Chapter 7

Summary and future perspectives

Cancer is one of the leading causes of premature deaths worldwide[1]. In 2018, this led to over 9.5 million deaths and the discovery of 18 million new cancer cases[2]. For the treatment of patients, we largely depend on natural products and their derivatives[3]. For example, etoposide is obtained from the plant lignan podophyllotoxin via hemisynthesis[4]. Podophyllotoxin is produced by plants of various species with the roots of *Podophyllum hexandrum* (Himalayan mayapple) being the most productive[5,6]. The excessive harvesting has resulted in the inclusion of *P. hexandrum* in the Convention of International Trade in Endangered Species[7]; therefore, alternative production routes toward podophyllotoxin have to be explored.

The research presented in this thesis focuses on production routes toward podophyllotoxin. To this end, a systematical literature review describing the lignan biosynthetic pathway toward podophyllotoxin in plants was written (**chapter 2**). Other topics discussed were the importance of podophyllotoxin derivatives and their development for chemotherapy; and the engineering approaches to produce podophyllotoxin in a heterologous system. For the latter a detailed knowledge of the previously described lignan biosynthetic pathway was necessary. Up to now, the majority of the enzymes in the lignan pathway are elucidated, except for the last enzyme that converts deoxypodophyllotoxin into podophyllotoxin.

For the *in vitro* production of podophyllotoxin, we first need to identify the enzyme responsible for the conversion of deoxypodophyllotoxin into podophyllotoxin in *P. hexandrum*. Deoxypodophyllotoxin can be converted to epipodophyllotoxin, the C-7 epimer of podophyllotoxin, by a cytochrome P450 enzyme[8,9]. Therefore, we assumed that a cytochrome P450 enzyme is responsible for the conversion of deoxypodophyllotoxin into podophyllotoxin in *P. hexandrum*. For catalytic activity, cytochrome P450 requires a NADPH-cytochrome P450 reductase as redox partner. In **chapter 3**, we searched for both enzymes in the publicly available *P. hexandrum* transcriptome database. To this end, we combined knowledge on cytochrome P450 transcript expression under stress conditions and sequence characteristics, such as highly conserved domain sequences in plant cytochrome P450s. In addition, we searched for proteins containing the highly conserved domain sequences for NADPH-cytochrome P450 reductases in the translated *P. hexandrum* transcriptome database. In total, six candidate cytochrome P450s and one candidate NADPH-cytochrome P450 reductase were found. We chose *Escherichia coli* as expression host for the expression of one of these cytochrome P450 candidates, CYP82D61, and NADPH-cytochrome P450 reductase from *P. hexandrum*. Subsequently, we showed conversion of deoxypodophyllotoxin into epipodophyllotoxin, when CYP82D61 was co-expressed with the endogenous NADPH-cytochrome P450 reductase or when a fusion protein of CYP82D61 and NADPH-cytochrome P450 reductase was expressed.

In addition, we showed that other plant NADPH-cytochrome P450 reductases were able to support the deoxypodophyllotoxin conversion of CYP82D61.

For the bacterial expression system, large quantities of deoxypodophyllotoxin are required as substrate; therefore, a large-scale extraction method for deoxypodophyllotoxin is necessary. In the Netherlands, deoxypodophyllotoxin can be obtained from the roots of the very common weed *Anthriscus sylvestris*[10]. In **chapter 4**, we investigated the extraction of deoxypodophyllotoxin from *A. sylvestris* roots with the environmentally friendly supercritical carbon dioxide extraction method. We showed that this method extracts 75 - 80 % of the total deoxypodophyllotoxin content, which is comparable to a single extraction by traditional Soxhlet. Furthermore, less unwanted polar components were extracted with the supercritical carbon dioxide method. To obtain large quantities of deoxypodophyllotoxin, we should focus not only on the extraction, but also on the deoxypodophyllotoxin content in *A. sylvestris* roots via plant breeding programs. The supercritical carbon dioxide extraction method is not suitable as quick screening method, therefore, we designed a quick small scale methanol vortex method for this in **chapter 4**.

Another way to obtain deoxypodophyllotoxin is via *in vitro* root cultures. In **chapter 5**, we induced shoots from *A. sylvestris* callus tissue. These shoots were rooted to obtain regenerated plant and root cultures. We were able to cultivate large batches of the root cultures in Erlenmeyer flasks and showed that they produced deoxypodophyllotoxin. A more economical system is the large scale cultivation of these roots in disposable bioreactors; therefore, we designed a novel type of wave-mixed disposable bioreactor that enables oxygen measurements at every spot in the bioreactor. The reactor system was evaluated with *Mucuna pruriens* cell suspension culture, which showed good growth and production of the pharmaceutical relevant L-DOPA.

