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Identification, characterisation and expression of early biosynthetic genes from Artemisia annua

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Chapter 5

Oxygen dependent biosynthesis of artemisinin precursors in yeast

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Abstract

The medicinal plant *Artemisia annua* L. (*A. annua*) contains artemisinin, a metabolite very effectively eradicating the malaria parasite in patients suffering from malaria. However, the concentration of this endoperoxide sesquiterpene lactone *in planta* is low (0,2-0,8% of the dry weight) and hence yield and availability of artemisinin are limited. It is therefore desirable to develop new strategies to cope with the shortage. One approach is to use genetically modified micro-organisms and that produce artemisinin or metabolic precursors of artemisinin that can be chemically converted to artemisinin. In this report, the suitability of *Saccharomyces cerevisiae* (*S. cerevisiae*) as a host for the heterologous production of artemisinin precursors was investigated. We focused on the possibilities to use a combination of heterologous, *A. annua* derived, and endogenous yeast genes and controlled environmental conditions to affect product specificity. Hereto, two key enzymes in the artemisinin pathway, amorpha-4,11-diene synthase and the cytochrome P450 Cyp71av1 were expressed in *S. cerevisiae*. The production of known intermediates from the artemisinin pathway was confirmed, but under restrictive oxygen conditions a shift in product pattern occurred from artemisinic acid to dihydroartemisinic acid. We postulate that under less optimal conditions for Cyp71av1 catalysis, an endogenous yeast reductase is able to reduce the exocyclic double bond of one of the enzyme intermediates, hence leading to reduced artemisinin precursors. For the first time, we show that Cyp71av1 can also catalyze the oxidation of dihydroartemisinic aldehyde to dihydroartemisinic acid. Dihydroartemisinic acid is a preferred product, as it is one step closer to artemisinin than artemisinic acid, and can be more easily chemically converted to artemisinin.

Introduction

Artemisia annua L. (*A. annua*) is an annual herb that produces the antimalarial compound artemisinin (Wallaart et al., 2000). Currently, artemisinin is recommended by the World Health Organization for use in combination drug therapies against malaria. Moreover, artemisinin displays few and relatively minor side effects in comparison to the traditionally used quinine. Consequently, there is an increasing demand for artemisinin. Due to the low production of the compound *in planta* (0,2-0,8% of the dry weight), there are many attempts to produce it in other ways. Chemical synthesis proved to be an impossible strategy due to the complex structure of the molecule and the consequent high costs (Rydén et al., 2007). A recent promising strategy, that may complement plant extraction, is synthetic biology (Bouwmeester et al., 1999; Ro et al., 2006; Rydén et al., 2009; Teoh et al., 2006; Teoh et al., 2007; Wallaart et al., 2001; Zhang et al., 2008). This approach requires identification of the biosynthetic genes in the artemisinin pathway and their expression in a suitable host. Initial biochemical studies were used to identify probable intermediates in the biosynthetic pathway (Bertea et al., 2005; Bouwmeester et al., 1999; Wallaart et al., 1999; Wallaart et al., 1999), which was followed by cloning of the responsible genes (Mercke et al., 2000; Ro et al., 2006; Teoh et al., 2006; Wallaart et al., 2001; Zhang et al., 2008). These efforts led to the identification of the terpene cyclase amorpha-4,11-diene synthase (Amds) which catalyses the first committed step of the biosynthetic pathway of artemisinin (figure 1) (Bouwmeester et al., 1999; Mercke et al., 2000; Wallaart et al., 2001). The second step is catalysed by the cytochrome P450 Cyp71av1 which oxidizes amorpha-4,11-diene at C12 to artemisinic acid in three consecutive steps (figure 1) (Ro et al., 2006, Teoh et al., 2006). Release of the product after the second oxidation step, artemisinic aldehyde, is allowing Dbr2, a carbon double bond reductase, to reduce the Δ11(13) carbon double bond, thereby producing dihydroartemisinic aldehyde (Zhang et al., 2008).

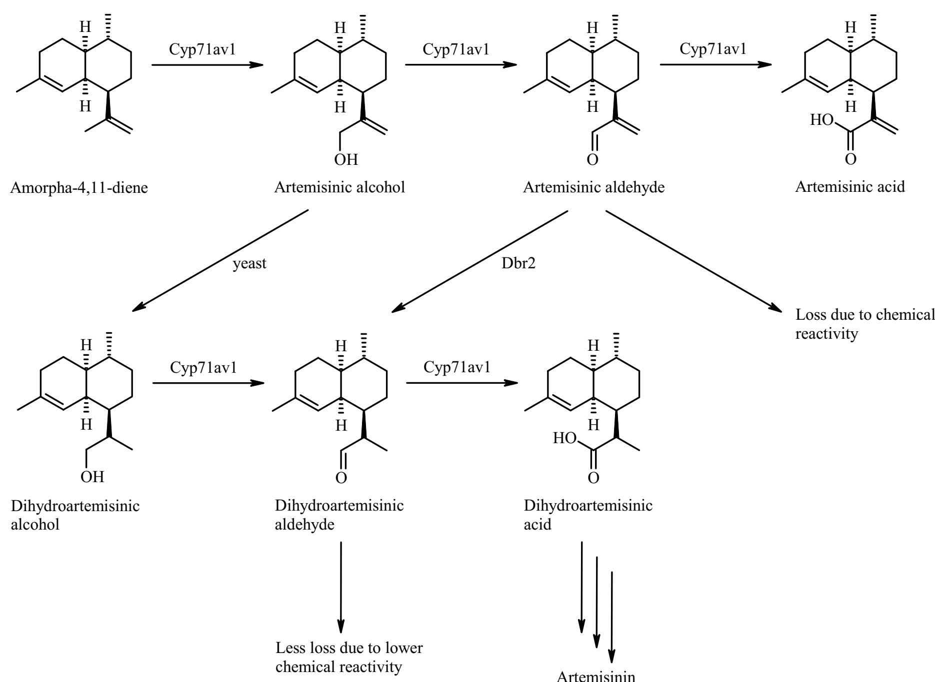


Figure 1. Production of dihydroartemisinic acid in yeast during low-oxygen conditions. Cyp71av1 oxidizes amorpha-4,11-diene to artemisinic alcohol, artemisinic aldehyde and artemisinic acid. In *A. annua*, the carbon double bond reductase Dbr2 reduces artemisinic aldehyde to dihydroartemisinic aldehyde. We show here that in yeast an unidentified enzyme, UDbr, converts artemisinic alcohol to dihydroartemisinic alcohol. We also show that Cyp71av1 can oxidize dihydroartemisinic alcohol to dihydroartemisinic aldehyde and dihydroartemisinic aldehyde to dihydroartemisinic acid, the closest known biosynthetic precursor of artemisinin.

Biochemical studies have indicated that conversion of dihydroartemisinic aldehyde to dihydroartemisinic acid occurs through soluble proteins rather than membrane bound cytochrome P450s (Bertea et al., 2005; Teoh et al., 2006), but the responsible gene has insofar not been identified. In contrast to these reported

results, we can show that a membrane bound cytochrome P450 from *A. annua* is able to efficiently convert dihydroartemisinic aldehyde to dihydroartemisinic acid.

With the availability of all these pathway genes, it should now be possible to create heterologous hosts to produce artemisinin or artemisinin precursors. The focus on artemisinin derived from the plant has in so far not relied so much on genetic engineering, as on growth condition improvement, harvesting methods and marker assisted breeding (Graham et al., 2010; Rydén et al., 2007). Approaches to enhance artemisinin production in *A. annua* include the transient expression of a transcription factor, which regulates the expression of amorpha-4,11-diene synthase (Ma et al., 2009). Furthemore, a stable expression of 3-hydroxy-3-methylglutaryl CoA reductase, an enzyme involved in precursor production for general terpene biosynthesis, lead to a 22% increase in artemisinin content compared to control plants (Aquil et al., 2009). Silencing of the squalene biosynthetic pathway, which competes with the artemisinin biosynthetic pathway for precursor molecules, led to a 3 fold increase in artemisinin production (Zhang et al., 2009). As part of a synthetic biology approach, much effort has been spent on improving the precursor-substrate availability in the heterologous microbial hosts. By increasing the production of early precursors that feed into the artemisinin biosynthetic pathway, a greater yield of the desired product may be obtained. For example, the flux through the mevalonate pathway in *Escherichia coli* (*E. coli*) (Martin et al., 2003; Pitera et al., 2007; Tsuruta et al., 2009) and *Saccharomyces cerevisiae* (*S. cerevisiae*) (Ro et al., 2006) has been optimized. Although hampered by stress related responses (Ro et al., 2008), the use of *S. cerevisiae* seems promising. By engineering the mevalonate pathway and introducing genes from the artemisinin pathway, a titer of 100 mg l^{-1} of artemisinic acid was obtained (Ro et al., 2006). Artemisinic acid in its turn can be chemically converted into artemisinin (Ro et al., 2006).

