Terpenoid, the largest group of specialized metabolites, exhibits a wide range of functional roles and bioactivities (1,2). The condensation of five-carbon terpenoid building blocks [isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP)], together with the further addition of IPP, leads to the generation of various sizes of common terpenoid precursors. These precursors are then converted into several types of terpenoid classified based on the number of C5-isoprene units in the structure, for example, hemiterpenoids (C5), monoterpenoids (C10), sesquiterpenoids (C15), diterpenoids (C20), sesterterpenoids (C25), and triterpenoids (C30).

Terpene synthases (TPSs) are a key enzyme in terpenoid biosynthesis. They play a major role in the catalyzation of the first step linearization/cyclization of common terpenoid precursors. TPSs are classified into two types based on their catalytic mechanism. Class I TPSs are known for the syntheses of hemi-, mono-, and sesquiterpenoids. Enzymes in this class contain one conserved aspartate-rich DDXXD motif and a consensus sequence of (N,D)D(L,I,V)X(S,T) XXXE, or NSE/DTE motif, which are important for the initiation of substrate ionization via metal-triggered diphosphate elimination (3,4). Class II TPSs are involved in the syntheses of di-, sester-, and triterpenoids. In contrast to class I, they use their highly conserved DXDD motif to protonate a substrate, thus initiating a carbon-carbon double bond or epoxide formation. TPSs that contain both DDXXD and DXDD motifs and exhibit either class I or class II catalytic activities are called bifunctional TPSs. This type of TPSs is involved in the synthesis of some diterpenoids from plants and fungi (4,5).

The unique functions of TPSs have led to the synthesis of more than 70,000 different terpenoids with multiple chiral centers and complex hydrocarbons (6). Some TPSs also have complex catalytic activities and synthesize multiple products. These have aroused the interest of scientists for a decade on the resolving of TPSs catalytic mechanism for further applications, such as improving the catalytic efficiency as well as the product specificity of the enzymes and metabolic engineering for the production of bioactive terpenoids. However, the molecular basis of the catalytic mechanism of TPSs, including the amino acid residues involved in the catalytic process, remains unknown.

Among the highly diverse terpenoids, the sesquiterpene lactone artemisinin, isolated from the Artemisia annua plant, is the most renowned terpene-based drug for its effective antimalarial activity. The first committed step in artemisinin biosynthesis is the cyclization of a common precursor of sesquiterpenoid farnesyl pyrophosphate (FPP) to amorpha-4,11-diene, catalyzed by a specific class I sesquiterpene synthase, amorpha-4,11-diene synthase (ADS) (7-9). After FPP is ionized to farnesyl cation, the next step of cyclization of this intermediate is believed to proceed via 1,6-ring closure generating a bisabolyl cation and subsequent 1,10-cyclization to a bicyclic amorphadien-11-yl cation. This amorphadien-11-yl cation intermediate...
is further regioselectively deprotonated to amorpha-4,11-diene (10,11).

In general, sesquiterpene synthases convert FPP into a high variety of hydrocarbon backbones, including linear, mono-, bi-, tri-, and tetra-cyclic. Many of them can produce multiple sesquiterpenoid products; thus the production of a specific sesquiterpenoid for further usage becomes more difficult. In contrast to other sesquiterpene synthases, ADS shows high fidelity and produces amorpha-4,11-diene as the major product of more than 90% (9). Normally, amino acid residues located within, surrounding, and distal to the active site, including DDXXD and NSE/DTE motifs, are involved in the reaction specificity of TPSs (12). With this in mind, Li, Fang and colleagues applied domain swapping and site-directed mutagenesis to study the molecular basis of the two-step cyclization process of ADS and successfully revealed a residue T399 responsible for the deprotonation of the amorphadien-11-yl cation to amorpha-4,11-diene. This work was published in Biochemical Journal several years ago (13).

As a continued investigation, the same group aimed to identify the catalytic residues involved in 1,6- and 1,10-cyclization by phylogeny-based site-directed substitution and recently published their work in Biochemical Journal once again (14). This time they aligned the amino acid sequence of ADS with those of other sesquiterpene synthases isolated from *A. annua*. Within this alignment, the different residues, as compared to ADS, in the catalytic pockets of α-bisabolol synthase (BOS) and germacrene A synthase (GAS) were then focused as both enzymes catalyze only 1,6- or 1,10-ring closure, respectively. After substituting several potential catalytic residues from BOS and GAS into the corresponding position of ADS, they found that a single-substituted ADS mutant (T296V) led to a loss of stabilization of the carbocation intermediate from a side chain hydroxy group of threonine and impaired 1,6-ring closure activity resulting in a production of more than 90% linear sesquiterpene product, (E)-β-farnesene. In case of 1,10-ring closure, tetra-substituted mutant (L374Y/L404V/L405I/G439S) dislocated and influenced the folding of bisabolyl cation intermediate in the active site, thus increasing the production of 1,6-cyclic bisabolyl-type sesquiterpene products to more than 80%, while lowering the yield of a bicyclic amorpha-4,11-diene. These results clearly indicate the major roles of residue T296 as well as residues L374, L404, L405, and G439 in the first 1,6- and the second 1,10-cyclization processes of ADS, respectively.

The ADS<sub>T447G</sub> mutant generated from the reciprocal substitution between BOS and ADS also lowered the production yield of amorpha-4,11-diene. Simulation of the ADS active site with amorphadien-11-yl cation suggested that residue T447 coordinates water molecules with its side chain hydroxy group to deprotonate H-12 or H-13 of this intermediate to amorpha-4,11-diene in the same manner with residue T399. This result indicates that not only T399, but residue T447 is also responsible for the last deprotonation of amorpha-4,11-diene formation. Single and double substitutions of these two residues with serine also increased the catalytic efficiency by approximately two- to three-fold higher turnover rate due to a more hydrophilic environment of serine than threonine, thus accelerating the dissociation rate of the product.

Several TPSs, which show high percent amino acid sequence identity and have similar catalytic mechanism to ADS, have been previously reported (15). However, none of them synthesize amorpha-4,11-diene as the major product. With the newly detected catalytic amino acid residues identified in this study, a guide to identify critical residues that determine the product specificity and to engineer TPSs with higher catalytic efficiency becomes available. In addition, besides adding extra copies of biosynthetic enzymes or other enzymes that can accelerate the metabolic flux (16-18), TPS engineering could also be an alternative powerful approach to enhance the synthetic biological production of valuable terpenoids in the future.

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**Footnote**

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