Chapter 2

Chemistry, biosynthesis and biological activity of artemisinin and related natural peroxides

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Adapted from: Bioactive Heterocycles III, series: Topics in Heterocyclic Chemistry (2007), vol. 9, p. 1-32

Abstract

Artemisinin is a heterocyclic natural product and belongs to the natural product class of sesquiterpenoids with an unusual 1,2,4 trioxane substructure. Artemisinin is one of the most potent antimalarial drugs available. It serves as a lead compound in the drug development process to identify new chemical derivatives with optimized antimalarial activity and improved bioavailability. In this review we report about the latest status of research on chemical and physical properties of the drug and its derivatives. We describe new strategies to produce artemisinin on a biotechnological level in heterologous hosts and in plant cell cultures. We also summarize recent reports on its pharmacokinetic profile. Furthermore we describe attempts to develop drug delivery systems to overcome bioavailability problems and to target the drug to *Plasmodium* infected erythrocytes as main target cells.

Chemistry

For thousands of years Chinese herbalists treated fever with a decoction of the plant called "qinghao", *Artemisia annua,* "sweet wormwood" or "annual wormwood", belonging to the family of Asteraceae. In the 1960s a program of the People Republic of China re-examined traditional herbal remedies on a rational scientific basis including the local qinghao plant. Early efforts to isolate the active principle were disappointing. In 1971 Chinese scientists followed an uncommon extraction route using diethyl ether at low temperatures obtaining an extract with a compound that was highly active *in vivo* against *Plasmodium berghei* in infected mice. The active ingredient was febrifuge, structurally elucidated in 1972, called mostly in China "qinghaosu", or "arteannuin" and in the west "artemisinin". Artemisinin, a sesquiterpene lactone, contains a peroxide group unlike most other antimalarials. It was also named artemisinine, but following IUPAC nomenclature a final "e" would suggest that it was a nitrogen-containing compound which is misleading and therefore that name is not favored today.

Artemisinin and its antimalarial derivatives belong to the chemical class of unusual 1,2,4-trioxanes. Artemisinin is poorly soluble in water and decomposes in other protic solvents, probably by opening of the lactone ring. It is soluble in most aprotic solvents and is unaffected by them at temperatures up to 150 °C and shows a remarkable thermal stability. This section will focus on biological and pharmaceutical aspects; synthetic routes to improve antimalarial activity and synthetic production of artemisinin derivatives with different substitution patterns are reviewed elsewhere (Woerdenbag, et al., 1994; Ziffer et al., 1997). Most of the chemical modifications were conducted to modify the lactone function of artemisinin to a lactol. In general alkylation, or a mixture of dihydroartemisinin epimers in the presence of an acidic catalyst, gave products with predominantly β-orientation, whereas acylation in alkaline medium preferentially yields α-orientation (figure 1). Artemether (figure 1.2)

as the active ingredient of Paluther® is prepared by treating a methanol solution of dihydroartemisinin with boron trifluoride-etherate, yielding both epimers. The main goal was to obtain derivatives that show a higher stability when dissolved in oils to enable parenteral use. The α-epimer is slightly more active (EC_{50} = 1.02 mg/kg b.w.) than the β-epimer (EC_{50} = 1.42 mg/kg) and artemisinin itself (EC_{50} = 6.2 mg/kg) (CCRG, 1982). Synthesis of derivatives with enhanced water solubility has been less successful. Sodium artesunate, Arsumax® (1.5) has been introduced in clinics, is well tolerated and less toxic than artemisinin.

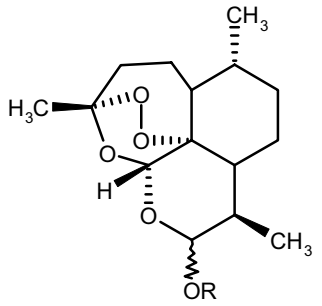


Figure 1. Artemisinin and its derivatives. (1.1) dihydroartemisin; R = H (α + ß), (1.2) artemether; R = CH_3 (ß), (1.3) arteether; R = CH_2CH_3 (ß), (1.4) artelinate; R = $CH_2C_6H_4COONa$ (ß), (1.5) artesunate; R = $COCH_2CH_2COONa$ (α).

Trioxane and peroxides in nature

Besides artemisinin more than 150 natural peroxides are known in Nature. The presence of the typical peroxide functions is not related to one natural product group and occurs as cyclic and acyclic peroxides in terpenoids, polyketides, phenolics and also alkaloids. Most stable are cyclic peroxides even under harsh conditions and artemisinin is a nice example for that. Artemisinin can be boiled or treated with sodium borohydride as base without degradation of the

peroxide function. In contrast acyclic peroxides are rather unstable, form hydrogen peroxides and are easily broken by metals or bases.

Most of the natural peroxides have been isolated from plants and marine organisms, and terpenoids have attracted most interest because of the structural diversity they cover. In an excellent review from Jung et al.(2003), an overview is given and it should be stressed out that *Scapania undulata*, which is a bryophyte in the Nothern parts of Europe, biosynthesize amorphane like natural products with a cyclic peroxide (figure 2.1) structurally related to the well known artemisinin. There is less information about the biological activity of natural peroxides from plant origins, but some reports indicate the use against helminth infections, rheumatic diseases and antimicrobial activity. Natural cyclic peroxides from marine source (figure 2) have been tested for a broad range of activities including antiviral (Aikupikoxide A), antimalarial, antimicrobial activity and cytotoxicity (figure 2.2). A second important natural product group is polyketides and it is interesting that all of the isolated polyketide-derived peroxides are from marine sources. Due to the high flexibility in the carbon chain and presence of hydroxy substituents, a high chemical diversity can be documented ranging from simple and short peroxides like haterumdioins in Japanese sponge *Plaktoris lita* to more complex structures with long chain derivatives like peroxyacarnoic acids from the sponge *Acarnus bicladotylota* (figure 2.3). Most of the polyketide-derived peroxides show a high cytotoxic activity and moderate activity against microorganisms.

As expected, due to chemical instability the number of acyclic peroxides is lower. Most of them occur as plant derived products, but also in soft corals like *Clavularia inflata*, hydroperoxides with potent cytotoxicity exist. Interestingly the bioactivity disappeared when the hydroperoxide function was deleted. It must be noted that most of natural hydroperoxides in plants are found in the group of saponins from *Panax ginseng* or *Ficus microcarpa* which are used in the ethnomedicine in South East Asia.

