LETTERS

Production of the antimalarial drug precursor artemisinic acid in engineered yeast

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Malaria is a global health problem that threatens 300–500 million people and kills more than one million people annually[1]. Disease control is hampered by the occurrence of multi-drug-resistant strains of the malaria parasite *Plasmodium falciparum*[2,3]. Synthetic antimalarial drugs and malarial vaccines are currently being developed, but their efficacy against malaria awaits rigorous clinical testing[4,5]. Artemisinin, a sesquiterpene lactone endoperoxide extracted from *Artemisia annua* L (family Asteraceae; commonly known as sweet wormwood), is highly effective against multi-drug-resistant *Plasmodium* spp., but is in short supply and unaffordable to most malaria sufferers[6]. Although total synthesis of artemisinin is difficult and costly[7], the semi-synthesis of artemisinin or any derivative from microbially sourced artemisinic acid, its immediate precursor, could be a cost-effective, environmentally friendly, high-quality and reliable source of artemisinin[8,9]. Here we report the engineering of *Saccharomyces cerevisiae* to produce high titres (up to 100 mg l^{-1}) of artemisinic acid using an engineered mevalonate pathway, amorphadiene synthase, and a novel cytochrome P450 monooxygenase (*CYP71AV1*) from *A. annua* that performs a three-step oxidation of amorpha-4,11-diene to artemisinic acid. The synthesized artemisinic acid is transported out and retained on the outside of the engineered yeast, meaning that a simple and inexpensive purification process can be used to obtain the desired product. Although the engineered yeast is already capable of producing artemisinic acid at a significantly higher specific productivity than *A. annua*, yield optimization and industrial scale-up will be required to raise artemisinic acid production to a level high enough to reduce artemisinin combination therapies to significantly below their current prices.

We engineered artemisinic-acid-producing yeast in three steps, by (1) engineering the farnesyl pyrophosphate (FPP) biosynthetic pathway to increase FPP production and decrease its use for sterols, (2) introducing the amorphadiene synthase gene (*ADS*) from *A. annua* into the high FPP producer to convert FPP to amorphadiene, and (3) cloning a novel cytochrome P450 that performs a three-step oxidation of amorphadiene to artemisinic acid from *A. annua* and expressing it in the amorphadiene producer (Fig. 1). The first committed reaction in artemisinin biosynthesis is catalysed by ADS[10], which has been characterized and used for *de novo* production of amorphadiene in *Escherichia coli*[11]. To test for improvements in FPP production, we expressed *ADS* under the control of the *GAL1* promoter on the pRS425 plasmid (see Supplementary Information for details). Yeast engineered with *ADS* alone produced a low quantity of amorphadiene (Fig. 2, strain EPY201, 4.4 mg l^{-1} amorphadiene).

To increase FPP production in *S. cerevisiae*, the expression of several genes responsible for FPP synthesis was upregulated, and one gene responsible for FPP conversion to sterols was downregulated. All of these modifications to the host strain were made by chromosomal integration to ensure the genetic stability of the host strain. Overexpression of a truncated, soluble form of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*tHMGR*)[12] improved amorphadiene production approximately fivefold (Fig. 2, strain EYP208). Downregulation of *ERG9*, which encodes squalene synthase (the first step after FPP in the sterol biosynthetic pathway), using a methionine-repressible promoter (P_{MET3})[13] increased amorphadiene production an additional twofold (Fig. 2, strain EPY225). Although *upc2-1*, a semi-dominant mutant allele that enhances the activity of *UPC2* (a global transcription factor regulating the biosynthesis of sterols in *S. cerevisiae*)[14], had only a modest effect on amorphadiene production when overexpressed in the EPY208 background (Fig. 2, strain EPY210), the combination of downregulating *ERG9* and overexpressing *upc2-1* increased amorphadiene production to 105 mg l^{-1} (Fig. 2, strain EPY213). Integration of an additional copy of *tHMGR* into the chromosome further increased amorphadiene production by 50% to 149 mg l^{-1} (Fig. 2, strain EPY219). Although overexpression of the gene encoding FPP synthase (*ERG20*) had little effect on total amorphadiene production (Fig. 2, strain EPY224), the specific production increased by about 10% owing to a decrease in cell density. Combining all of these modifications resulted in a strain (EPY224) able to produce 153 mg l^{-1} amorphadiene, a sesquiterpene production level nearly 500-fold higher than previously reported[15].

