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Side Products of Recombinant Amorpha-4,11-diene Synthase and Their Effect on Microbial Artemisinin Production

Jin-Quan Huang,■ Dong-Mei Li,■ Xiu Tian, Jia-Ling Lin, Lei Yang, Jing-Jing Xu, and Xin Fang*

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ABSTRACT: Amorpha-4,11-diene synthase (ADS) is the first committed enzyme in the biosynthesis of artemisinin. Artemisinin production by biobased fermentation is considered a reliable alternative pathway. Heterologously expressed ADS has been established to generate several minor products, including structural analogues of amorpha-4,11-diene, but their fate in fermentation is still unknown. Here, using chiral analysis, we found that ADS produces one of the analogues, amorpha-4-en-11-ol, as a pair of epimers. Labeling experiments revealed that ADS mutants yielded amorphene-type sesquiterpenes, indicating the co-occurrence of initial 1,6 and 1,10 cyclization of farnesyl diphosphate in a single enzyme. Interestingly, the immediate downstream oxidase CYP71AV1 had very low affinity to the side products of the recombinant ADS, including amorpha-4-en-7-ol, which is structurally similar to amorpha-4,11-diene. Our data uncover the complex catalytic mechanism of recombinant ADS and reveal a potential negative effect of the side products of recombinant ADS on the production of the artemisinin precursor in microbes.

KEYWORDS: *artemisinin, chiral analysis, cytochrome P450 monooxygenases, labeling, sesquiterpenes, specialized metabolism pathway, terpene synthases*

■ INTRODUCTION

Artemisia annua L. is a medicinal plant, from which artemisinin, a sesquiterpene lactone, was found as a well-known antimalarial drug against chloroquine-resistant strains of *Plasmodium falciparum*.[1] In 2002, the World Health Organization recommended artemisinin-based combinatorial therapies as the first-line treatment for uncomplicated malaria, which promoted the large-scale cultivation of *A. annua* as a pharmaceutical crop.[2−6] The effect of cultivation conditions and postharvest treatment on artemisinin accumulation have been extensively investigated.[2,3] However, the increasing demand for artemisinin and its low content *in planta* (0.01−1.4% dry weight) have led to the expansion of the cultivated area with *A. annua*.[2−6] Thus, it is essential to find pathways to augment the content of artemisinin in the native plant. Research achievements in the direction of high-artemisinin plant production include the establishment of mutant libraries,[7] construction of a tetraploid cultivar with high artemisinin content,[8] increasing the number of glandular trichomes where artemisinin is biosynthesized and accumulated,[9] overexpression of the transcription factor that positively regulates the artemisinin biosynthetic pathway in transgenic *A. annua* plants,[10,11] and so forth. Nevertheless, not all developed plant lines are trans-generationally stable.[12]

Alternatively, to meet the increasing demand for artemisinin supply, synthetic biological methods have been developed to produce a high-yielding precursor (artemisinic acid) in microorganisms such as *Escherichia coli* and yeasts, which is then chemically converted to artemisinin.[13−17] To date, six genes from *A. annua* have been engineered into yeast to produce artemisinic acid. Amorpha-4,11-diene synthase (ADS), a sesquiterpene synthase, catalyzes the conversion of C-15 farnesyl diphosphate (FDP) into the bicyclic amorpha-4,11-diene;[18−20] then, a cytochrome P450 (CYP71AV1),[21] a cytochrome P450 reductase (CPR1), and a cytochrome b_5 (CYB5) efficiently transform amorpha-4,11-diene into artemisinic alcohol. Alcohol dehydrogenase (ADH1) and aldehyde dehydrogenase (ALDH1) are responsible for the next two oxidation steps to generate artemisinic aldehyde and artemisinic acid, respectively (Figure 1).[17] This production strategy is land-saving and inexpensive and offers more controllable culture conditions as compared to the purification of artemisinin from *A. annua*.

The basic carbon skeleton of artemisinin is constructed by ADS. However, the heterologously expressed ADS yields 91% amorpha-4,11-diene and seven minor products: amorpha-4,7(11)-diene, amorpha-4-en-7-ol, amorpha-4-en-11-ol, (*E*)-*β*-farnesene, *α*-bisabolol, *β*-sesquiphellandrene, and *γ*-humulene.[14,19,22] The structures of amorpha-4,7(11)-diene, amorpha-4-en-7-ol, and amorpha-4-en-11-ol are similar to that of amorpha-4,11-diene. Mechanistically, the removal of a proton at C-12/C-13 (FDP numbering in Figure 1) of an amorpha-11-yl cation gives rise to amorpha-4,11-dieneamorpha-4,11-diene, whereas the extraction of a proton at C-10 (FDP numbering in Figure 1) generates amorpha-4,7(11)-diene, in

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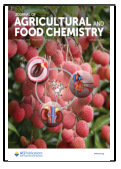

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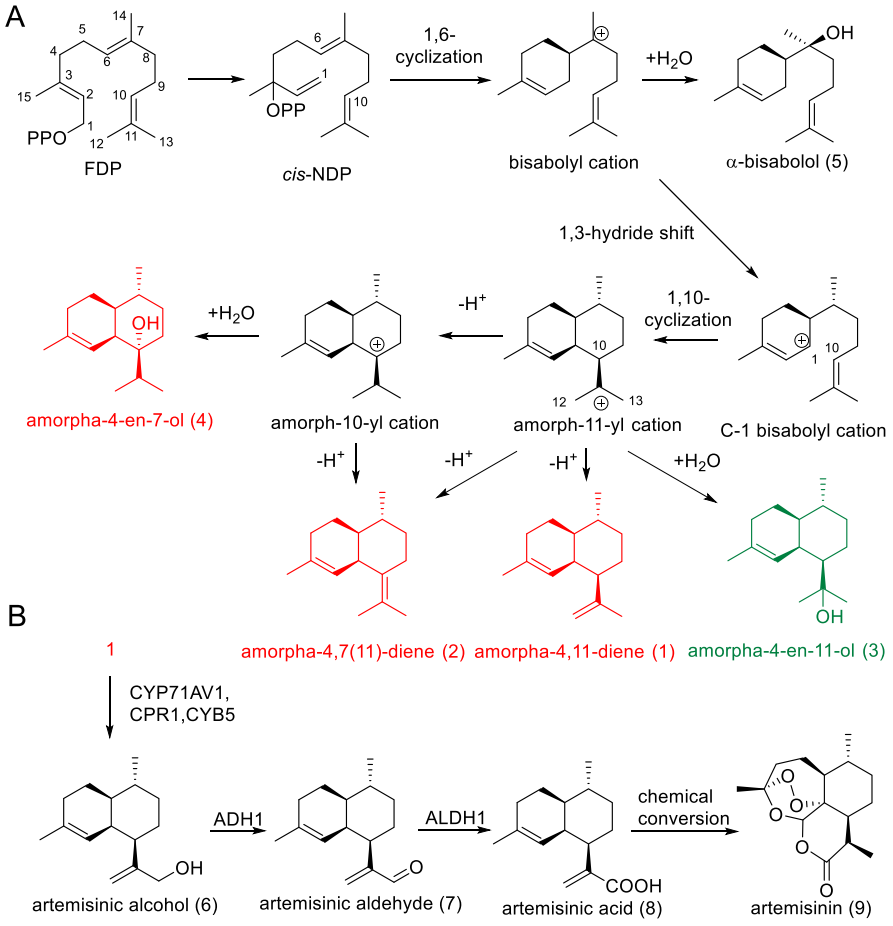

Figure 1. Proposed catalytic mechanism of recombinant ADS (A) and the oxidation of amorpha-4,11-diene to artemisinic acid in yeast and its chemical conversion to artemisinin (B).

addition to amorpha-4-en-11-ol and amorpha-4-en-7-ol, which are direct water capture products of the amorpha-11-yl cation and amorpha-10-yl cation, respectively (Figure 1). The structural similarity of these three compounds to amorpha-4,11-diene might lead to their oxidation by subsequent artemisinin biosynthetic pathway enzymes, producing analogues to artemisinin. However, the fate of these byproducts in fermentation has remained unclear due to their low content.

ADS has attracted considerable research attention as a key enzyme in involved artemisinin biosynthesis, particularly regarding due to its cyclization mechanism of action. The presence of β-sesquiphellandrene and α-bisabolol as side products of ADS supports the intermediacy of the bisabolyl cation in the cyclization mechanism of ADS.[19,22] The deuterium-containing FDP has been confirmed to be instrumental in mechanistic investigations of sesquiterpene synthases, in which both NMR and mass spectrometry (MS) analyses could provide the exact location and quantitative calculations of the incorporated deuterium atom in the production of sesquiterpenes and enable the elucidation of this complex cyclization mechanism.[23] The initial 1,6-ring closure pathway of amorpha-4,11-diene was unambiguously established using deuterium-labeled FDP as a chemical probe.[24,25] However, a quantum chemical study indicated that the germacradienyl cation intermediate derived from initial 1,10 cyclization may also exist as an alternative pathway of ADS catalysis, implying the existence of more complex catalytic mechanisms of ADS action.[26]

We recently established the molecular basis of the catalytic mechanism of ADS by mutagenesis of its catalytic pocket, which generated several mutants producing high amounts of ADS side products.[27,28] Notably, the M446A/T447G/A450E mutant yielded 15.9% amorpha-4,7(11)-diene and 28.4% amorpha-4-en-7-ol, while the T399L/M446A/T447G/A450E mutant produced 36.5% amorpha-4,7(11)-diene and 20.2% amorpha-4-en-7-ol. This research was aimed to determine the complex catalytic mechanism of ADS action and the fate of these amorpha-4,11-diene analogues during fermentation, by fully utilizing the high ratio of these analogues. The findings of this study provide novel insights into the potential production of artemisinin by microbial fermentation in the future.