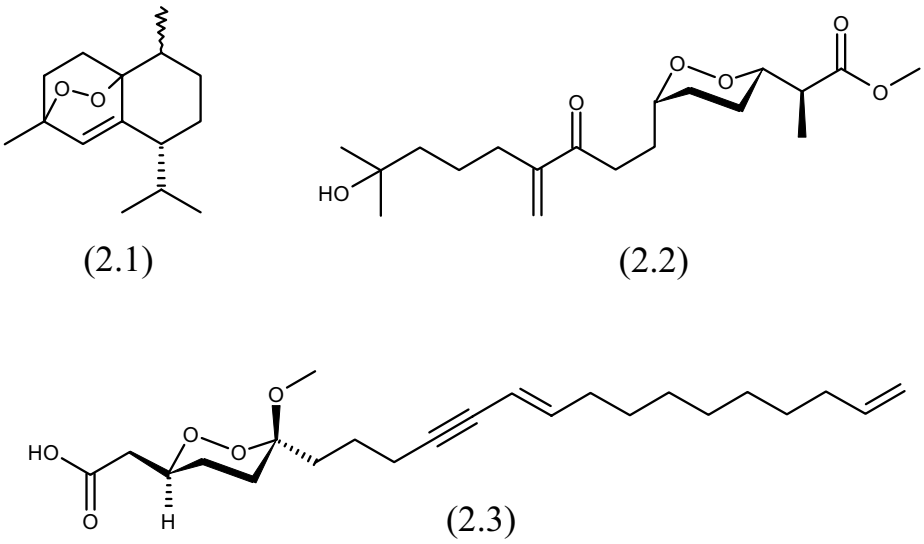


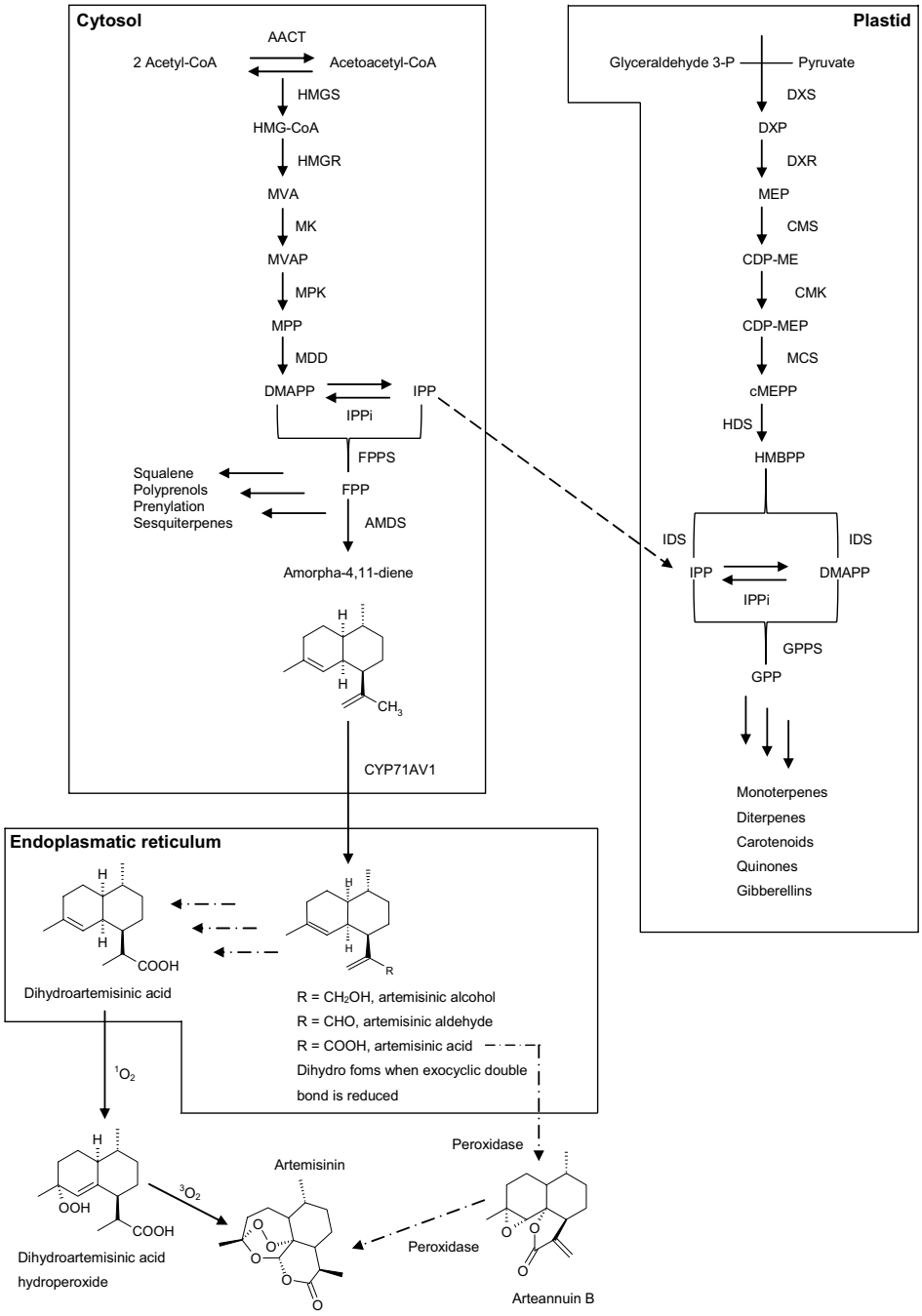
Figure 2. Natural peroxides. (2.1) Terpene type peroxide from *Scapania undulate*, (2.2) Aikupikoxide-D from *Diacarnus erythraenus*, (2.3) Peroxyacarnoic acid A from *Acarnus bicladotyloata*.

Biosynthesis

Biosynthesis in Artemisia annua

Biochemistry

There are two pathways employed in plants for production of isoprenoids, the 1-deoxy-D-xylulose 5-phosphate pathway (DXP) localized in the plastid and the mevalonate pathway which is precent in the cytosol (figure 3) (Yan Liu et al., 2005). These pathways are normally used to produce different sets of isoprenoids; sesquiterpenoids, sterols and triterpenoids among others being reserved for the mevalonate pathway, while the diterpenes and


Cytosol
AACT
2 Acetyl-CoA
Acetoacetyl-CoA
HMGS
HMG-CoA
HMGR
MVA
MK
MVAP
MPK
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MDD
DMAPP
IPP
IPPi
FPPS
Squalene
Polyprenols
Prenylation
Sesquiterpenes
FPP
AMDS
Amorpha-4,11-diene
CYP71AV1
Plastid
Glyceraldehyde 3-P
Pyruvate
DXS
DXP
DXR
MEP
CMS
CDP-ME
CMK
CDP-MEP
MCS
cMEPP
HDS
HMBPP
IDS
IDS
IPP
DMAPP
IPPi
GPPS
GPP
Monoterpenes
Diterpenes
Carotenoids
Quinones
Gibberellins
Endoplasmatic reticulum
COOH
Dihydroartemisinic acid
R = CH2OH, artemisinic alcohol
R = CHO, artemisinic aldehyde
R = COOH, artemisinic acid
Dihydro foms when exocyclic double bond is reduced
1O2
Peroxidase
OOH
COOH
3O2
Artemisinin
Dihydroartemisinic acid hydroperoxide
Peroxidase
Arteannuin B

Figure 3. Isoprenoid biosynthetic pathways in plant cells. In the cytosol the mevalonate pathway is represented, in the plastid the MEP pathway. Biosynthesis of artemisinin is depicted in detail. Long dash arrow depicts transport. Dash punctured arrow depicts unknown or putative enzymatic function. Single arrow depicts single reaction step. Multiple arrows depict several reaction steps. Shortenings of substrates: CDP-ME, 4-(Cytidine 5'-diphospho)-2-C-methyl-D-erythritol; CDP-MEP, 4-(Cytidine 5'-diphospho)-2-C-methyl-D-erythritol 2-phosphate; cMEPP, 2-C-Methyl-D-erythritol 2,4-cyclodiphosphate; DMAPP, Dimethylallyl diphosphate; DXP, 1-Deoxy-D-xylulose 5-phosphate; FPP, Farnesyl diphosphate; GPP, Geranyl diphosphate; HMBPP, 1-Hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate; HMG-CoA, 3S-Hydroxy-3-methylglutaryl-CoA; IPP, Isopentenyl diphosphate; MEP, 2-C-Methy-D-erythritol 4-phosphate; MPP, Mevalonate diphosphate; MVA, 3R-Mevalonic acid; MVAP, Mevalonic acid-5-phosphate. Shortenings of enzymes: AACT, Acetoacetyl-coenzyme A (CoA) thiolase; AMDS, Amorpha-4,11-diene synthase; CMK, 4-(Cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase; CMS, 2-C-Methyl-D-erythritol 4-phosphate cytidyl transferase; CYP71AV1, Cytochrome P450 71AV1; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; DXS, 1-deoxy-D-xyluose 5-phosphate synthase; FPPS, Farnesyl diphosphate synthase; GPPS, Geranyl diphosphate synthase; HDS, 1-Hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase; HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; HMGS, 3-hydroxy-3-methylglutaryl CoA synthase; IPPi, Isopentenyl diphosphate isomerase; MCS, 2- C-Methyl-D-erythritol 2,4-cyclodiphosphate synthase; MDD, mevalonate diphosphate decarboxylase; MK, mevalonate kinase; MPK, mevalonate-5-phosphate kinase.

monoterpenes are produced by the DXP pathway. However, there are recent evidences that the pathways to some extent crosstalk at the isopentenyl diphosphate (IPP) level (Yan Liu, et al., 2005).

The first committed step in the biosynthetic pathway of artemisinin is the cyclization of the general mevalonate pathway sesquiterpenoid precursor farnesyl diphosphate (FPP) into (1S, 6R, 7R, 10R)-amorpha-4,11-diene by amorpha-4,11-diene synthase (AMDS) (figure 4) (Chang et al., 2000; Mercke et al., 2000; Wallaart et al., 2001). The crystal structure of this sesquiterpene synthase is not known which is the situation for all but one plant sesquiterpene synthase: the 5-*epi*-aristolochene synthase from tobacco (Starks et al., 1997). In contrast, the mechanism behind the cyclization of FPP into amorpha-4,11-diene has been proven by Picaud et al. (2006) and Kim et al. (2006) through the use of deuterium labeled FPP (figure 4). Differing from the bicyclic sesquiterpene cyclases δ-cadinene synthase from cotton (Benedict et al., 2001) and pentalene synthase (Cane et al., 2003) which produce a germacrene cation as the first cyclic intermediate, Amds produces a bisabolyl cation. FPP is ionized and the paired diphosphate anion (OPP) is transferred to C3 giving (3R)-nerolidyl diphosphate. This intermediate allows rotation around the C2-C3 bond to generate a cisoid form. The cisoid form brings C1 in close proximity to C6 allowing a bond formation between these two carbon atoms thus resulting in the first ring closure and a bisabolyl cation. The formed cation is in equilibrium with its deprotonized uncharged form, which is interesting because it implies a solvent proton acceptor and stands in contrast to studies discussing properties of the active site of a investigated trichodiene synthase (Rynkiewicz et al., 2001).