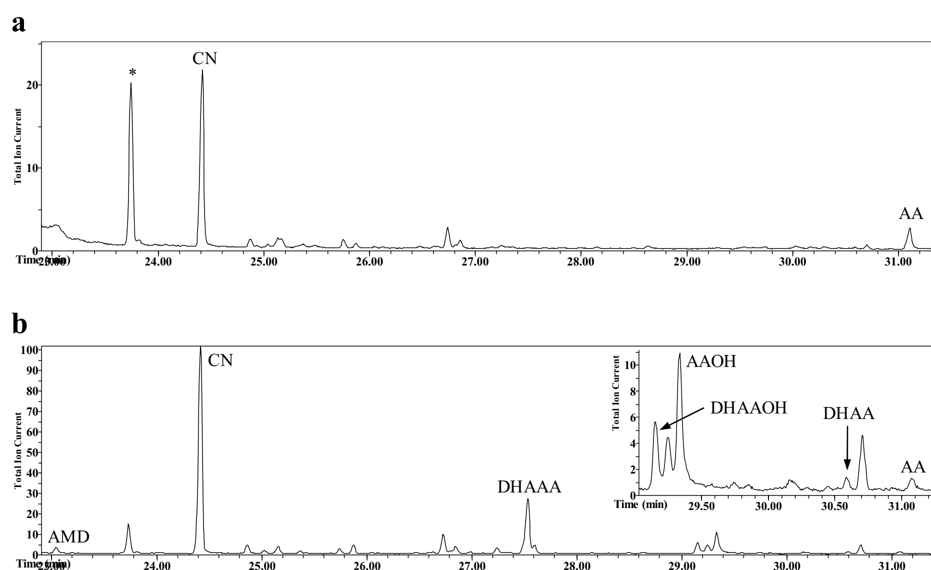
To further increase the production of artemisinin precursors by the heterologous host, it is necessary to reduce any stress factors as much as possible. Expression of heterologous genes introduces stress responses, which will drain resources from artemisinin precursor production. Therefore, it may be desirable to use endogenous genes which are capable of performing the same conversion as genes from the artemisinin pathway in *A. annua*. Here, we investigated whether *S. cerevisiae* is able to convert any intermediates in the artemisinin biosynthetic pathway by combining endogenous and heterologous genes. The endogenous metabolic possibilities of *S. cerevisiae* in artemisinin production were studied by cultivating the host in shake flask cultures with different atmospheric compositions. As gene expression in *S. cerevisiae* changes in response to alterations of atmospheric composition, it is possible that endogenous genes performing the desired conversions of artemisinin intermediates are up-regulated under such conditions. In addition, we show that Cyp71av1 which is a key enzyme in the artemisinin biosynthetic pathway, has a wider substrate specificity than originally reported (Ro et al., 2006; Teoh et al., 2006).

Results and Discussion

Microsomal conversion of artemisinin intermediates

As outlined in figure 1, the first committed precursor in the artemisinin biosynthetic pathway is amorpha-4,11-diene, which is oxidized by the cytochrome P450 Cyp71av1 to artemisinic alcohol, artemisinic aldehyde and artemisinic acid (Ro et al., 2006; Teoh et al., 2006). In *A. annua*, the exocyclic carbon double bond of artemisinic aldehyde is reduced by the reductase Dbr2 (Zhang et al., 2008). The product dihydroartemisinic aldehyde is reportedly further oxidized to dihydroartemisinic acid by cytosolic dehydrogenases (Teoh et al., 2007). Dihydroartemisinic acid is thought to be the closest precursor to artemisinin (Brown et al., 2004). Extracts of *A. annua* contain other artemisinin-like sesquiterpenoids, that may be

intermediates of the artemisinin pathway, but their biosynthesis and involvement in artemisinin formation remain obscure (Lommen et al., 2007; Lommen et al., 2006). Dihydroartemisinic alcohol is one of these compounds that is likely involved in the artemisinin pathway. Previously we have shown that microsomal fractions of *A. annua* were able to convert dihydroartemisinic alcohol to dihydroartemisinic aldehyde (Bertea et al., 2005). Due to the close chemical relationship between artemisinic alcohol and dihydroartemisinic alcohol, we hypothesized that Cyp71av1 can also convert dihydroartemisinic alcohol to dihydroartemisinic acid just as it can convert artemisinic alcohol to artemisinic acid. Microsomal fractions of Cyp71av1 expressing *S. cerevisiae* catalysed the conversion of amorpha-4,11-diene to artemisinic acid as previously published (figure 2a, supplemetary figure 1a) (Ro et al., 2006; Teoh et al., 2006).



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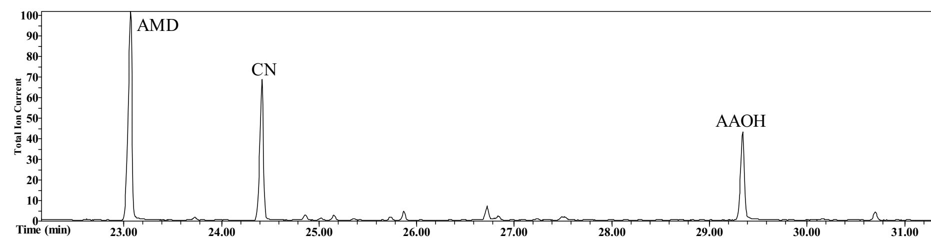

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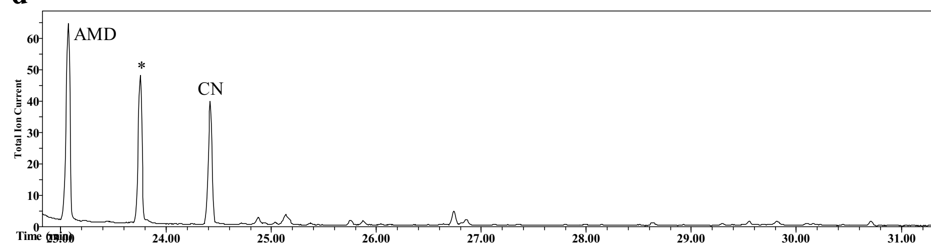

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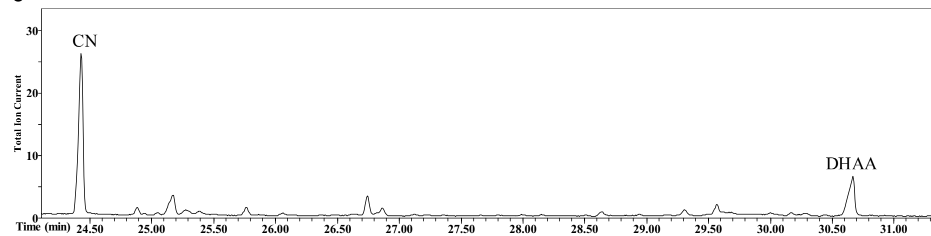

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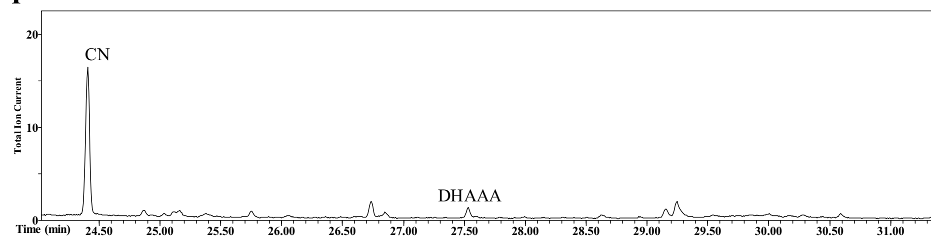

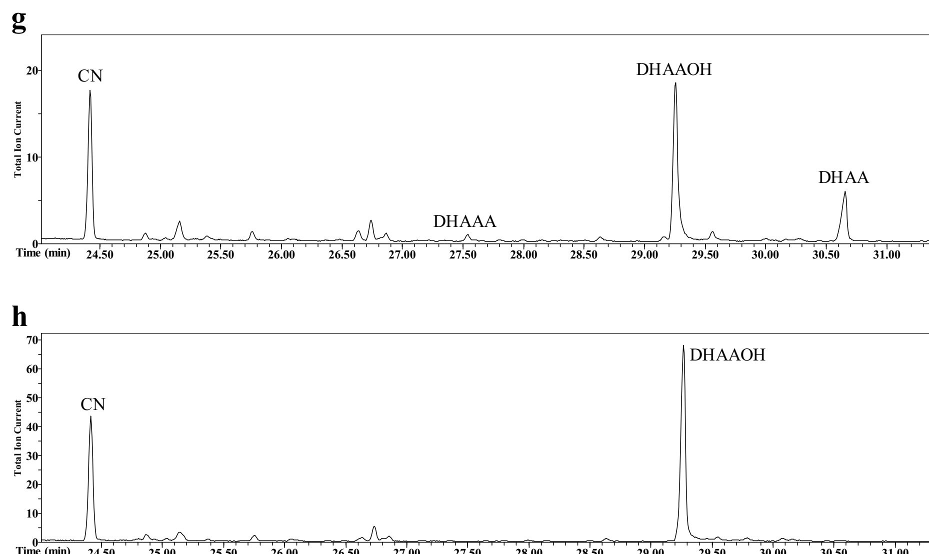

Figure 2. Microsomal conversion of artemisinin intermediates. AMD – amorpha-4,11-diene, AA – artemisinic acid, AAOH – artemisinic alcohol, DHAA – dihydroartemisinic acid, DHAAOH – dihydroartemisinic alcohol, DHAAA – dihydroartemisinic aldehyde, CN – *cis*-nerolidol, (*)-unknown.

a. Undiluted yeast microsomes over-expressing Cyp71av1 with AMD as substrate.

b. 10x diluted yeast microsomes over-expressing Cyp71av1 with AMD as substrate.

c. 100x diluted yeast microsomes over-expressing Cyp71av1 with AMD as substrate.

d. Empty vector control: Undiluted yeast microsomes with AMD as substrate.

e. Undiluted yeast microsomes over-expressing Cyp71av1 with DHAAA as substrate.

f. Empty vector control: Undiluted yeast microsomes with DHAAA as substrate.

g. Undiluted yeast microsomes over-expressing Cyp71av1 with DHAAOH as substrate.

h. Empty vector control: Undiluted yeast microsomes with DHAAOH as substrate.

