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Stabilisation of eudesmane cation by tryptophan 334 during aristolochene synthase catalysis†

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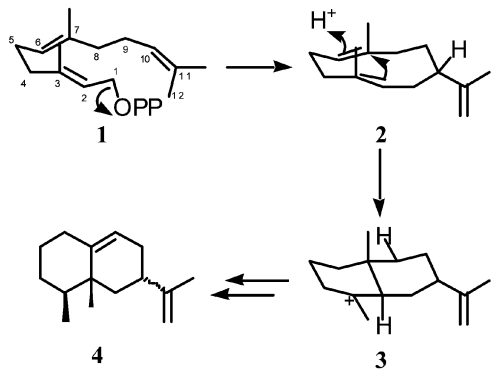
Analysis of the hydrocarbons produced during catalysis by mutants of aristolochene synthase from *Penicillium roqueforti* indicated that Trp 334 had a pivotal function for the efficient production of aristolochene from farnesylpyrophosphate most likely by stabilising the intermediate, eudesmane cation.

Sesquiterpene cyclases catalyse the cyclisation of the universal alicyclic precursor farnesyl pyrophosphate (**1**, FPP) to produce more than 300 different hydrocarbon skeletons often with high regio- and stereospecificity.[1–3] The solution of the X-ray structures of 5-*epi*-aristolochene synthase from *Nicotiana tabacum*,[4] pentalenene synthase from *Streptomyces UC5319*,[5] trichodiene synthase from *Fusarium sporotrichoides*[6] and aristolochene synthase from *Penicillium roqueforti*[7] revealed that they share the same terpenoid fold despite the absence of any significant sequence similarity.[8] Sesquiterpene cyclases must therefore serve as high fidelity templates for FPP, which subtly channel conformation and stereochemistry during the cyclisation reactions. Despite many mechanistic studies of FPP cyclisation, the molecular details of catalysis by sesquiterpene cyclases are only just beginning to emerge.[6,9–13]

Aristolochene synthase (AS) from *P. roqueforti* is a monomeric enzyme that catalyses the Mg^{2+}-dependent cyclisation of FPP to the bicyclic sesquiterpene aristolochene (**4**),[14] the precursor of fungal toxins such as PR-toxin, sporogen-AO1, phaseolinone, gigantenone, phomenone and bipolaroxin.[15,16] The correct folding of FPP within the active site of AS has been shown to be a critical determinant of the reaction pathway.[10,13] AS appears to bind the substrate in a conformation favouring attack of C1 by the double bond at C10–C11 subsequent to metal triggered expulsion of pyrophosphate (Scheme 1). Recent work supported the proposal[17] that AS catalyses the cyclisation of FPP to aristolochene *via* the intermediate *S*-germacrene A (**2**).[9] X-Ray crystallography suggested that the formation of germacrene A was followed by protonation at C6 and cyclisation through electron flow from the C2–C3 double bond to yield the bicyclic eudesmane cation (**3**). We have recently shown that Tyr 92 acts as the active site acid responsible for the formation of eudesmane cation.[9] A hydride shift from C2 to C3 of eudesmane cation followed by a methyl shift from C7 to C2 and deprotonation at C8 results in the formation of **4**.



Scheme 1 Formation of **4** *via* eudesmane cation **3** during AS catalysis.

The generation of eudesmane cation from germacrene A is an energetically demanding step. We have postulated that a hydrogen bonding network from Arg 200, which is exposed to the solvent at the top of the active site cleft, through Asp 203 and Lys 206 to Tyr 92, which is buried deeply in the hydrophobic environment of the active site, might enhance the acidity of the phenolic hydroxy group sufficiently to allow protonation of the C6–C7 double bond.[9] However, protonation of this double bond leads to the formation of an unstable carbocation. Inspection of the X-ray structure of AS suggested that the π-system of Trp 334 could interact favourably with the positive charge at C3 of the eudesmane cation.[7] This is an ideal strategy for the stabilisation of high-energy carbocationic intermediates[3] since it prevents the quenching of the positive charge, which might result from interaction with a nucleophilic intermediate. In order to address whether Trp 334 is indeed central for the formation of eudesmane cation we have produced ASW334F, ASW334L, and ASW334V, in which Trp334 was replaced with phenylalanine, leucine, and valine, respectively.

cDNAs for ASW334F, ASW334L, and ASW334V were constructed by site directed mutagenesis from a cDNA of wild-type AS isolated from *P. roqueforti*, expressed to high levels in *E. coli* BL21(DE3) and purified to homogeneity.[13] The steady-state kinetic parameters of the mutant proteins were measured by incubating them with [1-^{3}H]-FPP and determining the amount of hexane-extractable, tritiated products formed.[10] The Michaelis constants for ASW334F, ASW334L, and ASW334V were similar. Relative to the wild-type enzyme they were increased between 15- and 33-fold (Table 1). The turnover number k_{cat} for ASW334F was 0.16 min^{-1}, which is an 11-fold reduction relative to AS. The catalytic efficiency of ASW334F was reduced approximately 350-fold. A further reduction in k_{cat} was observed when hydrophobic but non-aromatic residues replaced Trp334 leading to an overall decrease in the catalytic efficiencies of ASW334L and ASW334V of more than 4 orders of magnitude.