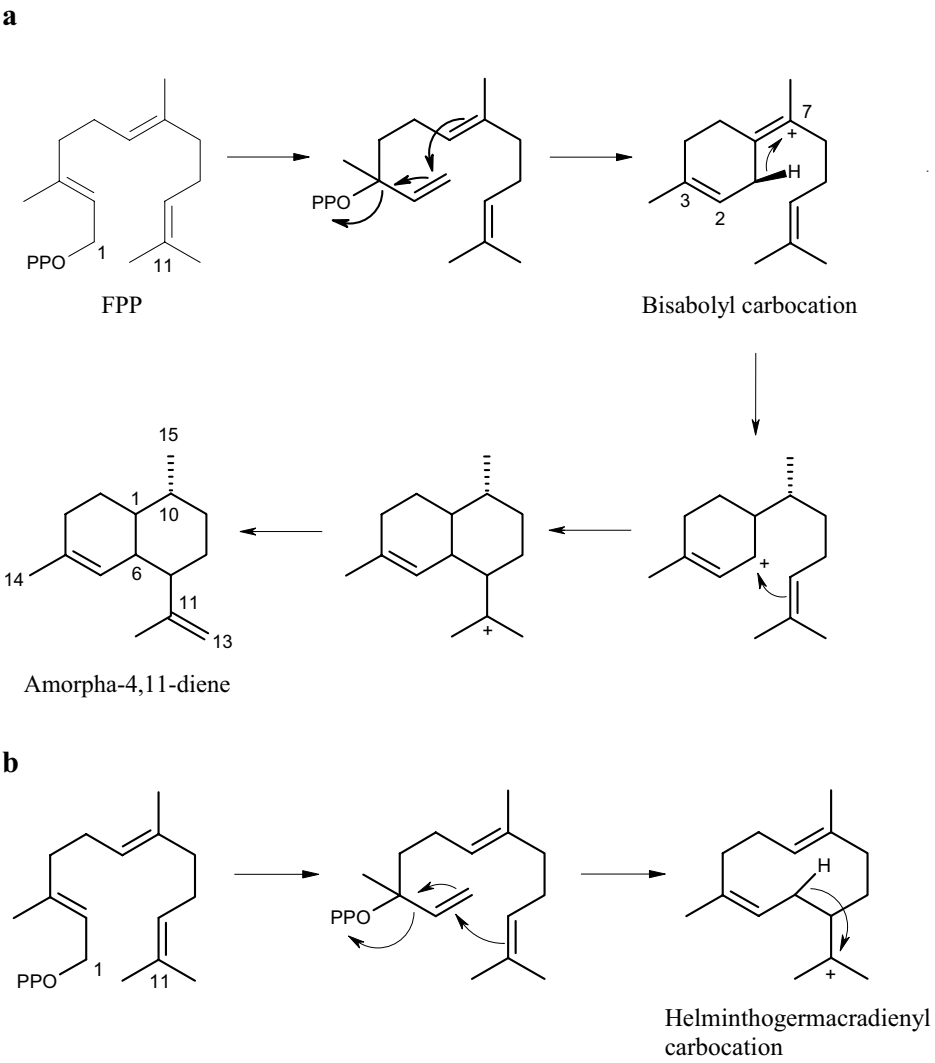

Figure 4. Commitment of FPP to amorpha-4,11-diene.
a. Cyclization of FPP to amorpha-4,11-diene by Amds as described by Kim et al. (2006) and Picaud et al. (2006).
b. Cyclization of FPP to helmonthogermacradienyl carbocation.

Rynkiewicz and Cane came to the conclusion that the active site is completely devoid of any solvent molecule which would prematurely quench the reaction. In a second report from the group Vedula et al. (2005), the authors draw the conclusion from their results that terpene cyclization reactions in general are governed by kinetic rather than thermodynamic rules in the step leading to formation of the carbocation. In the bisabolyl cation, an intermediate in the reaction towards amorpha-4,11-diene, a 1,3 hydride shift to C7 occurs leaving a cation with a positive charge at C1 (FPP numbering).Through a nucleophilic attack on C1 by the double bond C10-C11 the second ring closes to give an amorphane cation. Deprotonation on C12 or C13 (amorphadiene numbering) gives amorpha-4,11-diene.

The three-dimensional structures of three non-plant sesquiterpene synthases reveals a single domain composed entirely of α-helices and loops despite the low homology on amino acid sequence level (Caruthers et al., 2000; Lesburg et al., 1997; Rynkiewicz, et al., 2001). The secondary elements of 5-*epi*-aristolochene synthase, a plant sesquiterpene synthase, conforms to this pattern with the exception that it contains two domains solely composed of α-helices and loops. It is reasonable, but still a matter of debate, to extrapolate these data to the case of amorpha-4,11-diene synthase which probably will only display α-helices and loops once the crystal structure has been solved.

A further element shared by all sesquiterpene synthases is the need for a divalent metal ion as cofactor. The metal ion is essential for substrate binding but also for product specificity. The metal ions stabilize the negatively charged pyrophosphate group of farnesyl diphosphate as illustrated by the crystal structure of 5-*epi*-aristolochene synthase (Starks, et al., 1997). The highly conserved sequence (I, L, V)DDxxD(E) serves to bind the metal ions in all known terpene and prenyl synthases (figure 5) (Colby et al., 1993; Desjardins et al., 1993; Hohn et al., 1989; Math et al., 1992; Proctor

et al., 1993). A further interesting property among terpene synthases is that the active sites are enriched in relatively inert amino acids, thus it is the shape and dynamic of the active site that determines catalytic specificity (Greenhagen et al., 2006).

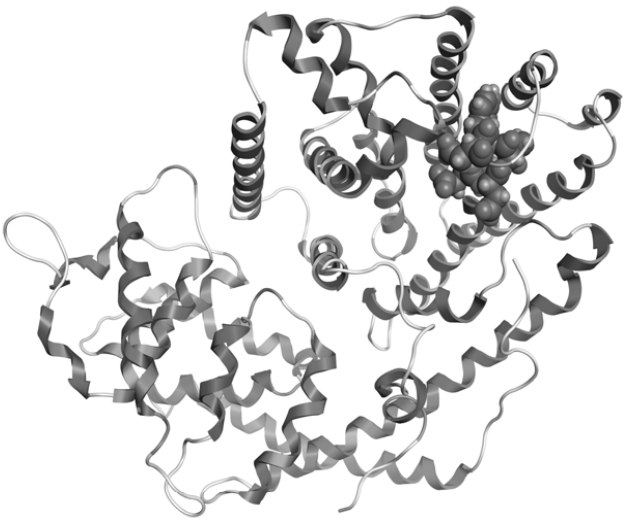

Figure 5. Computerized 3D-structure of amorpha-4,11-diene. Residues marked with space fill reprecentation belong to the conserved metal ion binding amino acid sequence IDDxxDD. We kindly thank Wolfgang Brandt, Leibniz Institute of Plant Biochemistry Halle/Germany for his 3D model of amorphadiene synthase (Amds).

Picaud et al. (2005) purified recombinant Amds and determined its pH optimum to 6.5. Several sesquiterpene synthases show maximum activity in this range, examples are tobacco 5-*epi*-aristolochene synthase (Bouwmeester et al., 2002; Vogeli et al., 1990), germacrene A synthase from chickory (Bouwmeester, et al., 2002) and nerolidol synthase from maize (Schnee et al., 2002). Terpenoid synthases are, however, not restricted to a pH optimum in this range. Intriguing examples are the two (+)-δ-cadinene synthase variants from cotton

which exhibit maximum activity at pH 8.7 and 7-7.5 respectively (Chen et al., 1996) and 8-*epi*-cedrol synthase from *A. annua* (Mercke et al., 1999) with pH optimum around 8.5-9.0. The authors further investigated the metal ion required as cofactor for Amds as well as substrate specificity. The kinetics studies revealed k_{cat}/K_m values of $2.1x10^{-3}$ μM^{-1} s^{-1} for conversion of FPP at the pH optimum 6.5 with Mg^{2+} or Co^{2+} ions as cofactors and a slightly lower value of $1.9x10^{-3}$ μM^{-1} s^{-1} with Mn^{2+} as a cofactor. These very low efficiencies are common to several sesquiterpene synthases but there are substantial differences reported. The synthase reached a k_{cat}/K_m value of $9.7x10^{-3}$ μM^{-1} s^{-1} for conversion of FPP at pH 9.5 using Mg^{2+} as a metal ion cofactor. This increase in efficiency is interesting and shows the broad window in which the enzyme can work, something that may prove to be industrially useable but which physiologically does not have a meaning in the plant. The increase in efficiency is not linear as the maximum activity of Amds is around pH 6.5-7.0 with a minimum at pH 7.5. The established pH optimum of 6.5 is in line with the range established for Amds isolated from *A. annua* leaves (Bouwmeester et al., 1999). Amds did not show any relevant activity in the presence of Ni^{2+}, Cu^{2+} or Zn^{2+}. In presence of Mn^{2+} as cofactor, Amds is capable of using geranyldiphosphate (GPP) as substrate although with very low efficiency (4.2 $x10^{-5}$ μM^{-1} s^{-1} at pH 6.5). Using Mn^{2+} as a cofactor also increased the product specificity of Amds to ~ 90% amorpha-4,11-diene with minor negative impact on efficiency. Under optimal conditions Amds was proven to be faithful towards the production of amorpha-4,11-diene from FPP, converting ~ 80% of the substrate into amorpha-4,11-diene, ~ 5% amorpha-4,7 (11)-diene and ~3.5 % amorpha-4-en-7-ol together with 13 other sesquiterpenes in minute amounts.