Furthermore, we were able to show that microsomes containing Cyp71av1 oxidized dihydroartemisinic alcohol and dihydroartemisinic aldehyde to dihydroartemisinic acid (figure 2e-h, supplementary figure 1e-h). It is thus theoretically possible that there is an additional pathway proceeding from artemisinic alcohol to dihydroartemisinic alcohol, rather than from artemisinic aldehyde to dihydroartemisinic aldehyde (figure 1). The conversion rates of artemisinic alcohol to artemisinic acid and dihydroartemisinic alcohol to dihydroartemisinic acid do not seem to differ significantly, as the final concentrations of the acids in the samples are similar in the 10x diluted microsomal fractions supplemented with amorpha-4,11-diene as substrate (table 1). However an exact kinetic analysis is difficult to conduct due to the mixed protein nature of microsomes and the losses of artemisinic aldehyde (table 1). Generally aldehydes are more reactive than alcohols, and it is likely that artemisinic aldehyde and dihydroartemisinic aldehyde, either supplied as substrate or formed enzymatically from earlier precursors, bind more to the microsomal fraction than artemisinic alcohol and dihydroartemisinic alcohol. The reactivity of artemisinic aldehyde is illustrated by the absence of the compound in the 10-fold diluted microsomal fractions with amorpha-4,11-diene as substrate, while all other intermediates up to artemisinic acid were detected. Furthermore, in the undiluted microsomal fractions which were supplemented with dihydroartemisinic aldehyde as substrate, it could not be detected anymore after the assay. The loss of artemisinic aldehyde is expected to be even more severe as it is chemically more reactive than dihydroartemisinic aldehyde.

Another interesting unexpected result is the detection of dihydroartemisinic alcohol, dihydroartemisinic aldehyde and dihydroartemisinic acid in a microsomal conversion assay with amorpha-4,11-diene as substrate (figure 2b, supplementary figure 1b). Previously, only the conversion products artemisinic alcohol, artemisinic aldehyde and artemisinic acid have been detected in

Table 1. Retention times and abundances of the internal standard *cis*-nerolidol and produced artemisinin intermediates in the microsomal assays. Dilution series of microsomes were mixed with amorpha-4,11-diene as substrate. Undiluted microsomes were incubated with either amorpha-4,11-diene, dihydroartemisinic aldehyde or dihydroartemisinic alcohol.

Chemical component	Retention time [min]	Microsomal fractions		
		Undiluted	10x dilution	100x dilution
		Terpenoid [µM]	Terpenoid [µM]	Terpenoid [µM]
Internal standard				
Cis-nerolidol	24.2	15	15	15
Metabolic intermediates, AMD as substrate				
Amorpha-4,11-diene	23.3	-	2.6	5.7
Artemisinic alcohol	29.2	-	2.8	4.0
Artemisinic acid	31.0	2.2	2.6	-
Dihydroartemisinic alcohol	29.1	-	2.7	-
Dihydroartemisinic aldehyde	27.3	-	3.0	-
Dihydroartemisinic acid	30.3	-	2.6	-
Metabolic intermediates, DHAAA as substrate				
Dihydroartemisinic acid	30.3	2.6	-	-
Metabolic intermediates, DHAAOH as substrate				
Dihydroartemisinic alcohol	29.1	2.7	-	-
Dihydroartemisinic aldehyde	27.3	2.1	-	-
Dihydroartemisinic acid	30.3	2.3	-	-

microsomal assays using amorpha-4,11-diene as substrate (Teoh et al., 2006). This suggests that *S. cerevisiae* microsomal preparations contain an unspecific Δ11(13) carbon double bond reductase (Udbr). The fact that both artemisinic acid and dihydroartemisinic acid are produced (figure 2b) suggests that competition occurs between Cyp71av1 and Udbr for the substrate artemisinic alcohol. In undiluted microsomal fractions, a complete conversion of amorpha-4,11-diene to artemisinic acid is observed (table 1). When microsomes are diluted 100-fold, the enzymes involved become a limiting factor and only artemisinic alcohol is produced from amorpha-4,11-diene. However, in a 10-fold diluted microsomal preparation, there is a window for production of the full range of artemisinin intermediates. This is probably explained by different kinetic properties of Cyp71av1 and Udbr. The results infer that a sufficient amount of artemisinic alcohol needs to accumulate before Cyp71av1 can recapture the molecules and further oxidize them to artemisinic aldehyde. Again, a minimal amount of artemisinic aldehyde must be reached before artemisinic aldehyde is oxidized to artemisinic acid. These enzyme-intermediate capture-release cycles create an opportunity for other non-specific enzymes, such as Udbr, to act on the molecules. Hence, as artemisinic alcohol accumulates, part of it will be reduced to dihydroartemisinic alcohol by Udbr and part of it will be oxidized to artemisinic aldehyde by Cyp71av1. However, the affinity of Cyp71av1 for artemisinic alcohol is greater than that of Udbr, as is evident from the 100x microsomal dilution assay, where only artemisinic alcohol is detected but no dihydroartemisinic alcohol. If the reverse situation would be true, that the affinity of Udbr for artemisinic alcohol would be greater than that of Cyp71av1, then we would have detected dihydroartemisinic alcohol in the sample.

Shake flask cultures

To confirm the relevance of Udbr in production of dihydroartemisinic acid *in vivo*, *S. cerevisiae* WAT11 was

transformed with c*yp71av1* and intact cells were incubated with amorpha-4,11-diene as substrate in shake flask cultures (figure 3, table 2, supplementary figure 2).