To create a strain that produced artemisinic acid from amorphadiene, we isolated genes encoding enzymes responsible for oxidizing amorphadiene to artemisinic acid in *A. annua*. Artemisinin is a sesquiterpene lactone derivative, which is the most widespread and characteristic class of secondary metabolites found in Asteraceae (also known as Compositae)[16]. We hypothesized that plants belonging to the Asteraceae family would share common ancestor enzymes for the early steps in the biosynthesis of sesquiterpene lactones, and therefore undertook a comparative genomic analysis of plants in the Asteraceae family. Previous cell-free assays have indicated that a cytochrome P450 monooxygenase (P450) catalyses the first regiospecific hydroxylation of amorphadiene (Fig. 1) in *A. annua*[17]. We thus retrieved P450-expressed-sequence tags (ESTs) from the Asteraceae EST-database generated from two Asteraceae crops, sunflower and lettuce (http://www.cgpdb.ucdavis.edu). Use of degenerate primers highly specific to the Asteraceae CYP71 and CYP82 subfamilies (the most abundant P450 subfamilies in Asteraceae) enabled the isolation

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of several unique P450 fragments from an *A. annua* trichome-enriched complementary DNA pool. Using BLAST[18] analyses of these P450 gene fragments against sunflower and lettuce ESTs, we identified a single *A. annua* P450 gene fragment that had surprisingly high sequence identity (85–88% at the amino-acid level) to ESTs of unknown function from both sunflower and lettuce. Sequence identity of this *A. annua* P450 fragment to other P450 fragments outside the Asteraceae family was much lower (~50% at the amino-acid level), indicating that this P450 is highly conserved in three distantly related genera in the Asteraceae family, but not in plants outside the Asteraceae family. This P450 gene was therefore a good candidate for a conserved Asteraceae sesquiterpene lactone biosynthetic enzyme.

The corresponding full-length P450 cDNA (*CYP71AV1*), encoding an open reading frame of 495 amino acids, was recovered from *A. annua*. Phylogenetic analysis showed that CYP71AV1 shares a close lineage with other P450s that catalyse the hydroxylation of monoterpenoids (CYP71D13/18; ref. 19), sesquiterpenoids (CYP71D20; ref. 20) or diterpenoids (CYP71D16; ref. 21), further suggesting the potential involvement of this P450 in terpenoid metabolism (Supplementary Information). For functional, heterologous expression of *CYP71AV1*, its native redox partner, NADPH: cytochrome P450 oxidoreductase (*CPR*), was also isolated from *A. annua*, and its biochemical function was confirmed *in vitro*. (Michaelis–Menten constants (K_m) for cytochrome *c* and NADPH were determined to be 4.3 ± 0.7 μM and 23.0 ± 4.4 μM (mean ± s.d., $n = 3$), respectively.)

Using *A. annua* CPR as a redox partner for CYP71AV1, we then investigated whether CYP71AV1 could catalyse the conversion of amorphadiene to more oxygenated products *in vivo*. The transgenic yeast strain EPY224 was transformed with a vector harbouring *CPR* and *CYP71AV1* under the control of galactose-inducible promoters. After galactose induction, the ether-extractable fraction of the yeast culture medium and cell pellet were analysed by gas chromatography mass spectrometry (GC–MS). A single chromatographic peak unique to EPY224 co-expressing *CYP71AV1* and *CPR* was detected in both the yeast culture medium and cell pellet, but was not present in control yeast (EPY224 expressing *CPR* only). However, more than 95% of this novel compound was associated with the cell pellet. In GC–MS analysis, the electron-impact mass spectrum and retention time of this compound were identical to those of artemisinic acid isolated from *A. annua* (Fig. 3). In a shake-flask culture, 32 ± 13 mg l^{-1} (mean ± s.d., $n = 7$) artemisinic acid was produced from EPY224 expressing *CYP71AV1* and *CPR*. Notably, the pathway intermediates, artemisinic alcohol and artemisinic aldehyde, were present at negligible levels in the culture medium and cell pellets of EPY224 engineered with *CYP71AV1* and *CPR*. (Artemisinic alcohol was present at less than 5% of the artemisinic acid in the cell pellet, and no artemisinic aldehyde was detected.)

