Probing the reaction mechanism of aristolochene synthase with 12,13-difluorofarnesyl diphosphate†

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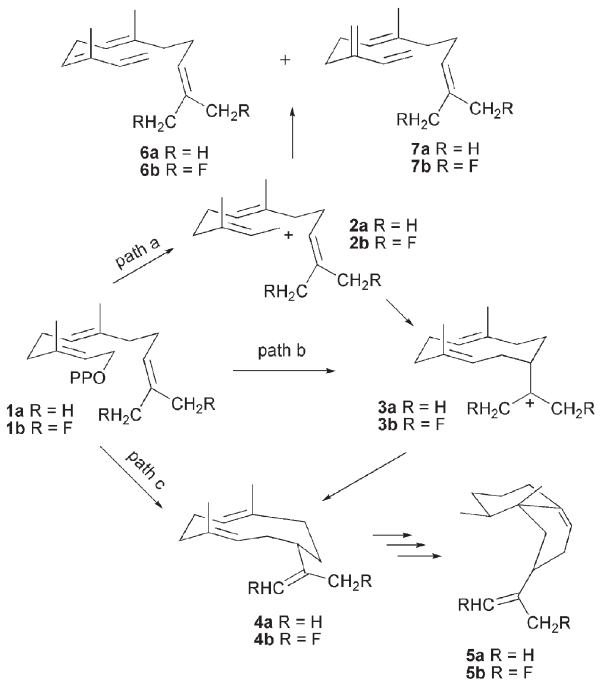
12,13-Difluorofarnesyl diphosphate, prepared using Suzuki–Miyaura chemistry, is a potent inhibitor of aristolochene synthase (AS), indicating that the initial cyclisation during AS catalysis generates germacryl cation in a concerted reaction.

The terpenoid family of natural products consists of many thousand compounds of enormous structural and stereochemical diversity that derive from only a small number of linear isoprenoid precursors. Sesquiterpene synthases, for instance, catalyse the cyclisation of the universal acyclic precursor farnesyl diphosphate (**1a**, FPP) to produce more than 300 different hydrocarbon skeletons, often with high regio- and stereospecificity.[1–4]

The solution of the X-ray crystal structures of 5 sesquiterpene synthases, namely 5-epi-aristolochene synthase from *Nicotiana tabacum*,[5] pentalenene synthase from *Streptomyces* UC5319,[6] trichodiene synthase from *Fusarium sporotrichoides*[7] and aristolochene synthase (AS) from both *Penicillium roqueforti*[8] and *Aspergillus terreus*,[9] revealed that, in spite of the absence of significant sequence similarities, they all adopt the mainly α-helical class I terpene fold. Sesquiterpene synthases therefore serve as high fidelity templates that subtly channel conformation and stereochemistry during the cyclisation reactions.

The mechanisms of the enzyme-catalysed cyclisations of sesquiterpenoids are inherently difficult to study spectrometrically and their elucidation has been largely dependent on the use of substrate mimics and site directed enzyme mutants. Mechanistic studies with substrate analogues suggested that AS converts **1a** into the bicyclic sesquiterpene aristolochene (**5a**) through an initial cyclisation to generate the intermediate (*S*)-germacrene A (**4a**) followed by protonation, 1,2 hydride and methyl shifts and stereospecific deprotonation from C8 (Scheme 1). Surprisingly little is known about the reaction mechanism of the formation of the decalin ring system that could be formed by several mechanistically distinct pathways. The results obtained with site-specific mutants of AS suggested that diphosphate ionisation led to the formation of farnesyl cation (**2a**) which was attacked by the C10–C11 π-bond to generate germacryl cation (**3a**) (path a, Scheme 1).[10,11] Alternatively, the formation of **3a** could take place in a concerted reaction in which farnesyl diphosphate ionisation is accompanied by electrophilic attack of C1 of FPP by the C10, C11 π-system (path b).[9] This mechanism is in agreement with the observation that the reaction of stereospecifically deuterated FPP