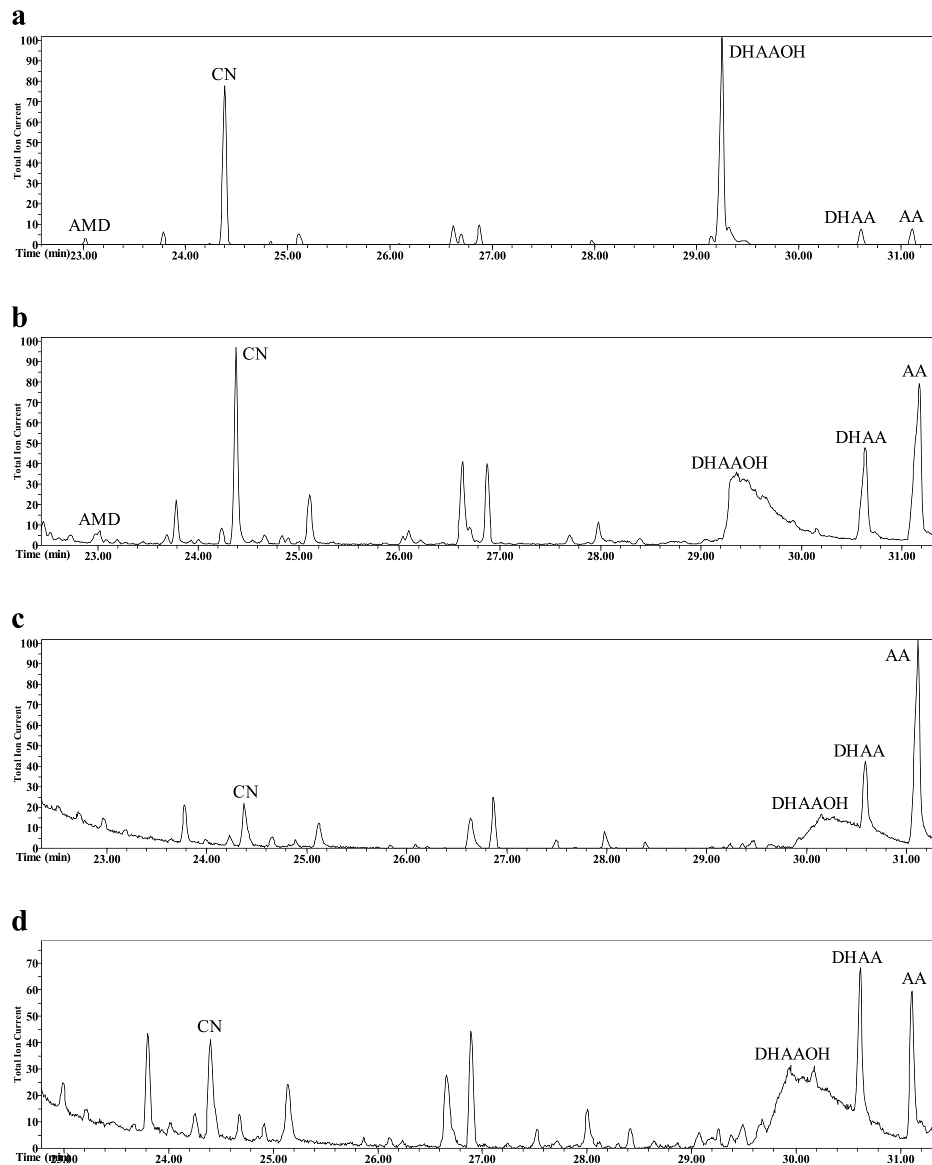

Figure 3. Shake flask cultures with *S. cerevisiae* WAT11. AMD – amorpha-4,11-diene,, AA – artemisinic acid, DHAA – dihydroartemisinic acid, DHAAOH – dihydroartemisinic alcohol, CN – *cis*-nerolidol.

a. *S. cerevisiae* WAT11 over-expressing Cyp71av1. Incubation in shaking flask with nitrogen atmosphere. AMD was added as substrate.
b. *S. cerevisiae* WAT11 over-expressing Cyp71av1. Incubation in shaking flask with atmosphere identical to normal air composition. AMD was added as substrate.
c. *S. cerevisiae* WAT11 over-expressing Cyp71av1. Incubation in shaking flask with loose cotton plug. AMD was added as substrate.
d. *S. cerevisiae* WAT11 over-expressing Cyp71av1. Incubation in shaking flask with tight cotton plug. AMD was added as substrate.

Table 2. Production of artemisinin intermediates in shake flask cultures with varying atmospheric compositions.

Culture conditions	AMD [mg/L]	DHAAOH [mg/L]	DHAA [mg/L]	AA [mg/L]
Shaking flask, nitrogen	5.5-6.1	10.1-10.9	6.7-7.4	6.6-7.5
Shaking flask, aired	4.2-4.9	7.2-7.8	6.0-6.5	6.9-7.6
Shaking flask, tight cotton plug	-	4.6-5.3	4.7-5.0	4.4-4.8
Shaking flask, loose cotton plug	-	3.9-4.7	4.6-5.1	4.8-5.4

To further test the hypothesis of enzymatic competition we included oxygen as a variable parameter, since this is expected to affect the efficiency of Cyp71av1 but not of Udbr. Moreover, gene expression in *S. cerevisiae* is known to drastically change with variations in atmospheric oxygen level (Rintala et al., 2009). The number of genes affected by oxygen limitation is also depending on the carbon source where galactose has been shown to have a stronger impact on the transcriptome than glucose (Lai et al., 2006). The results show a

trend towards higher titers of artemisinic acid than dihydroartemisinic acid in cultures that have more oxygen (table 2). This suggests that Cyp71av1 catalysis is less efficient under low oxygen availability and/or that the expression of Udbr is increased under these conditions. The volatile nature of the sesquiterpenes is evident as the amounts of artemisinin intermediates are significantly lower in flasks sealed solely with cotton. However, the trend of a higher titer of dihydroartemisinic acid compared to artemisinic acid in limited oxygen conditions is preserved.

Fermentation studies

To confirm the influence of oxygen on dihydroartemisinic acid production, *S. cerevisiae* WAT 11 expressing Amds and Cyp71av1 was grown under different atmospheric conditions by flushing the cultures with normal air or air mixed with nitrogen. Maximum oxygen level (100%) was defined as normal air entering the culture at a rate of 250 ml min under constant stirring at 350 rpm. 20% of this oxygen value was defined as low level oxygen condition and was achieved by mixing in nitrogen with air. Thus, an experimental setup with high versus low oxygen levels was created. In these fermentor studies, *S. cerevisiae* WAT11 transformed with *amds* and *cyp71av1* yielded 24 mg L^{-1} dihydroartemisinic alcohol in low level

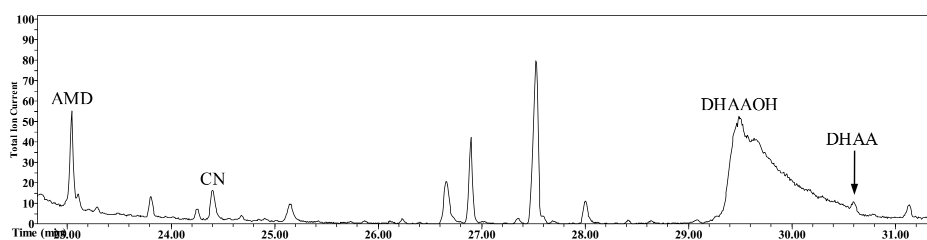

Figure 4. Fermentor study with *S. cerevisiae* WAT11 over-expressing Amds and Cyp71av. Sampling after 2 days incubation. AMD – amorpha-4,11-diene, AA – artemisinic acid, DHAA – dihydroartemisinic acid, DHAAOH – dihydroartemisinic alcohol.

Table 4. Production of DHAAOH in fermentor cultures.

Sample	DHAAOH [mg/L]
Cyp71av1, low oxygen concentration	24-25.9
Cyp71av1, high oxygen concentration	8.1-8.9

oxygen cultures (figure 4, table 3, supplementary figure 3). In contrast, fermentations performed in oxygen rich environment produced three times less dihydroartemisinic aldehyde (8 mg L^{-1}). As discussed above, the artemisinin intermediates are volatile and the lack of artemisinic acid and dihydroartemisinic acid in the fermentor samples may be explained by a combination of low production and escape of volatile intermediates with waste air. Therefore dihydroartemisinic alcohol, which is the first precursor to dihydroartemisinic acid, was accepted as an indicator for the capacity of dihydroartemisinic acid production. An attempt to trap the volatiles in an organic overlay of dodecane resulted in no detectable production of either artemisinic acid or dihydroartemisinic acid, but rather the accumulation of amorpha-4,11-dinene in the organic phase (data not shown).

Concluding remarks

In this communication we demonstrate for the first time the conversion of dihydroartemisinic alcohol to dihydroartemisinic aldehyde and dihydroartemisinic acid by Cyp71av1 (figure 2e-h; figure 3). We exploit this feature in a fermentation study and report that under conditions with limited oxygen concentration and galactose as sole carbon source, *S. cerevisiae* transformed with *amds* and *cyp71av1* is capable of reducing the early artemisinin intermediate artemisinic alcohol to dihydroartemisinic alcohol (figure 1). The corresponding enzyme has up to date not been found in *A. annua*.

Imposed heterologous genes impart stress on the host, and the stress reactions drain resources from production of the desired metabolite. One way of limiting the stress is to use as few heterologous genes as possible and combine the biosynthetic pathway with adequate endogenous enzymes instead. It is desirable to choose a host that contains genes compatible with the heterologous biosynthetic pathway. The finding that *S. cerevisiae* already expresses an aspecific carbon double bond reductase that performs a key step in the artemisinin biosynthesis, may open up possibilities to use one heterologous gene less in microbial artemisinin precursor production. The identity of Udbr remains obscure. Even so, it can be argued that Udbr belongs to a different protein family than Dbr2 (Zhang et al., 2008). Although the substrates of Dbr2 and Udbr are similar, the enzymes act on different chemical groups. Dbr2 reduces the exocyclic carbon double bond of artemisinic aldehyde, thereby producing dihydroartemisinic aldehyde, while Udbr reduce the ketone of dihydroartemisinic aldehyde to an alcohol. Dbr2 is classified into the same protein family as the yeast old yellow enzyme due to its sequence homology and its conformity with the general substrate pattern of α,β-unsaturated carbonyls (Williams et al., 2002). As Udbr is reducing the carbonyl double bond itself and not a distand carbon-carbon double bond, it is unlikely that it belongs to this vast family of proteins. Regardless of the identity of Udbr, the results show that it may be rewarding to thoroughly investigate the selection of hosts and growth conditions in a synthetic biology project. Some hosts may offer more benefits than others in terms of endogenous promiscuous enzymatic activities.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma or Difco. DNA polymerase was purchased from Finnzymes and restriction enzymes

from New England Biolabs. Artemisinin intermediates were synthesized as described by Bertea el al. (2005).

Media

SGal: 1% w/v casamino acids, 2% w/v galactose, 0.67% w/v yeast nitrogen base with ammonium sulfate without amino acids. SGlu: 1% w/v casamino acids, 2% w/v glucose, 0.67% w/v yeast nitrogen base with ammonium sulfate without amino acids. Agar plates were prepared by adding 1.5% w/v agar to the medium. YPGA: 1% w/v yeast extract, 1% w/v bactopeptone, 2 % w/v glucose, 200 mg l^{-1} adenine. YPL: 1% w/v yeast extract, 1% w/v bactopeptone, 2% w/v galactose.