Bertea et al.(2005) postulated that the main route to artemisinin is the conversion of amorpha-4,11-diene to artemisinic alcohol, which is further oxidized to artemisinic aldehyde (figure 3). The C11-C13 double bond in artemisinic aldehyde was then proposed to be reduced, giving dihydroartemisinic aldehyde which would upon

further oxidation give dihydroartemisinic acid. The authors supported their conclusion by demonstrating the existence of amorpha-4,11-diene, artemisinic alcohol, artemisinic aldehyde and artemisinic acid together with the reduced forms of the artemisinin intermediates in leaf- and glandular trichome microsomal pellets, by direct extraction from leaves and through enzyme assays. Interestingly, they could not show any significant conversion of artemisinic acid into dihydroartemisinic acid regardless of the presence of cofactors NADH and NADPH thus strengthening the hypothesis that reduction of the C11-C13 double bond occurs at the aldehyde level. In view of these results it is very likely that artemisinic acid is a dead end product which can not be converted into artemisinin in contrast with some literature (Woerdenbag et al., 1990), unless reduced to dihydroartemisinic acid.

Recently, two research groups cloned the gene responsible for oxidizing amorpha-4,11-diene in three steps to artemisinic acid (Ro et al., 2006; Teoh et al., 2006) (figure 3). This enzyme, a cytochrome P450 named Cyp71av1, was expressed in *Saccharomyces cerevisiae* (*S. cerevisiae*) and associated to the endoplasmatic reticulum. The isolation and application of this cytochrome P450 is described further in the section *Heterologous production in Saccharomyces cerevisiae*. Further research that will clarify whether additional cytochrome P450s or other oxidizing enzymes are precent in the native biosynthetic pathway and where the reduction of the C11-C13 double bond occurs are still open fields of exploration.

Several terpenoids including artemisinin and some of its precursors and degradation products, has been found in seeds of *A. annua* (Brown et al., 2003). In its vegetative state, secretory glandular trichomes (Mueller et al., 2004) are the site of production of artemisinin. In a recent paper by Lommen et al. (2006) the authors show that the production of artemisinin is a combination of enzymatic and non-enzymatic steps. The authors followed the production of artemisinin and its precursors on a level per leaf basis.

The results showed that artemisinin is always precent during the entire life cycle of a leaf, from appearance to senescence and that the quantity is steadily increasing as would be expected for an end product in a biosynthetic pathway. Interestingly, the immediate precursor to artemisinin, dihydroartemisinic acid (Sy, 2002) was more abundant than other precursors, indicating that the conversion of dihydroartemisinic acid into artemisinin is a limiting step. It was also shown that dihydroartemisinic acid is not converted to artemisinin directly. The authors argue, in line with other literature (Sy, 2002), that this might be due to a temporary accumulation of the putative intermediate dihydroartemisinic acid hydroperoxide (figure 3). The observation that artemisinin levels continued to increase at the same time as the numbers of glandular trichomes decreased further supports the idea that the final step of artemisinin formation is non-enzymatic. Wallaart et al. (1999) were able to show that conversion of dihydroartemisinic acid to artemisinin is possible when using mineral oil as reaction solvent instead of glandular oil (figure 3). By adding dihydroartemisinic acid and chlorophyll *a* to mineral oil and exposing the mixture to air and light, a conversion of 12% after 120 hours was achieved. In absence of mineral oil a conversion of 26.8% could be achieved. Wallaart et al. (1999) were later able to show that the hypothesized intermediate between dihydroartemisinic acid and artemisinin, dihydroartemisinic acid hydroperoxide, could be isolated from *A. annua* and upon exposure to air for 24 hours at room temperature yielded artemisinin and dihydro-*epi*-deoxyarteannuin B (figure 3).

Genetic versus environmental regulation of artemisinin production

The genetic regulation of the biosynthesis of artemisinin is poorly understood on the single pathway level. The situation further complicates as there are several FPP synthase (Fpps) and 3-hydroxy-3-methylglutaryl CoA reductase (Hmgr) isoforms making optimization options more versatile and complex. The active drug component in *A. annua* was isolated in the 1970s but it is only during

the last eight years that key enzymes in the committed biosynthetic pathway of artemisinin have been cloned and characterized (Chang et al., 2000; Mercke et al., 2000; Ro et al., 2006; Teoh et al., 2006; Wallaart et al., 2001) (figure 3). However, the genetic variation contributing to the level of artemisinin production has been investigated to some extent. The genetic variation is reflected in the existence of at least two chemotypes of *A. annua*. Wallaart et al. (2000) showed that plant specimens from different geographical origins had a different chemical composition of the essential oil during the vegetative period. The authors could distinguish one chemotype having a high content of dihydroartemisinic acid and artemisinin accompanied by a low level of artemisinic acid and a second chemotype represented by low artemisinin and dihydroartemisinic acid content together with a high level of artemisinic acid. With the aim to increase the artemisinin production the authors induced tetraploid specimens from normal high producing diploids using colchicine (Wallaart et al., 1999). This led to higher artemisinin content in the essential oil but to a 25% decrease in artemisinin yield per m^2 leaf biomass.

Only a few studies have been performed investigating the effect of singular genes on artemisinin production. Wang et al. (2004) overexpressed a flowering promoting factor (*fpf1*) from *Arabidopsis thaliana* in *A. annua* and observed 20 days earlier flowering compared with the control plants but could not detect any significant change in artemisinin production. From this it can be concluded that the event of flowering has no effect on artemisinin biosynthesis, an idea supported by a later study performed by the authors in which the early flowering gene constans from *A. thaliana* was overexpressed in *A. annua* (Wang et al., 2006). In contrast, when an isopentenyl transferase gene from *Agrobacterium tumefaciens* (*ipt*) was overexpressed in *A. annua*, the content of cytokinins, chlorophyll and artemisinin increased 2-3 fold, 20-60% and 30-70% respectively (Sa et al., 2001). By overexpression of endogenous FPP in *A. annua*, Han et al. (2006) established a maximum of 34.4% increase in

artemisinin content corresponding to 0.9% of the dry weight. Similarly, a 2-3 fold increase in artemisinin production was obtained using a FPP from *Gossypium arboreum* (Chen et al., 2000).

To assess the genetic versus environmental contributions to artemisinin production, quantitative genetics was applied by Dealbays et al. (2001). Variance manifested in a phenotype or a trait such as a chemotype is the sum of the genetic and environmental variance. The genetic variance can in its turn be divided into additive genetic variance, dominance variance and epistatic variance. Additive variance is a representation of the number of different alleles of a trait, dominance variance the relation between dominant and recessive alleles and epistatic variance the relation between allele and alleles at different loci. Broad-sense heritability of a trait is defined as the variation attributed to genetic variance divided by the total variance in trait. Ferreira et al. (1995) estimated in their experiments a broad-sense heritability of up to 0.98. Delabays et al. (2001) confirm the broad-sense heritability of artemisinin to be between 0.95 and 1 and that the dominance variance of 0.31 was precent in the experiment. This implies that there are great variations between the same alleles which support, besides a genetic based existence of chemotypes, a mass-breading selection program of *A. annua* to produce artemisinin high yielding crop. With the breeding program CPQBA-UNICAMP aiming at improvement of biomass yields, rates between leaves and stem, artemisinin content, and essential oil composition and yield in *A. annua*, genotypes producing 1.69 to 2.01 g/m^2 has been obtained (De Magalhaes et al., 2004).

Cell culture

One biotechnological research focus is to utilize hairy root cultures as a model of study and for production of artemisinin. Hairy roots are genetically and biochemically stable, are capable of producing a wide range of secondary metabolites, grow rapidly in comparison with the whole plant and can reach high densities (Flores et al., 1999;

Shanks et al., 1999). It is an interesting approach but is currently hampered by the difficulties in scaling up the production to industrial proportions. Scaling-up of *A. annua* hairy root cultures has been shown to produce complex patterns of terpenoid gene expression pointing towards the difficulty of obtaining a homogeneously producing culture (Souret et al., 2003). Souret et al. compare in their study the expression levels of four key terpenoid biosynthetic genes, (figure 3) Hmgr, 1-deoxy-D-xylulose 5-phosphate synthase (Dxs), 1-deoxy-D-xyluose 5-phosphate reductoisomerase (Dxr) and Fpps, in three different culture conditions: shake flask, mist bioreactor and bubble column bioreactor. In shake flask conditions all key genes were temporally expressed but only Fpps had a correlation with artemisinin production. This is not surprising as the terpenoid cyclase has often proven to be the rate limiting step in a terpenoid biosynthetic pathway. Expressions of the genes in both bioreactor types were similar or greater than the levels in shake flask cultures. In the bioreactors, the transcriptional regulation of all the four key genes were affected by the position of the roots in the reactors, but there was no correlation with the relative oxygen levels, light or root packing densities in the sample zones. Medium composition and preparation has been proven to affect the production of artemisinin in hairy root cultures. Wen et al. (2002) showed that the ratio of differently fixed nitrogen in Murashige and Skoog medium (MS medium) had a great impact on artemisinin level. The optimal initial growth condition of 20 mM nitrogen in the ratio 5:1 NO^{3-}/NH^{4+} (*w/w*) produced a 57% increase in artemisinin production compared to the control in standard MS medium. Weathers et al. (1997) determined optimal growth at 15 mM nitrate, 1.0 mM phosphate and 5% *w/v* sucrose with an 8 day old inoculum but the production of artemisinic acid was not detected using phosphate at higher concentrations than 0.5 mM. This implies that it is very difficult if even possible to optimize hairy root growth and terpenoid production at the same time, there has to be a trade off between biomass and product formation. As artemisinin is a secondary metabolite it is reasonable to assume that this compound will only be produced in