MATERIALS AND METHODS

Chemicals. (2*Z*,6*E*)-Farnesol, tris (tetrabutylammonium) hydrogen diphosphate, ammonium bicarbonate, Dowex 50WX8 (hydrogen form 100−200 mesh), and manganese dioxide were purchased from Sigma-Aldrich, USA; *s*-collidine and magnesium sulfate were purchased from Tokyo Chemical Industry, Japan; sodium borodeuteride, anhydrous lithium chloride, ammonium hydroxide, methanesulfonyl chloride, deuterium oxide, dry *N*,*N*-dimethylformamide, and dry acetonitrile were obtained from J&K Scientific, China.

Synthesis of Deuterium-Labeled FDPs. The [1,1-^{2}H]FDP was chemically synthesized as previously reported[29] with minor modifications. (2*Z*,6*E*)-Farnesol was oxidized by manganese dioxide in hexane into farnesal. After centrifugation and evaporation, farnesal was solved in deuterium oxide and reduced by sodium borodeuteride to obtain [1-^{2}H]-farnesol. Next, [1,1-^{2}H]-farnesol was generated by

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further oxidation and reduction, which was then transformed into [1,1-^{2}H]-farnesyl chloride by treatment with methanesulfonyl chloride in a mixed solution of dry *N*,*N*-dimethylformamide, anhydrous lithium chloride, and *s*-collidine at 0 °C under N_2 protection. We then performed gas chromatography−MS (GC−MS) analysis for determination of product yield and purity. [1,1-^{2}H]-Farnesyl chloride was dissolved in dry acetonitrile, and tris(tetrabutylammonium) hydrogen diphosphate was added to the reaction mixture at room temperature. After evaporation of the solvent, the resulting salt was dissolved in 25 mM ammonium bicarbonate solution and passed through a Dowex 50WX8 (hydrogen form 100−200 mesh) column. The resultant eluent was freeze-dried and redissolved in methanol and stored at −40 °C until use.

Heterologous Expression of ADS and CYP71AV1. ADS and its mutants were expressed in *E. coli* BL21 (DE3) using a pET32a expression vector, as previously reported.[28] A single colony was inoculated in 2 mL of LB medium containing 0.1 mg/mL of ampicillin and incubated for 5 h at 37 °C; *E. coli* was transferred to 50 mL of LB medium containing 0.1 mg/mL of ampicillin to grow until OD_{600} = 0.6 was reached and induction with 25 μM cedisopropyl-β-D-thiogalactopyranoside at 18 °C. The recombinant proteins obtained were purified with Ni-NTA resin (Qiagen, USA).

The open reading frame of CYP71AV1 was amplified with fast-*Pfu* DNA polymerase. The PCR products were then digested with *BamH*I and *Kpn*I and ligated into the pYeDP60 yeast expression vector. Next, using the lithium acetate method, plasmid DNA was introduced into *Saccharomyces cerevisiae* WAT11 harboring a chromosomal copy of the *Arabidopsis thaliana* cytochrome P450 reductase gene *ATR1*. Yeast growth, induction, and microsome preparation were performed as described earlier.[21,30]

Enzyme Assay. Recombinant ADS and its mutants were assayed in a volume of 450 μL ration buffer (25 mM Hepes (pH 7.0), 5 mM magnesium chloride, 5 mM dithiothreitol) with 40 μM deuterium-labeled or unlabeled FDP. To start the reaction, the purified recombinant enzyme (10 μg) was added, followed by incubation at 30 °C for 4 h and overlaid with hexane (500 μL). The reaction mixture was extracted by vortexing for 10 s and centrifuged at 12,000*g* for 10 min at 4 °C. The hexane part was subjected to GC−MS analysis or subsequent enzymatic oxidation after solvent removal.

For CYP71AV1, a 500 μL reaction buffer containing 25 mM Hepes (pH 7.0), 5 mM magnesium chloride, 5 mM dithiothreitol, and 10 mg of crude extract of microsome proteins was used. Reactions were initiated by adding NADPH and the substrate and were then left to proceed at 30 °C for 12 h. Microsomes from yeast harboring an empty vector were used as controls. The reaction mixture was quenched by the addition of hexane, and the extract was centrifuged at 12,000 g and subjected to GC−MS analysis.

Purification of Side Products of ADS. A large-scale incubation experiment was conducted to obtain a sufficient amount of ADS enzymatic side products. More specifically, 500 mL of reaction buffer containing 25 mM Hepes (pH 7.0), 5 mM magnesium chloride, 5 mM dithiothreitol, 80 μM FDP, and 20 mg of purified protein was used. The reaction mixture was overlaid with 20 mL of HPLC-grade hexane and incubated at 30 °C overnight.[23] Then, the mixture was extracted with 3 × 100 mL of hexane, and the combined extracts were evaporated using a stream of N_2. The residue was separated using a preparative thin layer chromatography (silica) plate using pentane and then 5% EtOAc/hexane as the developing solvent to yield two fractions. Fraction 1 was a mixture of the sesquiterpenes amorpha-4,11-diene and amorpha-4,7(11)-diene, which was, unfortunately, evaporated during the removal of the solvent by the aforementioned N_2 stream. Fraction 2 (2 mg) was obtained and identified as amorpha-4-en-7-ol by GC−MS analysis.

GC−MS Analysis. For GC−MS analysis, we used an Agilent 6890 Series GC system coupled to an Agilent 5973 Network Mass Selective detector. Helium at 1 mL/min was employed as the carrier gas. Splitless injection and an Agilent HP-5MS column (5% phenyl methyl siloxane; with a length of 30.0 m, a diameter of 250.00 μm, and a film thickness of 0.25 μm) were used. The hexane that contained the products was subjected to analysis by the following temperature program: an initial temperature of 60 °C (5 min hold), followed by an increase to 270 °C at 10 °C/min. Compounds were identified by comparisons of their mass spectral data with reference spectra in databases, including NIST (National Institute of Standards and Technology, Gaithersburg, MD, USA) and Wiley Online Library. The retention indices (RIs) of each peak were then calculated on the basis of the standard alkane mixture (C_{11}−C_{18}) and compared with those of reference compounds.

Enantiomeric analysis was performed using a chiral FS-hydrodex β-6TBDM capillary GC column. The temperature program started from 45 °C for 4 min, followed by ramping up by 2 °C until a final oven temperature of 180 °C was reached.[31]

RESULTS

Recombinant ADS Produces Amorpha-4-en-11-ol as a Pair of Diastereomers. Except for the major and predominant product, amorpha-4,11-diene,[22] the stereochemistry of other products of recombinant ADS has not been elucidated and hypothetically they may share the same stereochemistry with amorpha-4,11-diene. To further understand ADS activities, enantiomeric analysis by chiral GC was performed on the enzymatic products of a tetrasubstitution ADS mutant (T399L/M446A/T447G/A450E), which produces a high amount of byproducts.[28] Commercial (−)-α-bisabolol standard was added as a reference to verify the chiral GC method.

The chiral analysis resulted in a clear separation of (−)-α-bisabolol from α-bisabolol produced by the ADS mutant, which was proposed to be (+)-epi-α-bisabolol.[22] This result was similar to that of a previously reported chiral separation of (−)-α-bisabolol and (+)-epi-α-bisabolol[32] and confirmed the effectiveness of the chiral capillary GC method in differentiating diastereomers. All products of ADS, except for amorpha-4-en-11-ol,[22] exhibited a single peak in the chiral GC analysis, which supports the notion that they are optically pure. Strikingly, amorpha-4-en-11-ol yielded a double peak with a similar MS spectrum (Figure 2). This result strongly suggests that ADS catalyzed the formation of a pair of diastereomers of amorpha-4-en-11-ol, which could produce an MS spectrum with slight differences, as exemplified by cubenol and 1-*epi*-cubenol.[33] Next, we aimed to elucidate the exact stereochemistry of this pair of diastereomers. Previous reports showed that sesquiterpene synthases generated diastereomers, for example, TPS4 and TPS5, from different *Zea mays* cultivars, which yielded sesquithujene and 7-*epi*-sesquithujene, a pair of epimers, but with different ratios.[34] Amorpha-4-en-11-ol possesses four chiral centers with 16 possible stereoisomers, and a pair of epimers of amorpha-4-en-11-ol could be formed during cyclization.

Amorpha-4-en-11-ol is a minor product of both ADS and its mutant enzymes. Preparative-scale incubation was carried out to obtain sufficient material for chromatographic fractionations and NMR analyses. However, the extraction of the reaction mixture with hexane and the further purification by silica gel chromatography yielded only 2 mg of sesquiterpene alcohol that was confirmed to be amorpha-4-en-7-ol. The other sesquiterpene hydrocarbons were, unfortunately, evaporated by the N_2 stream used in the process of solvent removal. We, therefore, continued with an examination of the ring closure mechanism of the two isomers of amorpha-4-en-11-ol to determine the stereochemistry of this pair of diastereomers.

