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COMMUNICATION

Templating effects in aristolochene synthase catalysis: elimination *versus* cyclisation†

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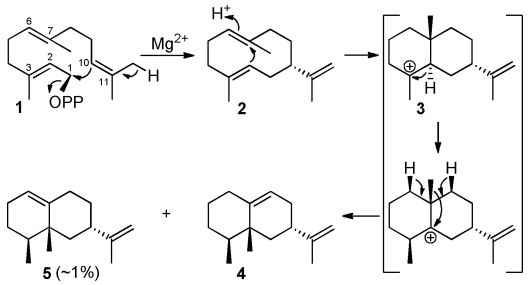
Analysis of the products generated by mutants of aristolochene synthase from *P. roqueforti* (PR-AS) revealed the prominent structural role played by the aliphatic residue Leu 108 in maintaining the productive conformation of farnesyl diphosphate to ensure C1–C10 (σ-bond) ring-closure and hence (+)-aristolochene production.

Aristolochene synthase from *P. roqueforti* (PR-AS) is a fungal sesquiterpene cyclase that catalyses the Mg^{2+}-dependent conversion of farnesyl diphosphate (FDP, **1**) *via* the neutral intermediate germacrene A (**2**), to (+)-aristolochene (**4**) and approximately 1% valencene (**5**) (Scheme 1).[1–3] Aristolochene is considered to be the biosynthetic precursor of several highly oxygenated mycotoxins including PR-toxin.[4] The mechanism for the conversion of FDP to **4** has been studied with modified substrates[1–3,5–10] and azasesquiterpenes that mimic postulated reaction intermediates such as eudesmane cation (**3**).[11,12] Single crystal X-ray analyses of the aristolochene synthases from *P. roqueforti*[13] and *A. terreus*[14,15] and structure-guided site directed mutagenesis studies[16–22] have uncovered active site residues responsible for binding and orienting the flexible substrate **1** towards its unique catalytically active conformation.[23,24] While the molecular recognition and subsequent Mg^{2+}-induced activation of the diphosphate group of **1** relies on charged or aliphatic amino acids, namely the D^{115}DVLE and N^{244}DIYSYDKE metal binding motifs of PR-AS, the well-defined reactive conformation of FDP, the stabilization of fleeting carbocations generated after diphosphate ionization[25] and the propagation of the cationic reaction cascade are thought to be templated by aromatic residues.[6,9,26] Indeed, previous work had shown the important stabilizing role played by Trp 334 in the energetically demanding formation of eudesmane cation (**3**) (Scheme 1).[19,27]

The structural roles of Tyr 92, Phe 112 and Phe 178 in defining the hydrophobic architecture at the bottom of the active site of PR-AS have recently been revealed.[17,20,21] In particular, it is worth mentioning the remarkable steric (and electronic) effect of both Tyr 92 and Phe 112 in enforcing the productive conformation of FDP (**1**) that brings about the initial C1–C10 σ-bond formation following or concomitant with the breakage of the C–O diphosphate bond. While the mutant PR-AS-Y92F displayed essentially a wild type product profile, replacement of Tyr 92 with residues of decreasing steric bulk (Tyr ≈ Phe > Val > Cys > Ala) led to a progressive accumulation of acyclic hydrocarbons (*i.e.* α- and β-farnesenes).[20] More specifically, inspection of a structural model of the enzyme–substrate complex (Fig. 1) suggests that the active site residues Phe 112 and Phe 178, in addition to being suitably oriented to stabilize a positive charge on C1,[13] could play an important role in anchoring the distal C10–C11 double bond in the precatalytic conformation of FDP (**1**) that dictates the correct orbital alignment necessary for C1–C10 σ-bond formation, and hence germacrene A (**2**) production. Our



Scheme 1 PR-AS-mediated conversion of FDP (**1**) to (+)-aristolochene (**4**) and valencene (**5**).

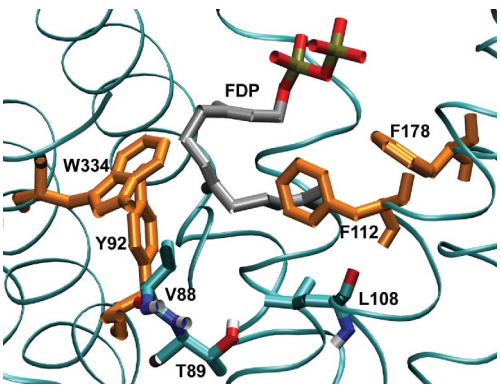


Fig. 1 Cartoon representation of PR-AS with FDP bound in the active site (1F1P.pdb).

