 1990;41:1405. doi:10.1039/c39900001405.

[82] Davin LB, Bedgar DL, Katayama T, Lewis NG. On the stereoselective synthesis of (+)-pinoresinol in Forsythia suspensa from its achiral precursor, coniferyl alcohol. Phytochemistry. 1992;31:3869-74. doi:10.1016/S0031-9422(00)97544-7.

[83] Burlat V, Kwon M, Davin LB, Lewis NG. Dirigent proteins and dirigent sites in lignifying tissues. Phytochemistry. 2001;57:883-97. doi:10.1016/S0031-9422(01)00117-0.

[84] Dima O, Morreel K, Vanholme B, Kim H, Ralph J, Boerjan W. Small glycosylated lignin oligomers are stored in arabidopsis leaf vacuoles. Plant Cell. 2015;27:695-710. doi:10.1105/tpc.114.134643.

[85] Petersen TN, Brunak S, von Heijne G, Nielsen H. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods. 2011;8:785-6. doi:10.1038/nmeth.1701.

[86] Chou KC, Shen HB, Ehrlich J, Hansen M, Nelson W, Glory E, et al. Plant-mPLoc: a top-down strategy to augment the power for predicting plant protein subcellular localization. PLoS One. 2010;5:e11335. doi:10.1371/journal.pone.0011335.

[87] Emanuelsson O, Nielsen H, Heijne G Von. ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. Protein Sci. 1999;8:978-84. doi:10.1110/ps.8.5.978.

[88] Schein AI, Kissinger JC, Ungar LH. Chloroplast transit peptide prediction: a peek inside the black box. Nucleic Acids Res. 2001;29:E82.

[89] Blum T, Briesemeister S, Kohlbacher O, Emanuelsson O, Brunak S, Heijne G von, et al. MultiLoc2: integrating phylogeny and gene ontology terms improves subcellular protein localization prediction. BMC Bioinformatics. 2009;10:274. doi:10.1186/1471-2105-10-274.

[90] Goldberg T, Hecht M, Hamp T, Karl T, Yachdav G, Ahmed N, et al. LocTree3 prediction of localization. Nucleic Acids Res. 2014;42:W350-5. doi:10.1093/nar/gku396.

[91] Schuler MA, Werck-Reichhart D. Functional genomics of P450s. Annu Rev Plant Biol. 2003;54:629-67. doi:10.1146/annurev.arplant.54.031902.134840.

[92] Gillam EMJ. Engineering cytochrome P450 enzymes. Chem Res Toxicol. 2007;21:220-31. doi:10.1021/tx7002849.

[93] Vasilev NP, Julsing MK, Koulman A, Clarkson C, Woerdenbag HJ, Ionkova I, et al. Bioconversion of deoxypodophyllotoxin into epipodophyllotoxin in *E. coli* using human cytochrome P450 3A4. J Biotechnol. 2006;126:383-93. doi:10.1016/j.jbiotec.2006.04.025.

[94] Kumari A, Singh HR, Jha A, Swarnkar MK, Shankar R, Kumar S. Transcriptome sequencing of rhizome tissue of Sinopodophyllum hexandrum at two temperatures. BMC Genomics. 2014;15:871. doi:10.1186/1471-2164-15-871.

[95] Magnússen SH. NOBANIS –Invasive Alien Species Fact Sheet -*Anthriscus sylvestris*. Database of the European Network on Invasive Alien Species. 2011.

[96] Hendrawati O, Woerdenbag HJ, Hille J, Quax WJ, Kayser O. Seasonal variations in the deoxypodophyllotoxin content and yield of *Anthriscus sylvestris* L. (Hoffm.) grown in the field and under controlled conditions. J Agric Food Chem. 2011;59:8132-9. doi:10.1021/jf200177q.

[97] Lee KH, Imakura Y, Haruna M, Beers SA, Thurston LS, Dai HJ, et al. Antitumor agents, 107. New cytotoxic 4-alkylamino analogues of 4′-demethyl-epipodophyllotoxin as inhibitors of human DNA topoisomerase II. J Nat Prod. 1989;52:606-13. doi:10.1021/np50063a021.

[98] Liu H, Liao JX, Hu Y, Tu YH, Sun JS. A highly efficient approach to construct (*epi*)-podophyllotoxin-4- *O* -glycosidic linkages as well as its application in concise syntheses of etoposide and teniposide. Org Lett. 2016;18:1294-7. doi:10.1021/acs.orglett.6b00216.

[99] Zong G, Barber E, Aljewari H, Zhou J, Hu Z, Du Y, et al. Total synthesis and biological evaluation of ipomoeassin F and its unnatural 11 *R* -epimer. J Org Chem. 2015;80:9279-91. doi:10.1021/acs.joc.5b01765.

[100] Yamaguchi Hi, Arimoto M, Nakajima S, Tanoguchi M, Fukada Y. Studies on the constituents of the seeds of Hernandia ovigera L. V Syntheses of epipodophyllotoxin and podophyllotoxin from desoxypodophyllotoxin. Chem Pharm Bull (Tokyo). 1986;34:2056-60. doi:10.1248/cpb.34.2056.