Almost all (>96%) of the synthesized artemisinic acid was removed from the cell pellet by washing with alkaline buffer (pH 9 Tris-HCl buffer supplemented with 1.2 M sorbitol), with less than 2% remaining in the washed cell pellet or culture medium. Thus, it seems that artemisinic acid is efficiently transported out of yeast cells but remains bound to the cell surface when it is protonated under acidic culture conditions. We used this feature to develop a one-step purification method: a single silica gel column chromatographic separation of ether-extracted artemisinic acid from the wash buffer routinely yielded >95% pure artemisinic acid. In a one-litre aerated bioreactor, 115 mg of artemisinic acid was produced, of which 76 mg was purified using this method. The ^{1}H and ^{13}C nuclear magnetic resonance spectra of this yeast-derived artemisinic acid were identical to those of artemisinic acid isolated from the leaves of *A. annua*, and are consistent with previously reported values[22,23]. We can therefore confirm that structurally authentic artemisinic acid is synthesized by

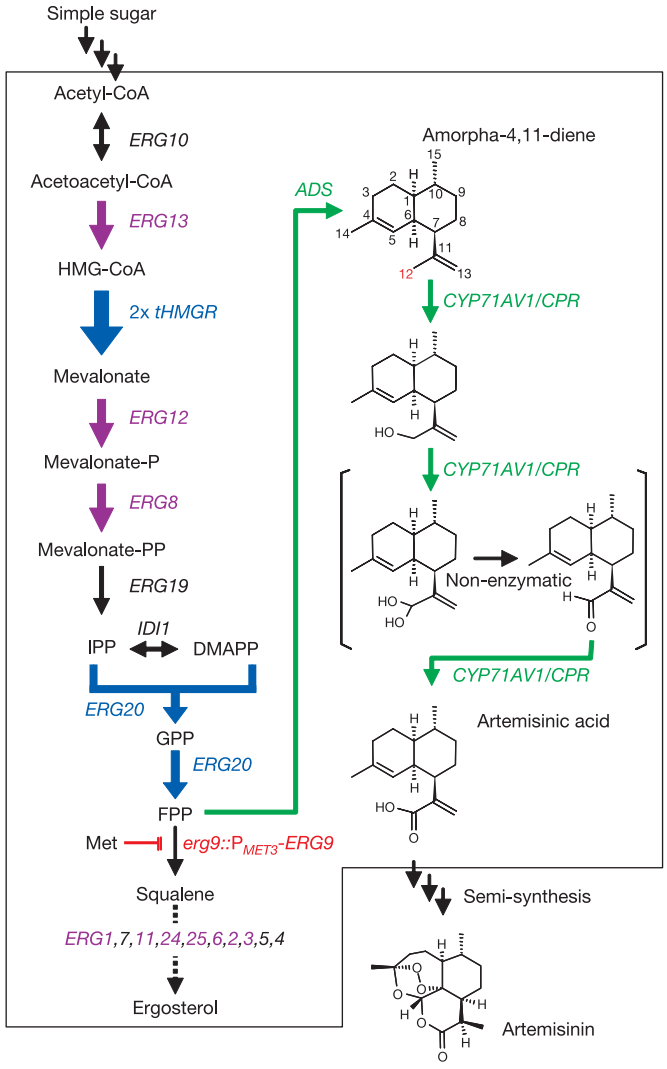

Figure 1 | Schematic representation of the engineered artemisinic acid biosynthetic pathway in *S. cerevisiae* strain EPY224 expressing *CYP71AV1* and *CPR*. Genes from the mevalonate pathway in *S. cerevisiae* that are directly upregulated are shown in blue; those that are indirectly upregulated by *upc2-1* expression are in purple; and the red line denotes repression of *ERG9* in strain EPY224. The pathway intermediates IPP, DMAPP and GPP are defined as isopentenyl pyrophosphate, dimethyl allyl pyrophosphate and geranyl pyrophosphate, respectively. Green arrows indicate the biochemical pathway leading from farnesyl pyrophosphate (FPP) to artemisinic acid, which was introduced into *S. cerevisiae* from *A. annua*. The three oxidation steps converting amorphadiene to artemisinic acid by *CYP71AV1* and *CPR* are shown.