Strains and plasmids

Escherichia coli (*E.coli*) XL-1 Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{R})]) (Stratagene) and the vector pGEMT (Promega) was used for molecular cloning and sequencing. The construction of the *Saccharomyces cerevisiae* (*S. cerevisiae*) strain WAT11, a derivative of the W303-B strain (*MAT a*; *ade*2–1; *his*3–11,-15; *leu*2–3,-112; *ura*3–1; *can*^{R}; *cyr*$^{+}$) expressing the ATR1 *Arabidopsis thaliana* NADPH–P450 reductase has been described (Truan et al., 1993; Urban et al., 1997). *S. cerevisiae* WAT11 was used as destination host and the vectors pYeDP60 and pYeDP80 for protein expression (Pompon et al., 1996). The episomal high copy expression vectors pYeDP60 and pYeDP80 are induced by galactose and confer adenine and tryptophane or uracil auxotrophy.

Cloning of amds and cyp71av1

Amds was a gift from Mattijs Julsing (Department of Pharmaceutical biology, University of Groningen). Primers 1 and 2 were used to

amplify *amds* with the added restriction sites BamHI and EcoRI (table 5).

Table 5. List of primers used in the study. Underlined nucleotides reprecent restriction sites.

No. primer	Sequence 5' to 3'	Restriction site
1	5' gggatccatgtcacttacagaagaaaaacc 3'	Bam HI
2	5' ggaattctcatatactcataggataaacgag 3'	EcoRI
3	5' cggcggatccatggcactctcactgaccacttcca 3'	Bam HI
4	5' cggcggtaccctagaaacttggaacgagtaacaactcagcctttc 3'	KpnI

The reaction conditions recommended by the manufacturer of Phusion DNA polymerase (Finnzymes) were followed using the PCR program: Initial denaturation for 1 min at 98°C, 30 cycles at 98°C for 10 sec, 70°C for 20 sec, 72°C for 30 sec and a final extension step at 72°C for 10 min. The construct was cloned into pGEMT (Promega), propagated in *E. coli* XL1-Blue and sequenced. A confirmed construct was subcloned from pGEMT by restriction with BamHI and EcoRI and ligated into the expression vector pYeDP80. The construct pYeDP80-*amds* was transformed into *E. coli* XL1-Blue and confirmed by restriction analysis.

EST sequences of a glandular trichome specific cDNA library (Bertea et al., 2006) were compared with Genbank sequences using batch BlastX (NCBI) with standard settings. Interesting candidate cytochrome P450s were extended to full length using the EST library described above. The full length gene of *cyp71av1* (Ro et al., 2006; Teoh et al., 2006), was obtained and subcloned into the sequencing vector pGEMT using the primers 3 and 4 listed in table 1. *Cyp71av1* was picked up from the cDNA library by following the reaction conditions recommended by the manufacturer of Phusion DNA polymerase (Finnzymes) and the PCR program: Initial denaturation for 1 min at 98°C, 30 cycles at 98°C for 10 sec, 70°C for 20 sec,

72°C for 30 sec and a final extension step at 72°C for 10 min. The construct was propagated in *E. coli* XL1-Blue and confirmed with sequencing. Thereafter, *cyp71av1* was restricted from pGEMT using BamHI and KpnI and ligated into the expression vector pYeDP60. The construct pYeDP60-*cyp71av1* was transformed into *E. coli* XL1-Blue and confirmed by restriction analysis.

Microsomal isolation and conversion assays

The constructs *pYeDP60* and *pYeDP60-cyp71av1* were transformed into *S. cerevisiae* WAT11 using the LiAc method (Maniatis et al., 1982). Mutants were selected on SGal agar plates supplemented with 100 µg ml^{-1} ampicillin. Colonies were checked for the presence of the constructs by yeast colony PCR (Sambrook et al., 2001). Small scale cultures of 50 ml SGal were initiated with *S. cerevisiae* WAT11 containing either *pYeDP60* or *pYeDP60-cyp71av1*. *S. cerevisiae* WAT11 transformed with the empty vector was used as negative control. The seed cultures were incubated for 40 hours at 28°C with shaking. Following incubation the cultures were centrifuged and the pellets were resuspended in 250 ml YPL medium. Microsomes were isolated using the method described by Pompon et al. (1996) and stored in 200 µl aliquots at -80°C. Protein content was estimated to 10.7-10.9 mg ml^{-1} in Bradford assays using bovine serum albumin as standard (Biorad). Conversion assays were performed using 200 µl microsomes in 800 µl assay buffer (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1mM DTT, 4% w/v glycerol, 5 µM FAD, 5 µM FMN, 2 mM glucose 6-phosphate, 1 unit glucose 6-phosphate dehydrogenase, 1mM β-NADPH and 30 µM amorpha-4,11-diene, dihydroartemisinic aldehyde or dihydroartemisinic alcohol. Dilution series contained 2.1 mg, 0.21 mg and 21 µg microsomal protein. Assays were incubated for 2 hours at 30°C with gentle shaking. Reactions were stopped on ice. 15 µM *Cis*-nerolidol was added as internal standard. The assay was then extracted 3 times with 1 ml ethyl acetate followed by a final extraction with 1.5 ml ethyl acetate. The combined organic extractions were dried with

anhydrous Na_2SO_4 and evaporated to 200 µl under a gentle flow of N_2. Samples were analyzed by GC-MS as described below. Peaks were identified using authentic standards. Product yields were calculated based on the internal standard peak area and calibration curves made from authentic standards.

Shake flask studies

5 ml aliquots of SGlu were inoculated with appropriate combinations of *S. cerevisiae* WAT11 pYeDP60, pYeDP80, *pYeDP60-cyp71av1* or *pYeDP80-amds*. The seed cultures were incubated at 30°C with gentle shaking until an OD_{600} of 0.5 was reached. Main cultures were initiated by adding seed culture to a calculated final OD_{600} of 0.05 to 50 ml SGal in 250 ml shake flasks with airtight glass stoppers or cotton plugs. The atmosphere was adjusted by adding 350 ml l^{-1} air or nitrogen gas for 1 min to flasks sealed with glass stoppers. The atmosphere of these cultures was refreshed by flushing the flasks for 2 min with 350 ml l^{-1} air or nitrogen after 24 h of incubation. Cultures sealed with loose or tight cotton plugs were used without any atmospheric modification. All cultures were incubated at 30°C with shaking for 48 h. Thereafter the cultures were cooled for 30 min at 4°C and extracted with 15 ml ethyl acetate. To 2 ml of this organic phase, 0.83 µg *cis*-nerolidol was added as internal standard. The samples were filtered and dried using a small column containing silica and anhydrous Na_2SO_4 which was eluted with an additional 1 ml of ethyl acetate. Before GC-MS analysis, as described below, the samples were concentrated to approximately 500 µl under a gentle flow of N_2. Product yields were calculated based on the internal standard peak area and calibration curves made from authentic standards.

Fermentation studies

100 ml SGlu seed cultures were initiated by inoculation with appropriate combinations of *S. cerevisiae* WAT11 pYeDP60,

pYeDP80, *pYeDP60-cyp71av1* or *pYeDP80-amds*. Cultures were incubated at 30°C with shaking until OD_{600} 4.0 was reached. Bench top fermentors (New Brunswick Scientific One BioFlo 310) with a total volume of 5 l were used in the fermentation studies. The level of dissolved oxygen (DO_2) was calibrated to 100% in the medium at 30°C by stirring at 350 rpm and adding air at a flow of 250 ml min^{-1}. Fermentor cultures were started by adding seed cultures to a calculated value of OD_{600} of 0.05 in 3 l SGal. By mixing in nitrogen with air as well as automatically adjusting the stirring speed, DO_2 was kept at either 100% or 20% of the calibrated initial value. Thus, an environment was created which was either rich in oxygen (100% DO_2) or low in oxygen (20% DO_2) content. Incubation was maintained for 48 hours. From the cultures 55 ml samples were extracted with 30 ml ethyl acetate spiked with 15 µM of the internal standard *cis*-nerolidol. The samples were subsequently extracted twice more with 30 ml ethyl acetate. The organic phases were combined, and the water removed with anhydrous Na_2SO_4. The extracts were concentrated in a vacuum centrifuge to approximately 8 ml. A small aliquot was analyzed by GC-MS, as described below. Product yields were calculated based on the internal standard peak area and calibration curves made from authentic standards.

GC-MS

GC-MS analysis was performed on an Agilent 6890 series gas chromatograph coupled to a JEOL JMS-SUN 200 mass selective detector in electron impact mode (70 eV) equipped with a guard column (1 m x 0.25 mm i.d., GL-Sciences) and a HP-5MS analytical column (30 m x 0.25 mm i.d., 0.25 µm film). The temperature program was set to an initial temperature of 60°C followed by a first temperature gradient of 3°C min^{-1} to 220°C and then a second gradient of 30°C min^{-1} to 300°C with an endpoint hold of 4 min. Helium flow was set to 0.7 ml min-1, detector temperature and injector temperature to 250°C using 500 scans min^{-1} with a scan range of 40-350 m/z. A persistent problem with peak tailing of

dihydroartemisinic alcohol in *in vivo* assays could not be solved, but this did not hamper reliable peak integration.

