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Amorpha-4,11-diene synthase: Mechanism and stereochemistry of the enzymatic cyclization of farnesyl diphosphate

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Abstract

Recombinant amorpha-4,11-diene synthase from *Artemisia annua*, expressed in *Escherichia coli*, was incubated with the deuterium-labeled farnesyl diphosphates, (1*R*)-[1-^{2}H]FPP, (1*S*)-[1-^{2}H]FPP, and [1,1-$^2H_2$]FPP. GC–MS analysis of amorpha-4,11-diene formed from the deuterated FPPs shows that the deuterium atoms are retained in the product. Furthermore, analysis of the MS-spectra obtained with the differently labeled substrate indicates that the H-*1si*-proton of FPP is transferred during the cyclization reaction to carbon 10 of amorphadiene while the H-*1re*-proton of FPP is retained on C-6 of the product. Proton NMR and COSY experiments proved that the original H-*1si*-proton of FPP is located at C-10 of amorpha-4,11-diene as a result of a 1,3-hydride shift following initial 1,6-ring closure. The results obtained support the previously suggested mechanism for the cyclization of farnesyl diphosphate by amorph-4,11-diene synthase involving isomerization of FPP to (*R*)-nerolidyl diphosphate (NPP), ionization of NPP, and C-1,C-6-ring closure to generate a bisabolyl cation, followed by a 1,3-hydride shift, 1,10-ring closure to generate the amorphane skeleton, and deprotonation at either C-12 or C-13 to afford the final product (1*S*,6*R*,7*R*,10*R*)-amorpha-4,11-diene.

Keywords: Sesquiterpenes; Amorpha-4,11-diene synthase; Enzyme mechanism; Deuterated substrate; GC–MS; Proton NMR; COSY

Sesquiterpenoids are a structurally diverse class of isoprenoids found in plants, fungi, and some bacteria, which play a variety of physiological and ecological roles, including plant–plant, plant–insect, and plant–pathogen interactions. The structural diversity and stereochemical complexity of the C_{15}-isoprenoid skeletons of sesquiterpenoids are remarkable. More than 300 types of cyclic sesquiterpenes have been characterized to date and each is derived from the common acyclic precursor, farnesyl diphosphate (**1**, FPP).[1] Sesquiterpene synthases are key branch point enzymes in the biosynthesis of these compounds. Several sesquiterpene synthases have been cloned from plants and the deduced amino acid sequences show high identity/similarity to one another [1–9]. Numerous plant sesquiterpene synthase sequences have been recognized on the basis of this sequence similarity, but the nature of the cyclization products is as yet unknown. Our understanding of the relationship between protein structure and product selectivity of highly homologous sesquiterpene synthases is still limited.

We recently reported the molecular cloning and biochemical characterization of amorpha-4,11-diene synthase from *Artemisia annua*, which catalyzes the cyclization of farnesyl diphosphate to (1*S*,6*R*,7*R*,10*R*)-amorpha-4,11-

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[1] *Abbreviations used:* ADS, amorpha-4,11-diene synthase; AMU, atomic mass unit; FPP, farnesyl diphosphate; GPP, geranyl diphosphate; NPP, nerolidyl diphosphate; IMAC, immobilized metal affinity chromatography.

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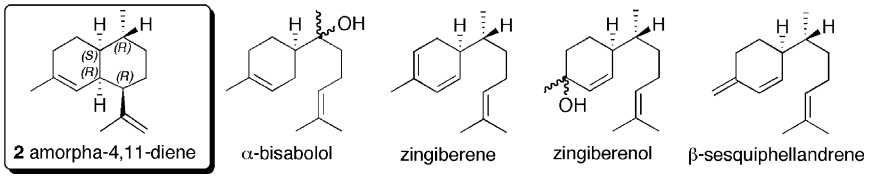

Fig. 1. Amorpha-4,11-diene (**1**) and bisabolane-type co-products of the ADS-catalyzed cyclization of FPP in the presence of Mg^{2+}.