We discussed the possibilities to produce podophyllotoxin or related lignans by *in vitro* systems. Another route toward podophyllotoxin focused on improving the cultivation conditions of *P. hexandrum* to ensure a sustainable supply of *P. hexandrum* roots. In **chapter 6**, we investigated whether *P. hexandrum* could be cultivated in a glasshouse in the Netherlands. To this end, we determined the biomass and podophyllotoxin production of plants cultivated under various conditions. We investigated the influence of soil type, temperature and treatment with the plant hormone, methyl jasmonate. Biomass and podophyllotoxin production per plant were higher if the water drainage control of the soil was high and the temperature was kept low. Furthermore, the podophyllotoxin production in the roots was increased upon treatment of the leaves with methyl jasmonate.

Overall, we demonstrated various production routes toward podophyllotoxin. One route is via the precursor deoxypodophyllotoxin, which can be obtained by extraction of deoxypodophyllotoxin from *A. sylvestris* roots by the supercritical carbon dioxide extraction method; or production of deoxypodophyllotoxin by *A. sylvestris* roots cultures in a disposable bioreactor system. Subsequently, deoxypodophyllotoxin can be converted into (epi)podophyllotoxin in a recombinant *E. coli* system. As an alternative, we suggest controlled cultivation of *P. hexandrum* in a glasshouse in the Netherlands.

Future perspectives

This thesis describes several production routes toward podophyllotoxin, which still need to be further developed to become economically feasible. The controlled cultivation of *P. hexandrum* could be a potential replacement for harvesting *P. hexandrum* populations in nature. We believe improvement of the podophyllotoxin yield is possible by further optimizing the cultivation conditions by investigating other soil types and biotic factors, such as water and (UV) light[5,6,11–15].

An alternative way to produce podophyllotoxin is via its precursor deoxypodophyllotoxin; however, we need a cytochrome P450 enzyme for the conversion of deoxypodophyllotoxin into podophyllotoxin. If this is possible, then it would be interesting to cultivate *A. sylvestris* as a crop either on the field or as root culture to produce deoxypodophyllotoxin. We believe that a few factors should be considered before large-scale cultivation of *A. sylvestris* is economically feasible. A plant breeding program is necessary to increase the deoxypodophyllotoxin content in *A. sylvestris* roots. Additionally, the green supercritical carbon dioxide extraction method for the extraction of deoxypodophyllotoxin from *A. sylvestris* roots should be scaled-up to industrial dimensions, like the decaffeination of tea and coffee[16]. An alternative route is the production of deoxypodophyllotoxin via *A. sylvestris* root cultures in a disposable bioreactor system. Although, deoxypodophyllotoxin production should be improved by investigating various culturing conditions. Furthermore, the efficiency of the supercritical carbon dioxide extraction method to extract deoxypodophyllotoxin from *A. sylvestris* root cultures should be assessed.

The other part of the thesis focused on using a heterologous host for the production of (epi)podophyllotoxin. We discussed the conversion of deoxypodophyllotoxin into (epi) podophyllotoxin by *P. hexandrum* cytochrome P450 and NADPH-cytochrome reductase in *E. coli*. Another route interesting to explore would be the production of (epi)podophyllotoxin via transgenic *A. sylestris* root cultures transformed with *Agrobacterium tumefaciens* carrying

CYP82D61. Shortening the chemical synthesis route toward etoposide[17,18] is possible by adding CYP71B54 to one of the systems to convert deoxypodophyllotoxin directly into (−)-4′-demethylepipodophyllotoxin as was shown before in tobacco leaves[9]. More challenging is the expression of the complete lignan pathway toward (epi)podophyllotoxin or (−)-4′-demethylepipodophyllotoxin in a heterologous host. In literature, the production of deoxypodophyllotoxin in recombinant tobacco leaves from phenylalanine was reported with high yield[19]; however, further conversion to (−)-4′-demethylepipodophyllotoxin showed production in the nanogram range and should be improved or repeated in another production host. Another possibility is a culturing system producing deoxypodophyllotoxin via recombinant tobacco leaves or *A. sylvestris* root cultures and subsequently conversion of deoxypodophyllotoxin by *E. coli* or chemical synthesis to (epipodophyllotoxin)[20].

Overall, technically all methods discussed can be performed, but process optimization, up-scaling and economic analysis are necessary to determine which routes are economically feasible for the future.

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