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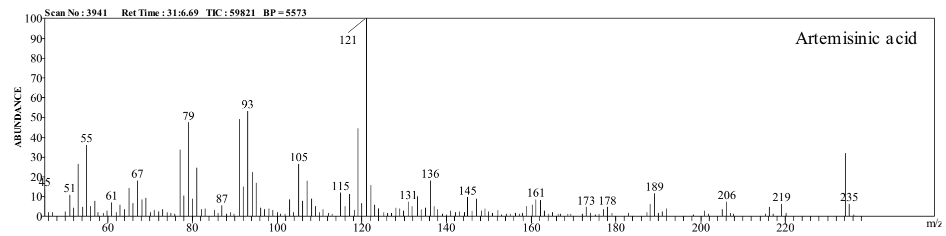
Supplementary figures

a



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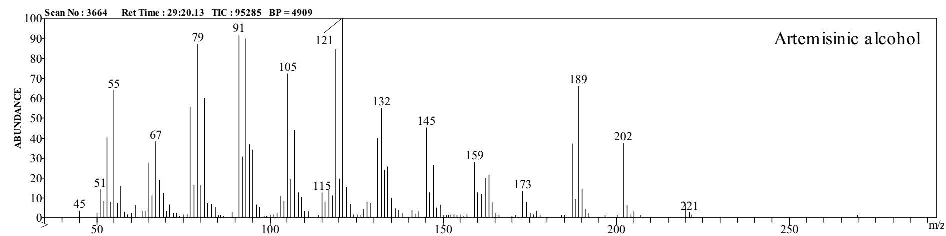

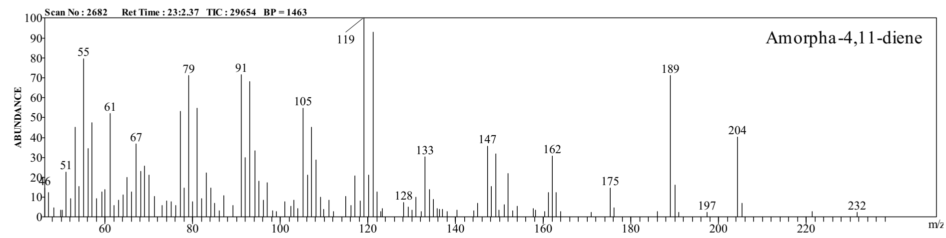

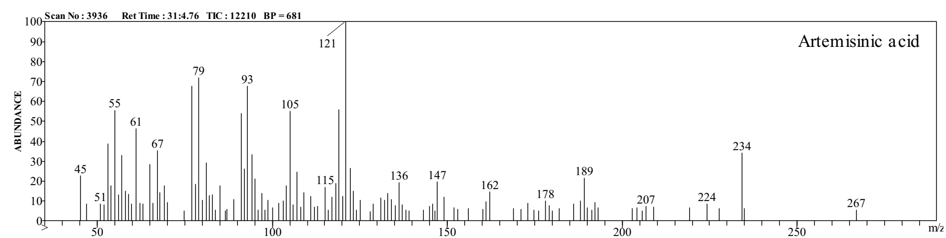

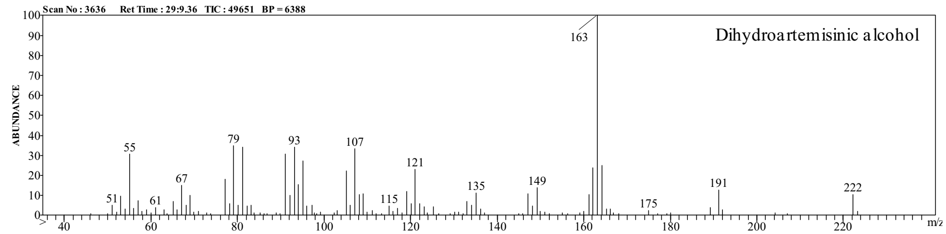

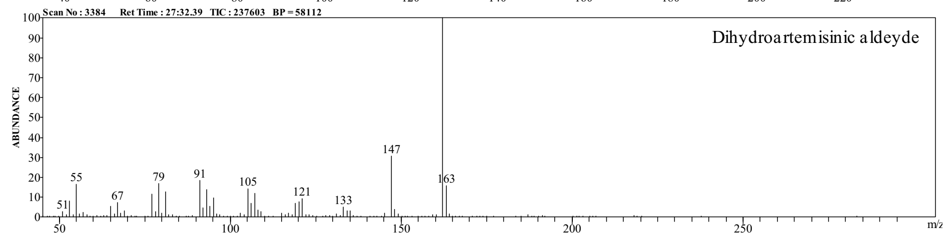

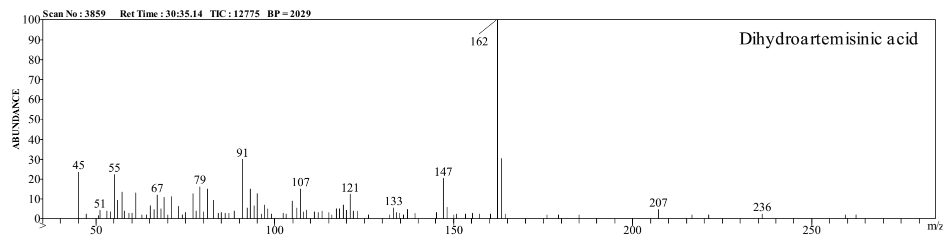

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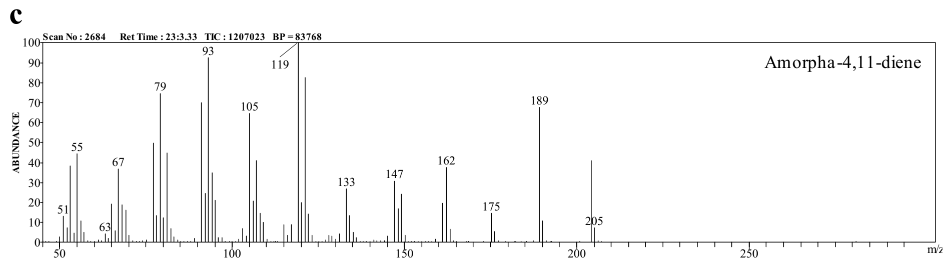

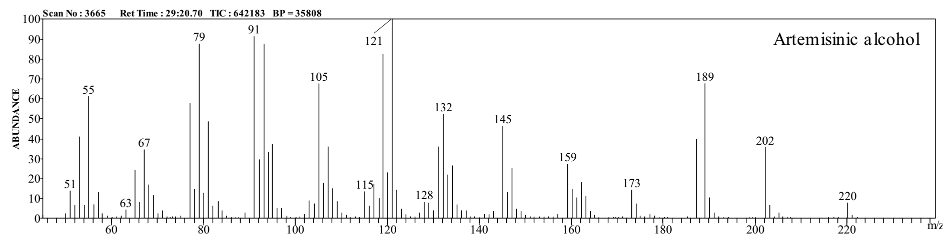

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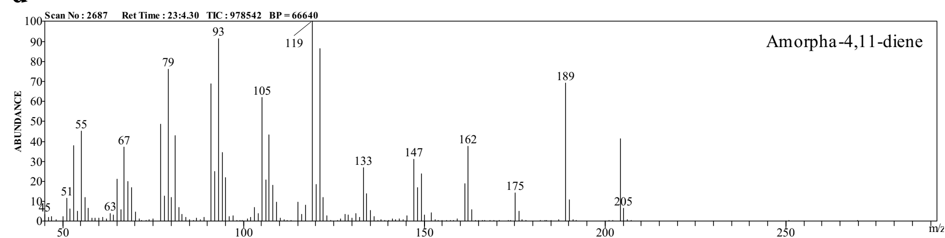

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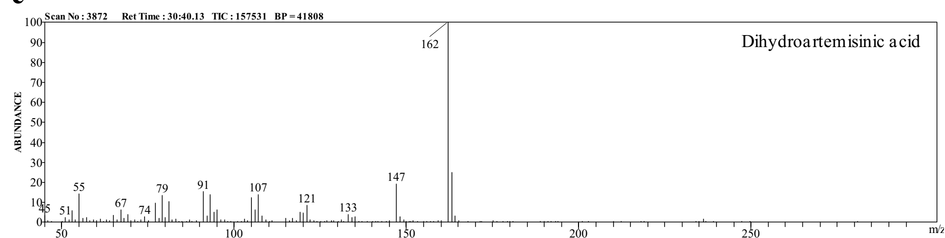