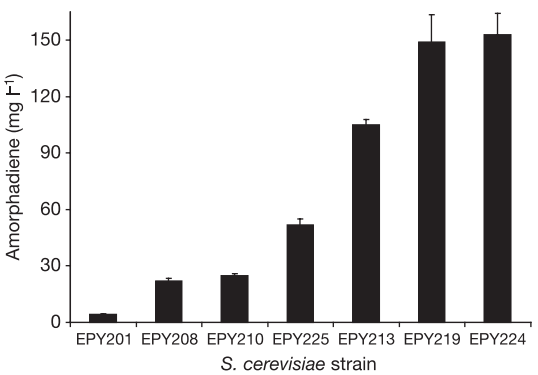

Figure 2 | Production of amorphadiene by *S. cerevisiae* strains. The various *S. cerevisiae* strains are described in the text. Cultures were sampled after 144 h of growth, and amorphadiene levels were quantified. Data, shown as total production, are mean ± s.d. ($n = 3$).

transgenic yeast *de novo*. The transgenic yeast produced artemisinic acid at a biomass fraction comparable to that produced by *A. annua* (4.5% dry weight in yeast compared to 1.9% artemisinic acid and 0.16% artemisinin in *A. annua*) but over a much shorter time (4–5 days for yeast versus several months for *A. annua*). As such, the specific productivity of the engineered yeast strain is nearly two orders of magnitude greater than *A. annua*.

Three-step oxidations by P450 enzymes have been previously reported in plant hormone gibberellin biosynthetic pathways[24,25]. We conducted *in vitro* enzyme assays to identify whether CYP71AV1 catalyses all three oxidation reactions from amorphadiene to artemisinic acid. Microsomes were isolated from *S. cerevisiae* strain YPH499 expressing either *CPR* alone or *CPR* and *CYP71AV1*, and incubated with pathway intermediates (amorphadiene, artemisinic alcohol or artemisinic aldehyde) (Fig. 4). Microsomes from the CPR control did not catalyse the conversion of any pathway intermediate to more oxidized products, whereas efficient conversion of amorphadiene, artemisinic alcohol and artemisinic aldehyde to the final product artemisinic acid was detected in microsomes containing CYP71AV1 and CPR. These *in vitro* assays demonstrate unambiguously that recombinant CYP71AV1 is able to catalyse three oxidation reactions at the C12 position of amorphadiene. Previous *in vitro* enzyme assays using *A. annua* protein extract have suggested that soluble alcohol and aldehyde dehydrogenases and a C11,13 double-bond reductase (which acts on the aldehyde) are involved in artemisinin biosynthesis[17]. Although we cannot exclude a catalytic role for additional alcohol and aldehyde dehydrogenases in artemisinin synthesis in *A. annua*, the efficient *in vivo* conversion of amorpha-4,11-diene to artemisinic acid by recombinant CYP71AV1 indicates that the membrane-bound, multifunctional CYP71AV1 is a key contributor to artemisinin biosynthesis.

In summary, we have created a strain of *S. cerevisiae* capable of producing high levels of artemisinic acid by engineering the FPP biosynthetic pathway to increase FPP production and by expressing amorphadiene synthase, a novel cytochrome P450 and its redox partner from *A. annua*. Given the existence of known, relatively high-yielding chemistry for the conversion of artemisinic acid to artemisinin or any other derivative that might be desired[8,9], microbially produced artemisinic acid is a viable source of this potent family of antimalarial drugs. Upon optimization of product titres, a conservative analysis suggests that artemisinin combination therapies could be offered significantly below their current prices (see Supplementary Information). In addition to cost savings, this bioprocess should not be subject to factors like weather or political climates that may affect plant cultivation. Furthermore, artemisinic acid from a microbial source can be extracted using an environmentally friendly process without worrying about contamination by other terpenes that are produced by plants, thereby increasing the ease with which it can be produced while reducing purification costs.