Scheme 1 Possible mechanisms for the initial cyclisation during catalysis by aristolochene synthase.

occurred with inversion of configuration at C1.[12] In path c, cyclisation is initiated by deprotonation from C12[13] and concurrent bond formation between C10 and C1. Since it has become increasingly apparent that only a few amino acid substitutions can be sufficient to remodel the active site template provided by terpene synthases, thereby often dramatically altering the reaction products, a detailed mechanistic understanding of the reaction catalysed by these enzymes is central to their further evolution *in vitro* or *in vivo* for the generation of novel products.[14]

Fluoro analogues of prenyl diphosphates have recently been used as powerful reagents to explore the mechanism of the enzyme-catalysed cyclisations to terpenoid products.[15] Fluoro substituents do not greatly affect the binding affinities as a consequence of size and shape but at the same time exert a strong influence on the electronic environment at the site of replacement in that they stabilize cations on the α-carbon by π-donation but exert a destabilizing inductive effect on cations located on the β-carbon. Here we report the synthesis of 12,13-difluorofarnesyl diphosphate (**1b**) and its effects on AS-catalysis.

Due to the electronic effects of fluoro substituents on carbocations described above, incubation of 12,13-difluorofarnesyl

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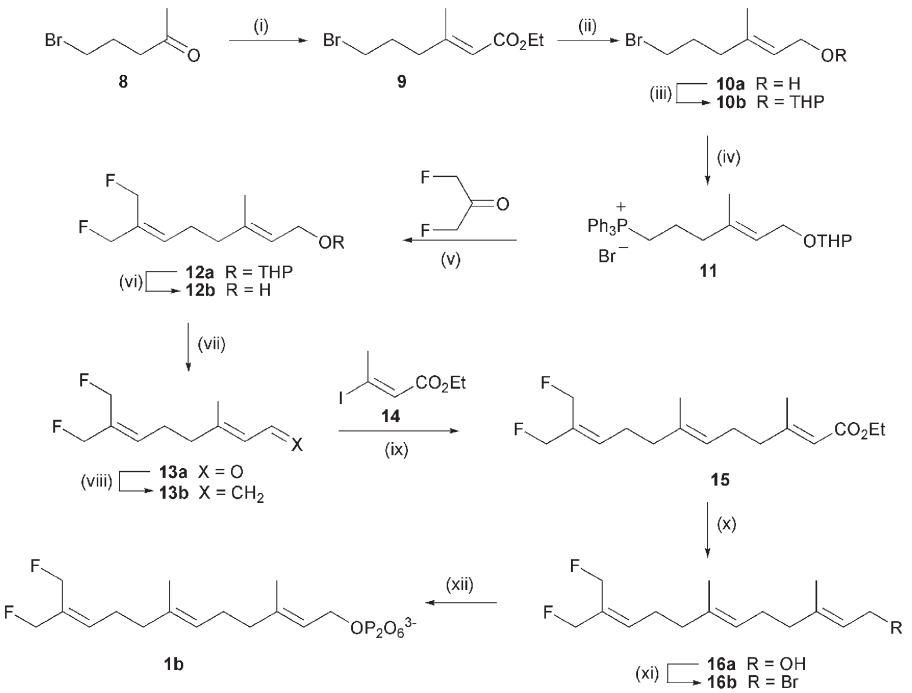

Scheme 2 Synthesis of 12,13-difluorofarnesyl diphosphate **1b**. *Reagents and conditions*: (i) triethyl phosphonoacetate, NaH, THF, 0 °C, 68%; (ii) DIBAL-H, THF, −78 °C, 98%; (iii) 3,4-DHP, CH_2Cl_2, *p*-TsOH, 98%; (iv) PPh_3, CH_3CN, Δ; (v) LiHMDS, THF, −78 °C, 87%; (vi) PPTS, EtOH, 55 °C, 97%; (vii) TPAP, NMO, CH_3CN, 82%; (viii) CH_3PPh_3Br, n-BuLi, THF, 78%; (ix) 9-BBN, THF then **14**, $PdCl_2dppf$, NaOH, $AsPh_3$, THF, 50 °C, 28%; (x) DIBAL-H, THF, −78 °C, 68%; (xi) NEt_3, MsCl, −45 °C then LiBr; (xii) $(Bu_4N)_3HP_2O_7$, CH_3CN then DOWEX ion exchange Bu_4N^+/NH_4^+,10%.