We used deuterium-labeled FDP as a substrate to investigate the cyclization mechanism of amorpha-4-en-11-ol. We supposed that if amorpha-4-en-11-ol shares the same

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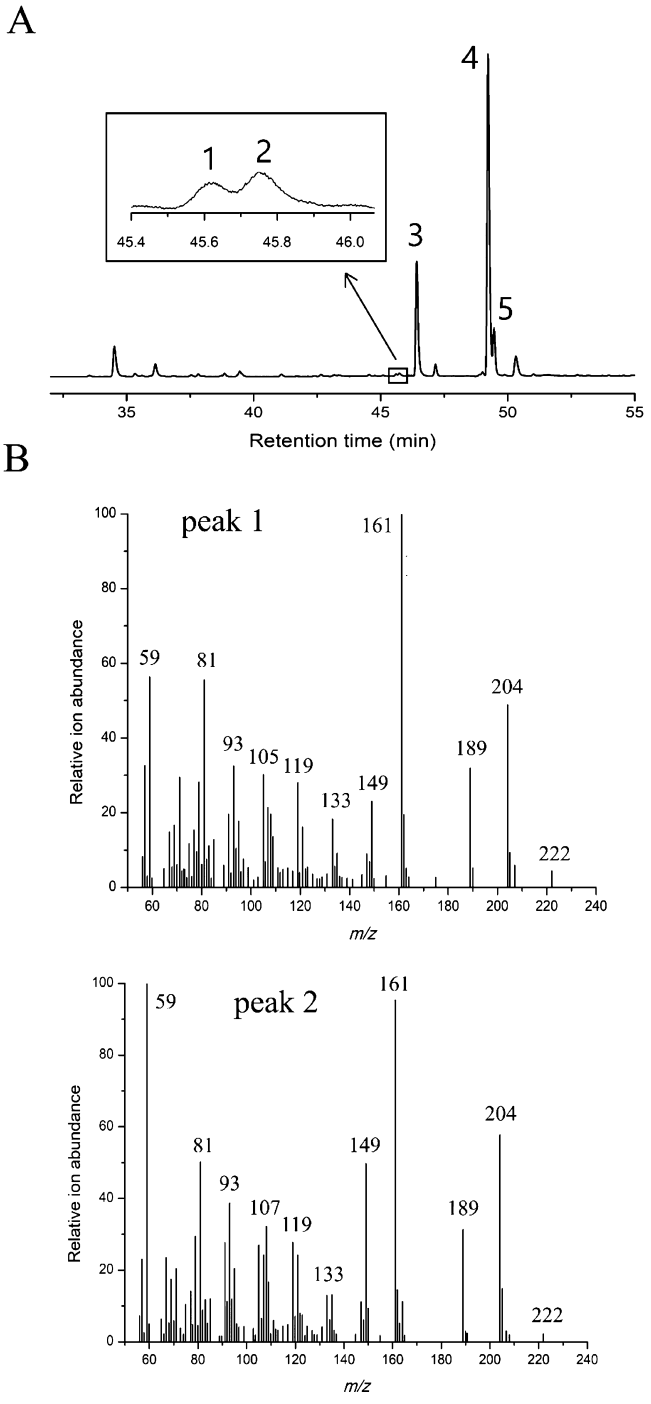

Figure 2. Chiral analysis of amorpha-4-en-11-ol produced by recombinant ADS. (A) Chiral separation of amorpha-4-en-11-ol into two peaks 1 and 2. Peaks numbered with 3, 4, and 5 represent amorpha-4-en-7-ol, (−)-α-bisabolol, and α-bisabolol produced by recombinant ADS, respectively; (B) mass spectra of peaks 1 and 2.

cyclization mechanism with amorpha-4,11-diene, the deuterium positions of the labeled amorpha-4-en-11-ol should be identical to those of the labeled amorpha-4,11-diene, which could be determined by ^{1}H and ^{2}H NMR at C-6 and C-10 (amorpha-4,11-diene numbering, Figure 1), two of the four chiral centers.[24,25] The fragment peaks of these two centers could provide details of their stereochemistry.

The ADS M446A/T447G/A450E mutant produces a high amount of aberrant proton extraction products, and its turnover rate is close to that of the wild-type recombinant ADS.[28] Incubation of this mutant enzyme with [1,1-^{2}H]-FDP yielded amorpha-4-en-11-ol labeled with deuterium atoms, which shows a molecular ion at m/z 224, comparable to an unlabeled molecular ion at m/z 222, implying that both deuterium atoms were retained in the product (Figure 3). Previously, the sites of deuterium atoms were elucidated through analysis of the fragment peaks of labeled and unlabeled amorpha-4,11-diene.[25] The peaks at m/z 94 and 93 of labeled and unlabeled amorpha-4,11-diene formed from breaking of C-6/C-7 and C1/C10 bonds (amorpha-4,11-diene numbering, Figure 3). This outcome suggests the location of one deuterium atom at C-6 and the peaks at m/z 123 and 121, derived from the cleavage of both C-6/C-7 and C-9/C-10 bonds (amorpha-4,11-diene numbering, Figure 3) of the labeled and unlabeled amorpha-4,11-diene, which indicates the position of another deuterium atom at C-10.[25] Notably, all these peaks were present in the labeled and unlabeled amorpha-4-en-11-ol (Figures 3 and 4), suggesting that the two labeled deuterium atoms were also anchored at C-6 and C-10.

To obtain information on the stereochemistry of these two deuterium atoms, we analyzed the fragment peaks. The most notable difference in the mass spectra of the labeled and unlabeled amorpha-4-en-11-ol is that the latter yielded a strong M-H_2O peak at m/z 204, whereas the former at 205 (Figures 3 and 4), suggesting the loss of one labeled atom occurred during dehydration. To yield this fragment, the hydroxyl group at either H-6 or H-10 should have shared the same direction and should have been in proximity in the epimers. We analyzed all four pairs of epimers, namely, 1(R/S)-amorpha-4-en-11-ol, 10(R/S)-amorpha-4-en-11-ol, 6(R/S)-amorpha-4-en-11-ol, and 7(R/S)-amorpha-4-en-11-ol, by molecular modeling and found that only the deuterium-labeled 6(R)-amorpha-4-en-11-ol and 6(S)-amorpha-4-en-11-ol could generate the desired M-H_2O peak at m/z 205 since the hydroxyl group in both molecules is close to the deuterium at C-10. Thus, this pair of epimers was tentatively assigned as 6(R/S)-amorpha-4-en-11-ol.

ADS Mutant Could Yield Amorphene-type Compounds through Initial 1,10 Cyclization of FDP. To date, no amorphene-type side products have been detected in an ADS enzymatic reaction.[19,22] However, a quantum chemical study proposed that both amorphene and amorphadiene types of sesquiterpenes could be produced by a single enzyme.[26]

Mechanistically, compounds of the amorphene and amorphadiene types are formed by initial 1,10 and 1,6 cyclization of FDP, respectively, which could be distinguished by labeling experiments.[33,35] Both amorphenes and amorphadienes contain an isopropenyl moiety that produces a peak at m/z 161 as the isopropyl group is fragmented from the core structure. Using [1,1-^{2}H]-FDP as a substrate, a deuterium atom is labeled at the isopropenyl group of amorphenes based on its cyclization mechanism, which indices the characteristic m/z 162 peak in the MS spectrum. On the other hand, amorphadienes retain both deuterium atoms at the bicyclic molecule core and generate an ion peak at m/z 163.

We incubated ADS and its mutants with [1,1-^{2}H]-FDP and analyzed the GC−MS data. A trace amount of the minor product yielded by the ADS Q518L mutant showed a strong ion peak at m/z 162 (Figure 5), and its unlabeled counterpart had an MS spectrum similar to that of β-copaene, based on NIST database searches; its RI (1430) also matched with that