When the hexane extractable materials produced by ASW334F, ASW334L, and ASW334V were further analyzed by GC-MS (Fig. 1),[9,13] the importance of residue 334 of AS for the production of aristolochene became apparent. AS had been reported previously to produce ~92% aristolochene, ~8% germacrene A and a small amount of valencene, which is produced by abstraction of a proton from C6 rather than from C8 in the final step of aristolochene production.[9] When Trp 334 was replaced with phenylalanine, the relative production of **2** was increased to 9.4% and that of **4** reduced to 85.5% (Table 1). **2** was identified as germacrene A from its mass spectrum by comparison with the spectrum in the Wiley library[18] and with that of an authentic sample.‡ Co-injection with an authentic sample confirmed its identity. While the catalytic competence of ASW334F was significantly diminished, it still produced

† Electronic Supplementary Information (ESI) available: GC profiles of co-injections of germacrene A from ASW334F and ASW334V with authentic germacrene A; mass spectra of germacrene A produced by ASW334V and of an authentic sample; mass spectra of valencene produced by ASW334F and of an authentic sample. See http://www.rsc.org/suppdata/cc/b3/b306867f/

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Table 1 Kinetic constants for and relative amounts of hydrocarbon products formed by AS, AS W334F, ASW334F, ASW334V and ASW334L

	Kinetic data[a]			Relative amounts of products			
Enzyme	K_M/μM	k_{cat}/s^{-1}	k_{cat}/K_M/s^{-1} M^{-1}	**2**	**4**	**5**	Others
AS[9]	2.3 ± 0.5	0.03 ± 0.01	13043 ± 2989	7.5	91.5	0.4	0
W334F	66.8 ± 5.8	(2.65 ± 0.7) × 10^{-3}	37.1 ± 6.3	9.4	85.5	4.7	0.4
W334V	33.6 ± 5.2	(9.15 ± 1.0) × 10^{-5}	2.8 ± 0.5	95.3	4.7	0	0
W334L	74.8 ± 17	(2.25 ± 0.2) × 10^{-5}	0.3 ± 0.1	100	0	0	0

[a] Standard errors of mean were determined from a minimum of 3 measurements.

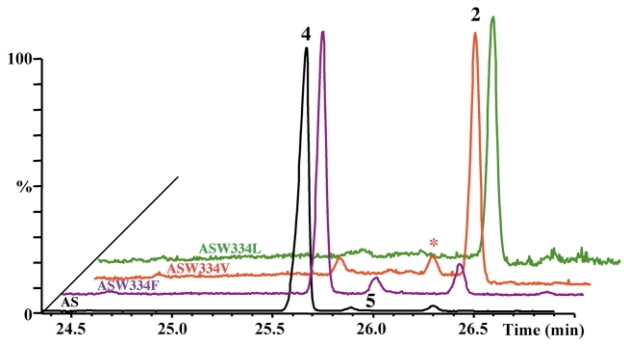

Fig. 1 Total ion chromatograms from GC-MS analysis of the products of catalysis by AS, ASW334F, ASW334V and ASW334L. (*: non-sesquiterpene contaminant.)

aristolochene with only slightly reduced specificity. ASW334F also produced an increased amount of valencene (**5**) (Fig. 1 and Table 1), which was identified by comparison of its mass spectrum with that of an authentic sample,‡ and 0.4% of an unidentified hydrocarbon of mass 204 (Fig. 1).

While the smaller aromatic ring of Phe appeared not to stabilise the developing positive charge in the transition state preceding eudesmane cation as well as the indole ring of tryptophan, stabilisation was nevertheless sufficient for the production of significant amounts of aristolochene. However, when Trp 334 was replaced with Val, 95.3% of the hexane extractable products were germacrene A, while the remainder was aristolochene (Table 1). When Leu replaced Trp, germacrene A was the only product. In the absence of the stabilising interaction with the aromatic π-system, the formation of eudesmane cation was prevented by an exceedingly high energy barrier.

The very low rate constants for ASW334L and ASW334V suggest that Trp 334 also affected the production of germacrene A from FPP in the first step of AS catalysis. If the overall rate of germacrene A formation had only been controlled by product release from the active sites of ASW334L and ASW334V, then k_{cat} would reflect the rate of this physical step. The rate of germacrene A release from AS could be estimated, but would be too low to account for the formation of 7.5% germacrene A by the wild-type enzyme. The speed of germacrene A formation from FPP must therefore be controlled at least in part by Trp334. The π-system of the indole ring of Trp334 could contribute to the stabilisation of the positive charge on C1. Previous evidence has suggested that cyclisation of FPP to germacrene A is not a concerted process, but rather proceeds in a stepwise fashion *via* an allylic cation.[13] Alternatively, the size of the side chain of residue 334 might be important to orientate the pyrophosphate leaving group in a way that allows optimal orbital overlap with the π-orbitals of the neighbouring double bond and the C2–C3 double bond.

In summary, the work described here established that Trp 334 in aristolochene synthase played a central role in the production of aristolochene by stabilising the positive charge of eudesmane cation. When this aromatic residue was replaced with an aliphatic amino acid, only small amounts of aristolochene were produced and the main reaction product was germacrene A. While the possibility that germacrene A is a side product of AS catalysis could not be excluded, these observations were in agreement with the proposal that germacrene A is an intermediate of AS catalysis. Together with the previous observations addressing the functional role of Tyr 92 and the results obtained with mechanism based inhibitors,[17,19] our findings support strongly the reaction mechanism proposed above (Scheme 1) for the production of aristolochene from FPP.

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Notes and references

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