diphosphate with AS would be expected to generate different outcomes for the three cyclisation pathways of Scheme 1. For path a, the destabilizing effect of the two fluoro substituents on the putative carbocation on C11 should lead to the accumulation of the 12,13-difluorofarnesyl cation (**2b**) and the formation of 12,13-difluoro-(*E*,*E*)-α-farnesene (**6b**) and 12,13-difluoro-(*E*)-β-farnesene (**7b**) through deprotonation from C4 or C15. For a reaction where phosphate departure occurs simultaneously with electron flow from the C10, C11 double bond, 12,13-difluorofarnesyl diphosphate would be expected to act as a competitive inhibitor. In pathway c, the acidifying effect of the fluoro substituents on the protons on C12 and C13 should allow the reaction to proceed, ultimately generating 12,13-difluoroaristolochene (**5b**).

12,13-Difluorofarnesyl diphosphate (**1b**) was synthesised from bromoketone **8** (Scheme 2).[16] Compound **8** was initially subjected to a Horner–Emmons reaction with triethyl phosphonoacetate to generate bromoester **9** with good stereoselectivity ($E : Z = 11 : 1$).[17] Reduction of the ester with DIBAL-H in THF at −78 °C followed by protection of the resulting alcohol with a THP group gave **10b** in near quantitative yield. This compound was converted to the phosphonium bromide salt **11** and then treated with 1,3-difluoroacetone in a Wittig reaction to give **12a** in 87% yield. The THP ether was removed and the alcohol **12b** converted to the 1,3-diene **13b** by successive TPAP oxidation and Wittig reaction. A Suzuki–Miyaura coupling with the crotonyl iodide **14**[18] resulted in the production of ester **15**, which was converted to the diphosphate **1b** by reduction to the alcohol and diphosphorylation.[19] It is worthy of note here that attempts to prepare the ester **15** by the more conventional chain extension chemistry of Weiler and Sum[20] failed since the use of lithium dimethylcuprate resulted in loss of fluorine atoms, most likely by elimination of hydrogen fluoride.

Incubation of **1b** with purified recombinant AS under the standard reaction conditions previously used for the conversion of **1a** to aristolochene[11] did not lead to any hexane extractable products as judged by GC-MS analysis, even after prolonged incubation. Since 12,13-difluorofarnesyl diphosphate clearly did not act as a substrate for AS, the steady-state kinetic parameters of purified recombinant AS were measured in the presence of varying amounts of **1b** by incubation with [1-^{3}H]-FPP and monitoring the formation of tritiated, hexane extractable products.[21] A double reciprocal plot indicated that **1b** was a reversible competitive inhibitor of AS (Fig. 1). The measured K_I of 0.8 ± 0.2 μM was comparable to the Michaelis constant for AS catalysis (K_M = 2.3 μM) reported previously, indicating that the fluoro substituents do not have a negative effect on the affinity of the enzyme for this compound. For comparison, farnesyl thiodiphosphate,[22,‡] a compound known to act as a potent inhibitor of undecaprenyl diphosphate synthase from *E. coli* and *S. aureus*,[22–24] was also tested and found to also act in a competitive fashion, albeit with much reduced affinity (K_I = 10 μM).

