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Enzymatic synthesis of natural (+)-aristolochene from a non-natural substrate†

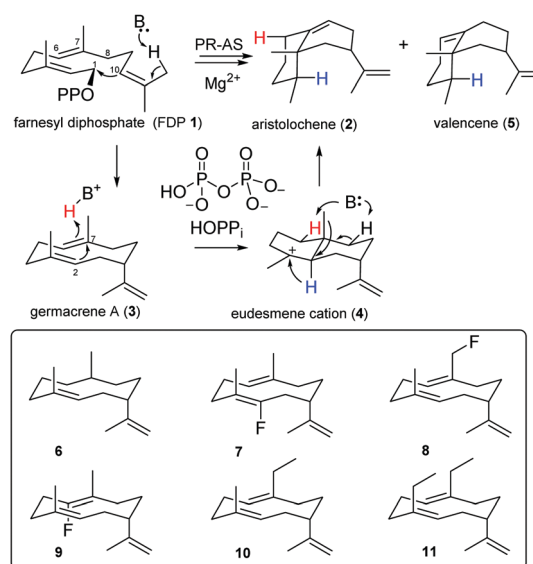
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The sesquiterpene cyclase aristolochene synthase from *Penicillium roquefortii* (PR-AS) has evolved to catalyse with high specificity (92%) the conversion of farnesyl diphosphate (FDP) to the bicyclic hydrocarbon (+)-aristolochene, the natural precursor of several fungal toxins. Here we report that PR-AS converts the unnatural FDP isomer 7-methylene farnesyl diphosphate to (+)-aristolochene via the intermediate 7-methylene germacrene A. Within the confined space of the enzyme's active site, PR-AS stabilises the reactive conformers of germacrene A and 7-methylene germacrene A, respectively, which are protonated by the same active site acid (most likely HOPP_i) to yield the shared natural bicyclic intermediate eudesmane cation, from which (+)-aristolochene is then generated.

Terpenoids are found in all forms of life and comprise one of the largest and most structurally diverse families of natural products.^{1–4} Sesquiterpenoids are biosynthesized from the universal linear C15-precursor farnesyl diphosphate (FDP, **1**), which is converted in electrophilic cyclization reactions of often exquisite specificity to > 300 mostly cyclic hydrocarbon products in class I sesquiterpene synthase catalysed processes.⁵ The electrophilic nature and stereochemical precision of these cascade reactions have been probed *in vitro* with FDP isotopologues^{6–11} as well as with substituted FDPs that carry fluoro,^{12–16} methyl^{17,18} and phenyl substituents.¹⁹ The ability of sesquiterpene synthases to bind such synthetic FDP analogues as competitive inhibitors^{13,19,20} and occasionally as substrates^{11,12,14,16–18} demonstrates their plasticity and catalytic flexibility, properties that have been exploited for the synthesis of several complex unnatural molecules.^{12,14,17,21}

Aristolochene synthase from *Penicillium roquefortii* (PR-AS) efficiently (92%) catalyses the cyclisation of **1** to the bicyclic

eremophilane hydrocarbon (+)-aristolochene (**2**), the precursor of several highly oxygenated mycotoxins such as PR-toxin.^{8,22} This cyclisation, which generates two 6-membered rings and introduces two double bonds and three stereo-centres with high specificity,^{23–25} goes through the neutral intermediate germacrene A (**3**), a flexible cyclodecadiene hydrocarbon that undergoes reprotonation to eudesmane cation (**4**),^{24,25} most likely by proton transfer from the by-product diphosphate (HOPP_i).^{26–28} Rearrangement of **4** by way of a hydride and a methyl shift is followed by a selective deprotonation at C8 to yield (+)-aristolochene (**2**) (Scheme 1) together with a small amount of ~1% valencene (**5**), the double bond isomer of **2** that results from deprotonation at C6 rather than C8. PR-AS also releases ~7% germacrene A (**3**) prior to protonation of the central C6,C7-double bond of **3**, possibly as a consequence of a conformational change prior to eudesmane cation formation.^{24,25}



Scheme 1 Biosynthesis of (+)-aristolochene (**2**) and unnatural germacrene A (**3**) and eudesmane cation (**4**) from the corresponding FDP analogues (**6–11**).