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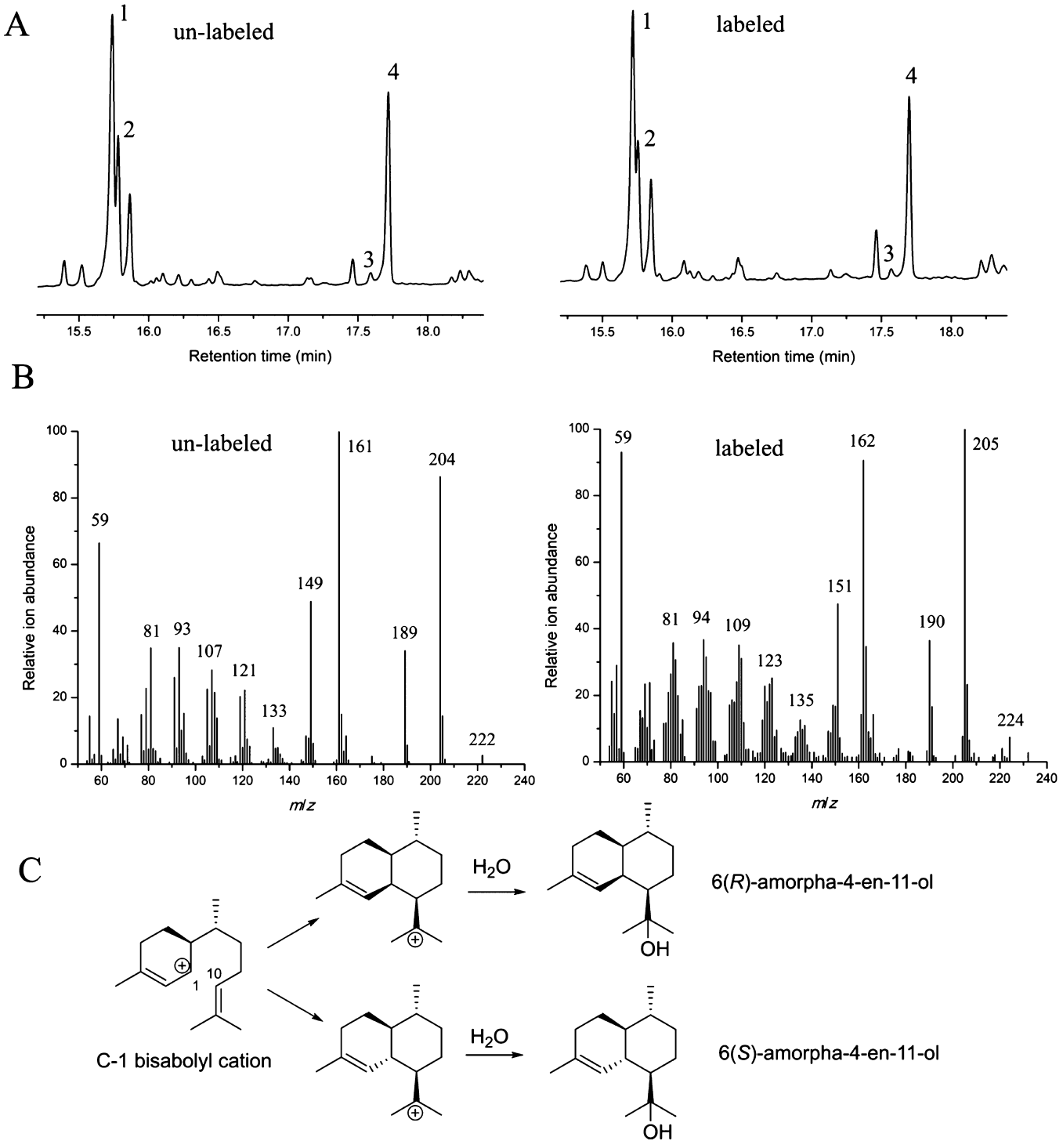

Figure 3. Labeling experiment reveals that recombinant ADS catalyzes FDP into a pair of epimers, 6(*R*/*S*)-amorpha-4-en-11-ol (tentatively). (A) GC−MS traces of products formed from FDP and [1,1-^{2}H]-FDP by the ADS M446A/T447G/A450E mutant. For peak numbers (compounds), see Figure 1; (B) mass spectra of amorpha-4-en-11-ol generated from FDP and [1,1-^{2}H]-FDP; and (C) proposed biosynthesis of 6(*R*/*S*)-amorpha-4-en-11-ol.

of the reference (1430).[33] Therefore, the ADS Q518L mutant produced both amorphene- and amorphadiene-type sesquiterpenes.

CYP71AV1 Shows Affinity to Amorpha-4,11-diene as a Substrate. CYP71AV1 catalyzes the oxidation steps of amorpha-4,11-diene. The specificity of CYP71AV1 has been evaluated using several sesquiterpenes,[21] yet its activity toward the side products of recombinant ADS, including amorpha-4,7(11)-diene, amorpha-4-en-7-ol, and amorpha-4-en-11-ol, which are structurally similar to amorpha-4,11-diene, has not been examined. The low ratio of these side products in the ADS enzymatic assay could have hindered the detection of this type of CYP71AV1 activities.

The ADS M446A/T447G/A450E and T399L/M446A/T447G/A450E mutants produced high amounts of amorpha-4,7(11)-diene and amorpha-4-en-7-ol,[28] which provided an

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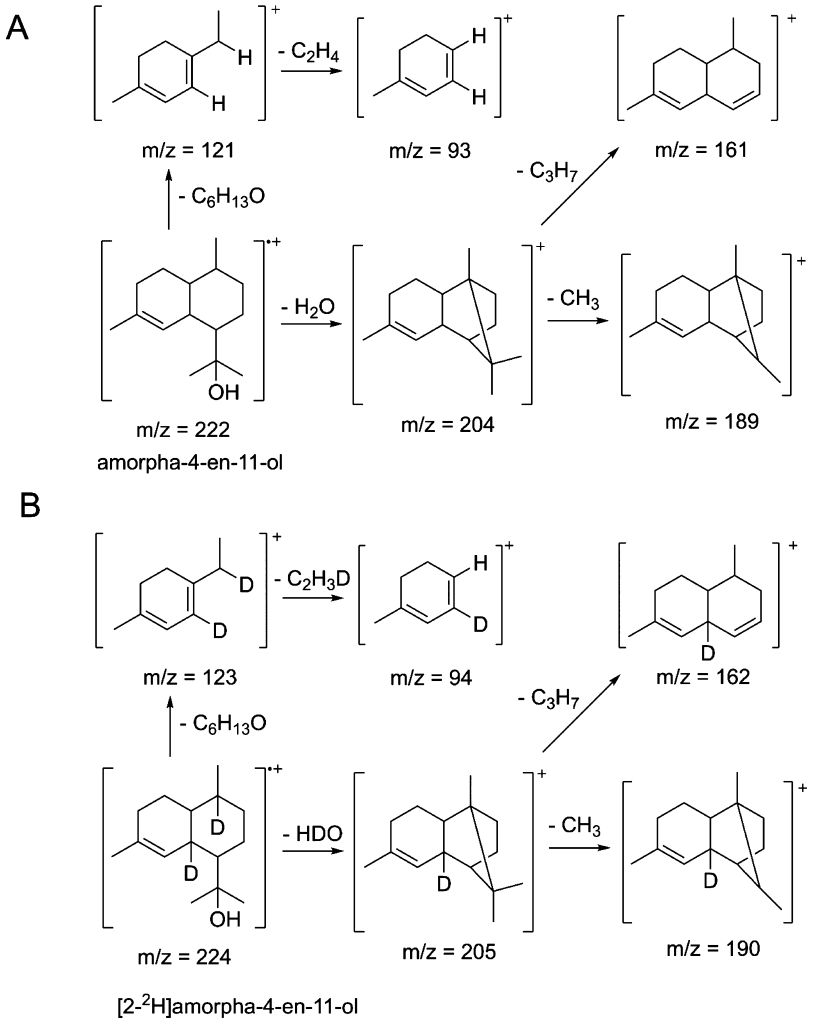

Figure 4. Proposed fragmentation schemes. (A) Amorpha-4-en-11-ol and (B) deuterium-labeled amorpha-4-en-11-ol.

opportunity to evaluate the activity of CYP71AV1 toward these byproducts. We detected no other oxidized products except for artemisinic alcohol (Figure 6B,C). Because the kinetic parameters could not be obtained in the assay,[36] the current results suggest a very low turnover rate or affinity of CYP71AV1 to these side products. Thus, of the possible substrates evaluated in these experiments, amorpha-4,11-diene was preferred. Furthermore, we evaluated the activity of CYP71AV1 toward our isolated pure amorpha-4-en-7-ol. However, in contrast to the microsomes from yeast harboring the empty vector, CYP71AV1-containing microsomes could not consume the substrate (Figure 6D,E), and no hydroxylation products of amorpha-4-en-7-ol were detected. Thus, we concluded that CYP71AV1 showed negligible activities toward the side product amorpha-4-en-7-ol, which is structurally different from amorpha-4,11-diene only in terms of the presence of a hydroxyl group and the absence of a double bond. This result indicated the high specificity of CYP71AV1 to amorpha-4,11-diene. To verify the effectiveness of the experiment, CYP71AV1-containing microsomes were incubated with the products generated by recombinant ADS as a positive control, which converted amorpha-4,11-diene into artemisinic alcohol (Figure 6A).

