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Tyrosine 92 of aristolochene synthase directs cyclisation of farnesyl pyrophosphate†

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A mutant of Aristolochene Synthase (AS), in which Tyr 92 was replaced by Val, produced the alicyclic β-(*E*)-farnesene as the major product, indicating that cyclisation of FPP is controlled by Tyr 92 in AS.

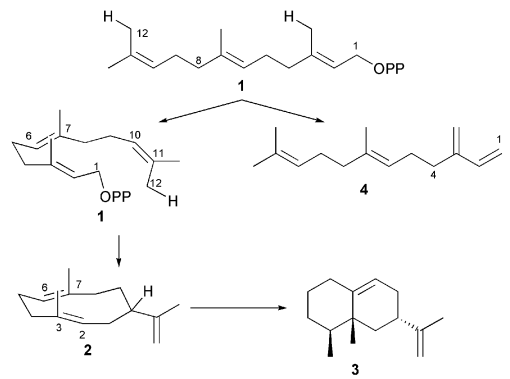
Sesquiterpene cyclases are a ubiquitously expressed family of proteins capable of converting the universal acyclic precursor farnesyl pyrophosphate (FPP, **1**) into more than 300 different cyclic sesquiterpene products.[1,2] Product diversity is to a large extent controlled by the proper folding of **1** in the active sites of the enzymes. After substrate binding and folding, the reactions are often initiated by metal cation triggered expulsion of pyrophosphate followed by a sequence of cyclisations and rearrangements of exquisite regio- and stereo-specificity. The reaction sequence is terminated by deprotonation of or water capture by the final cation.

Aristolochene synthase (AS) from *P. roqueforti* is a monomeric enzyme of 39 kD that catalyses the cyclisation of **1** to (+)-aristolochene (**3**) (Scheme 1), the biosynthetic precursor of several toxins in filamentous fungi including PR-toxin, gigantenone, sporogen-AO1, phaseolinone, phomenone, and bipolaroxin.[3,4] The structure of AS has been solved to 2.5 Å by X-ray crystallography.[5] Investigations of the mechanism of AS catalysis[6–9] and X-ray crystallography[5] suggested that **1** was bound in the active site of AS in a conformation, which, subsequent to (or concurrent with) metal triggered pyrophosphate expulsion, favoured attack of C1 by the C10–C11 bond (Scheme 1). Reprotonation of germacrene A (**2**) at C6, cyclisation and hydride and methyl migrations generated **3**. The precise folding of **1** leading to the proper spatial orientation of the C10–C11 double bond and the breaking carbon–oxygen bond ensures optimal reactivity for cyclisation and prevents the competing reaction of pyrophosphate elimination by deprotonation from the C3 methyl-group to produce the alicyclic sesquiterpene β-(*E*)-farnesene (**4**). **4** is used extensively by both plants and insects for communication[10] while mammals appear to use **4** as a pheromone.[11] Interestingly, β-(*E*)-farnesene synthase from *M. piperita* is homologous to many sesquiterpene cyclases including 5-*epi*-aristolochene synthase (EAS) from *N. tabacum*,[10,12] the tertiary structure of which closely resembles that of AS.[5,13]

We have investigated the mechanisms by which AS controls the specificity of the initial cyclisation of **1** leading to **2** and prevents the formation of **4**. Inspection of the X-ray structure of AS[5] suggested that the relative bulkiness of Tyr 92 might be a key determinant of proper folding of **1** in the active site of the enzyme. Tyr 92 is positioned close to the C6–C7 double bond and might direct folding of **1** leading to cyclisation. Previous results have shown that Tyr 92 acts as the active site acid which protonates the C6–C7 double bond of germacrene A to induce the generation of aristolochene.[14] In order to determine whether Tyr 92 also directed proper folding of **1**, we have produced ASY92V, in which a valine residue replaced Tyr 92 in aristolochene synthase. Substitution of the bulky side chain of Tyr with the much smaller Val was predicted to reduce the efficiency of the cyclisation to **2**. As a consequence the mutant enzyme was predicted to act as a β-(*E*)-farnesene synthase.

ASY92V was constructed by site directed mutagenesis from a cDNA of AS in plasmid pZW04.[15] ASY92V was efficiently expressed in *E. coli* and purified to apparent homogeneity.[5] The CD-spectra of WT-AS and ASY92V matched closely indicating that the two proteins had similar secondary structures (data not shown). The steady state kinetic parameters of ASY92V were determined by incubation with [1-^{3}H]-FPP and monitoring the formation of tritiated, hexane extractable products.[16] The K_M and k_{cat} values for ASY92V were determined as 4.86 μM and 2.51×10^{-4} s^{-1}, respectively. Relative to the wild-type enzyme, ASY92V displayed a 2-fold increase of K_M, while the turnover number k_{cat} was reduced by approximately two orders of magnitude.[14] The catalytic efficiency of ASY92V was relatively low (51.65 M^{-1} s^{-1}). However, even wild-type AS is not a fast enzyme (k_{cat}/K_M = 13,043 M^{-1} s^{-1})[14] and it appears that terpene cyclases have been optimised by nature for product specificity rather than speed.

The hexane extractable materials produced by ASY92V were also analysed by GC–MS, which indicated that despite its low activity ASY92V produced terpenes specifically.[17] The GC-trace revealed that the mutant enzyme produced several products of mass 204 (Fig. 1). The minor products **5**, **6**, **7** and **8**, which had already been observed in the case of ASY92F,[14] constituted only 18% of the total amount of the hexane extractable material (Table 1). They were identified by



Scheme 1 Formation of **3** and **4** during ASY92V catalysis.

