Chapter 1

Introduction and scope of the thesis

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Terpenoids and their role in nutrition and medicine

Terpenoids belong to the largest class of natural products with important medicinal and industrial properties, and today approximately 25,000 terpenoid structures have been elucidated and isolated from plants, microorganisms, insects and various marine organisms (Gershenzon et al., 2007). Despite the enormous structural complexity, terpenoids are constructed from two basic isoprene building blocks, isopentenyl pyrophosphate and dimethylallyl pyrophosphate, which can be coupled in nearly any way as defined by the enzymatic setup in a species.

Terpenoids serve a number of different functions e. g., steroids for membrane fluidity and hormone signaling, carotenoids for photosynthesis in plants and as antioxidant agents (Howitt et al., 2006), quinones for electron transport (Berry, 2002) and dolichol for protein glycosylation (Lehrman, 2007). Especially from plants and marine invertebrates have many terpenoids with significant pharmaceutical importance been identified. Indeed, the terpenoids encompass a vast number of bioactive compounds like artemisinin, paclitaxel and menthol; most of these molecules are used in pharmaceutical, cosmetic or food industries. Compounds such as limonene, menthol and carotenoids are available in discrete quantities in Nature, and hence they can easily be extracted and purified. However, terpenoids such as the antiplasmodial artemisinin and the anti-cancer chemotherapy drug paclitaxel are produced by one dedicated species respectively and furthermore in small quantities. Such limitations cause supply problems. This has sparked an interest in using biotechnology to solve the situation.

Metabolic engineering of rare and complex compounds such as artemisinin or paclitaxel is a new concept in synthetic biology. The strategy is considered a novel avenue for the production of rare and high-cost natural products. The underlying idea encompasses transfer of a complete plant terpenoid pathway to microorganisms to

optimize the biosynthetic production rate by fermentation. However, pathways in both bacteria and fungi meant to be used as target hosts are regulated through complex networks. Furthermore, the regulation of the terpenoid pathway is far from simple and control mechanisms are rather unknown. In **chapter 5**, the use of yeast as a heterologous host for production of artemisinin intermediates is described.

The keen interest in artemisinin, a sesquiterpene lactone which is extracted from the sweet wormwood plant *Artemisia annua* (*A. annua*), emanates from the potent antimalarial property of the molecule, and its lack of side effects (Li et al., 2010). Furthermore, artemisinin has been shown to reduce cancer by inducing apoptosis, disrupting cell migration and cell cycle arrest as well as inhibiting angiogenesis (Firestone et al., 2009). Moreover, due to development of resistance against traditional antimalarial medicines such as chloroquine, artemisinin combination therapies are left as the sole remaining treatment of the disease (Eastman et al., 2009). Artemisinin combination therapies are therefore the first-line treatments for uncomplicated *Plasmodium falciparum* (*P. falciparium*) malaria in most malaria-endemic countries. With 150-300 children lost to malaria each hour and approxiamtely a toll of 2 million deaths a year, malaria has been put forward as one of the most urgent catastrophies by the World Health Organization (Breman, 2009). This has been followed up by an intense research in how artemisinin can be synthesized, most efficiently isolated from the plant, in how the yield *in planta* can be increased and the latetst approach aimed at heterlogous production of artemisinin (**chapter 2**).

Metabolic engineering of the terpenoid pathway

The concept of metabolic engineering

Metabolic engineering is considered as one of the major concepts in biotechnology. At the moment, genetic engineering allows the

transfer of a biosynthetic pathway to any selected host. The isoprenoid pathway is an example on how biosynthetic relevant genes can be reassembled from different biological sources (e.g. plants, bacteria and fungi) in a heterologous microorganism. Engineering of plant terpenoids into microbial hosts has been focused mainly on isoprenoid derived compounds such as carotenoids (Schmidt-Dannert et al., 2000), artemisinin and paclitaxel (Newman, et al., 2006; Withers et al., 2007). The production of carotenoids and artemisinin demonstrates that complex natural products can be produced by microbial fermentation with yields approaching commercial relevance. Development of a microbial production platform for the biosynthesis of complex terpenoids offers large-scale and cost-effective industrial production via fermentation, which is independent from climate (too dry, too wet) and cultivation risks (pest controls, weeds, soil conditions). Well characterized and genetically fairly easy to manipulate heterologous hosts such as *Escherichia coli* (*E. coli*) and *Saccharomyces cerevisiae* (*S. cerevisiae*) allow very specific engineering of biosynthetic pathways for increased yields and generating novel compounds. Although extensive engineering is frequently required to successfully reconstitute biosynthetic pathways in these hosts, the potential to manipulate the biosynthesis is a significant advantage when compared to engineering in poorly characterized host strains or non microbial systems.