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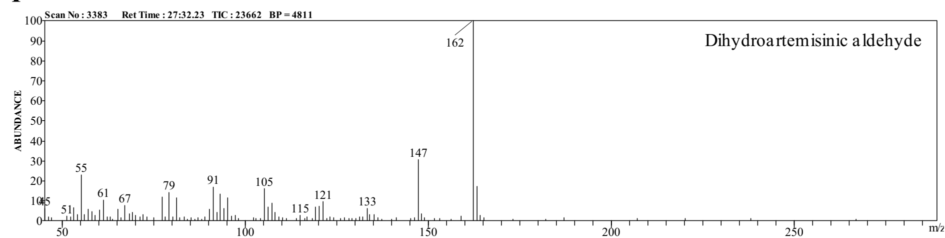

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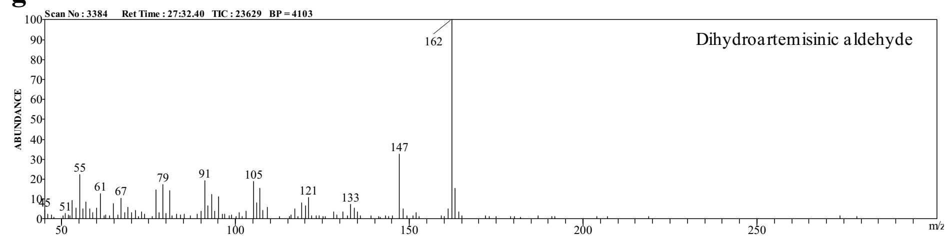

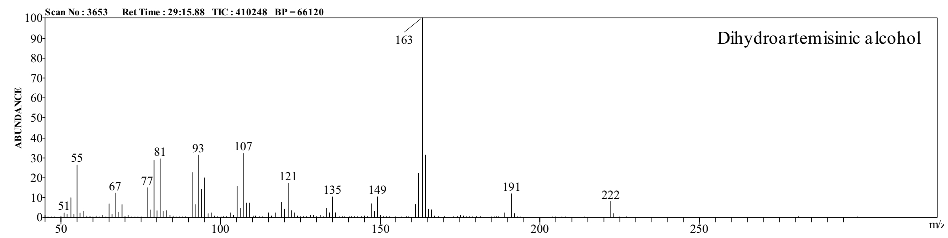

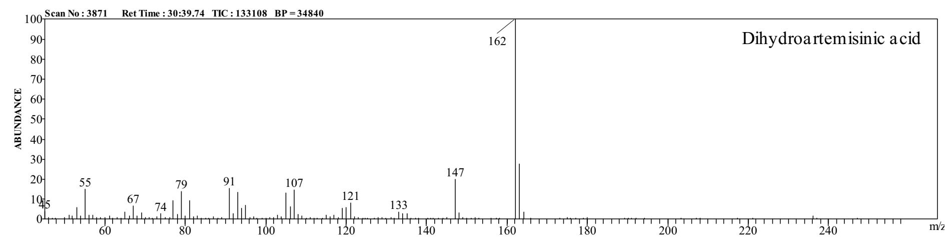

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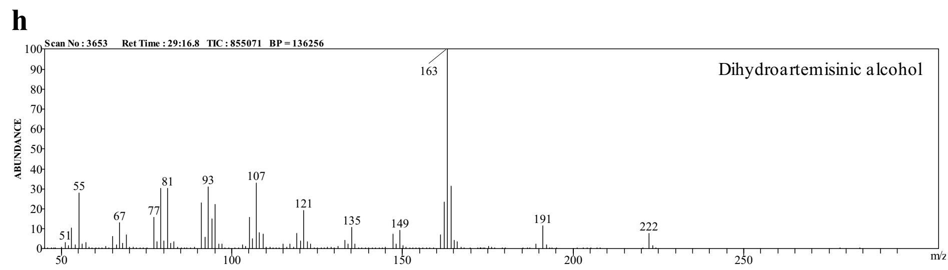

Supplementary figure 1.

Mass spectra for GC chromatograms from yeast microsomal assays.

a. Undiluted yeast microsomes over-expressing Cyp71av1 with amorpha-4,11-diene as substrate.
b. 10x diluted yeast microsomes over-expressing Cyp71av1 with amorpha-4,11-diene as substrate.
c. 100x diluted yeast microsomes over-expressing Cyp71av1 with amorpha-4,11-diene as substrate.
d. Empty vector control: Undiluted yeast microsomes with amorpha-4,11-diene as substrate.
e. Undiluted yeast microsomes over-expressing Cyp71av1 with dihydroartemisinic aldehyde as substrate.
f. Empty vector control: Undiluted yeast microsomes with dihydroartemisinic aldehyde as substrate.
g. Undiluted yeast microsomes over-expressing Cyp71av1 with dihydroartemisinic alcohol as substrate.
h. Empty vector control: Undiluted yeast microsomes with dihydroartemisinic alcohol as substrate.

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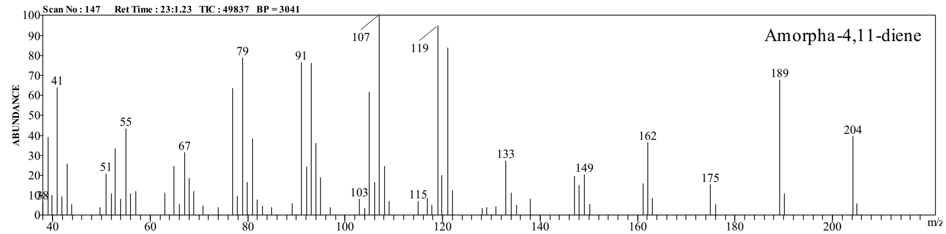

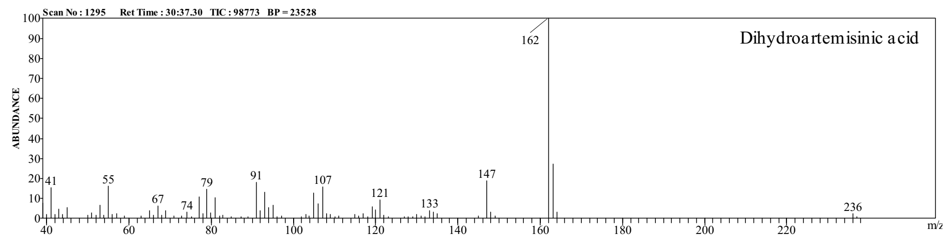

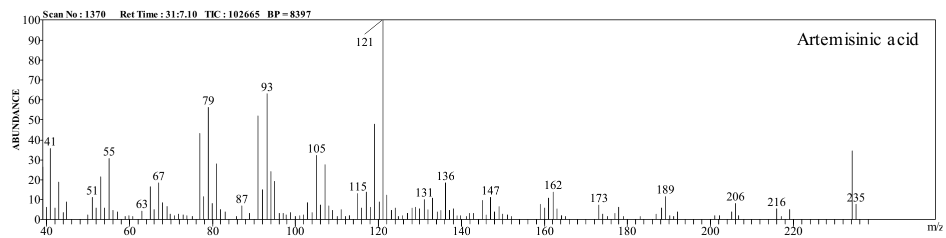

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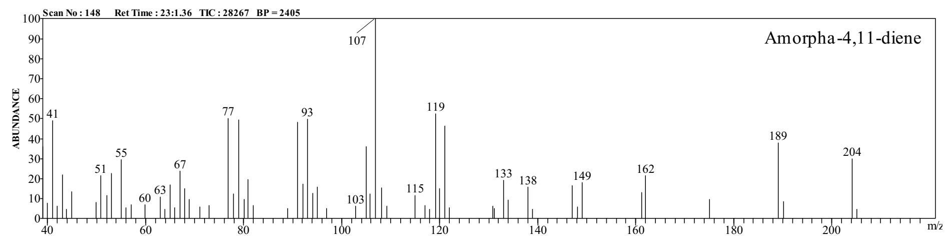