The observation that **1b** acts as an inhibitor of AS suggests that the cyclisation of FPP occurs along path b in which farnesyl diphosphate ionisation is accompanied by electrophilic attack of C1 by the C10, C11 π-bond with inversion of configuration at C1 resulting in the formation of germacryl cation. This conclusion is different from that reached previously from experiments with site-specific mutant AS-F112A, in which Phe 112 was replaced with alanine, leading to the production of 36% (*E*)-β-farnesene (**7a**) and 53.5% (*E*,*E*)-α-farnesene (**6a**) in addition to a small amount of

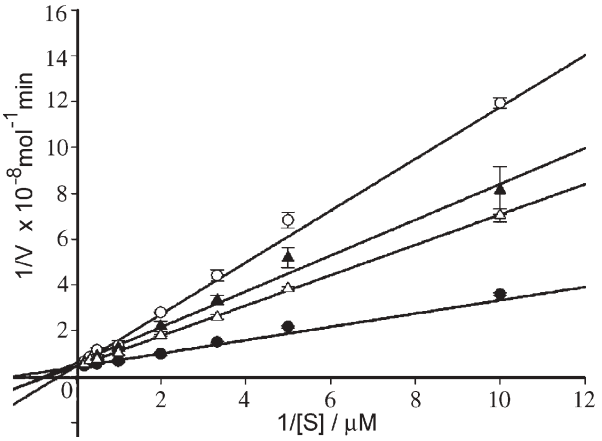

Fig. 1 Double reciprocal plot for inhibition of AS catalysed turnover of **1a** by **1b** at 0 (●), 0.4 μM (△), 0.6 μM (▲) and 1 μM (○) of **1b**.

germacrene A (**4a**).[11] The presence of these linear sesquiterpenoids suggested that farnesyl cation **2a** accumulated during catalysis in the mutant enzyme due to the absence of stabilization through interaction with the aromatic ring of Phe 112 of germacryl cation and the transition state preceding it. The results presented here suggest that in addition to stabilizing carbocation **3a** through its aromatic ring, the presence of Phe 112 (together with other active site residues) may also exert a stereoelectronic effect on the reaction through its steric bulk by aligning the π-orbital of the C10, C11 double bond with the breaking C1–O bond of the diphosphate group. When the size of this side chain is reduced by replacing the benzyl group with the smaller methyl group, this alignment may no longer be optimal, thereby preventing the immediate quenching of the developing positive charge on C1 and leading to the accumulation of farnesyl cation (**2a**) and the production of the linear farnesenes **6a** and **7a**.

According to this argument, **1b** should act as a substrate of the mutant enzyme AS-F112A since the less hindered rotation around the C1–O bond of **1b** in the mutant should lead to the formation of 12,13-difluorofarnesyl cation (**2b**) and hence of the difluorinated farnesenes, **6b** and **7b**. One major and two side products with the molecular ion peaks of *m*/*z* of 240 expected for difluorinated sesquiterpenoids were detected in the gas chromatogram of the hexane extractable products of incubations of the AS-F112A with **1b** (ESI). While the low activity of AS-F112A even with the unfluorinated substrate (**1a**)[11] allowed for the production of only a small amount of fluorinated products and prevented the detailed molecular characterization of the products, this experiment nevertheless confirmed that AS-F112A could indeed turnover **1b**. This result strongly supported the proposal that AS catalyses the breaking of the ester bond in **1a** and the formation of the cyclic germacryl cation (**3a**), at least in part, by facilitating optimal orbital overlap between the C10–C11 and C1–O bonds in **1a** through the templating effect of bulky active site residues such as Phe 112.

It appears therefore that the interpretation of results from site-specific mutants of terpene cyclases requires some caution, since the change of only a single amino acid in their active site can alter their templating potential. Such local geometrical changes may be inherently difficult to detect since they may not affect the global fold of the proteins but nevertheless lead to the synthesis of novel products through subtle alterations of the reaction pathway. The strong influence on the reactivity exerted by fluoro substituents without significantly affecting size and shape may make these reagents more suitable than mutagenesis experiments to explore the chemistry of terpene synthases. However, only through a combination of experimental approaches will the intricate details of this masterpiece in combinatorial chemistry be deciphered.

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Notes and references

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