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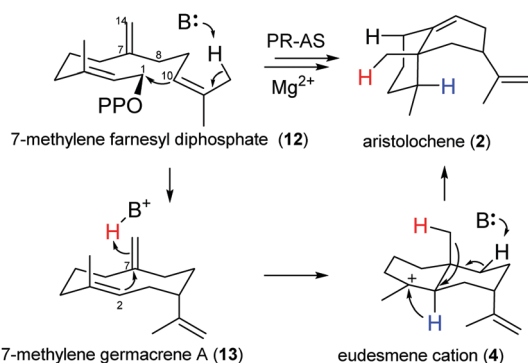
† Electronic supplementary information (ESI) available: Synthesis of FDP and 7-methylene-FDP, gas chromatograms, MS spectra, ¹H-NMR spectra and molecular calculations. See DOI: 10.1039/c6cc08164a



The intermediacy of eudesmane cation (**4**) was inferred from site directed mutagenesis experiments^{29,30} and inhibition studies with ammonium and iminium salt analogues of **4**.^{31,32} Evidence for the formation of germacrene A (**3**) was first obtained when PR-AS was challenged with 6,7-dihydroFDP, which was turned over to the corresponding dihydrogermacrene A (**6**).²¹ Similarly, the PR-AS catalysed turnover of several fluoro- and methyl-substituted FDP analogues to the corresponding altered germacrene A analogues (**6–11**)^{12,14,17,21} illustrated the synthetic potential of sesquiterpene synthases for organic synthesis.^{11,14,16–18}

If HOPP_i protonates germacrene A (**3**) and its corresponding base (⁻OPPi) acts to abstract a proton from eudesmane cation (**4**) in the final step of PR-AS catalysis^{27,28} (Scheme 1), the synthetic space accessible to PR-AS might potentially be expandable and 7-methylene FDP (**12**), an unnatural FDP isomer that the enzyme will never be exposed to in the host organism, could be efficiently converted to aristolochene by reprotonation of the C7,C14 double bond of the intermediate 7-methylene germacrene A (**13**) to yield eudesmane cation (**4**) (Scheme 2), accomplishing for the first time a one-step enzymatic synthesis of a structurally complex natural sesquiterpene (**2**) from a non-natural, synthetic precursor. Comparison of the relative reaction rates of the hydration for a series of simple olefins in aqueous sulfuric acid³³ suggests that both **3** and **13** should have similar reactivity towards protonation under general acid catalysis.

To assess the feasibility of the PR-AS catalysed cyclisation of 7-methylene-FDP (**12**) to **2**, quantum mechanical geometry optimisation and docking into the enzyme active were performed. Previously, it was suggested that **3** exists as a mixture of three conformers in solution with the UU conformer (enforced by PR-AS, Fig. 1) as the predominant form (52%).³⁴ Molecular modeling, in which the hydrocarbon chain was confined to the approximate active site volume, confirmed that like for **3**, the most stable conformation of **13** is the UU form. Upon relaxation *in vacuo*, distances between C2 and C7 in **3** and **13** are ~3.3 Å. When the volume accessible to **3** and **13** is constrained, marked shortening of the C2–C7 distances were observed to less than 2.5 Å (Fig. 1). Docking of the constrained structures of **3** and **13** in the (highly homologous) closed active site of aristolochene synthase from *A. terreus*³⁵ (Fig. 2) demonstrates that both fit in the active site pocket in similar conformations and in an



Scheme 2 The PR-AS catalysed conversion of 7-methylene-FDP (**12**) to (+)-aristolochene (**2**).

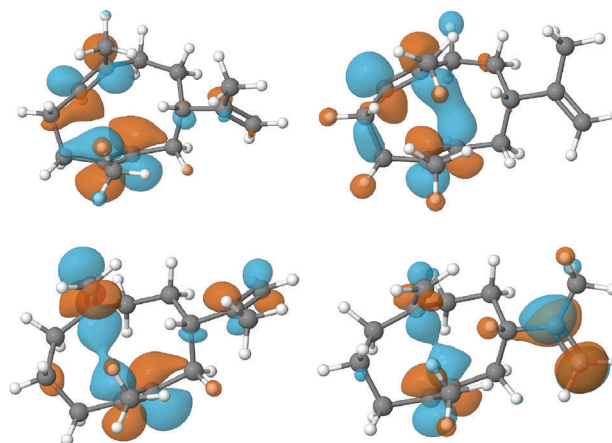


Fig. 1 Geometries and LUMOs of **3** (above) and **13** (below) in their reactive UU conformations, after unconstrained optimisation (vacuum) (left) and constrained optimisation and docking into the enzyme active site (right). Under constrained volume conditions **3** and **13** become more similar and the 2,7-contact shorter than the 2,6 contact (see also Fig. S2, ESI[†]).

orientation consistent with the expected reaction path, with *e.g.* ⁻OPPi abstracting a proton from **1** or **12**. As an indicator of the direction of the second ring closure reaction (Scheme 1), we modeled the LUMOs of **3** and **13** both *in vacuo* and in the enzyme environment. Upon constrained optimisation of the vacuum relaxed structures, the nodal configurations markedly changed for both **3** and **13**, and they remain similar when the enzyme environment is taken into account. The calculated LUMOs highlight the favourable orbital overlap across the ring and demonstrate the feasibility of the acid-catalysed C2,C7 ring closure for both **3** and **13** inside PR-AS (Fig. 1).

