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Dual role for phenylalanine 178 during catalysis by aristolochene synthase†

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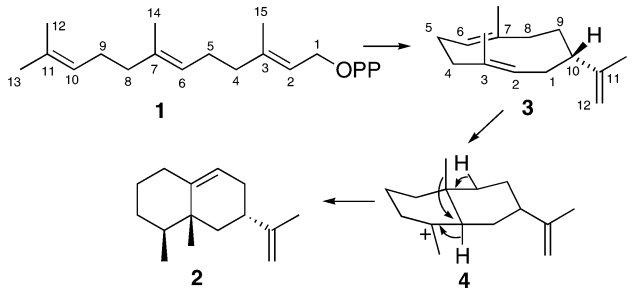
A mutant of aristolochene synthase, in which Phe 178 was replaced by Val, produced significant amounts of α- and β-farnesene as well as α and β-selinene and selina-4,11-diene, suggesting that Phe 178 is involved in the stabilisation of transition states preceding germacrene A and following eudesmane cation.

Aristolochene synthase (AS) from *Penicillium roqueforti* catalyses the Mg^{2+}-dependent cyclisation of farnesyl pyrophosphate (**1**, FPP) to the bicyclic sesquiterpene (+)-aristolochene (**2**), the parent hydrocarbon of a large number of fungal toxins including PR-toxin.[1–4] Biochemical labelling and site directed mutagenesis studies have supported a mechanism in which FPP initially cyclises to (*S*)-germacrene A (**3**),[5–7] which undergoes a further cyclisation to generate the bicyclic eudesmane cation (**4**) (Scheme 1). Eudesmane cation is then converted to **2** through a hydride and a methyl shift followed by deprotonation from C8. The active site of AS provides the template for the folding of FPP in a conformation favouring the formation of **2** and at the same time preventing the formation of alternate reaction products.[8,9] AS promotes cyclisation, methyl and hydride shifts as well as protonation and deprotonation reactions and prevents premature quenching of the extremely reactive carbocationic intermediates through exclusion of water.

Recent studies have shed some light on the molecular mechanisms employed by AS to chaperone the reaction intermediates along only one of many possible reaction pathways with exquisite specificity. Tyr 92 of AS acts as the active site acid responsible for the protonation of the C6–C7 double bond of germacrene A that is necessary for the formation of **4** through electron flow from the C2–C3 double bond.[6,9] Trp 334 also facilitates the energetically demanding generation of **4** through interactions between the π-system of its indole ring and the developing positive charge on C3.[10]

Inspection of the X-ray structure of AS,[11] which was obtained in the absence of a substrate analogue or inhibitor, suggested that the π-system of Phe 178 might also contribute to the stabilisation of the positive charge on C3 of eudesmane cation. In addition, Phe 178 appeared ideally placed to stabilise carbocations on C2 and C1. Indeed, stabilisation of the developing positive charge on C1 by Phe 178 in the first step of catalysis had been suggested previously.[11] The conversion of FPP to germacrene A proceeds in a stepwise fashion by way of the intermediate farnesyl cation **5** (Scheme 2).[6,8] To address the function of Phe 178 during AS catalysis, we have produced ASF178Y and ASF178V, in which Phe 178 was replaced by tyrosine and valine, respectively.



Scheme 1 Mechanism for the formation of aristolochene from FPP.

cDNAs for ASF178Y and ASF178V were generated by site directed mutagenesis from a cDNA of wild type AS which had been isolated from *P. roqueforti*. The mutants were expressed to high levels in BL21(DE3)pLysS cells and purified to apparent homogeneity.[8] The hexane extractable products of the conversion of FPP by the mutant enzymes were analysed by GC-MS.[12] AS had previously been reported to produce ~92% aristolochene (**2**), ~8% germacrene A (**3**) and a small amount of valencene (**6**), which resulted from deprotonation from C6 rather than C8 in the final step of the reaction.[6] A similar distribution of products was found for ASF178Y, which converted FPP into ~86% **2**, ~11% **3** and ~3% **6** (Table 1). The steady state kinetic parameters of ASF178Y were determined by incubation with [1–^{3}H]-FPP and monitoring the formation of tritiated, hexane extractable products.[13] The K_M value of 5.1 μM for ASF178Y was similar to that reported previously for the wild type enzyme (Table 1). The turnover number was reduced approximately 30-fold resulting in an overall reduction of the catalytic efficiency, k_{cat}/K_M, of almost two orders of magnitude. The *p*-hydroxybenzene ring of tyrosine in ASF178Y could clearly substitute for the benzene ring of phenylalanine and direct the substrate along the reaction coordinate to generate close to wild type products, albeit with reduced speed.