significant amounts when the primary needs of the tissue have been covered. An extended phase of biomass formation would mean a procrastinated production of secondary metabolites. Interestingly, Weathers et al. (1997) found that artemisinic acid was not detected when arteannuin B was produced (figure 3). They suggest that artemisinic acid is degraded by a peroxidase to arteannuin B which can be converted into artemisinin (Dhingra et al., 2001). This together with another observation that an oligosaccharide elicitor from the mycelial wall of an endophytic *Colletotrichum* sp. B501 promoted artemisinin production in *A. annua* hairy roots together with greatly increased peroxidase activity and cell death makes it tempting to see a peroxidase in the biosynthetic pathway from (dihydro)artemisinic acid to artemisinin (Wang et al., 2002). Dhingra et al. (2001) purified and characterized an enzyme capable of performing the peroxidation reaction converting arteannuin B to artemisinin (figure 3). Conversion was estimated to be 58% of the substrate on molar basis. Sangwan et al. (1993) was able to show conversion of artemisinic acid into arteannuin B and artemisinin using horse radish peroxidase and hydrogen peroxide on cell free extracts from immature *A. annua* leaves. On the other hand, it has been shown in chickory that the lactone ring formation in (+)-costunolide is dependent on a cytochrome P450 hydroxylase using germacrene acid as substrate (de Kraker et al., 2002).

Sugars are not only energy sources but also function as signals in plants (Loreti et al., 2006). Wethers et al. (2004) performed a study in which autoclaved versus filter sterilized media were used with the conclusions that filter sterilized media give higher biomass and more consistent growth results as well as better replicatable terpenoid production results although the yields of these secondary metabolites decreased. The authors explain the inconsistent results accompanying autoclaved media with variable hydrolysis of sucrose. By carefully choosing nutrient composition, light quality and bioreactor type the artemisinin production level can reach up to approximately 500 mg/l (Liu, et al., 1998; Liu et al., 1997). Ploidity

is another factor to consider. De Jesus-Gonzales and Weathers produced tetraploid *A. annua* hairy roots by treating normal diploid parents with colchicine and thereby obtained a tetraploid hairy root producing six times more artemisinin than the diploid versions. Tetraploid plants have also been made using colchicine which led to a 39% increase in artemisinin production averaged over the whole vegetation period compared to diploid wild type plants (Wallaart, et al., 1999).

Heterologous biosynthesis

There are currently two main research strategies for production of artemisinin that are intensively pursued. One is to increase production in the plant by bioengineering or through breeding programs; the second strategy is to utilize microorganisms in artificial biosynthesis of artemisinin. The group focusing on plant improvement brings forward the advantages of low production costs and easy handling disregarding infestation and pest problems and additional costs for containment to prevent ecological pollution. The group favoring heterologous production of artemisinin in microorganisms admits higher production costs at the moment compared with artemisinin isolated from the plant but points to the advantages of efficient space versus production ratio, complete production and quality control and continuous supply of artemisinin possible only with sources not dependent on uncontrollable factors such as weather. In the two following chapters we give examples of the progress of the heterologous production of isoprenoid and artemisinin precursors in microorganisms.

Heterologous production in Escherichia coli

Of the two isoprenoid biosynthetic pathways that exist (figure 3), the Dxp is used by most prokaryotes for production of IPP and dimethylallyl diphosphate (DMAPP) (Boucher et al., 2000; Rohdich, et al., 2002). With the knowledge of the genes involved in the DXP

pathway available, several groups have studied the impact of changed expression levels of these genes on the production of reporter terpenoids. Farmer et al. reconstructed the isoprene biosynthetic pathway in *Escherichia coli* (*E. coli*) to produce lycopene which was used as an indication of increase or decrease in isoprenoid production levels (Farmer et al., 2001). By overexpressing or inactivating enzymes involved in keeping the balance of pyruvate and glyceraldehyde 3-phosphate (G3P) the authors established that directing flux from pyruvate to G3P increased lycopene production making the available pool of G3P the limiting precursor to isoprenoid biosynthesis. Kajiwara et al. (1997) showed that overexpression of IPP lead to increased production of their terpenoid reporter molecule beta carotene. Kim et al. (2001) investigated the influence of Dxs, Dxr, plasmid copy number, promoter strength and strain on production of the reporter terpenoid lycopene and were able to show a synergistic positive effect upon overexpression of both genes. These kinds of strategies have all led to a moderate increase in production of terpenoid reporter molecules. Martin et al. (2003) hypothesized that the limited increase in isoprene production could be attributed to unknown endogenous control mechanisms. By introducing the heterologous mevalonate pathway from *S. cerevisiae* into *E. coli* these internal controls were bypassed and isoprenoid precursors reached a toxic level. Introduction of a codon optimized Amds alleviated this toxicity and led to production of amorpha-4,11-diene at the level of 24 μg caryophyllene equivalent/ml (Martin et al., 2003; Newman et al., 2006). Genes, such as transcriptional regulators, that are not directly involved in the isoprenoid biosynthetic pathway have also been shown to have a similar impact on production levels of terpenoid reporter molecules (Kang et al., 2005). This can be expected as the isoprenoid biosynthetic pathway is tightly intertwined with the energy metabolism of the cell. The design strategy of the construct used can have a great influence on precursor production as showed by Pfleger et al. (2006). By tuning intergenic regions in the mevalonate operon constructed by Martin et al. (2003), a seven fold

increase in mevalonate production was recorded compared with the starting operon conditions. Brodelius et al. (2002) went a step beyond manipulating isolated genes, singular or multiple, in the biosynthesis of isoprenoids. By fusing Fpps isolated from *A. annua* and *epi*-aristolochene synthase from tobacco, the extreme proximity and therefore very short diffusion path led to a 2.5 increase in *epi*-aristolochene compared to solitary *epi*-aristolochene synthase. Heterologous production of cyclized terpenoids is efficient but the following modification to form oxygenated plant terpenoids in *E. coli* seems to be a great bottleneck. Carter et al. (2003) engineered GPP biosynthesis coupled with the monoterpene cyclase limonene synthase, cytochrome P450 limonene hydroxylase, cytochrome P450 reductase and carveol dehydrogenase in *E. coli* with the expectation of producing the oxygenated limonene skeleton (-)-carvone. Production of the unoxygenated intermediate limonene reached 5 mg/l but no oxygenated product was detected. The authors argue that this limitation could be due to cofactor limitations and membrane structural limitations in *E. coli* compared to plants. Hence, several research groups have turned to yeast for heterologous expression of complex biosynthetic pathways.

Heterologous production in Saccharomyces cerevisiae

Fungi use the mevalonate pathway to produce all their isoprenoids. Lessons learned on manipulation of the genes involved in the yeast mevalonate pathway, were useful to increase the production of isoprenoids in *E. coli* as discussed above. Jackson et al. (2003) used *epi*-cedrol synthase converting FPP as a reporter gene for ispoprenoid production. By overexpressing a truncated version of Hmgr in a *S. cerevisiae* mutant (*Upc2-1*, upregulates global transcription activity) taking up sterol, which is a major terpenoid product, from the media an increase from 90µg/l to 370µg/l *epi*-cedrol was obtained. Overexpression of a native Fpps gene did not, however, improve levels of *epi*-cedrol. As in the attempt of heterologous production of the oxygenated terpenoid *epi*-cedrol

(Carter et al., 2003) in *E. coli*, an attempt to reconstruct early steps of taxane diterpenoid (taxoid) metabolism in *S. cerevisiae* produced taxadiene but did not proceed with cytochrome P450 hydroxylation steps (Dejong et al., 2006). Membrane structural limitations or co-factor limitations such as NADPH can not explain this result. The authors discussed that poor expression of the heterologous plant cytochrome P450 genes might be an explanation to this pathway restriction. Another angle mentioned by the authors is a possible inefficient coupling and interaction between the endogenous yeast NADPH-cytochrome P450 reductase and the plant cytochrome P450 hydroxylase, thus severely limiting the transfer of electrons to the cytochrome P450 hydroxylase and as a consequence premature termination of the pathway. Ro et al. (2006) introduced several genetic modifications in *S. cerevisiae* and were able to produce the oxygenated terpenoid artemisinic acid at 100 mg/l titre. This was achieved by optimized oxygen availability, downregulation of squalene synthase (*Erg9*) thus reducing endogenous consumption of the FPP pool, introduction of the *Upc2-1* mutation, overexpression of Fpps and a catalytic form of Hmgr, inducible expression of Amds, cytochrome P450 71av1 and a cytochrome P450 reductase from *A. annua*. More than 50 mg/l amorphadiene was produced in yeast engineered for overexpression of truncated Hmgr and Amds in a *Upc2*-1 yeast mutant genetic background. An additional two to threefold increase in amorphadiene level was obtained through knock out of squalene synthase but a marginal increase was harvested with additional overexpression of Fpps. Teoh et al. (2006) showed that oxygenation of amorphadiene to artemisinic alcohol and artemisinic alcohol to artemisinic acid was possible at proof of principle levels using cytochrome P450 71av1 and a cytochrome P450 reductase from *Arabidiopsis thaliana* (Urban et al., 1997). Takahashi et al. (2006) chose a similar strategy to create a yeast platform for production and oxygenation of terpenes. *S. cerevisiae* mutated in squalene synthase (*Erg9*) and capable of efficient aerobic uptake of ergosterol (*Sue*) from the culture media, produced 90mg/l farnesol, which is the dephosphorylated form of FPP and is