After expressing the PR-AS cDNA in *E. coli* (ESI[†]), GC-MS analysis of incubations of 7-methylene FDP (**12**) with purified recombinant PR-AS revealed the *in vitro* formation of a 5:3 mixture of two major products (Fig. 3). Comparison of the elution profiles in the gas chromatograms and MS fragmentation patterns from parallel incubations of **1** and **12** with PR-AS and *Solidago canadensis* germacrene A synthase (GAS)³⁶ indicated that the

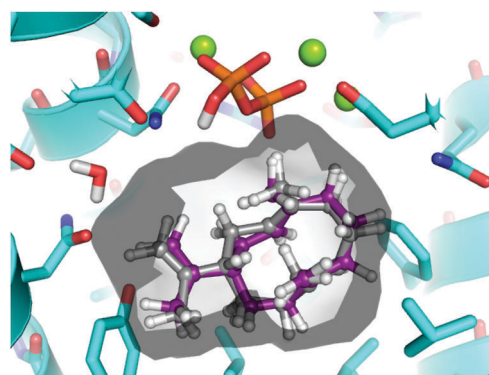


Fig. 2 Constrained geometries of **3** (grey carbons) and **13** (purple carbons) as docked in the active site of aristolochene synthase from *A. terreus* (cyan carbons; PDB ID: 4KVY, chain A). The solvent accessible surface of the enzyme active site is shown. For clarity, hydrogen atoms are removed from protein residues.



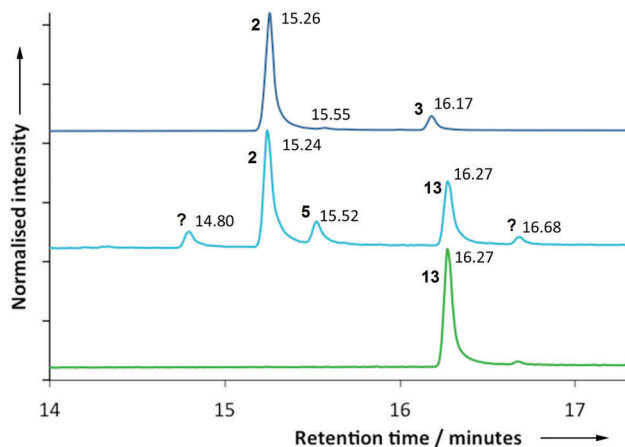


Fig. 3 Gas chromatographic analyses of the products produced from incubations of PR-AS with FDP (**1**) (blue, top) and 7-methylene FDP (**12**) (light blue, middle). For comparison, the result from an incubation of germacrene A synthase (GAS) with 7-methylene FDP (**12**) is also shown (green, bottom).

two products generated by PR-AS from **12** were aristolochene (**2**) (50.1%, 15.24 minutes) and the germacrene A analogue **13** (30.1%, 16.27 minutes). In addition to **2** and **13** three minor products were formed (Fig. 3) that made up 19.8% of the total amount of products. Their identity was not unambiguously determined but the possibility that they were linear 7-methylene farnesenes was ruled out by comparison of the GC chromatograms and MS spectra of the products obtained from the reaction of **12** with *Mentha X piperita* (*E*)- β -farnesene synthase (EBFS).³⁷ The GC retention times and comparison with the MS of the products formed from incubations of **1** with PR-AS suggested that the compound that eluted at 15.55 (*ca.* 10%) min was valencene (**5**) (Fig. 3). The production of **2** from diphosphate **12** suggests that both reactions go *via* eudesmane cation (**4**) (Schemes 1 and 2). In the case of **13**, the *exo* double bond may be positioned slightly less favourably for protonation so that **13** is longer lived in the active site than **3** and more germacrenoid **13** (30%) is released than is the case for **3** (7%). Alternatively, **13** might bind less tightly to the active site of PR-AS. Nevertheless, the similarity of **1** and **12** and the plasticity of PR-AS lead to similar active site conformations for both substrates so that the initial 1,10-ring closure reactions to **3** and **13** occur along closely related reaction paths. The molecular modeling results demonstrate the importance of constraining the germacrene A intermediates **3** and **13** in the active site for the protonation of the *endo*-C6,C7 and *exo*-C7,C14 double bonds.

The investigation reported here demonstrates the remarkable versatility of PR-AS, which effectively turns over an unnatural synthetic precursor to the natural products aristolochene (**2**) and valencene (**5**). The PR-AS catalysed conversion of the unnatural substrate **12** to (+)-aristolochene (**2**) illustrates the power of substrate engineering for the *in vitro* synthesis of structurally complex natural products utilizing sesquiterpene synthases as biocatalysts. Our *in silico* work also highlights the importance of constraining the reactants for the reactivity of intermediates towards the generation of complex terpene products, an effect that has first been seen in the relatively easy, albeit less specific

biomimetic cyclisation reactions of squalene in the absence of enzyme, where the hydrophobic effect leads to restrictions of the conformational space accessible to the hydrocarbon chain.³⁸ For shorter chain isoprenoid diphosphates such as geranyl diphosphate, farnesyl diphosphate and geranylgeranyl diphosphate on the other hand, the enzyme is needed to act as a folding template for efficient product formation.

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