When a valine residue replaced Phe 178, the catalytic efficiency of the resulting mutant enzyme, ASF178V, was reduced by a further two orders of magnitude, mostly due to a reduction in the turnover number (Table 1). The K_M of ASF178V was 11.2 μM and the k_{cat} 2.1 × 10^{-5} s^{-1}. Analysis of the hexane extractable products revealed that the presence of the isopropyl side chain in place of the aromatic ring of Phe 178 led to the formation of only 10.8% **2** (Table 1). (*S*)-Germacrene A (**3**), the enantiomer produced by wild-type AS, made more than 50% of the total amount of products. Its stereochemistry was determined through the analysis of the configuration of the β-elemene produced in the heat-induced Cope rearrangement of germacrene A by enantioselective gas-chromatography[14] and comparison with (+)-β-elemene, obtained from the incubation of AS with FPP, as well as a racemic sample of β-elemene.†

The increased formation of germacrene A by ASF178V suggested that Phe 178 was involved in the stabilisation of a transition state following this intermediate. The formation of 5.7% α-selinene (**7**), 9.1% β-selinene (**8**) and 2.1% selina-4,11-diene (**9**) was observed (Table 1). These bicylic hydrocarbons, which were identified from their mass spectra and comparison with those of authentic samples, resulted from eudesmane cation through proton loss from C2, C4 and C15 (Scheme 2). The formation of significant amounts of selinenes by ASF178V suggested that Phe 178 was involved in the stabilisation of the developing positive charge on C2 during AS catalysis. In addition, the bulkiness of Phe 178 might

† Electronic supplementary information (ESI) available: total ion chromatograms from GC-MS analyses of the products of AS, ASF178Y and ASF178V catalysis; mass spectra of the products generated by ASF178Y and ASF178V and those of authentic samples. GC-traces and reaction schemes for the production of β-elemenes; figure indicating the relative positions of F178, Y92 and W334 within the active site of AS. See http://www.rsc.org/suppdata/cc/b4/b407932a/

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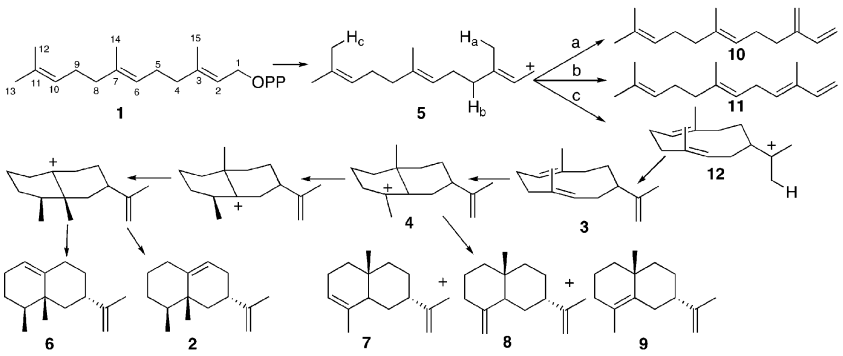

Scheme 2 Products formed by ASF178V.

Table 1 Kinetic parameters of and distribution of products formed by AS, ASF178Y and ASF178V

	Kinetic parameters			Product distribution							
Enzyme	$k_{cat} \times 10^2/s^{-1}$	$K_M/\mu M$	$k_{cat}/K_M/s^{-1}\ M^{-1}$	**2**	**3**	**6**	**7**	**8**	**9**	**10**	**11**
AS[6]	3 ± 2	2.3 ± 0.5	13 043 ± 2 989	91.5	7.5	<1					
ASF178Y	0.1 ± 0.049	5.1 ± 1.4	196.1 ± 110	86.4	10.7	2.7					
ASF178V	0.0021 ± 0.0004	11.2 ± 0.18	1.88 ± 0.32	10.8	54.1	5.2	5.7	9.1	2.1	9.2	2.7

help promote the 1,2-hydride shift. In the absence of the aromatic group, the stabilisation of the transition state following **4** appeared to be reduced, eventually leading to aberrant deprotonation and formation of α- and β-selinene as well as selina-4,11-diene.

Interestingly, ASF178V also produced relatively large amounts of the two alicyclic sesquiterpenes (*E*)-β-farnesene (**10**) and (*E*,*E*)-α-farnesene (**11**) (Table 1). **10** and **11** formed approximately 9.2% and 2.7% of the total amount of hexane extractable products (Table 1). These linear products, which were identified from their mass spectra and through comparison of their mass spectra and retention times with those of authentic samples, and the significantly reduced catalytic activity of ASF178V supported an involvement of Phe 178 in the cyclisation of FPP to germacrene A. However, it appeared that in addition to stabilising the developing positive charge on C1 of farnesyl-cation, which might be, at least in part, the basis of the reduction of the reaction rate of ASF178V, Phe 178 also controlled the barrier height in a step between farnesyl cation and germacrene A. When an isopropyl-group replaced the aromatic side chain of Phe 178 the conversion of farnesyl-cation to germacrene A was slowed and the production of aberrant **10** and **11** through deprotonation from C4 and C15 increased.

The results reported here together with previously published data[6,8] suggest that the cyclisation of FPP to germacrene A might involve two cationic intermediates. After expulsion of the pyrophosphate group of FPP to generate farnesyl-cation (**5**), cyclisation leads to germacryl-cation (**12**), which carries the positive charge on C11 (Scheme 2). Proton loss from C12 results in the production of **3**.

In summary, Phe 178 appears to fulfil a dual role during AS catalysis. The efficient conversion of FPP to germacrene A depends on the interaction of the positive charges on C1 and C11 with the π-system of Phe 178. In addition, Phe 178 promotes the conversion of eudesmane cation to aristolochene through stabilisation of the developing positive charge on C2 and induction of the hydride shift from C2 to C3 of **4**.

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13 ASF178Y and ASF178V were assayed in a total volume of 250 μl containing 2.5 μM enzyme in Buffer A and [1–^{3}H]-FPP (0.8–180 μM). After termination of the reactions by addition of 200 μl of 100 mM EDTA (pH 7.25) the samples were extracted three times with *n*-hexane and vortexed with 0.5 g of silica. The combined hexane extracts were analysed for radioactivity as described[8].
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