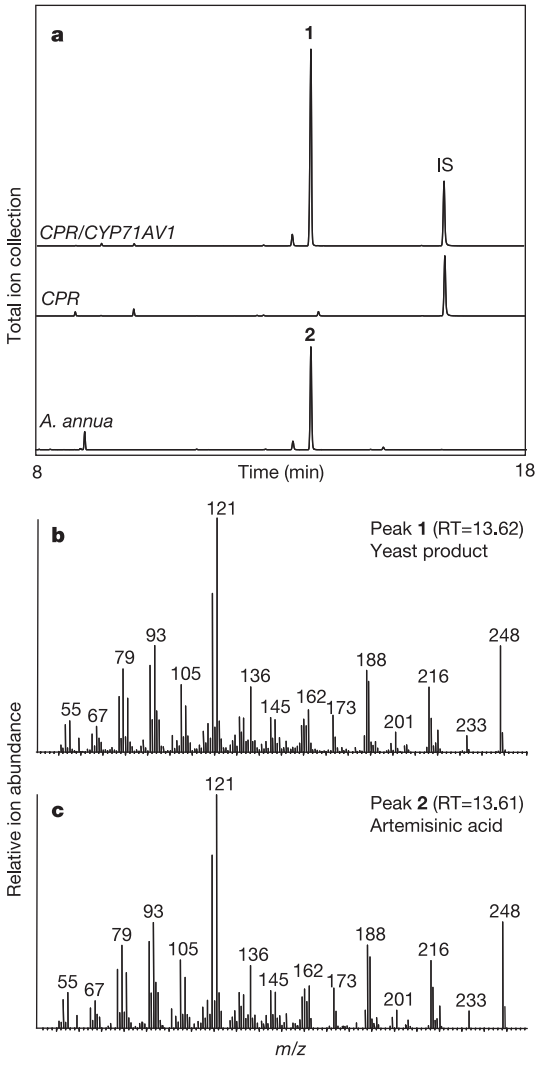

Figure 3 | GC–MS analysis of artemisinic acid produced from *A. annua* and transgenic yeast. **a**, Cell pellets from *S. cerevisiae* strain EPY224 expressing *CPR* or *CPR* and *CYP71AV1* were washed using an alkaline buffer followed by acidification and ether extraction. Artemisinic acid was extracted from *A. annua* leaves using hexane. Methyl esters of both samples were prepared with trimethylsilyl-diazomethane before GC–MS analysis. The internal standard (IS) is the methyl ester of 4-octylbenzoic acid. **b**, **c**, Mass spectra and retention times of artemisinic acid from yeast (**b**) and *A. annua* (**c**). RT, retention time (in min).

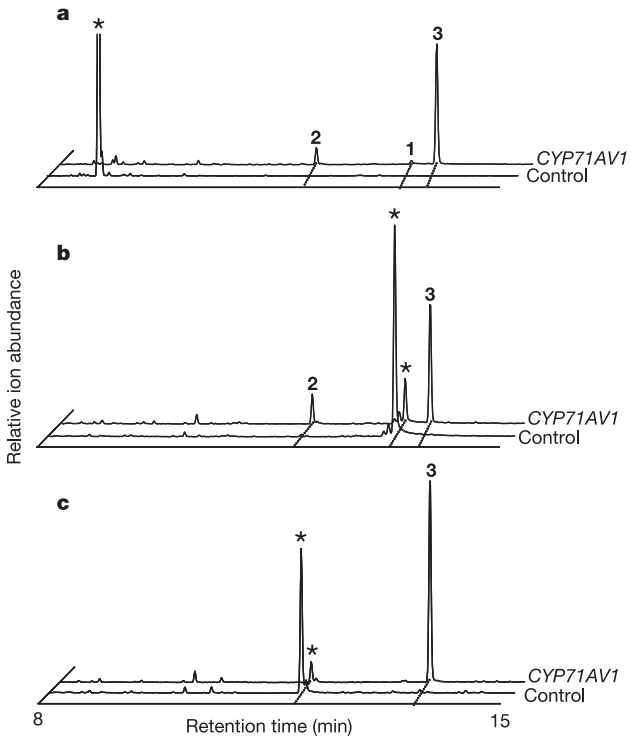

Figure 4 | *In vitro* enzyme assays for CYP71AV1 activities. Microsomes were isolated from *S. cerevisiae* strain YPH499 expressing *CPR* (control) or *CPR* and *CYP71AV1* (*CYP71AV1*). **a**–**c**, For each enzyme assay, 10 μM amorphadiene (**a**), 25 μM artemisinic alcohol (**b**) or 25 μM artemisinic aldehyde (**c**) was used. Chromatographic peaks for the substrates used are indicated with asterisks. Ether-extractable fractions were derivatized and analysed by GC–MS in selective ion mode (*m/z*: 121, 189, 204, 218, 220 and 248). Enzymatic products are as indicated: 1, artemisinic alcohol (retention time 13.20 min); 2, artemisinic aldehyde (retention time 11.79 min); 3, artemisinic acid (retention time 13.58 min, detected as methyl ester).

METHODS

Detailed descriptions of the methods used in the generation and characterization of *S. cerevisiae* EPY strains, the cloning of *CYP71AV1* and *CPR*, and the semi-synthesis of artemisinic alcohol and artemisinic aldehyde are provided in Supplementary Information.

Chemicals and plant material. Authentic artemisinic acid was purchased from Apin Chemicals or extracted from *A. annua* leaves with hexane, as described in Supplementary Information. *A. annua* plants were started from seeds (Sandeman Seeds) and grown in a greenhouse at the University of California, Berkeley.