DISCUSSION

Field production of *A. annua* remains the main commercial source of artemisinin. Nevertheless, microbial systems have been engineered to produce artemisinic acid efficiently, which is considered a successful example of producing valuable natural products by a synthetic biological approach. ADS is responsible for the initial branching step in the biosynthetic pathway of artemisinin, which catalyzes the formation of amorpha-4,11-diene. However, the heterologously expressed ADS in *E. coli* could generate several minor products in addition to the obtained 91% of amorpha-4,11-diene, among which amorpha-4,7(11)-diene, amorpha-4-en-7-ol, and amorpha-4-en-11-ol are structurally analogous to amorpha-4,11-diene. Thus, it might be possible that enzymes participating in subsequent reactions could use these analogues to finally produce artemisinin derivatives. As these compounds are present in low concentrations during fermentation, their fate escaped our attention in previous studies. Here, we used our recently generated ADS mutants, which produced a high amount of amorpha-4,7(11)-diene and amorpha-4-en-7-ol, to investigate whether these compounds could be used as substrates of CYP71AV1. Our results showed that CYP71AV1 had negligible activities toward these byproducts, and they were volatilized from the aqueous medium during fermentation as was the case with amorpha-4,11-diene.[14]

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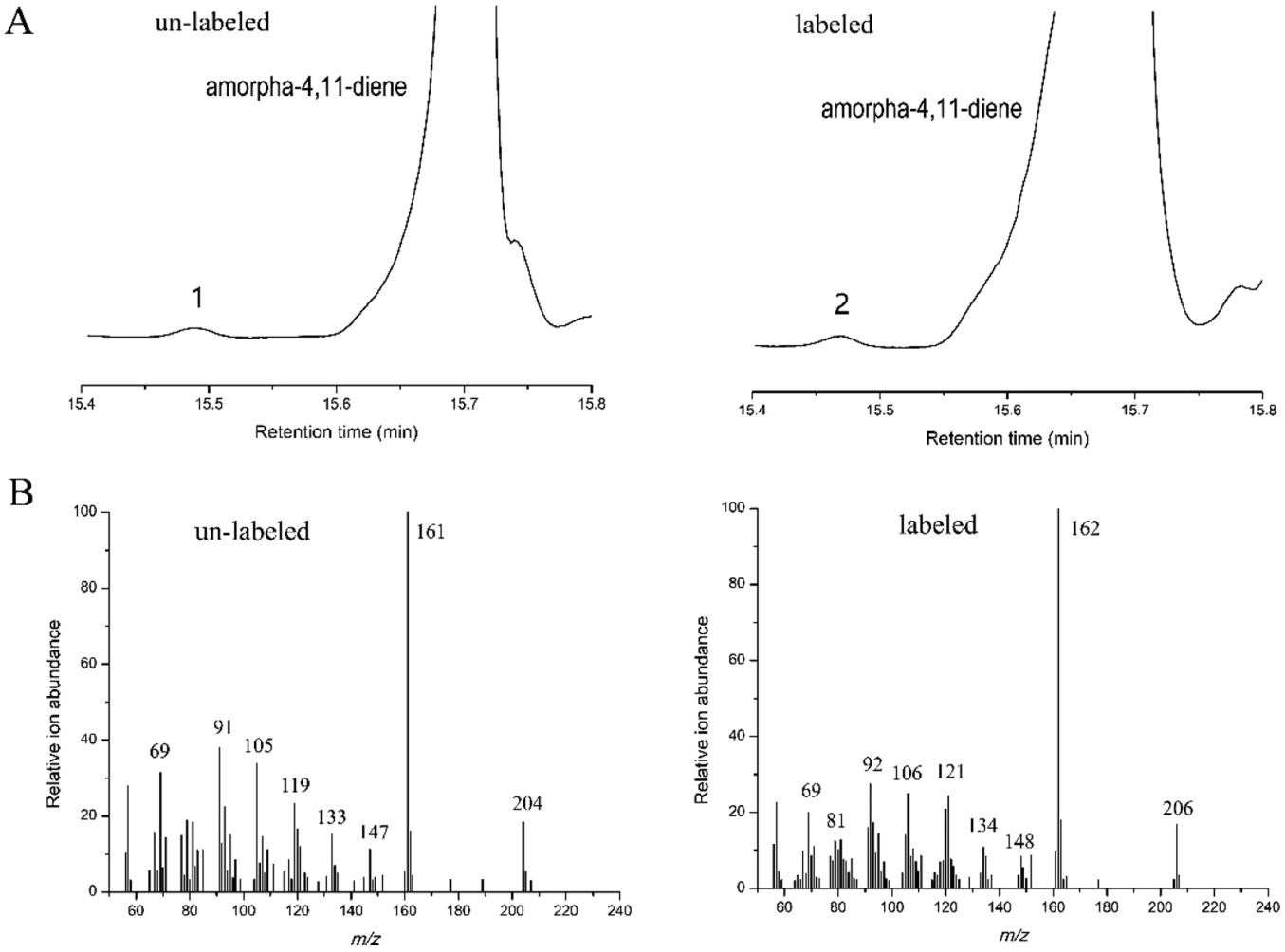

Figure 5. ADS Q518L mutant yielded amorphene-type sesquiterpene. (A) GC−MS traces of products formed from FDP and [1,1-^{2}H]-FDP by the ADS Q518L mutant, which shows an additional minor product at peaks 1 and 2. The huge peak is amorpha-4,11-diene. (B) Mass spectra of amorphene-type sesquiterpene generated from FDP and [1,1-^{2}H]-FDP.

Although our results excluded the possible impurities introduced by these side products, their occurrence reduced the yield of artemisinin. Thus, it is important to improve the fidelity of heterologously expressed ADS for more effective production of the artemisinin precursor by fermentation, which is a long-overlooked issue as research has been focused mainly on increasing the yield of amorpha-4,11-diene in *E. coli* cultures.[37−39]

To address this research gap, an in-depth understanding of the promiscuous activity of ADS, or more generally, of sesquiterpene synthases activities, is necessary. ADS represents a type of sesquiterpene synthases that generate dominant products as intermediates in the biosynthesis of defensive natural products (often phytoalexins). For example, 5-*epi*-aristolochene synthase[40] in tobacco and δ-cadinene synthase[41] in cotton produce 79.8% of the 5-*epi*-aristolochene and 100% of the δ-cadinene needed for the biosynthesis of capsidiol and gossypol, respectively. By contrast, another type of sesquiterpene synthase was found to yield a number of fragrance or resin products, such as δ-selinene synthase and γ-humulene synthase from conifer, yielding 34 and 52 products as principal components of conifer resin, respectively.[42] From a mechanistic point of view, sesquiterpene synthase provides a template for a cyclization cascade. Thus, the active pocket of a specific enzyme is relatively small and could precisely control the reaction cascade, resulting in the generation of one product. On the other hand, a promiscuous enzyme, which has a much larger pocket, accommodated multiple substrates or intermediate conformations, resulting in the formation of a larger number of products.[43,44] The tightly designed active pocket of a high-fidelity synthase such as Cop6 from *Coprinus cinereus* ensured its product specificity under different reaction conditions.[44] However, the product profile of a promiscuous sesquiterpene synthase varies according to the conditions of its environment. For example, Cop4 from *C. cinereus* showed strikingly distinct product profiles at different temperatures, pH values, and binding metal ions.[44] Similarly, 5-*epi*-aristolochene synthase produced 87.6 and 66.2% of 5-*epi*-aristolochene at 0 and 42 °C, respectively.[40] The replacement of Mg^{2+} with Mn^{2+} or Co^{2+} as the divalent metal ion in ADS significantly increased the production of amorpha-4,11-diene, probably due to the larger size of Mn^{2+} and Co^{2+}, which forced a tighter fitting of the substrate or intermediate into the active pocket of ADS.[22] Therefore, designing a smaller and more tightly controlled active pocket of ASD by protein engineering may be a reliable approach to improve ADS product specificity.

An earlier *in vitro* experiment showed that the specificity of ADS varied according to its reaction condition.[22] Therefore,

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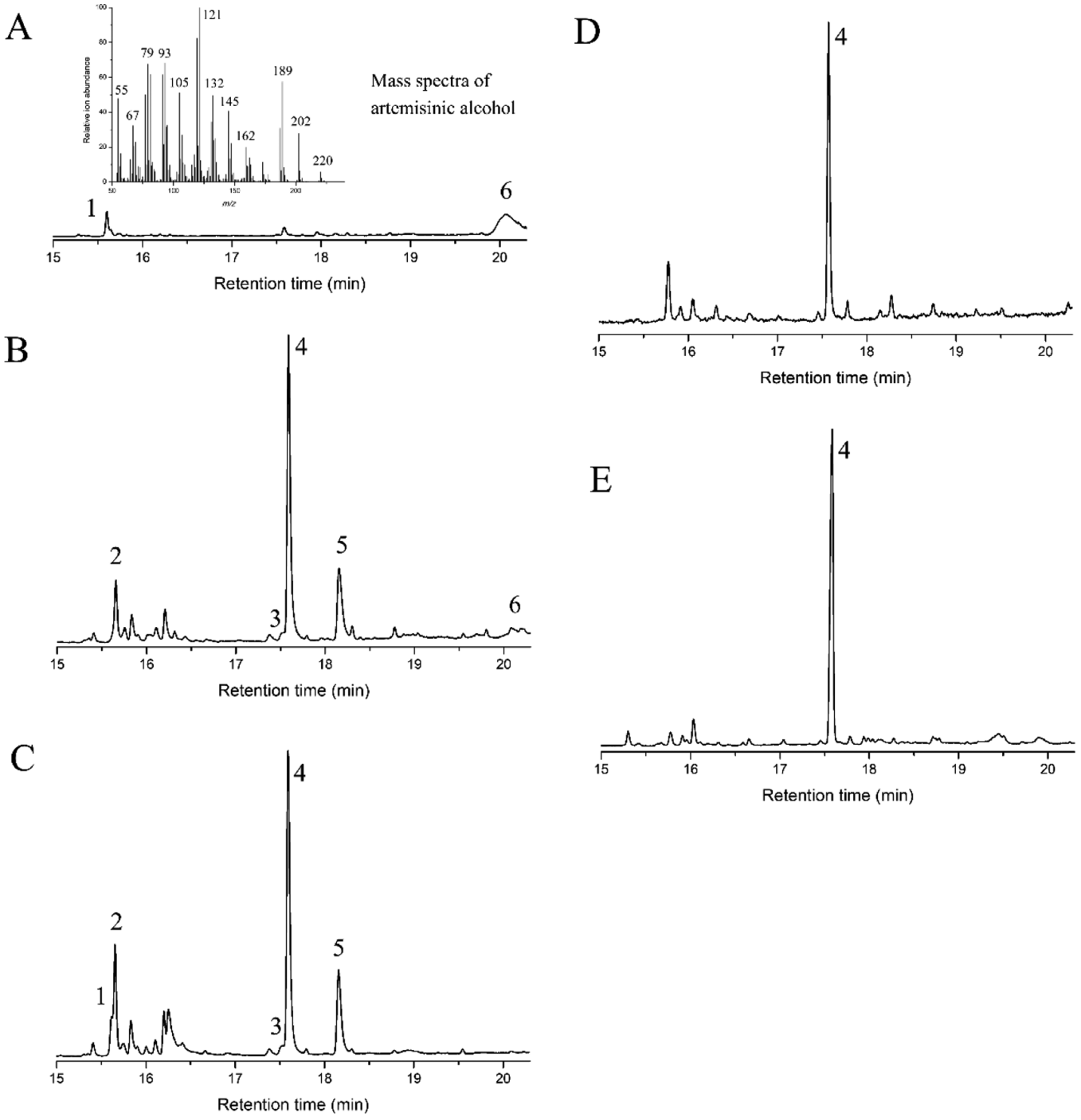