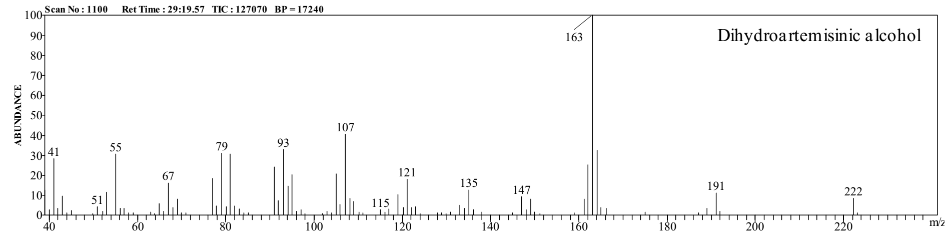

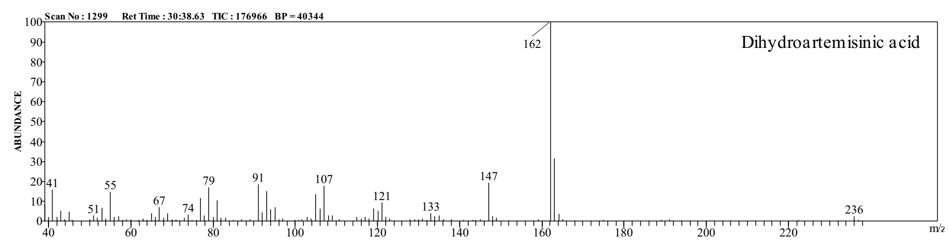

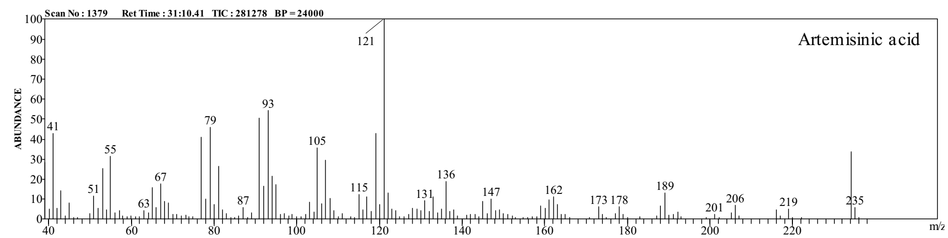

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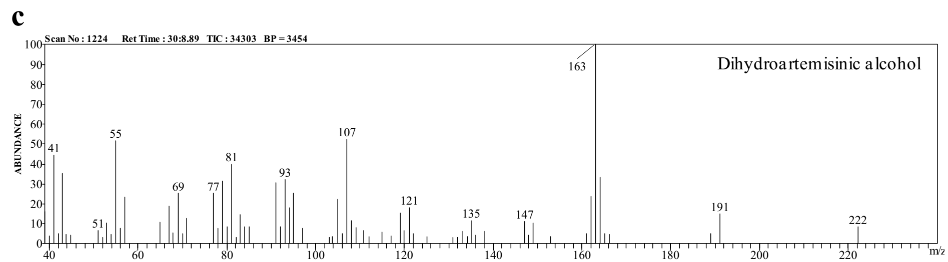

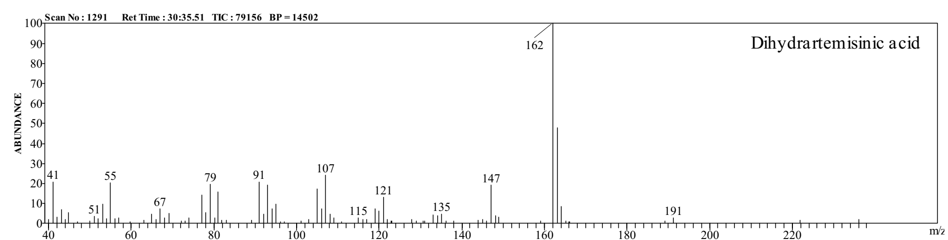

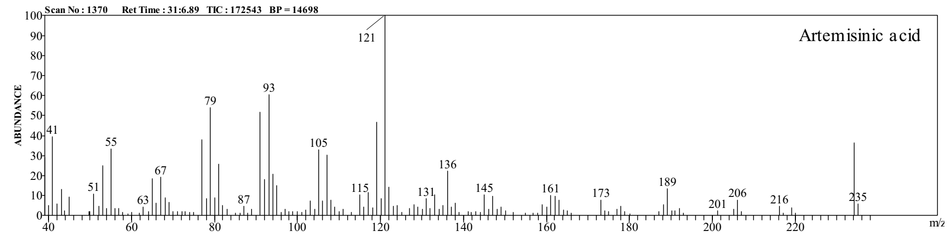

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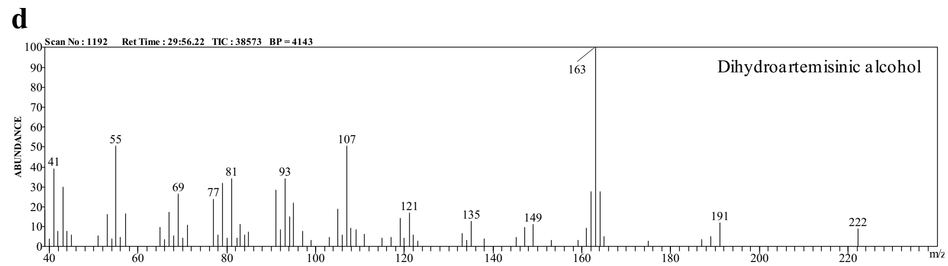
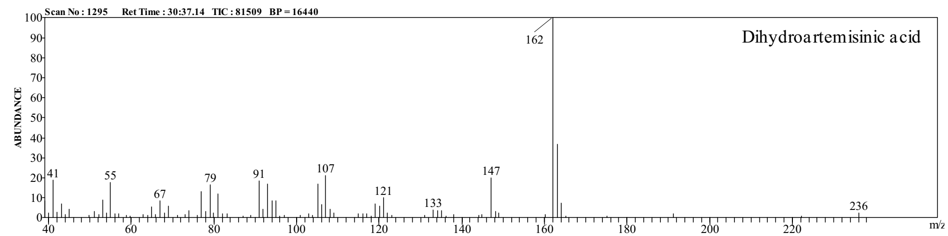
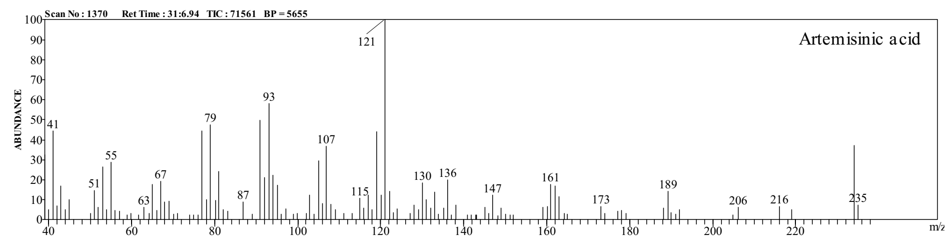


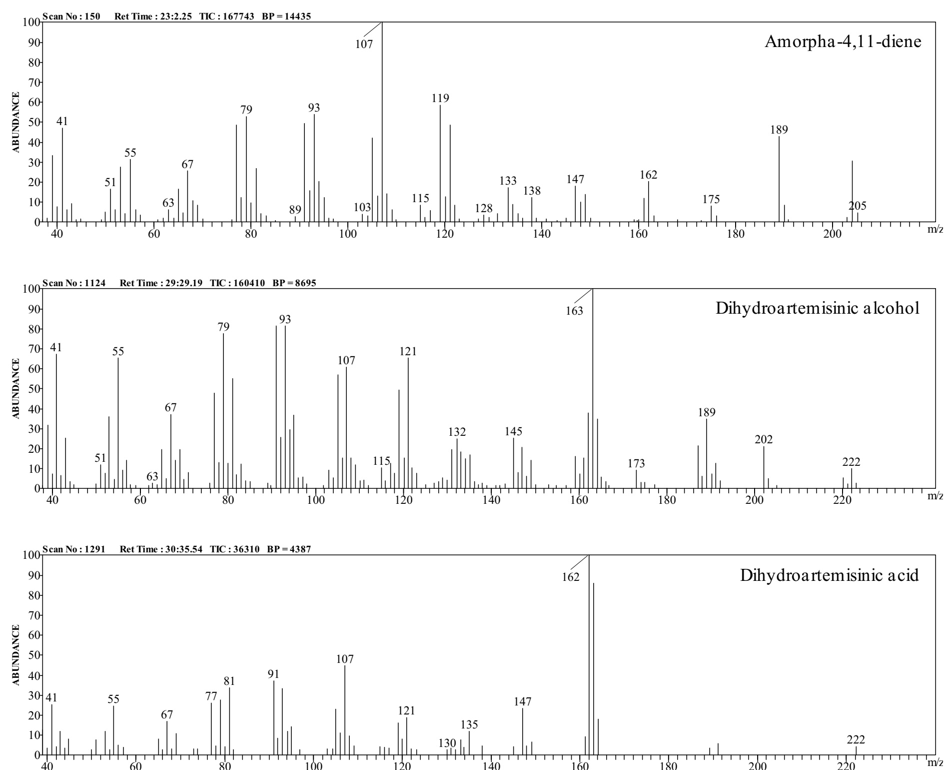




Supplementary figure 2.

Mass spectra for GC chromatograms from shake flask cultures.

a. *S. cerevisiae* WAT11 over-expressing Cyp71av1. Incubation in shaking flask with nitrogen atmosphere. Amorpha-4,11-diene was added as substrate.
b. *S. cerevisiae* WAT11 over-expressing Cyp71av1. Incubation in shaking flask with atmosphere identical to normal air composition. Amorpha-4,11-diene was added as substrate.
c. *S. cerevisiae* WAT11 over-expressing Cyp71av1. Incubation in shaking flask with loose cotton plug. Amorpha-4,11-diene was added as substrate.
d. *S. cerevisiae* WAT11 over-expressing Cyp71av1. Incubation in shaking flask with tight cotton plug. Amorpha-4,11-diene was added as substrate.



Supplementary figure 3.
Mass spectra for GC chromatograms from fermentor study.