unaccessible for cyclization through terpene synthases. This mutant was, when engineered with various single terpene synthases, capable of producing around ~80- ~100 mg/l sesquiterpene varying with terpene synthase introduced. After additional engineering with hydroxylases, up to 50 mg/l hydroxylated terpene and 50 mg/l unmodified terpene product were obtained. Knocking out a phosphatase (*Dpp*1) known to dephosphorylate FPP (Faulkner et al., 1999) and additional upregulation of the catalytic activity of Hmgr did not yield an increase in terpene production compared to the *Erg9/Sue* yeast mutant. The authors note that a larger part of the farnesol is phosphorylated in a D*pp*1 mutant and as FPP function as a negative feedback signal on the mevalonate pathway it suppress the flux of carbon through the isoprene pathway (Gardner et al., 1999; Takahashi et al., 2006). Inserting a terpene cyclase diverts the pool of FPP and relieves the feedback inhibition which leads to an increase in carbon flux through the pathway almost matching the E*rg9/Sue* yeast mutant. In the E*rg9/Sue* mutant, a low but continuous flow through the mevalonate pathway leads to higher production of terpenoids. Takahashi et al. (2006) also illustrate the importance of design strategy of the expression vectors for optimal terpene production. Physically separating the cytochrome P450 reductase and the cytochrome P450 hydroxylase, led to a very low yield of oxygenated terpenoid. On the other hand, expression vectors where the reductase preceded the hydroxylase gene on the same plasmid yielded approximately 50% coupling of oxygenation to hydrocarbon. Physically linking the terpene synthase with the hydroxylase was unsuccessful using both N-terminal and C-terminal fusion. Lindahl et al. (2006) showed that there are great differences in the production of amorphadiene depending on genomic or episomal expression. The authors compared production of amorpha-4,11-diene using the terpenoid synthase cloned in the high-copy number galactose inducible yeast plasmid pYeDP60 with the terpenoid synthase using the same galactose inducible promoter integrated into the genome of *S. cerevisiae* CEN PK113-5D. It was found that the yeast with an integrated Amds grew at the same rate as the wild type while the

yeast carrying Amds episomaly had a slightly lower growth rate, yet the episomal system produced 600µg/l amorpha-4,11-diene compared to 100 µg/l for the integrated system. This is an expected result which shows that in the case of integrated Amds the enzyme activity is the limiting factor while in the episomal system substrate availability is the limiting factor.

Growth of Artemisia annua in the fields and in controlled environments

Studies have been made where the intrinsic capacity of *A. annua* to produce artemisinin under various environmental conditions were explored. Ram et al. (1997) performed a study in which *A. annua* was grown with varying plant densities during the winter-summer season of one year in semiarid-subtropical climate with no interculture and no fertilization. At a population density of 2.22×10^5 plants/ha 7.4 kg of artemisinin were obtained and 91 kg of essential oil. By increasing the plant density 2, 4 and 8-fold an increase of artemisinin by 1.5, 2 and 2.5 fold was observed at the same oil yield level. Interestingly, the suppression of weeds was positively correlated with the increase in artemisinin production. Weed, however, does not seem to be a trigger for artemisinin production as treatment of *A. annua* with herbicides did remove weeds but did not influence artemisinin yields (Bryson et al., 1991). Kumar et al. (2004) showed that multiple harvesting of *A. annua* grown in the subtropical Indo-Gangatic plains, not surprisingly, increased the total yield of artemisinin but also increased the production of artemisinin in leaves as averaged over the separate sampling events. This trend was more expressed the later in the year the seeds were sown, confirming a study performed by Ram et al. (1997). The effect of post-harvest treatment of *A. annua* on artemisinin content was investigated by Laughlin (2002) in a study using *A. annua* grown and harvested in temperate maritime environment in Tasmania. The experiments included drying of the cut off plants *in situ*, in the shadow, indoors in the dark or in a 35°C oven (used as comparison

base). Drying *in situ* did not give any concentration difference in artemisinin content compared to oven treatment. The authors noted a trend for sun-, shade- and dark drying for 21 days to give higher artemisinin levels than oven drying although artemisinic acid levels were unaffected.

Under greenhouse controlled conditions, Ferreira (2007) investigated the impact of acidity and macronutrient deficiency on biomass and artemisinin yield. Acidic soil and low levels of nitrogen, phosphor and potassium reduced, as expected, leaf biomass to 6.18 g/plant. Providing lime to increase pH and addition of the macronutrients nitrogen, phosphor and potassium gave a biomass of 70.3 g/plant. Potassium deficiency was shown to have the least negative effect on biomass accumulation and most positive effect on artemisinin production. Plants grown under potassium deficient conditions were compared with plants grown under full addition of lime and macronutrients. This comparison did not detect any significant change in artemisinin production between the two growth conditions. The author concludes that under mild potassium deficiency conditions, similar production of artemisinin can be obtained per ha as when fertilizing the soil with potassium. Potassium fertilization can thus be omitted in acidic soil growth conditions, decreasing the production costs as stated by the author but would also decrease the environmental pressure.

Synthesis of artemisinin, derivatives and new antiplasmodial drugs

Ever since artemisinin was isolated as the active compound against malaria, organic chemists have been trying and succeeding to produce the drug in the reaction flask. This has been performed with variable success but the general conclusion is still that it is a great scientific achievement but economically not attractive. A recent synthetic route to artemisinin involves 10 reaction steps from (+)-isolimonene to (+)-artemisinin with a final yield of a few percent

(Yadav et al., 2003). Yet this result is considered a success in terms of yield and stereochemistry precision. In contrast, conversion of artemisinic acid into artemisinin is simple and can be done with photooxygenation in organic solvent (Roth et al., 1989). Sy et al. (2002) describe in their study the role of the 12-carboxylic acid group in spontaneous autooxidation of dihydroartemisinic acid to artemisinin. The mechanism is further developed in the accompanying paper by the authors (Sy et al., 2002). Artemisinin, however, has very poor solubility in both oil and water and is despite its antiplasmodial activity therefore not suitable as a drug. The development of artemisinin derivatives and completely synthetic analogues is described in a review by Ploypradith (2004). Among the first tries to improve the solubility of artemisinin were to replace the ketone with other bigger polar groups forming ester derivates of artemisinin. Depending on the attached groups, the first generation derivates showed solubility in either oil or water. The derivates sodium artesunate and artelinic acid are still in use due to their efficiency in clearing severe malaria infections. However, these first generation derivates are labile in acid environment, have a short half life and some derivates has shown to have neurotoxic effects. The second generation of semi-synthetic analogues were produced from artemisinin or artemisinic acid with the goals of improvement in metabolic and chemical stability, bioavailability and half-life. Two main streams were developed in the second generation of semi-synthetics. One group retained the acetal C10-oxygen, the other line developed further on the line converting this oxygen to a carbon to increase the stability in acidic conditions. Of these two groups there are monomers and dimers. The dimers are interesting not only because they have a high antiplasmodial activity, but also because of their antineoplastic features.

Artemisinin with its crucial endoperoxide bridge is not the only natural compound exhibiting antiplasmodial activity. An example of the biosynthesis of antiplasmodial endoperoxidic compounds is plakortin, a simple 1,2-dioxane derivative, which is produced by the

marine sponge *Plakortis simplex* (Fattorusso et al., 2006). This compound shows activisty against chloroquine-resistant strains of *Plasmodium falciparum* (*P. falciparum*) at submicromolar level.

Several synthetic simplifications have been made as the knowledge of the mode of action of artemisinin has developed. In a review by Ploypradith (2004), selected strategies are reported. One line is to omit the lactone ring which is considered to be less important if at all for antiplasmodial activity. Molecules that completely abandon the structure of artemisinin and its precursor only retaining the peroxide bond as a crucial functional pharmacophore are numerous. These molecules are easy to make but unfortunately display significantly reduced activity against malaria compared with artemisinin. As discussed in the introduction they have a short half-life and poor chemical stability. A further dimension added in the synthesis of synthetic aniplasmodials was the idea to add multiple endoperoxide bridges within a molecule ring rather than adding them up as dimers with a linker in between. These tetraoxacykloalkanes showed an several fold increase in efficiency against malaria compared to artemisinin yet had a lower toxicity in mouse models. Design and synthesis of selected tetraoxanes are described in an article by Amewu et al. (2006).

