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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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Probing Eudesmane Cation−π Interactions in Catalysis by Aristolochene Synthase with Non-canonical Amino Acids

Juan A. Faraldos, Alicja K. Antonczak, Verónica González, Rebecca Fullerton, Eric M. Tippmann, and Rudolf K. Allemann*

School of Chemistry, Cardiff University, Main Building, Park Place, Cardiff CF10 3AT, United Kingdom

Supporting Information

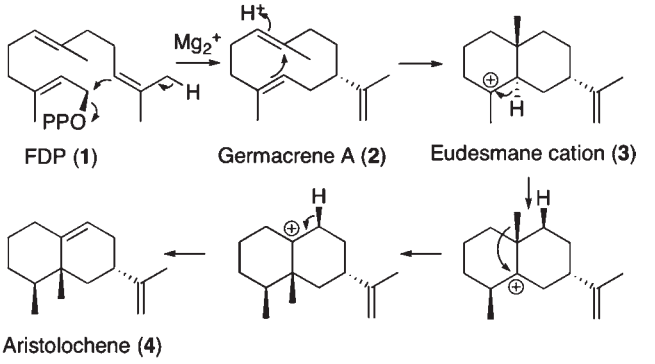
ABSTRACT: Stabilization of the reaction intermediate eudesmane cation (**3**) through interaction with Trp 334 during catalysis by aristolochene synthase from *Penicillium roqueforti* was investigated by site-directed incorporation of proteinogenic and non-canonical aromatic amino acids. The amount of germacrene A (**2**) generated by the mutant enzymes served as a measure of the stabilization of **3**. **2** is a neutral intermediate, from which **3** is formed during PR-AS catalysis by protonation of the C6,C7 double bond. The replacement of Trp 334 with *para*-substituted phenylalanines of increasing electron-withdrawing properties led to a progressive accumulation of **2** that showed a good correlation with the interaction energies of simple cations such as Na^+ with substituted benzenes. These results provide compelling evidence for the stabilizing role played by Trp 334 in aristolochene synthase catalysis for the energetically demanding transformation of **2** to **3**.

The fungal aristolochene synthases from *Penicillium roqueforti* (PR-AS)[1] and *Aspergillus terreus* (AT-AS)[2] and the related tobacco 5-*epi*-aristolochene synthase (TEAS)[3] catalyze the magnesium-dependent cyclization and cationic rearrangements of farnesyl diphosphate (FDP, **1**) to the bicyclic sesquiterpenes aristolochene (**4**) and *epi*-aristolochene, the parent eremophilene hydrocarbon scaffolds of oxygenated mycotoxins and phytoalexins such as PR-toxin[4] and capsidiol.[5] Work with FDP substrate analogues,[6,7] mechanism-based inhibitors,[8] ammonium and iminium cation mimics of presumed carbocationic intermediates (e.g., **3**),[9] and aristolochene synthase mutants,[11,12] as well as stereochemical analyses with deuterated forms of FDP[10] and X-ray protein crystallographic studies,[13−15] supports a catalytic mechanism with the neutral macrocycle germacrene A (**2**) and the transient eudesmane cation (**3**) as intermediates (Scheme 1).

Unlike most biological reaction mechanisms, which go through neutral and anionic intermediates, terpene biosynthesis is essentially carbocationic in nature.[16,17] The key question in all terpene synthase-mediated cyclization cascades is how high-energy carbocations are stabilized within the "mild" environment provided by the active sites of these enzymes. Negatively charged amino acids are largely ruled out since their interaction with the carbocationic intermediates might lead to alkylation and hence suicide inhibition. The X-ray crystal structure of terpene cyclases[17] indicated that their active sites are lined with aromatic amino acids (Figure 1), thereby providing unreactive, hydrophobic environments for cation stabilization. Widespread in molecular biology and essential for molecular recognition,[18−20] cation−π interactions arise mostly from cation-induced polarization contributions[21] and the electrostatic attraction between ions (often alkali or tetraalkylammonium cations) and the large permanent quadrupole of aromatic rings.[18]

On the basis of X-ray structural analysis of recombinant PR-AS, it has been proposed that the indole ring of the active-site residue Trp 334 could be responsible for stabilization of **3**