Selecting microorganisms for engineering terpenoid pathways

Commonly the host *E. coli* and *S. cerevisiae* are favored in metabolic engineering, but other hosts can be used as well. Both laboratory domestic organisms share the advantages of having been studied over decades and the elucidation of their genome, transcriptome and metabolome are nearly complete. However, *in silico* models of these organisms do not exist (Feist et al., 2009). In theory, the possibility exists to adapt every host for the production of natural compounds, but as various organisms exhibit different genetic properties, they

also react distinctively to genetic manipulations and to the introduced recombinant genes. Although nearly all organisms are able to produce terpenoid structures (Goldstein et al., 1990; Hunter, 2007), their strategy and diversity varies dramatically between species. Synthetic biology provides the tools to overcome these problems and to explore the rich variety of enzymes by which nature is capable of creating all imaginable structures. This can be exploited by metabolic engineers: by selecting the right host for recombinant expression, it is possible to increase production levels by minimal adaption of the genetic system. This strategy was investigated as described in **chapter 5**.

Engineering the secondary terpenoid metabolism

In contrast to the primary metabolism, no standard biological components exist which would fit for all different secondary pathways. Secondary metabolite routes are per definition species specific and therefore we have to expect a high diversity in structures between organisms in the terpenoid biosynthesis (Fischbach et al., 2007). Artemisinin is an excellent example since *A. annua* produces low amounts of artemisinin, while its relatives *Artemisia absinthium* (Van Nieuwerburgh, et al., 2006) and *Artemisia afra* (Van der Kooy et al., 2008) synthesize no artemisinin at all. The properties of many terpenoids make them interesting for recombinant production, although many pathways are yet too complex or enzymes unknown. This thesis reports on the isolation and functional characterization of enzymes from the artemisinin biosynthetic pathway (**chapter 3-6**).

Case study artemisinin

The activity of artemisinin against the malaria parasite *P. falciparium* has been well established. In addition, it has been proven that artemisinin acts by selective inhibition of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA). The same study showed that artemisinin contributes to a change in membranous structures in the

cytoplasm of parasites inside erythrocytes (Eckstein-Ludwig, et al., 2003). Examples of derivatives of artemisinin include artemether and artemisone, which are on the market or currently under development (Haynes, et al., 2006). An overview of artemisinin derivatives is described in **chapter 2**.

The low production yield of artemisinin from *A. annua* (0.01-1 % of dry weight) has led to difficulties in managing the demand, while offering an acceptable price for most patients (Liu et al., 2006; Mutabingwa, 2005). The wish to improve the overall supply of artemisinin at a reduced market price has encouraged interest in molecular biology and biochemistry of artemisinin biosynthesis (figure 1) (Bertea, et al., 2005; Bouwmeester, et al., 1999; Covello et al., 2007; Mercke et al., 2000; Ro, et al., 2006). The biosynthesis starts with the cyclization of farnesyl diphosphate to amorpha-4,11-diene by amorpha-4,11-diene synthase (Amds) (Kim, et al., 2006; Picaud, et al., 2006; Wallaart et al., 2001). Various terpene synthases are known to convert the terpenoid backbone into pathway specific structures, however some of them are less specific and can produce more than one structure (Christianson, 2008). In the first step a divalent metal, generally magnesium, is used to stabilize the pyrophosphate group in similar terpenoid backbones, such as geranyl diphosphate or farnesyl diphosphate. After this first committed enzymatic step towards secondary relevant terpenoids, the pyrophosphate is lost and more species specific enzymes (e.g. cytochromes) create the broad diversity of structures (Covello, et al., 2007; Croteau et al., 2005).

Subsequent enzymatic steps in the biosynthetic pathway of artemisinin can possibly be performed in different orders. The main likely conversion scheme *in planta* for production of artemisinin include the oxidation of amorpha-4,11-diene to artemisinic alcohol followed by a further oxidation at C12 from alcohol to aldehyde by the cytochrome P450 Cyp71av1. In the next step, the carbon double