diene (**2**) [8,10]. Studies by Bouwmeester et al. [11] established that amorphadiene synthase mediates a key step in the biosynthesis of the antimalarial endoperoxide artemisinin. Furthermore, these authors established the structure and stereochemistry of enzymatically produced amorpha-4,11-diene [11]. We have previously proposed a mechanism for the formation of amorpha-4,11-diene (**2**) [10] that posits an initial 1,6-ring closure to generate a bisabolyl cation intermediate, based on the generation of a number of bisabolane-type by-products in the enzymatic reaction including α-bisabolol (1.6% of total sesquiterpenoids), (+)-zingiberene (0.2%), zingiberenol (0.4%), and (+)-β-sesquiphellan- drene (1.8%) [10] (Fig. 1). The formation of these products may be explained by premature quenching of the cyclization cascade that leads to amorphadiene (**2**). On the other hand, no germacrane-type sesquiterpenes could be detected in the enzymatic assay mixture. Furthermore, the acyclic monoterpene substrate, geranyl diphosphate (GPP), is converted to the cyclic compounds α-terpineol, limonene, and terpinen-4-ol by ADS, in contrast to the action of tobacco *epi*-aristolochene synthase, which only produces the acyclic products linalool and myrcene when incubated with GPP (B.T. Greenhagen, S.G. Koenig, L. Olofsson, P.E. Brodelius, J.P. Noel, J. Chappell, unpublished) (Fig. 2). The cyclization of FPP to *epi*-aristolochene by the tobacco enzyme has been shown to involve the intermediacy of the 10-membered ring germacrene A [12].

The enzymatic cyclization of FPP to other 4,10-dimethyl-7-isopropyloctalins such as cubenene [13], *epi*-cubenol [13,14], γ-cadinene [15], and δ-cadinene [16] has been suggested or shown to proceed through a common helminthogermacradienyl intermediate, which is formed by an initial 1,10-ring closure (Fig. 3). We now describe experiments with recombinant amorpha-4,11-diene synthase using deuterium-labelled FPPs, that confirm the proposed intermediacy of a bisabolyl cation in the cyclization reaction and and the operation of a 1,3-hydride shift during the carbocationic cyclization cascade.

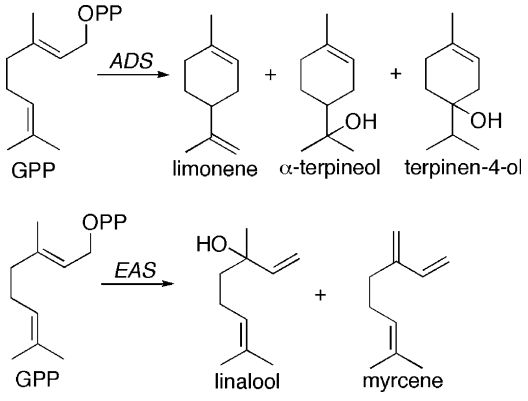

Fig. 2. Cyclization or solvolysis of the anomalous substrate geranyl diphosphate by ADS or *epi*-aristolochene synthase (EAS).

Materials and methods

Reagents

[1-^{3}H]FPP (0.59 TBq/mmol) was purchased from Amersham-Pharmacia Biotech. IPTG, FPP, and kanamycin were obtained from Sigma. All other biochemicals and reagents were purchased from commercial sources. (1*R*)-[1-^{2}H]FPP and (1*S*)-[1-^{2}H]FPP, synthesized as described by Cane et al. [17], were a gift from Dr. W. König through Dr. H. Bouwmeester. [1,1-$^2H_2$]FPP was synthesized as previously described [13,17]. Semisynthetic amorpha-4,11-diene, synthesized from artemisinic acid, was kindly supplied by Dr. Ben Jansen.

Production of recombinant ADS

For production of recombinant ADS, *E. coli* BL21 (DE3) cells were transformed with bacterial expression

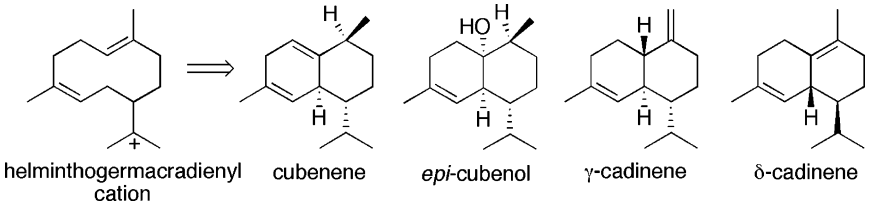

Fig. 3. Formation of 4,10-dimethyl-7-isopropyl octalin sesquiterpenes via a helmintho-germacradienyl cation (1,10-ring closure).