Scheme 1. Biosynthesis of (+)-Aristolochene



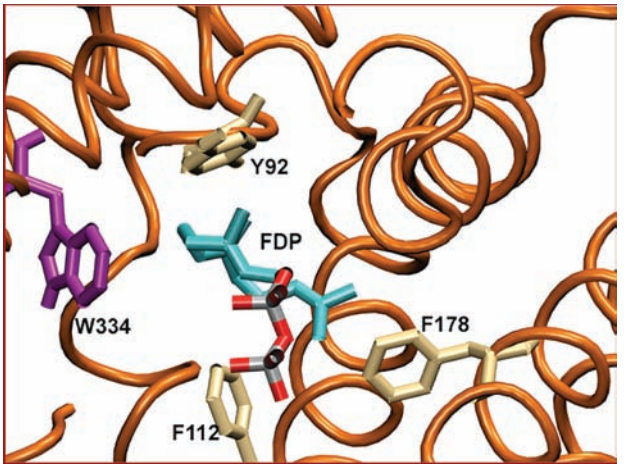


Figure 1. Sketch of the active site of PR-AS, indicating the position of Trp 334 and a modeled conformation of FDP (PDB code: 1F1P).[13]

Received: June 25, 2011
Published: August 04, 2011

Table 1. Kinetic Parameters and Product Distributions for WT PR-AS and PR-AS-W334 Natural Mutants

enzyme	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M ($min^{-1}\mu M^{-1}$)	**4** (%)[a]	**2** (%)
WT[b]	5.04	0.53	9.51	92	8
W334F[c]	0.38	0.28	1.40	82	18
W334Y	0.43	1.03	0.42	62	38
W334H	0.02	0.13	0.14	10	90
W334L[c]	0.16	0.75	0.22	2	98

[a] Includes percentage of valencene; for details, see Supporting Information [b] Values taken from ref 9a. [c] See also ref 11b.

Table 2. Kinetic Parameters and Product Distibutions for $(His)_6$-WT PR-AS and $(His)_6$-PR-AS-W334 Non-canonical Substitutions

enzyme	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M ($min^{-1}\mu M^{-1}$)	**4** (%)[a]	**2** (%)
His-tag WT	3.18	0.93	3.41	96	4
W334naphthyl	0.09	1.48	0.06	78	22
W334*p*-(Cl)F	0.01	0.32	0.03	57	43
W334*p*-(CF_3)F	0.01	0.88	0.01	31	69
W334*p*-(NO_2)F	0.02	1.02	0.02	23	77

[a] Includes percentage of valencene; for details, see Supporting Information.

through cation−π interactions (Figure 1).[13] This hypothesis was investigated with PR-AS mutants, PR-AS-W334F, PR-AS-W334L, and PR-AS-W334V, which lack the aromatic side chains required for stabilization of **3**.[11b] While the substitution of Trp 334 with phenylalanine had only a small effect on the product distribution (Table 1), replacement of Trp 334 with leucine or valine derailed the enzymatic reaction at the germacrene A stage, a strong indication of the pivotal role of Trp 334 in the formation of **3**. The failure of exogenous diphosphate to enhance the binding affinity of the aza-analogue of cation **3**[9a] has previously been interpreted as evidence against the formation of carbocation−diphosphate anion pairs[22] in the PR-AS active site as an alternative electrostatic strategy[23,24] to stabilize **3**.

Tryptophan is ideal for cation−π stabilization and is often found at potential cation−π binding sites[18] such as the active site of terpene synthases. Amino acid sequence alignments (Supporting Information) and X-ray structural analyses[17] reveal that many fungal and plant sesquiterpene synthases maintain a conserved tryptophan residue at their catalytic sites. Therefore, the replacement of the active site residue Trp 334 with natural (Table 1) or non-canonical (Table 2) amino acids with lower π-electron densities than tryptophan might be expected to decrease the rate of the chemical reaction and likely change the product profile due to a reduction in stabilizing cation−π interactions. This effect is clearly observed with the PR-AS-W334F mutant, where a 10% increase in the amount of **2** and a concomitant reduction in the catalytic efficiency (k_{cat}/K_M) were observed relative to the wild-type enzyme (Table 1). Histidine is unlikely[18] to be found at presumed cation−π binding sites since its protonated form at physiological pH would effectively preclude cation−π interactions; indeed, the W334H mutation led to an almost complete suppression of aristolochene synthase activity, as evidenced by the 11-fold increase in **2** production and 70-fold decrease in catalytic efficiency (Table 1). Finally, aristolochene production was all but abolished when leucine replaced Trp 334.[11b]