Analytics

The detection and structural elucidation of terpenes has been hampered by the often very low amounts and complex mixtures formed in plants. The honing of extraction methods and analytical methods has increased the ease and speed with which these problems can be solved. The choice of extraction protocol greatly influence the yield and composition of the isolated product as well as the cost and time factors (Christen et al., 2001). Peres et al. (2006) compare soxhlet, ultrasound-assisted and pressurized liquid extraction of terpenes, fatty acids and vitamin E from *Piper gaudichaudianum* Kunth. The authors conclude that the method pressurized liquid

extraction decrease the total time of extraction, solvent use and handling compared to the other two methods. Furthermore, it was determined that pressurized liquid extraction was more efficiently extracting terpenes than the other two methods. Lapkin et al. (2006) compare extraction of artemisinin using hexane, supercritical carbon dioxide, hydrofluorocarbon HFC-134a, several ionic liquids and ethanol. Hexane was found to be simple and at a first glance most cost efficient but is characterized by lower rates and efficiency compared to all other methods including safety and environmental impact issues. The new techniques based on supercritical carbon dioxide, hydrofluorocarbon HFC-134a and ionic liquids consistently showed faster extraction cycles with higher recovery in addition to enhanced safety and decreased negative impact on the environment compared to hexane and ethanol extraction. With some process optimization, the authors predict that ionic liquid and HFC-134a extraction can compete with hexane extraction also on economical terms. Christen et al. (2001) compare in their review article the extraction techniques supercritical fluid extraction, pressurized solvent extraction and microwave-assisted extraction and the detection methods gas chromatography, tandem mass spectrometry, HPLC-UV, -EC and -MS as well as ELISA and capillary electrophoresis. The use of evaporative light scattering detector is mentioned as a tool for detection of non-volatile non-chromophoric compounds. Common to all these methods is the trend toward mild operating conditions to avoid degradation of the analytes, isolation of one compound in complex mixtures and time and price reduction compared to traditional extraction methods. ELISA is accurate and is usable for screening of large plant populations but is laboursome and expensive compared to standard GC and HPLC based methods (Ferreira et al., 1996). It is likely that this method will win stronger support in assessing the drug susceptibility of *P. falciparum* (Kaddouri et al., 2006). A simple, fast and selective method of quantification of artemisinin and related compounds was developed by Van Nieuwerburgh et al. (2006). This method makes use of HPLC-ESI-TOF-MS/MS technology and has a recovery of >97% for

all measured analytes. Peng et al. (2006) compared the use of GC-FID and HLPC-ELSD for detection of artemisinin in leaves. Both methods are valuable for routine measurements as they are cheap, easy to use and do not require derivatization of artemisinin for detection. Both methods had a high sensitivity at ng level and produced reproducible results of artemisinin from field plants with a correlation coefficient of $r^2 = 0.86$ between the two methods. Another interesting simple and rapid method circumventing the problems with thermolability, lack of chromophoric or fluorophoric groups, low concentration *in vivo* and interfering compounds *in planta* of artemisinin detection is the method developed by Chen et al. (2002). Artemisinin is converted on-line to the strongly absorbing compound Q292 through treatment with NaOH. The obtained product is analyzed with capillary electrophoresis in 12 minutes, allowing a sampling frequency of 8 h^{-1}. With this work, Chen et al. (2002) show that it is possible to determine artemisinin content based on the unstable UV-absorbing compound Q292 thus omitting the traditional time-consuming step of acidic conversion of Q292 to the stable UV-absorbing compound Q260 before analysis. A HPLC-MS method in selective ion mode developed by Wang et al. (2005) is another interesting cheap, sensitive and fast method for detection and quantification of artemisinin in crude plant extracts. The obtained linearity of detection in this method is about 5-80 ng/ml for artemisinin with an analyze time of 11 min per sample.

An old method revived is the use of thin layer chromatography plates for detection of sesquiterpenoids (Bhandari et al., 2005; Klayman et al., 1984). While this kind of detection is qualitative and preferably used as quick determination of yes/no cases, more comprehensive and qualitative methods are needed for research purposes.

Ma et al. (2007) made a finger print of the volatile oil composition of *A. annua* by using two dimensional gas chromatography time-of-flight mass spectrometry. With this method, approximately 700 unique peaks were detected thereof 303 tentatively identified (Ma, et

al., 2007). As a comparison, only 61 peaks could be detected using GC. This type of comprehensive metabolic fingerprinting will ease detection of genes directly or indirectly relevant for the biosynthesis of artemisinin in experiments utilizing gene upregulation or downregulation mechanisms.

There is some discussion about synergistic effects on clearing of the parasite *P. falciparum* from infected patients using extracts from *A. annua*. Bilia et al. (2002) describe the importance of flavonoids in interaction between artemisinin and hemin. Hemin is thought to play a role in activation of artemisinin. It is thus valuable to develop a method which can analyze artemisinin and flavonoids simultaneously. Bilia et al. (2006) developed a method based on HPLC/diode-array-detector/MS delivering just that.

Medicinal use

The mode of action of artemisinin is subject to intense research (Drew et al., 2006; Hoppe et al., 2004; Krishna et al., 2004; Messori et al., 2004; O'Neill et al., 2005; Posner et al., 2004; Rafiee et al., 2005; Schmuck et al., 2002). Currently, the hypothesis supporting radical ion formation from artemisinin on the peroxide bridge is favored.

Traditionally, artemisinin is administered as a tea infusion. With the advent of combination therapies using artemisinin as an isolated compound it is necessary to compare the kinetic characteristics of each delivery method. Rath et al. (2004) studied the pharmacokinetics and bioavailability of artemisinin from tea and oral solid dosage forms. Interestingly, artemisinin was absorbed faster from herbal tea preparations than from oral solid forms, supporting the importance of flavonoids as synergistic factors. Nevertheless, bioavailability was similar in both treatments. As only about 90 mg artemisinin was contained in 9 g *A. annua* and as uptake of artemisinin through the human gut is very poor, only about 240

ng/ml was detected in plasma, a tea infusion is not recommended by the authors as a replacement for modern formulations in malaria therapy. This confirms the study of pharmacokinetics of artemisinin performed by Duc et al. (1994). Duc et al. (1994) propose to increase the dose of artemisinin until adequate plasma levels are reached to compensate for poor bioavailability and rapid elimination as no adverse effects were detected. This might prove a risky strategy as artemisinin-induced toxic brainstem encephalopathy has been observed in a patient treated for breast cancer with artemisinin (Panossian et al., 2005). The adverse effects were reversible and no permanent damage could be detected. Toxicity of antimalarials including artemisinin derivatives is described in a review article by Taylor et al. (2004). Mueller et al. (2004) studied the efficacy and safety of the use of *A. annua* as tea against uncomplicated malaria in a pilot study. Treatments were efficient but still less efficient compared to the traditional quinine, an average of 74% were cleared after 7 days of treatment compared to 91% treated with quinine. As the authors note, recrudescence rates were high in the groups treated with artemisinin and they therefore recommend combination therapies which is in line with the recommendation from WHO. However, the choice of combination partner in the combination therapies is a delicate question which is exemplified in the study of Sisowath et al. (2005). In a recent review article the mechanism behind antimalarial drug resistance is covered (White, 2004). Interestingly, resistance can be reversed (Henry et al., 2006). It is obvious that the clearance of the parasite through tea preparations will depend on the amount of artemisinin present in the plant as only approximately 40% of the available artemisinin in the plant was recovered in tea infusions as shown in another study by Mueller et al. (2000). Here it was demonstrated that malaria infested patients who were given tea preparations for 2-4 days showed a recovery of 92% within 4 days, a remarkable improvement compared with the previous mentioned study (Mueller et al., 2004).

An overview of older (up to 1999) artemisinin derivatives is given in the article by Dhingra et al. (2000). All these derivatives were developed with the aim of obtaining a more efficient remedy against malaria. However, more recently artemisinin and derivatives have been attributed other intriguing functions than antiplasmodial activities. Romero et al. (2006) describe in a study on flaviviruses the antiviral property of artemisinin. Zhou et al. (2005) observed the derivate 3-(12-β-artemisininoxy) phenoxyl succinic acid (SM735) to be strongly immunosuppressive *in vitro* and *in vivo*. Artemisinin derivatives have also been shown to have strong antineoplastic properties (Disbrow et al., 2005; Efferth, 2006; Efferth et al., 2002; Liu et al., 2005).

Pharmacokinetics

A characteristic of artemisinin and its related endoperoxide drugs is the rapid clearance of parasites in the blood in almost 48 hours. Titulaer et al. (1990) obtained pharmacokinetic data for the oral, intramuscular and rectal administration of artemisinin to volunteers. Rapid but incomplete absorption of artemisinin given orally occurs in humans with a mean absorption time of 0.78 h with an absolute bioavailability of 15 % and relative bioavailability of 82%. Peak plasma concentrations at a given dose are reached after 1-2 h and the drug is eliminated after 1 to 3 hours. The mean residence time after intramuscular administration was three times that when given orally. Other routes of administration, for example rectal or transdermal, are of limited success, but for the treatment of convulsive malaria in children artemether in a rectal formulation is favored. Artesunate acts as a prodrug that is converted to dihydroartemisinin. When given orally the first pass mechanism in the gut wall takes places metabolizing half of the administered dose. Oral artemether is rapidly absorbed reaching maximum blood levels (C_{max}) within 2-3 hours. Intramuscular artemether is rapidly absorbed reaching C_{max} within 4-9 hours. It is metabolized in the liver to the demethylated derivative dihydroartemisinin. The elimination is rapid, with a half-

life time ($T_{1/2}$) of 4 hours. In comparison, dihydroartemisinin has a $T_{1/2}$ of more than 10 hours. The degree of binding to plasma proteins varies markedly according to the species considered. The binding of artemether to plasma protein was 58% in mice, 61% in monkeys and 77% in humans. Radioactive labelled artemether was found to be equally distributed in plasma as well as in red blood cells indicating an equal distribution of free drug between cells and plasma.

