Concluding remarks and future perspectives
Medicinal plant research is a crucial discipline in medicines research and currently receives increasing interest from academia and industry. Although modern synthetic drugs are the mostly used medicines in developed countries, the use of herbal drugs in the western world is well accepted and a continuously high demand of plant material and extracted natural products is observed. Plants are used as a source for the starting material of semi-synthetic drugs and new chemical entities as well. Therefore, today’s focus of medicinal plant biotechnology is more on studying biosynthetic pathways and increasing diversity via combinatorial biosynthesis. Approximately 40% of the pharmaceutically applied lead compounds for the production of synthetic drugs today is derived from natural sources. For the supply of several of these drugs, the industry is fully dependent on the isolation of compounds from plant material. Only 10% of the used medicinal plant species are cultivated; the majority comes from wild collections. Harvesting from the wild may becomes a major problem as known e.g. for Podophyllum, Cimicifuga and Taxus species, and may cause loss of genetic diversity and habitat destruction. Compounds currently isolated from these species, respectively podophyllotoxin and paclitaxel, are of high interest for healthcare, which justifies the development of alternative strategies for their production. The same applies for artemisinin for which the costs for production should be reduced to make the drug generally accessible for the treatment of malaria in the developing countries. This thesis highlights possibilities for the use of bioconversion and combinatorial biosynthesis strategies for the production and development of plant natural products, at different levels in biosynthetic pathways.

The choice for a specific host organism depends on several aspects. For bioconversion and combinatorial biosynthesis studies, microorganisms are mostly used. There are several benefits of using microorganisms instead of plants or plant cell cultures, including their fast replication, low costs of cultivation, and the possibility for bioprocessing on an industrial scale. Next to that genetic modification is a rather simple procedure and widely accepted, especially in contrast to plants.

The presence of an endogenous pathway in a host can also be a reason to use a specific organism. For the terpenoids, especially artemisinin, most experience has been obtained with Escherichia. coli and Saccharomyces cerevisiae. In E. coli the endogenous DOXP pathway has been used to produce sesquiterpenoids [45], but an artificial MVA pathway has been constructed as well [12]. Yeast cells produce higher levels of endogenous terpenoids, which make them probably more suitable for the heterologous production of plant terpenoids. The total biosynthesis of hydrocortisone in S. cerevisiae is a good example of the use of the endogenous sterol biosynthesis for the production of a heterologous compound [236]. This thesis highlights the opportunity to use B. subtilis as a host cell for the production of terpenoids. The levels of isoprene produced by this bacterium, suggests the strong induction of the DOXP-pathway and the identification and further characterization of the genes involved can be used to optimize the production of isoprenoids even more. For industrial bioprocessing the Gram-positive organism B. subtilis has often been used, both for the production of heterologous proteins and for the industrial synthesis of small molecules such as riboflavin [237, 238]. The widespread experience with this organism renders the combination with an optimized endogenous biosynthetic pathway for terpenoids into an attractive option.

For a heterologous production the elucidation of biosynthetic pathways of plant secondary metabolites and the identification of enzymes and genes involved are of high importance.
The isolation and identification of these genes is laborious, but contemporary genomic and proteomic approaches result in a plethora of candidate genes and enzymes possibly involved in the biosynthesis of natural products. Knowledge about the catalytic mechanism of a plant enzyme can be used to optimize the efficiency by gene technology. For amorphadiene synthase we investigated the usefulness of a computational model in this respect, but the elucidation of crystal structures would give more precise information. A synthetic gene encoding the same enzyme expressed in E. coli has been described [12]. By optimizing the codons of the gene with the codon usage of the bacterial host, this artificial gene resulted in higher expression levels. However, overexpression of plant genes in a host is probably not as easy as it seems. Problems can arise with protein folding, solubility of the protein, the lack of compartmentalization, lack of modification and proteolytic degradation. Another aspect future research has to focus on is the regulation of the production of the secondary metabolites in plants and in the microbial host organism, because of the differences between the complex plant cell and the host cell. Overproduction of heterologous proteins and metabolites can easily cause damage to the host cell. More sophisticated expression systems should circumvent this metabolic burden. Several studies, e.g. on the biosynthesis of carotenoids, already described this [93, 95-97].

The last aspect of combinatorial biosynthesis described in this thesis is the use of enzymes, which are not originally related to the biosynthesis, for the bioconversion of natural products. The hypothesis seems to be in contradiction with the observation that plant secondary metabolites are special because of the regio- and enantioselectivity of the enzymes involved. However, there is a possibility for the use of cytochrome P450 enzymes for specific bioconversions. In plants these enzymes play a major role in the bioconversion of backbone structures, which are a result of specific plant enzymes. Human cytochrome P450 3A4 can be used to mimic these plant enzymes or to metabolize specific plant compounds to obtain new derivatives. In order to create a more efficient bioconversion system and to come to lower costs, the E. coli system described in this thesis have to be optimized or another host organism has to be used, which contains an endogenous NADPH- cytochrome P450 reductase system. In this respect, S. cerevisiae seems to be a good choice. For the hydroxylation of deoxypodophyllotoxin it would also be an option to develop transgenic Anthriscus sylvestris plants or plant cell cultures containing the human CYP3A4 gene. Although it has to be taken into account that the use of transgenic plants is more complex and less accepted by the community.

In future work the use of directed evolution techniques can also be applied for the optimization of the human cytochrome P450 enzymes. This approach can be used for the development of a more efficient enzyme or to generate novel biocatalysts by altering the substrate specificity of the enzyme. Random mutagenesis, selection of mutants, and computational approaches have been described for human cytochrome P450 enzymes (reviewed by [239]). Especially, the results obtained with the bacterial enzymes CYP450 BM3 and CYP450cam illustrate the potential of engineering these enzymes.

In conclusion, the results described in this thesis show good strategies for novel combinatorial biosynthesis approaches, which can be used as alternative production systems for structurally complex compounds. This approach can save endangered plant
species and reduce the costs for the production of currently expensive drugs. Next to this, the combination of selected genes which would never occur spontaneously in nature can be used to produce new natural products and derivatives of existing compounds. Combinatorial biosynthesis is a novel and potentially valuable strategy for the production of natural products and its development is just at the beginning. The tools described in this thesis will certainly become of increasing importance in the field of natural product research in the near future.