GC–MS analysis of amorphadiene. Amorphadiene production by the various strains was measured by GC–MS using a dodecane layer to trap volatile amorphadiene (see Supplementary Information for details). Amorphadiene (90% pure) was prepared by fermentation using an *E. coli* strain[11], and was used to construct a standard curve to determine amorphadiene production levels.

***In vivo* production, purification and chemical analysis of artemisinic acid.** Pre-cultured EPY224 strains transformed with pESCURA::*CPR* or pESCURA::*CPR/CYP71AV1* were inoculated at an absorbance of 0.05 at 600 nm (A_{600}) in 25 ml synthetic defined medium lacking histidine, leucine, methionine and uracil, and supplemented with 0.2% dextrose, 1.8% galactose and 1 mM methionine. After 120 h of culture at 30 °C, the cells were centrifuged and the cell pellet was washed using 50 mM Tris-HCl buffer (pH 9). The buffer was acidified to pH 2 using 2 M HCl, and extracted with ethyl acetate spiked with 4-octyl benzoic acid (10 μg ml^{-1}). The extracts were derivatized by 50 μl of 2 M TMS-diazomethane (Aldrich) with 10% methanol. For qualitative analysis by GC–MS, the product was purified by silica gel column chromatography eluted with ether and pentane (1:1).

Products were analysed using a gas chromatography mass spectrometer (70 eV, Agilent Technologies) equipped with a DB5 capillary column (0.25 mm internal diameter × 0.25 μm × 30 m, J&W Scientific). The gas chromatography oven programme used was 80 °C (held for 2 min), 20 °C min^{-1} ramp to 140 °C, product separation by a 5 °C min^{-1} increment up to 220 °C. For quantification by gas chromatography-flame ionization detection, samples were analysed without column purification using the same gas chromatography oven programme.

Fermentation and product analyses. A one-litre bioreactor (New-Brunswick Scientific) was used to culture the transgenic yeast strain for 93 h at 30 °C. Yeast cells were induced with 2% galactose at an A_{600} of 1.7, and the final cell density reached an A_{600} of 5.0. The dissolved oxygen level was maintained at 40% by altering agitation speed from 100 to 500 r.p.m. and sparging air at 0.5 l min^{-1}. Artemisinic acid was removed from the cell pellet by an alkaline wash as before, and purified through a silica column eluted with 78% hexane, 20% ethyl acetate and 2% acetic acid. The structure of the isolated artemisinic acid (>95% purity) was analysed by ^{1}H and ^{13}C NMR using a 500 MHz NMR spectrometer (Bruker DRX-500) in the College of Chemistry NMR Facility at the University of California.

***In vitro* enzyme assays.** A one-litre culture of *S. cerevisiae* YPH499 transformed with pESCURA::*CPR* or pESCURA::*CPR/CYP71AV1* was induced with 2% galactose for 24 h. Microsomes were purified by polyethylene glycol precipitation followed by an additional ultracentrifugation step to remove cytosolic protein contamination, as previously described[26]. Approximately 500 μg of total microsomal protein was used in a 1-ml reaction containing 100 mM potassium phosphate buffer pH 7.5, 10 or 25 μM substrate, 100 μM NADPH and an NADPH regeneration system (5 mM glucose-6-phosphate and two units of glucose-6-phosphate dehydrogenase). Reactions were incubated for 2 h at 24 °C with gentle agitation, acidified to pH 2, and extracted with ethyl ether. Products were separated using the same gas chromatography oven programme as above. Selective ion mode (SIM), including six ions characteristic to the products (121, 189, 204, 218, 220 and 248), was used for detection.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions D.-K.R., E.M.P. and J.D.K. designed the project and experiments. D.-K.R., E.M.P., Y.S., M.C.Y.C., S.T.W. and J.K. performed experiments. K.J.F. conducted NMR analysis of artemisinic acid. J.M.N. and R.S. semi-synthesized artemisinic alcohol and artemisinic aldehyde. T.S.H. performed bioinformatics analysis of the Compositae EST-database. M.O., R.A.E. and K.A.H. provided technical assistance. D.-K.R., E.M.P., K.L.N. and J.D.K. wrote the paper. All authors discussed the results and commented on the manuscript.

Author Information *Artemisia annua CYP71AV1* and *CPR* gene sequence information has been deposited in GenBank under accession numbers DQ268763 and DQ318192, respectively. Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare competing financial interests: details accompany the paper at www.nature.com/nature. Correspondence and requests for materials should be addressed to J.D.K. (keasling@berkeley.edu).