Figure 6. CYP71AV1 has affinity to amorpha-4,11-diene as a substrate. GC−MS traces of products: (A) recombinant ADS and CYP71AV1-containing microsomes, and MS spectra of peak 6, which is identical to that of artemisinic alcohol ref 60; (B) ADS T399L/M446A/T447G/A450E mutant and CYP71AV1-containing microsomes; (C) ADS T399L/M446A/T447G/A450E mutant and microsomes from the yeast harboring the empty vector; (D) amorpha-4-en-7-ol and CYP71AV1-containing microsomes; and (E) amorpha-4-en-7-ol and microsomes from the yeast harboring the empty vector. For peak numbers 1−4 (compounds), see Figure 1. Peaks numbered with 5 and 6 denote α-bisabolol and artemisinic alcohol, respectively.

the host environment may affect the activity of ADS during fermentation. *E. coli* and yeasts are hosts used mainly for microbial production of the precursor of artemisinin, whereas other organisms have been used for amorpha-4,11-diene generation, including *Streptomyces avermitilis*,[45] *Bacillus subtilis*,[46] *Synechococcus elongatus* PCC 7942,[47] *Rhodobacter sphaeroides*,[48] and *Aspergillus nidulans*.[49] However, it is still unclear whether the expression of ADS in these hosts, except for *E. coli* and *A. nidulans*, also induces the production of similar side products. The yield of amorpha-4,11-diene in *E. coli* cultures was 89%,[14] which is close to the reported 91%[22] achieved by an *in vitro* enzymatic reaction. The expression of ADS in *A. nidulans* produces only small amounts of amorpha-4,11-diene but high quantities of linear and monocyclic sesquiterpenes. However, the recombinant ADS expressed in *Nicotiana benthamiana* produces a single byproduct, amorpha-

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4,7(11)-diene (3%), in an *in vitro* enzymatic reaction.[50] Clearly, the expression of ADS in different hosts manifests distinct product specificity, which suggests that the host environment could significantly impact the product profile, which is in agreement with the promiscuity nature of ADS. Additionally, the proteins expressed in bacterial systems may differ from those eukaryotic cells in terms of protein folding and post-translational modifications, possibly leading to functional differences. Probably, the ADS expressed in *E. coli* has a larger active site pocket than that in plants, which allows FDP folding in multiple conformations to yield such side products. Thus, careful selection of organisms is another important approach to be considered for the improvement of ADS specificity.

Translational fusion of thioredoxin to a sesquiterpene synthase ensured the proper folding and stability of enzymes.[51] Fusion tags or fusion proteins have been previously used to improve the heterologous expression of ADS in host microorganisms,[46,52] In certain cases, fusion tags or proteins in a sesquiterpene synthase could distort the overall structure of the enzyme and may affect its proper function.[51] Considering the promiscuity nature of ADS, tag fusion may be more likely to influence its performance and thus should be carefully used.

The cyclization mechanism of ADS has been thoroughly investigated by different methods, including deuterium-labeled chemical probe[24,25] and mutant enzymes.[27,28,53−55] Here, we found that one of the side products of ADS, amorpha-4-en-11-ol, is a pair of epimers, probably due to the attack of the C-1 bisabolyl cation on the C-10/C-11 double bond (FDP numbering, Figure 3C) from the up and down sides during the second ring closure. We propose that amorpha-4-en-11-ol is generated through a deviation from the amorpha-4,11-diene cyclization pathway in the C-1 bisabolyl cation, rather than from the amorph-11-yl cation (Figure 3C). In fact, sesquiterpene synthesis was reported to generate the same product through different cyclization pathways, such as recombinant patchoulol synthase that produces patchouli alcohol, through two distinct cyclization intermediates in two subpockets.[23] Natural products may harbor more than one chiral center to exist as stereoisomers, which frequently occurs in terpenoids[56] and alkaloids.[57] Generally, one enzyme produces one type of a stereoisomer, such as *Arabidopsis* TPS10 and TPS14 which yield (−)-(3*R*)-linalool and (+)-(3*S*)-linalool, respectively.[58] Our findings represent a rare case in which one enzyme generates a pair of epimers as products. Furthermore, the ADS Q518L mutant produced both amorphene- and amorphadiene-type sesquiterpenes, implying that ADS may recruit both initial 1,6 and 1,10 cyclization mechanisms to produce amorpha-4,11-diene, which is consistent with the results of a previous quantum chemical study.[26] The present findings further reveal the catalytic promiscuity of ADS.

Among our previously generated mutant ADS[27,28,] and those produced by other researchers,[53−55] only the M446A/T447G/A450E and T399L/M446A/T447G/A450E mutants produced high quantities of amorpha-4,7(11)-diene and amorpha-4-en-7-ol that could be used for evaluations of CYP71AV1 substrate specificity. Interestingly, these two mutants have impaired functions in regioselective deprotonation, which is similar to the case with mutated taxadiene synthase with shifted deprotonation product profile including more taxa-4(20)-11(12)-diene.[59]

AUTHOR INFORMATION

Corresponding Author

Xin Fang − *State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, P. R. China*; orcid.org/0000-0002-8561-1518; Phone: +86-871-65223070; Email: xinfang@mail.kib.ac.cn

Authors

Jin-Quan Huang − *Yunnan University, Kunming 650091, P. R. China; National Key Laboratory of Plant Molecular Genetics, Institute of Plant Physiology and Ecology/CAS Center for Excellence in Molecular Plant Sciences, Chinese Academy of Sciences, Shanghai 200032, P. R. China*

Dong-Mei Li − *Yunnan University, Kunming 650091, P. R. China; State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, P. R. China*

Xiu Tian − *National Key Laboratory of Plant Molecular Genetics, Institute of Plant Physiology and Ecology/CAS Center for Excellence in Molecular Plant Sciences, Chinese Academy of Sciences, Shanghai 200032, P. R. China*

Jia-Ling Lin − *National Key Laboratory of Plant Molecular Genetics, Institute of Plant Physiology and Ecology/CAS Center for Excellence in Molecular Plant Sciences, Chinese Academy of Sciences, Shanghai 200032, P. R. China; School of Life Science and Technology, ShanghaiTech Universit, Shanghai 200031, P. R. China*

Lei Yang − *Shanghai Key Laboratory of Plant Functional Genomics and Resources, Plant Science Research Center, Shanghai Chenshan Botanical Garden, Shanghai 201602, P. R. China*

Jing-Jing Xu − *Shanghai Key Laboratory of Plant Functional Genomics and Resources, Plant Science Research Center, Shanghai Chenshan Botanical Garden, Shanghai 201602, P. R. China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jafc.0c07462

Author Contributions

■J.-Q.H. and D.-M.L. contributed equally to this study.

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Notes

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■ ABBREVIATIONS

GC−MS, gas chromatography−mass spectrometry; NMR, nuclear magnetic resonance; *m/z*, mass-to-charge ratio; (*R*/*S*), Cahn−Ingold−Prelog chemical configuration