Based on previous *ab initio* calculations, the results obtained with the PR-AS-W334Y mutant were slightly surprising. Calculations suggest that Phe and Tyr should have similar potential to stabilize neighboring cations.[25,26] The W334Y-PR-AS mutant showed an increase in **2** and a 3-fold decrease in catalytic efficiency when compared to PR-AS-W334F (Table 1). These results exemplify the highly directional nature of the non-covalent cation−π interaction[18a,21c,25a,27] and may suggest that the PR-AS-W334Y enzyme enrolls, via the phenolic hydroxyl group, in a hydrogen-bonding network that prevents the orientation of the aromatic ring for optimal cation−π stabilization along the C6 axis of the ring.[27] Taken together with the similar Michaelis constants (K_M) obtained for PR-AS-W334X (X = F, Y, H, and L) and the remarkable and progressive decrease (F ≈ Y > L > H) in the reaction rates (k_{cat}) rates, the data seem in good agreement with the proposed non-bonding interaction between cation **3** and Trp 334.

However, rather than being a consequence of altered cation−π interactions in the mutants enzymes, the premature termination of the reaction cascade at the germacrene A stage could be caused simply by changes to the active-site architecture that prevent protonation of **2**. To probe the physicochemical significance of cation−π interactions for the stabilization of the transition state leading to **3**, a series of non-canonical aromatic amino acids with severely reduced π-electron densities (i.e., with lower cation−π binding energies)[25] were introduced into PR-AS in place of Trp 334. If the production of **2** by the mutants described above (Table 1) were merely a consequence of a reduction of steric bulk (W > Y ≈ F > H ≈ L)[28] at position 334, any bulky aromatic mutant would be expected to re-establish aristolochene production. On the other hand, if the π-electron density of residue 334 facilitates formation of **3**, a progressive increase in **2** production would be expected with decreasing π-electron density, even if, at the same time, the bulk of residue 334 increases. In this scenario, replacement of residue 334 with amino acids possessing lower cation−π binding abilities should have a stronger effect on the turnover rate of the reaction than on the binding affinity (K_M), in good agreement with the results obtained for the canonical replacements described above (Table 1).

Since the number of proteinogenic amino acids capable of cation−π interactions is limited, an array of non-canonical amino acids with diverse electronic properties was introduced into PR-AS in a site-specific manner using the amber suppression method with orthogonal aminoacyl tRNA synthetase/amber suppressor tRNA pairs.[29] The TAG mutation was introduced into the PR-AS gene at the position corresponding to Trp 334. Additionally, a genetically encoded C-terminal hexa-histidine affinity tag was incorporated to facilitate purification and removal of truncated products. In all, four PR-AS mutants with non-canonical amino acids were generated, purified, and compared to a PR-AS variant that was allowed to terminate at the newly installed stop codon (PR-AS-W334TAG). As expected, this truncated protein was inactive, revealing the importance of the last C-terminal nine amino acids for PR-AS catalysis.

Replacement of tryptophan by *para*-substituted phenylalanines with strong electron-withdrawing substituents (X_{para} = Cl, CF_3, NO_2) had only minor effects on the K_M values of the reaction but led to approximately 30-fold decreases of k_{cat} relative to PR-AS-W334F (X_{para} = H) for all W334X_{para}F mutants (Table 2). Interestingly, the k_{cat} values for W334*p*-(Cl)F,