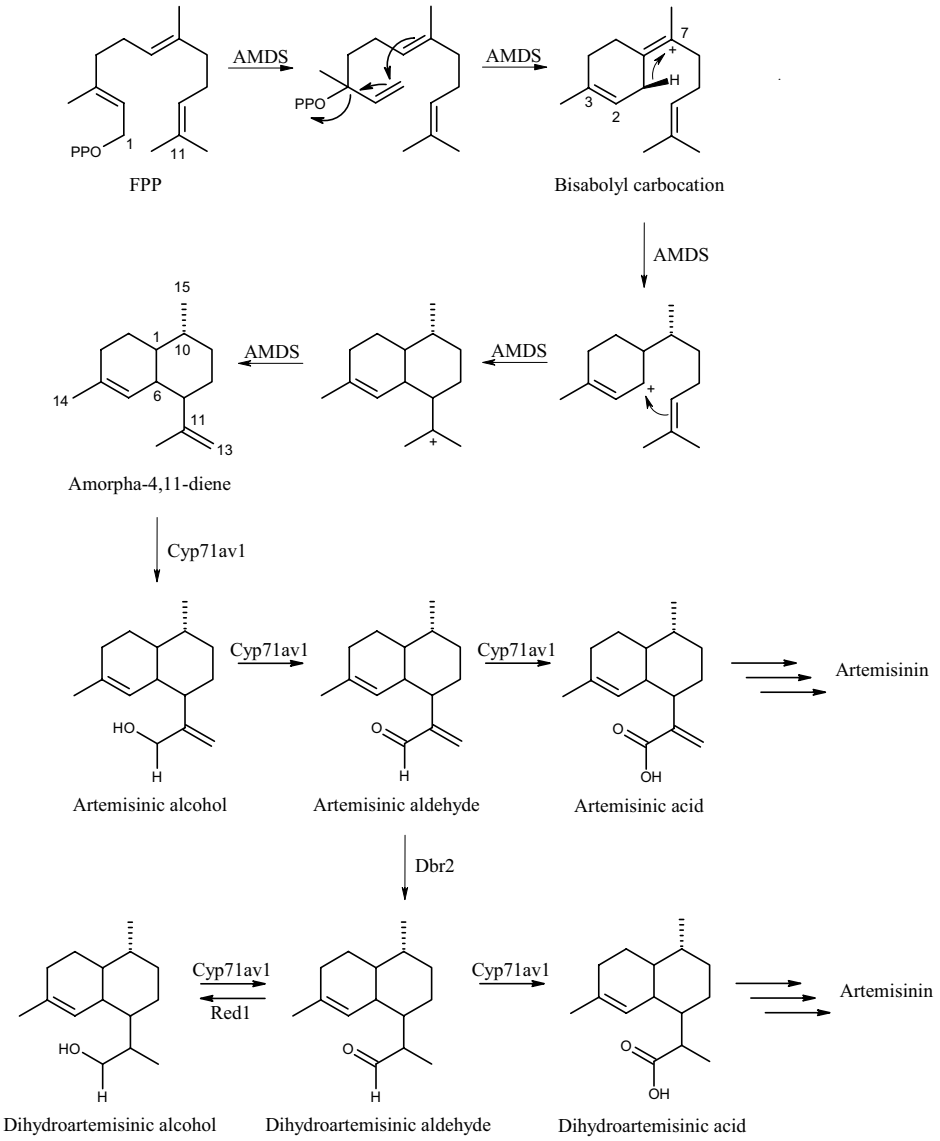

Figure 1. **Biosynthesis of artemisinin in *A. annua.*** The pathway is a summary based on chapters 2-5 as well as the literature mentioned therein. AMDS – Amorpha-4,11-diene synthase, Cyp71av1 – Cytochrome P450 71av1, Dbr2 – Double bond reductase 2, FPP – Farnesyl diphosphate, Red1 – Reductase 1.

bond at C11-C13 is reduced forming dihydroartemisinic aldehyde by the artemisinic aldehyde reductase (Dbr2), followed by conversion of dihydroartemisinic aldehyde to dihydroartemisinic acid by Cyp71av1. The formation of the 1,2,4-trioxane moiety is not fully understood and is suggested to occur patially in a non-enzymatic manner (Covello, et al., 2007; Ro, et al., 2006; Rydén et al., 2007). Identification of these enzymes is essential for the production of artemisinin in heterologous systems. These issues are further discussed in **chapter 2**.

The value of synthetic biology was proven when the two genes Amds and Cyp71av1 were expressed in yeast. The transgenic yeast produced more artemisinic acid in contrast to relative plant biomass (4.5% dry weight in yeast compared to 1.9% artemisinic acid and 0.16% artemisinin in *A. annua*) and in a shorter time period (4–5 days for yeast versus several months for *A. annua*) (Ro, et al., 2006). When these two enzymes were co-expressed in yeast together with Dbr2, dihydroartemisinic acid was produced at a level of 15.7 (+/- 1.4) mg/L culture and the related artemisinic acid was produced at levels of 11.8 mg/L (Zhang, et al., 2008). The production level of artemisinic acid was reduced compared to the mutant without the artemisinic aldehyde reductase (29.4 mg/L), due to production of dihydroartemisinic acid. This is preferred because dihydroartemisinic acid is chemically converted to artemisinin both easier and cheaper than artemisinic acid.

Perspectives

In the future, metabolic engineering will provide a third alternative to plant cell culture and chemistry to cover the high demand of the pharmaceutical drug artemisinin for a reasonable price. Pathway engineering will provide more benefits compared to traditional approaches: stereo-chemically complex natural products such as artemisinin can hardly be synthesized by conventional synthetic

chemistry. This approach still needs to be adapted to minimize unwanted side products in the synthesis. A biosynthetic production can be performed at room temperature, under buffer media conditions and under atmospheric pressure. This procedure is termed white biotechnology and it supports the transition from classical "dirty" organic chemistry. It is gaining support worldwide, where companies strongly associated with chemistry are investing in alternatives based on evolved proteins to improve industrial processes and create novel compounds (Ernst&Young, 2007). Upcoming white biotechnology will benefit from synthetic biology and metabolic engineering and change the way to synthesize even small terpenoids. A summary of the results presented in this thesis along with future perspectives is presented in **chapter 7** and as a Dutch summary in **chapter 8**.

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