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Table 1 Steady state kinetic parameters and relative amounts of hydrocarbons produced by PR-AS, PR-AS-V88A, PR-AS-V88F, PR-AS-T89A and PR-AS-T89F

Enzyme	K_M (μM)	k_{cat} ($\times 10^{-3} s^{-1}$)	k_{cat}/K_M ($\times 10^{-3} s^{-1} \mu M^{-1}$)	**4** (%)	**2** (%)	**5** (%)
PR-AS	0.53 ± 0.2	84 ± 5	158.49 ± 0.71	91.5	7.5	1
PR-AS-V88A	0.64 ± 0.2	2.5 ± 0.1	3.91 ± 0.49	86.2	11.6	2.2
PR-AS-V88F	0.70 ± 0.1	0.8 ± 0.08	1.14 ± 0.24	18.4	57.8	23.8
PR-AS-T89A	1.75 ± 0.6	6.6 ± 0.3	3.77 ± 0.19	93.4	4.4	2.2
PR-AS-T89F	0.50 ± 0.1	1.1 ± 0.2	2.20 ± 0.43	67.6	27.2	5.2

previous mutagenesis experiments indicate that the mutant PR-AS-F112A did not form cyclic hydrocarbons but rather generated linear farnesenes as the predominant (~90%) reaction products, while PR-AS-F178V produced some acyclic sesquiterpenes (12%); as expected, farnesene production (63%) was re-established in the double mutant PR-AS-F112A-F178V.[17] These observations are in good agreement with previous X-ray structure-based mechanistic inferences[13] and highlight the importance of Tyr 92 and Phe 112 in defining the active site contour of PR-AS that ultimately directs (+)-aristolochene (**4**) production from a unique conformation of **1**.

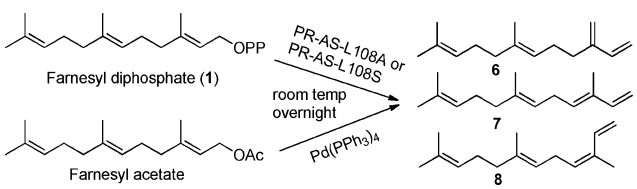
Here we report for the first time the involvement of aliphatic active site residues in PR-AS catalysis.[28] A careful analysis of the active site of PR-AS indicated a series of aliphatic residues that might play a part in maintaining the correct orientations of Tyr 92 and Phe 112 that ultimately modulate the productive conformation of FDP (**1**). Of particular interest,[29] given the close spatial relationships between Val 88 and Tyr 92, and Leu 108 and Phe 112 respectively, was the aliphatic triad formed by Val 88, Thr 89 and Leu 108 that constitutes the lowest hydrophobic part of the active site of PR-AS (Fig. 1). In order to evaluate the contributions of these amino acids to the productive folding of FDP (**1**) and their possible interactions with Tyr 92 and Phe 112, amino acid replacements in positions 88, 89 and 108 were constructed, the resulting cDNAs expressed in *E. coli* and the corresponding proteins purified.[16]

In addition to the reduced catalytic efficiency often observed with mutants of (sesqui)terpene synthases,[25,26] the replacement of Val 88 with alanine led to the production of a PR-AS mutant that displayed a product profile remarkably similar to that of the wild-type enzyme (Table 1). PR-AS-T89A produced a slightly larger amount of the bicyclic aristolochene (**4**) and valencene (**5**) than the parent PR-AS. These results seem to rule out Thr 89 as the active site residue responsible for maintaining, *via* the long range interactions Tyr 92/Val 88/Thr 89 or Thr 89/Leu 108/Phe 112, the catalytic orientation of Tyr 92 and Phe 112. The failure of PR-AS-V88A in abolishing aristolochene production and the farnesene synthase activity of PR-AS-Y92A suggest contributions to the orientation of Tyr 92 from 2nd or 3rd tier amino acids outside the 15 Å wide and 20 Å deep active site cleft[13] (as recently revealed in *epi*-aristolochene biosynthesis[30,31]). Alternatively, possible (intermolecular) non-covalent π–π interactions[32,33] and/or C6(H)–π contacts[33,34] between Tyr 92 and the C6–C7 double bond of FDP could constitute the molecular force(s) needed for bending the C1–C8 segment of **1** to enforce the reactive conformation of FDP (**1**). Indeed, (7*R*)-6,7-dihydrofarnesyl diphosphate has been shown to be a potent inhibitor of aristolochene synthase (K_i = 0.18 μM).[3] Furthermore, substitution of Tyr 92 by histidine (K_M = 0.38 ± 0.2 μM) or tryptophan (K_M = 0.42 ± 0.06 μM), two other aromatic residues known to engage in protein–ligand π-interactions,[35–38] did not alter the affinity of the substrate and produced exclusively cyclic hydrocarbons arising from the expected C1–C10 ring closure (see ESI†).

Notably, the substitution of either Val 88 or Thr 89 by the bulkier phenylalanine lead to an increase in germacrene A production, which was particularly strong (*ca.* 60%) for PR-AS-V88F (Table 1). Given that Val 88 is only approximately 3.5 Å from Trp 334, a residue directly involved in PR-AS catalysis through (eudesmane) cation–π-interactions,[19,27] we reason that the unusual accumulation of germacrene A (**2**) may be a consequence of a change in the active site contour of PR-AS-V88F that precludes the proper orbital alignment between Trp 334 and eudesmane cation (**3**) required for cation–π stabilization.[39]

The leucine rich segment L^{107}LTVLF present in PR-AS is conserved as L^{82}LTVLF in aristolochene synthase from *A. terreus* (AT-AS).[14,15] Inspection of the 2.1 Å resolution single crystal X-ray structure of the farnesyl diphosphate complex of AT-AS[15] revealed, as previously observed in the PR-AS substrate complex,[13] that the distal C10–C11 double bond of **1** is neatly anchored between Phe 87 and Phe 153 (Phe 112 and Phe 178 in PR-AS), and that Leu 83 is in close proximity to Phe 87. These observations suggest that Leu 108 of PR-AS, as well as Leu 83 of AT-AS, may be directly involved in catalysis, or indirectly through the interaction with the proximal Phe 112 residue. However, since PR-AS-F112A released almost exclusively (~90%) linear farnesenes as reaction products,[17] a direct involvement of Leu 108 in catalysis seemed unlikely.