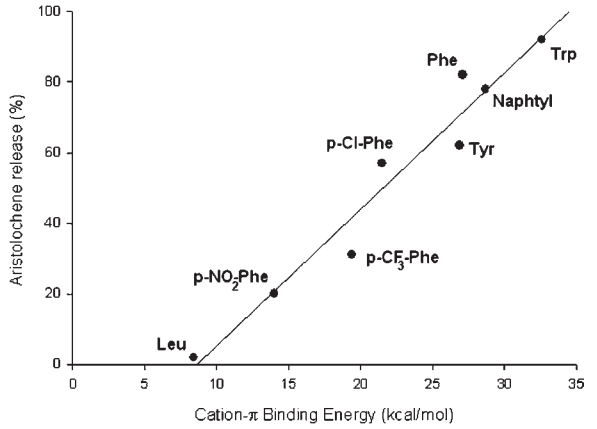


Figure 2. Relationship between the cation−π binding energies and the percentage of aristolochene produced by PR-AS, PR-AS-W334Phe, PR-AS-W334naphtyl, PR-AS-W334Tyr, PR-AS-W334Leu, PR-AS-W334*p*-(Cl)Phe, PR-AS-W334*p*-(CF_3)Phe, and PR-AS-W334*p*-(NO_2)Phe (R^2 = 95). Binding energies were taken from the literature.[25,26] The value of 8.5 kcal/mol for leucine corresponds to the calculated binding energy of the Na^+−cyclohexane pair.[25]

W334*p*-(CF_3)F, and W334*p*-(NO_2)F were similar to that observed for PR-AS-W334H, a mutant in which cation−π interactions are precluded due to protonation of the imidazole ring. Hence, as suggested by the results in Table 1, the relative amounts of **2** produced by the mutants seem unambiguously associated with the progressively decreased π-electron density rather than with the inherent bulk of the PR-AS-W334X_{para}F (or PR-AS-W334X) enzymes. Had steric bulk of amino acid 334 been the driving force for progression through the catalytic cycle, substituents such as *p*-(NO_2) and *p*-(CF_3) might have been expected to increase the amount of aristolochene. The fact that the introduction of the substituents led to a decrease in aristolochene production indicates the importance of electronic effects on stabilization of the transition state leading to **3**. Indeed, substitution of tryptophan by phenylalanine (PR-AS-W334F, Table 1) or by a bicyclic aromatic amino acid (PR-AS-W334-naphthyl, Table 2), two aromatic residues of similar cation−π binding energy,[25] led to the production of comparable amounts of aristolochene (82 and 78%). Likewise, the reduction in aristolochene produced by PR-AS-W334naphthyl relative to the wild-type enzyme correlates with the reduction in the cation−π binding energy of the naphthyl group relative to the indole ring.[25] The groups of Dougherty[25] and Houk[26] have calculated binding energies of sodium cation to substituted benzene rings. A plot of the percentage of aristolochene produced by PR-AS and its mutants against their Na^+−π binding energies showed a good correlation (Figure 2), indicating a strong contribution from the indole ring of Trp 334 to the energetically demanding conversion of **2** to **3** in PR-AS catalysis.

Similar to Trp 334, it is likely that the PR-AS active-site residues Tyr 92, Phe 112, and Phe 178 (Figure 1) are also involved in the stabilization of carbocationic intermediates along the reaction pathway outlined in Scheme 1.[11] In addition, sequence alignments of plant terpene synthases, together with the high structural similarities of all class I terpenoid synthase folds, suggest that aromatic quadrupoles are generally important for the stabilization of cationic transition states and intermediates in terpene biosynthesis. For instance, Trp 273 of TEAS,[30] which shows no homology to PR-AS, is in a position similar to that of Trp 334 in PR-AS; hence, **3** in TEAS is most likely stabilized through interaction with the π-system of Trp 273. Cation−π interactions have recently been shown to be involved in the cationic polycyclization cascade catalyzed by a squalene−hopene cyclase.[31]

In summary, the results reported here provide strong support for the significance of cation−π interactions for the energetically demanding stabilization of transition states and reaction intermediates in sesquiterpene synthase chemistry. Active sites rich in aromatic residues provide the ideal environment for the tight and selective binding of carbocations in the non-polar environment required for the synthesis of hydrocarbon products.

■ ASSOCIATED CONTENT

Supporting Information. Experimental procedures including DNA manipulations, protein purification, GC-MS data, kinetic plots, and amino acid sequence alignments. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
allemannrk@cf.ac.uk

■ ACKNOWLEDGMENT

The authors gratefully acknowledge Prof. Ryan Mehl for reagents to incorporate non-canonical amino acids and the financial support of the United Kingdom's Biotechnology and Biological Sciences Research Council through grant BB/G003572/1 (R.K.A., E.M.T.).

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