■ REFERENCES

(1) Klayman, D. Qinghaosu (Artemisinin) − an antimalarial drug from China. *Science* **1985**, *228*, 1049−1055.
(2) Ferreira, J. F. S. Nutrient deficiency in the production of artemisinin, dihydroartemisinic acid, and artemisinic acid in Artemisia annua L. *J. Agric. Food Chem.* **2007**, *55*, 1686−1694.
(3) Ferreira, J. F. S.; Luthria, D. L. Drying affects artemisinin, dihydroartemisinic acid, artemisinic acid, and the antioxidant capacity of Artemisia annua L. leaves. *J. Agric. Food Chem.* **2010**, *58*, 1691−1698.
(4) Jessing, K. K.; Juhler, R. K.; Strobel, B. W. Monitoring of artemisinin, dihydroartemisinin, and artemether in environmental matrices using high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS). *J. Agric. Food Chem.* **2011**, *59*, 11735−11743.
(5) Ding, F.; Ma, T.; Hao, M.; Wang, Q.; Chen, S.; Wang, D.; Huang, L.; Zhang, X.; Jiang, D. Mapping worldwide environmental suitability for Artemisia annua L. *Sustainability* **2020**, *12*, 1309.
(6) Ikram, N. K. B. K.; Simonsen, H. T. A review of biotechnological artemisinin production in plant. *Front. Plant Sci.* **2017**, *8*, 1966.
(7) Pandey, N.; Meena, R. P.; Rai, S. K.; Pandey-Rai, S. In vitro generation of high artemisinin yielding salt tolerant somaclonal variant and development of SCAR marker in Artemisia annua L. *Plant Cell, Tissue Organ Cult.* **2016**, *127*, 301−314.
(8) Banyai, W.; Sangthong, R.; Karaket, N.; Inthima, P.; Mii, M.; Supaibulwatana, K. Overproduction of artemisinin in tetraploid Artemisia annua L. *Plant Biotechnol. Nar* **2010**, *27*, 427−433.
(9) Singh, N. D.; Kumar, S.; Daniell, H. Expression of β-glucosidase increases trichome density and artemisinin content in transgenic Artemisia annua plants. *Plant Biotechnol. J.* **2016**, *14*, 1034−1045.
(10) Yu, Z.-X.; Li, J.-X.; Yang, C.-Q.; Hu, W.-L.; Wang, L.-J.; Chen, X.-Y. The jasmonate-responsive AP2/ERF transcription factors AaERF1 and AaERF2 positively regulate artemisinin biosynthesis in Artemisia annua L. *Mol. Plant* **2012**, *5*, 353−365.
(11) Lu, X.; Zhang, L.; Zhang, F.; Jiang, W.; Shen, Q.; Zhang, L.; Lv, Z.; Wang, G.; Tang, K. AaORA, a trichome-specific AP2/ERF transcription factor of Artemisia annua, is a positive regulator in the artemisinin biosynthetic pathway and in disease resistance to Botrytis cinerea. *New Phytol.* **2013**, *198*, 1191−1202.
(12) Delabays, N.; Simonnet, X.; Gaudin, M. The genetics of artemisinin content in Artemisia annua L. and the breeding of high yielding cultivars. *Curr. Med. Chem.* **2001**, *8*, 1795−1801.
(13) Martin, V. J. J.; Pitera, D. J.; Withers, S. T.; Newman, J. D.; Keasling, J. D. Engineering a mevalonate pathway in Escherichia coli for production of terpenoids. *Nat. Biotechnol.* **2003**, *21*, 796−802.
(14) Newman, J. D.; Marshall, J.; Chang, M.; Nowroozi, F.; Paradise, E.; Pitera, D.; Newman, K. L.; Keasling, J. D. High-level production of amorpha-4,11-diene in a two-phase partitioning bioreactor of metabolically engineered Escherichia coli. *Biotechnol. Bioeng.* **2006**, *95*, 684−691.
(15) Ro, D.-K.; Paradise, E. M.; Ouellet, M.; Fisher, K. J.; Newman, K. L.; Ndungu, J. M.; Ho, K. A.; Eachus, R. A.; Ham, T. S.; Kirby, J.; Chang, M. C. Y.; Withers, S. T.; Shiba, Y.; Sarpong, R.; Keasling, J. D. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* **2006**, *440*, 940−943.
(16) Westfall, P. J.; Pitera, D. J.; Lenihan, J. R.; Eng, D.; Woolard, F. X.; Regentin, R.; Horning, T.; Tsuruta, H.; Melis, D. J.; Owens, A.; Fickes, S.; Diola, D.; Benjamin, K. R.; Keasling, J. D.; Leavell, M. D.; McPhee, D. J.; Renninger, N. S.; Newman, J. D.; Paddon, C. J. Production of amorphadiene in yeast, and its conversion to dihydroartemisinic acid, precursor to the antimalarial agent artemisinin. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, E111−E118.
(17) Paddon, C. J.; Westfall, P. J.; Pitera, D. J.; Benjamin, K.; Fisher, K.; McPhee, D.; Leavell, M. D.; Tai, A.; Main, A.; Eng, D.; Polichuk, D. R.; Teoh, K. H.; Reed, D. W.; Treynor, T.; Lenihan, J.; Jiang, H.; Fleck, M.; Bajad, S.; Dang, G.; Dengrove, D.; Diola, D.; Dorin, G.; Ellens, K. W.; Fickes, S.; Galazzo, J.; Gaucher, S. P.; Geistlinger, T.; Henry, R.; Hepp, M.; Horning, T.; Iqbal, T.; Kizer, L.; Lieu, B.; Melis, D.; Moss, N.; Regentin, R.; Secrest, S.; Tsuruta, H.; Vazquez, R.; Westblade, L. F.; Xu, L.; Yu, M.; Zhang, Y.; Zhao, L.; Lievense, J.; Covello, P. S.; Keasling, J. D.; Reiling, K. K.; Renninger, N. S.; Newman, J. D. High-level semi-synthetic production of the potent antimalarial artemisinin. *Nature* **2013**, *496*, 528−532.
(18) Bouwmeester, H. J.; Wallaart, T. E.; Janssen, M. H. A.; van Loo, B.; Jansen, B. J. M.; Posthumus, M. A.; Schmidt, C. O.; De Kraker, J.-W.; König, W. A.; Franssen, M. C. R. Amorpha-4,11-diene synthase catalyses the first probable step in artemisinin biosynthesis. *Phytochemistry* **1999**, *52*, 843−854.
(19) Mercke, P.; Bengtsson, M.; Bouwmeester, H. J.; Posthumus, M. A.; Brodelius, P. E. Molecular cloning, expression, and characterization of amorpha-4,11-diene synthase, a key enzyme of artemisinin biosynthesis in Artemisia annua L. *Arch. Biochem. Biophys.* **2000**, *381*, 173−180.
(20) Chang, Y.-J.; Song, S.-H.; Park, S.-H.; Kim, S.-U. Amorpha-4,11-diene synthase of Artemisia annua: cDNA isolation and bacterial expression of a terpene synthase involved in artemisinin biosynthesis. *Arch. Biochem. Biophys.* **2000**, *383*, 178−184.
(21) Teoh, K. H.; Polichuk, D. R.; Reed, D. W.; Nowak, G.; Covello, P. S. Artemisia annua L. (Asteraceae) trichome-specific cDNAs reveal CYP71AV1, a cytochrome P450 with a key role in the biosynthesis of the antimalarial sesquiterpene lactone artemisinin. *FEBS Lett.* **2006**, *580*, 1411−1416.
(22) Picaud, S.; Olofsson, L.; Brodelius, M.; Brodelius, P. E. Expression, purification, and characterization of recombinant amorpha-4,11-diene synthase from Artemisia annua L. *Arch. Biochem. Biophys.* **2005**, *436*, 215−226.
(23) Faraldos, J. A.; Wu, S.; Chappell, J.; Coates, R. M. Doubly deuterium-labeled patchouli alcohol from cyclization of singly labeled [2-$^2H_1$]farnesyl diphosphate catalyzed by recombinant patchoulol synthase. *J. Am. Chem. Soc.* **2010**, *132*, 2998−3008.
(24) Kim, S.-H.; Heo, K.; Chang, Y.-J.; Park, S.-H.; Rhee, S.-K.; Kim, S.-U. Cyclization mechanism of amorpha-4,11-diene synthase, a key enzyme in artemisinin biosynthesis. *J. Nat. Prod.* **2006**, *69*, 758−762.
(25) Picaud, S.; Mercke, P.; He, X.; Sterner, O.; Brodelius, M.; Cane, D. E.; Brodelius, P. E. Amorpha-4,11-diene synthase: mechanism and stereochemistry of the enzymatic cyclization of farnesyl diphosphate. *Arch. Biochem. Biophys.* **2006**, *448*, 150−155.
(26) Hong, Y. J.; Tantillo, D. J. A tangled web-interconnecting pathways to amorphadiene and the amorphene sesquiterpenes. *Chem. Sci.* **2010**, *1*, 609−614.
(27) Li, J.-X.; Fang, X.; Zhao, Q.; Ruan, J.-X.; Yang, C.-Q.; Wang, L.-J.; Miller, D. J.; Faraldos, J. A.; Allemann, R. K.; Chen, X.-Y.; Zhang, P. Rational engineering of plasticity residues of sesquiterpene synthases from Artemisia annua: product specificity and catalytic efficiency. *Biochem. J.* **2013**, *451*, 417−426.
(28) Fang, X.; Li, J.-X.; Huang, J.-Q.; Xiao, Y.-L.; Zhang, P.; Chen, X.-Y. Systematic identification of functional residues of Artemisia annua amorpha-4,11-diene synthase. *Biochem. J.* **2017**, *474*, 2191−2202.
(29) Cane, D. E.; Oliver, J. S.; Harrison, P. H. M.; Abell, C.; Hubbard, B. R.; Kane, C. T.; Lattman, R. Biosynthesis of pentalenene and pentalenolactone. *J. Am. Chem. Soc.* **1990**, *112*, 4513−4524.
(30) Pompon, D.; Louerat, B.; Bronine, A.; Urban, P. Yeast expression of animal and plant P450s in optimized redox environments. *Methods Enzymol.* **1996**, *272*, 51−64.

https://dx.doi.org/10.1021/acs.jafc.0c07462
J. Agric. Food Chem. 2021, 69, 2168−2178