To explore the possible function of Leu 108 in enforcing the orientation of the side chain of Phe112 to place the distal C11–C10 double bond of FDP (**1**) in the required position for efficient C1–C10 cyclization, a series of PR-AS-L108X mutants (X = Ala, Ser, Val and Phe) was constructed. The replacement of Leu 108 with serine or alanine led to an almost complete suppression of the native aristolochene synthase activity and to the formation of a 5 : 10 : 1 mixture of (*E*)-β- (**6**), (*E*,*E*)-α- (**7**) and (*E*,*Z*)-α-farnesenes (**8**) (Scheme 2) as the predominant reaction products.



Scheme 2 (*E*)-β-Farnesene (**6**), (*E*,*E*)-α-farnesene (**7**) and (*E*,*Z*)-α-farnesene (**8**) from **1** and farnesyl acetate (see ESI† and Table 2 for relative amounts of products).

Table 2 Steady state kinetic parameters and relative amounts of sesquiterpenes produced by PR-AS, PR-AS-L108A, PR-AS-L108S, PR-AS-L108V and PR-AS-L108F

Enzyme	K_M (μM)	k_{cat} ($\times 10^{-3}$ s^{-1})	k_{cat}/K_M ($\times 10^{-3}$ s^{-1} μM^{-1})	**4** (%)	**2** (%)	**5** (%)	**6** (%)	**7** (%)	**8** (%)
PR-AS	0.53 ± 0.2	84 ± 5	158.49 ± 0.71	91.5	7.5	1	0	0	0
PR-AS-L108A	0.70 ± 0.2	0.9 ± 0.2	1.29 ± 0.47	6.8	13.9	[a]	26.9	48.2	4.1
PR-AS-L108S	0.99 ± 0.1	9.5 ± 0.2	9.60 ± 0.10	9.4	14.6	[a]	21.5	49.1	5.3
PR-AS-L108V	2.03 ± 0.3	5.7 ± 0.3	2.81 ± 0.07	88.3	8	1	0	0	2.3
PR-AS-L108F	1.76 ± 0.1	3.6 ± 0.1	2.05 ± 0.03	74.1	13.8	12	0	0	0

[a] Trace amounts.

Clearly, the flexible prenyl chain of FDP binds to the altered active sites of PR-AS-L108A and PR-AS-L108S in a more extended conformation, in which C1 and C10 are no longer held sufficiently close for macrocyclization. Interestingly, the product distributions observed with PR-AS-L108A and PR-AS-L108S were similar to that obtained from the unselective Pd(0)-catalyzed elimination of farnesyl acetate under mild conditions (Scheme 2 and ESI†).[40]

Since the reduction in the van der Waals volume induced by the L108A and L108S replacements is expected to compromise a possible interaction between Phe 112 and the aliphatic Leu 108 that would lead to an increase of cyclic products (Table 2), it is tempting to suggest that Leu 108 interacts with Phe 112 to establish the active site-contour that enforces the productive conformation of FDP essential for aristolochene biosynthesis. In agreement with this proposal, the replacement of Leu 108 with valine, which has almost twice the van der Waals volume of alanine, resulted in a product distribution that closely matched that of PR-AS (in addition to a small amount (~2%) of the acyclic **8**). Finally, replacement of Leu 108 with the significantly larger phenylalanine led to the accumulation of cyclic products (**4**, **2** and **5**) arising from the native C1–C10 ring closure. Taken together, these observations suggest that the productive, quasi-cyclic precatalytic conformation of FDP (**1**) is maintained so long as sufficient steric bulk is available. These observations underline the important structural and functional role of the aliphatic residue Leu 108 for aristolochene synthase.

In conclusion, our results indicate the importance of the active site template provided by PR-AS for the outcome of the reaction and demonstrate for the first time the involvement in PR-AS catalysis of an aliphatic residue (Leu 108), which is deeply buried in the lower, hydrophobic active site cleft. Several aromatic (Tyr 92, Phe 112 and possibly Phe178) and aliphatic (Leu 108) amino acids cooperate to enforce a conformation of FDP (**1**), in which after diphosphate ionisation, cyclization is preferred over deprotonation. However, simple amino acid substitutions can perturb the PR-AS template and alter the electronic environment so as to favour the production of alternative (acyclic) sesquiterpenes, uncovering new catalytic activities that highlight the delicate balance between the stereoelectronic control required to achieve the desired chemical outcome and the evolvability of the sesquiterpene synthase scaffold.

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