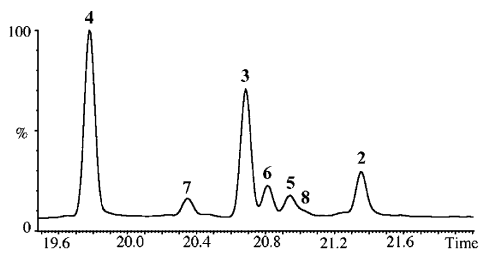


Fig. 1 GC-trace of hydrocarbons produced during ASY92V catalysis.

† Electronic supplementary information (ESI) available: GC-profiles of co-injections of **4** from ASY92V and authentic material and mass spectra of **4** produced by ASY92V and an authentic sample. See http://www.rsc.org/suppdata/cc/b2/b206517g/

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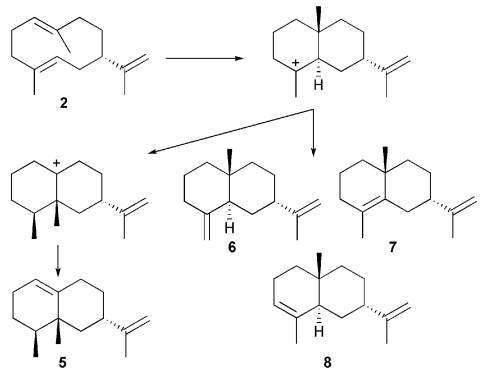
Table 1 Relative amounts of products formed from **1** by wild-type AS and ASY92V[17]

Product number Name	**2** Germacrene A	**3** Aristolochene	**4** β-(*E*)-Farnesene	**5** Valencene	**6** β-Selinene	**7** Selina-4,11-diene	**8** α-Selinene
Retention time /min	21.37	20.68	19.78	20.92	20.81	20.37	21.03
AS[14]	7.5	91.5	0	0.4	0	0	0
ASY92V	11.2	28.2	42.6	6.1	6.9	4.0	1.0



Scheme 2 Formation of products **5**, **6**, **7** and **8** during ASY92V catalysis.

comparison of their mass spectra with those in the Wiley and NIST libraries[18] and by co-injections with authentic valencene (**5**) and α- (**8**) and β-selinene (**6**).†‡ They are the products of erroneous deprotonations (Scheme 2). Germacrene A is known to undergo relatively facile acid-catalysed cyclisation to selinenes in solvents such as chloroform.[19,20] However, no evidence for such a rearrangement was obtained in hexane, which was the solvent in our studies.[21] Hydrocarbons **6**, **7**, and **8** appeared therefore to be true enzymatic products of ASY92V. The relative amount of **2** was slightly increased when compared to the wild-type enzyme (Table 1).[14] **2** was identified from its mass-spectrum and by comparison with authentic material.[14,22]‡

While aristolochene still constituted approximately 28% of the total amount of hexane extractable products a new major product was formed with a retention time of 19.78 min. Comparison of the mass-spectrum of this product with the entries in the Wiley and NIST libraries[18] suggested that this material was β-(*E*)-farnesene. Co-injection of authentic β-(*E*)-farnesene‡ and the products produced by ASY92V led to an increase in the intensity of the peak corresponding to **4** which made up almost 43% of the products generated by ASV92V. The replacement of a single amino acid in the active site of AS had therefore converted the enzyme into a β-(*E*)-farnesene synthase. Both the catalytic activity and the specificity of the mutant enzyme were reduced. However, it is noteworthy in this context that many natural terpene cyclases also have relatively low specificities. Multiple product formation may be a consequence of the electrophilic reaction mechanism employed by these enzymes in which highly reactive carbocationic intermediates are generated. The most extreme cases reported so far are δ-selinene synthase and γ-humulene synthase, for which the major name-giving products represent only just over 25% of the total amount of products formed.[23] β-(*E*)-farnesene synthase isolated from peppermint has been shown to produce **4** as a major product.[10] However, it also produced approximately 7% of cyclic sesquiterpenes of the cadinene-type.

The results reported here establish that Tyr 92 in aristolochene synthase plays a pivotal role in forcing **1** into the reactive conformation necessary for efficient cyclisation to **2**. When the relatively bulky residue is replaced with the smaller isopropyl group of valine, **4** is produced as the major product. The mutant enzyme still bound FPP efficiently through the unaltered DDVIE motif located at the top of the active site. This motif binds the pyrophosphate group of the substrate through the catalytically important Mg^{2+} ion. However, the conformation of the hydrophobic portion of **1** within the active site of ASY92V appeared to be altered leading to preferred deprotonation of the methyl group on C3 rather than cyclisation through the double bond at C10–C11. Tyr 92 of AS therefore controls the reactivity of **1** by forcing C1 and C10 into close proximity thereby favouring cyclisation to **2**.

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Notes and references

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16 ASY92V was assayed in a total volume of 250 μl (10 mM Tris, pH 7.5, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 15% glycerol (v/v) and 0.8–160 μM of [1-^{3}H]-FPP). The reaction was initiated by addition of 50 μl of enzyme (2.5 μM final concentration), terminated by the addition of 200 μl of 100 mM EDTA, pH 7.25 and extracted with 1.8 ml of *n*-hexane in the presence of 0.5 g of silica.
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