(31) Felicetti, B.; Cane, D. E. Aristolochene synthase: mechanistic analysis of active site residues by site-directed mutagenesis. *J. Am. Chem. Soc.* **2004**, *126*, 7212−7221.
(32) Attia, M.; Kim, S.-U.; Ro, D.-K. Molecular cloning and characterization of (+)-epi-bisabolol synthase, catalyzing the first step in the biosynthesis of the natural sweetener, hernandulcin, in Lippia dulcis. *Arch. Biochem. Biophys.* **2012**, *527*, 37−44.
(33) Garms, S.; Köllner, T. G.; Boland, W. A multiproduct terpene synthase from Medicago truncatula generates cadalane sesquiterpenes via two different mechanisms. *J. Org. Chem.* **2010**, *75*, 5590−5600.
(34) Köllner, T. G.; Schnee, C.; Gershenzon, J.; Degenhardt, J. The variability of sesquiterpenes emitted from two Zea mays cultivars is controlled by allelic variation of two terpene synthase genes encoding stereoselective multiple product enzymes. *Plant Cell* **2004**, *16*, 1115−1131.
(35) Benedict, C.; Alchanati, I.; Harvey, P. J.; Liu, J.; Stipanovic, R. D.; Bell, A. A. The enzymatic formation of δ-cadinene from farnesyl diphosphate in extracts of cotton. *Phytochemistry* **1995**, *39*, 327−331.
(36) Greenhagen, B. T.; Griggs, P.; Takahashi, S.; Ralston, L.; Chappell, J. Probing sesquiterpene hydroxylase activities in a coupled assay with terpene synthases. *Arch. Biochem. Biophys.* **2003**, *409*, 385−394.
(37) Anthony, J. R.; Anthony, L. C.; Nowroozi, F.; Kwon, G.; Newman, J. D.; Keasling, J. D. Optimization of the mevalonate-based isoprenoid biosynthetic pathway in Escherichia coli for production of the anti-malarial drug precursor amorpha-4,11-diene. *Metab. Eng.* **2009**, *11*, 13−19.
(38) Shukal, S.; Chen, X.; Zhang, C. Systematic engineering for high-yield production of viridiflorol and amorphadiene in auxotrophic Escherichia coli. *Metab. Eng.* **2019**, *55*, 170−178.
(39) Wei, Q.; He, S.; Qu, J.; Xia, J. Synthetic multienzyme complexes assembled on virus-like particles for cascade biosynthesis in cellulo. *Bioconjugate Chem.* **2020**, *31*, 2413−2420.
(40) O'Maille, P. E.; Chappell, J.; Noel, J. P. Biosynthetic potential of sesquiterpene synthases: alternative products of tobacco 5-epi-aristolochene synthase. *Arch. Biochem. Biophys.* **2006**, *448*, 73−82.
(41) Chen, X.-Y.; Chen, Y.; Heinstein, P.; Davisson, V. J. Cloning, expression, and characterization of (+)-delta-cadinene synthase: a catalyst for cotton phytoalexin biosynthesis. *Arch. Biochem. Biophys.* **1995**, *324*, 255−266.
(42) Steele, C. L.; Crock, J.; Bohlmann, J.; Croteau, R. Sesquiterpene synthases from grand fir (Abies grandis). Comparison of constitutive and wound-induced activities, and cDNA isolation, characterization, and bacterial expression of delta-selinene synthase and gamma-humulene synthase. *J. Biol. Chem.* **1998**, *273*, 2078−2089.
(43) Christianson, D. W. Structural biology and chemistry of the terpenoid cyclases. *Chem. Rev.* **2006**, *106*, 3412−3442.
(44) Lopez-Gallego, F.; Agger, S. A.; Abate-Pella, D.; Distefano, M. D.; Schmidt-Dannert, C. Sesquiterpene synthases Cop4 and Cop6 from Coprinus cinereus: catalytic promiscuity and cyclization of farnesyl pyrophosphate geometric isomers. *ChemBioChem* **2010**, *11*, 1093−1106.
(45) Komatsu, M.; Uchiyama, T.; Omura, S.; Cane, D. E.; Ikeda, H. Genome-minimized Streptomyces host for the heterologous expression of secondary metabolism. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 2646−2651.
(46) Zhou, K.; Zou, R.; Zhang, C.; Stephanopoulos, G.; Too, H. P. Optimization of amorphadiene synthesis in Bacillus subtilis via transcriptional, translational, and media modulation. *Biotechnol. Bioeng.* **2013**, *110*, 2556−2561.
(47) Choi, S. Y.; Lee, H. J.; Choi, J.; Kim, J.; Sim, S. J.; Um, Y.; Kim, Y.; Lee, T. S.; Keasling, J. D.; Woo, H. M. Photosynthetic conversion of CO2 to farnesyl diphosphate-derived phytochemical (amorpha-4,11-diene and squalene) by engineered cyanobacteria. *Biotechnol. Biofuels* **2016**, *9*, 202.
(48) Orsi, E.; Mougiakos, I.; Post, W.; Beekwilder, J.; Dompè, M.; Eggink, G.; van der Oost, J.; Kengen, S. W. M.; Weusthuis, R. A. Growth-uncoupled isoprenoid synthesis in Rhodobacter sphaeroides. *Biotechnol. Biofuels* **2020**, *13*, 123.
(49) Lubertozzi, D.; Keasling, J. D. Expression of a synthetic Artemesia annua amorphadiene synthase in Aspergillus nidulans yields altered product distribution. *J. Ind. Microbiol. Biotechnol.* **2008**, *35*, 1191−1198.
(50) Kanagarajan, S.; Muthusamy, S.; Gliszczyńska, A.; Lundgren, A.; Brodelius, P. E. Functional expression and characterization of sesquiterpene synthases from Artemisia annua L. using transient expression system in Nicotiana benthamiana. *Plant Cell Rep.* **2012**, *31*, 1309−1319.
(51) Nguyen, T.-D.; MacNevin, G.; Ro, D.-K. De novo synthesis of high-value plant sesquiterpenoids in yeast. *Methods Enzymol.* **2012**, *517*, 261−278.
(52) Pramastya, H.; Xue, D.; Abdallah, I. I.; Setroikromo, R.; Quax, W. J. High level production of amorphadiene using Bacillus subtilis as an optimized terpenoid cell factory. *New Biotechnol.* **2021**, *60*, 159−167.
(53) Li, Z.; Gao, R.; Hao, Q.; Zhao, H.; Cheng, L.; He, F.; Liu, L.; Liu, X.; Chou, W. K. W.; Zhu, H.; Cane, D. E. The T296V mutant of amorpha-4,11-diene synthase is defective in allylic diphosphate isomerization but retains the ability to cyclize the intermediate (3R)-nerolidyl diphosphate to amorpha-4,11-diene. *Biochemistry* **2016**, *55*, 6599−6604.
(54) Abdallah, I. I.; Czepnik, M.; van Merkerk, R.; Quax, W. J. Insights into the three-dimensional structure of amorpha-4,11-diene synthase and probing of plasticity residues. *J. Nat. Prod.* **2016**, *79*, 2455−2463.
(55) Abdallah, I. I.; van Merkerk, R.; Klumpenaar, E.; Quax, W. J. Catalysis of amorpha4,11-diene synthase unraveled and improved by mutability landscape guided engineering. *Sci. Rep.* **2018**, *8*, 9961.
(56) Fang, X.; Di, Y. T.; Zhang, Y.; Xu, Z. P.; Lu, Y.; Chen, Q. Q.; Zheng, Q. T.; Hao, X. J. Unprecedented Quassinoids with Promising Biological Activities from Harrisonia perforate. *Angew. Chem., Int. Ed.* **2015**, *54*, 5592−5595.
(57) Li, X.-H.; Cao, M.-M.; Zhang, Y.; Li, S.-L.; Di, Y.-T.; Hao, X.-J. Fluevirines A−D, four new securinega-type alkaloids from Flueggea virosa. *Tetrahedron Lett.* **2014**, *55*, 6101−6104.
(58) Ginglinger, J.-F.; Boachon, B.; HÖfer, R.; Paetz, C.; KÖllner, T. G.; Miesch, L.; Lugan, R.; Baltenweck, R.; Mutterer, J.; Ullmann, P.; Beran, F.; Claudel, P.; Verstappen, F.; Fischer, M. J. C.; Karst, F.; Bouwmeester, H.; Miesch, M.; Schneider, B.; Gershenzon, J.; Ehlting, J.; Werck-Reichhart, D. Gene coexpression analysis reveals complex metabolism of the monoterpene alcohol linalool in Arabidopsis flowers. *Plant Cell* **2013**, *25*, 4640−4657.
(59) Edgar, S.; Li, F.-S.; Qiao, K.; Weng, J.-K.; Stephanopoulos, G. Engineering of Taxadiene synthase for improved selectivity and yield of a key taxol biosynthetic intermediate. *ACS Synth. Biol.* **2017**, *6*, 201−205.
(60) Bertea, C. M.; Freije, J. R.; van der Woude, H.; Verstappen, F. W.; Perk, L.; Marquez, V.; De Kraker, J.-W.; Posthumus, M. A.; Jansen, B. J.; de Groot, A.; Franssen, M. C.; Bouwmeester, H. J. Identification of intermediates and enzymes involved in the early steps of artemisinin biosynthesis in Artemisia annua. *Planta Med.* **2005**, *71*, 40−47.

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