From the toxicological point of view artemisinin seems to be a safe drug for the use in humans. In animal tests neurotoxicity has been documented, but as yet this side effect has not been reported in humans (Merali et al., 1991). A major disadvantage of the artemisinin drugs is the occurrence of recrudescence when given in short monotherapy. So far no resistance has been observed clinically although it has been induced in rodent models in vivo. The mechanism of action is different from the other clinically used antimalarials. Artemisinin drugs act against the early trophozoite and ring stages, they are not active against gametocytes, and it affects blood- but not liver-stage parasites. The mode of action is explained by haem or Fe^{2+}, from parasite digested haemoglobin, catalysing the opening of the endoperoxide ring and forming free radicals. Malaria parasites are known to be sensitive to radicals because of the lack of enzymatic cleaving mechanisms. The mechanism of action and the metabolism of reactive artemisinin metabolites is shown in figure 6.

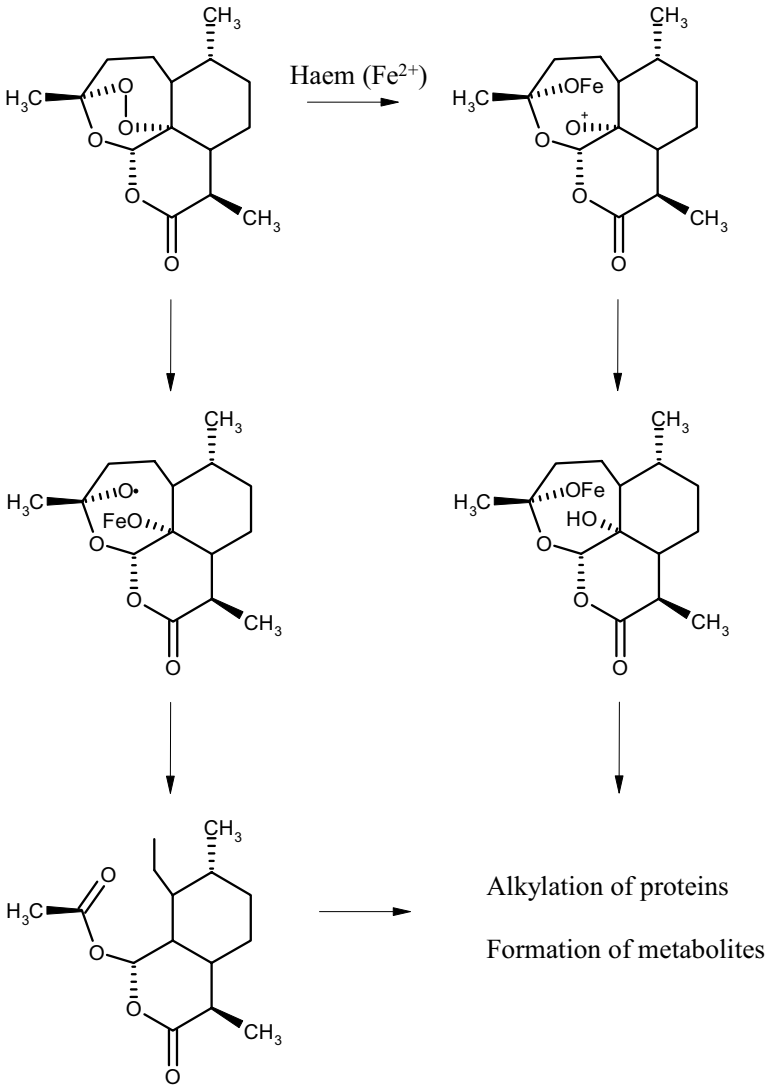

Figure 6. Mechanism of action of artemisinin drugs. Active metabolites and formation of reactive epoxide intermediates (van Agtmael et al., 1999).

Drug delivery

Drug delivery of artemisinin and its derivatives is not that easy as known for intracellular microorganisms like *Leishmania* sp., *Mycobacterium tuberculosis* or *Listeria monogynes*. *Plasmodium falciparum* and related species are facultative intracellular parasites which mainly persist in erythrocyte as host cells. Drug targeting of infected erythrocytes is not well known and it seems not to be a major area of interest for pharmaceutical technology to identify new strategies to deliver artemisinin or other antiplasmodial drugs to this target site. Literature search revealed no publication using liposomes, microemulsions, nanoemsulsions, microparticles or nanoparticles for targeting or drug delivery. Most formulation strategies have been focused on the improvement of the poor solubility of artemisinin (< 5 mg/l H_2O). One interesting approach has been published in detail documenting the approach to increase solubility with cyclodextrines. Cyclodextrines are cyclic oligosaccharides consisting of six, seven or eight glucose molecules forming α-, β-, or γ-cyclodextrine, respectively. Cyclodextrines form pores with an inner diameter ranging from 0.5 to 0.8 nm where lipophilic drugs may be incorporated, thereby increasing their distribution in water. While the lipophilic compound is shielded inside, hydroxyl groups on the outer surfaces create an overall hydrophilic character for this inclusion complex. For experimental purposes, artemisinin has been formulated with different cyclodextrines to improve its solubility and oral absorption leading to increased bioavailability (Wong et al., 2003). Solubility diagrams indicated that the complexation of artemisinin (85%, 40%, and 12%, α-, β-, or γ-cyclodextrine respectively) and the three different types of cyclodextrines occurred at a molar ratio of 1:1, and showed a remarkable increase in artemisinin solubility (Wong et al., 2003). In a bioavailability study from the same authors β-, or γ-cyclodextrines seem to be superior to commercial Artemisinin 250 and increased oral bioavailability with a mean of 782 ng h/ml to 1329 and 1131 ng h/ml (β-, or γ-cyclodextrine respectively). But still the poor solubility was a critical

parameter for significantly improved oral bioavailability (Wong et al., 2001).

Conclusion

Artemisinin is a potent antimalarial drug belonging to the chemical class of sesquiterpenoid endoperoxide lactones. Its poor solubility in water and organic phases has led to a focus on development of derivatives towards increased solubility, metabolic and chemical stability and bioavailability (Ploypradith, 2004). The first generation of artemisinin derivatives had as a common feature the replacement of the ketone with bigger polar groups to form ester derivatives (figure 1). Among these, sodium artesunate and artelinic acid are still in use. Unfortunately other common features of the first generation artemisinin derivatives are instability in acid environment and short half life. Some derivatives also have a neurotoxic effect. The second generation of semi-synthetic artemisinin derivatives target improved metabolic and chemical stability, bioavailability and half-life. In parallel with the progressing understanding of the mode of action of artemisinin, synthetic simplified antimalarial compounds have been developed. Several promising candidates based on synthetic simplified molecules containing multiple peroxide bridges within one ring which show higher activity against malaria and lower toxicity compared with artemisinin have been reported (Ploypradith, 2004).

Two genes have been isolated from the biosynthetic pathway of artemisinin: the first committed step in the pathway, amorpha-4,11-diene synthase and the next enzyme in the pathway, the cytochrome P450 71av1 which catalyzes three consecutive oxygenation steps on the amorphane sesquiterpene skeleton yielding artemisinic acid (figure 3, figure 4) (Ro et al., 2006; Teoh et al., 2006). This opens up for molecular biotechnology strategies aiming towards artificial biology making use of heterologous gene expression in optimized hosts and improvement of artemisinin yield in transgene *A. annua*

through genetic engineering. With the knowledge of nucleotide sequences, protein functions and characteristics, evolution of the genes identified in the biosynthetic pathway is a possible and logical next step to follow for increased levels of the artemisinin precursors amorpha-4,11-diene and oxygenated forms thereof. Great improvement in yield of amorpha-4,11-diene and other early precursors has been made with the aid of genetic engineering and optimization of culture conditions. There are currently two main research lines followed in parallel with the third line favoring artificial biology strategies with the aim of increased artemisinin production compared to the wild type plant: The use of cell cultures, a field which combines culture optimization and genetic engineering and the second line which employs traditional plant breeding through which the genetic dominance over environmental impact on artemisinin production is exploited. All strategies show potential for substantial improvement and it is currently not settled which if any approach is the better one in terms of economy, environmental impact, yield, safety and production flow. The recent developments in detection and separation technologies of terpenoids should aid a swift progress in screening mutants and complex networks in which the artemisinin biosynthesis pathway is embedded.

The traditional administration of artemisinin as a tea of the plant *A. annua* is a cheap, easily accessible source for malaria plagued countries but is an unreliable cure due to the fact that artemisinin level *in planta* is very low and varies considerably between plants and batches. Additionally, absorption through the human gut is rapid but inefficient and liver induction of cytochromes P450s will not allow repeated drug courses. The most efficient administration is intramuscular injection as the drug then has a mean residence time three times longer than orally administrated drug. As intramuscular administration requires medical personnel, is painful and generally disliked by patients, other delivery strategies is an urgent research field together with techniques increasing the solubility of artemisinin such as the use of cyclodextrines (Wong et al., 2003).

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