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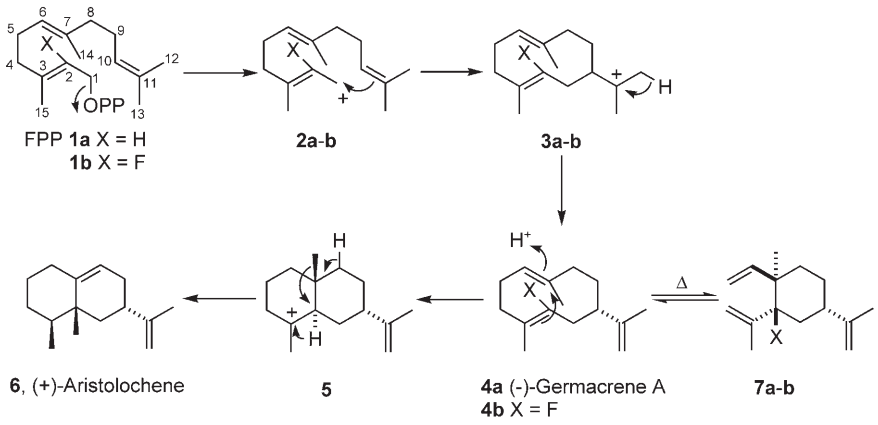
Aristolochene Synthase-Catalyzed Cyclization of 2-Fluorofarnesyl-Diphosphate to 2-Fluorogermacrene A

David J. Miller, Fanglei Yu, and Rudolf K. Allemann*[a]

*The mechanism of the conversion of (E,E)-farnesyl diphosphate (FPP, **1a**) to aristolochene (**6**) catalyzed by aristolochene synthase from* Penicillium roqueforti *has been proposed to proceed through the neutral intermediate germacrene A (**4a**). However, much of the experimental evidence is also in agreement with a mechanism in which germacrene A is not an intermediate in the predominant mechanism that leads to the formation of aristolochene, but rather an off-pathway product that is formed in a side reaction. Hence, to elucidate the mechanism of FPP cyclisation the substrate analogue 2-fluoroFPP (**1b**) was synthesized, and upon incubation with aristolochene synthase was converted to a single pentane extractable product according to GC-MS analysis. On the basis of NMR analyses this product was identified as 2-fluorogermacrene A (**4b**). Variable temperature ^{1}H NMR spectroscopy indicated the existence of two conformers of **4b** that were in slow exchange at −60 °C, while at 90 °C the two isomers gave rise to averaged NMR signals. In the major isomer (~75%) the methyl groups on C3 and C7 were most likely in the down–down orientation as had been observed for other (E,E)-germacranes. This work suggests that after an initial concerted cyclisation of FPP to germacryl cation deprotonation leads to the formation of germacrene A, and provides compelling evidence that germacrene A is indeed an on-pathway product of catalysis by aristolochene synthase.*

Introduction

Terpene synthases are a fascinating class of enzymes due to their almost unique ability to direct the formation and rearrangement of carbocationic species.[1] Sesquiterpene synthases, many of which share a common mainly α-helical fold, transform the shared substrate, farnesyl diphosphate (FPP, **1a**) with often exquisite regio- and stereochemical control into more than three hundred different hydrocarbon scaffolds that form the structural basis of the large family of sesquiterpenoids with many tens of thousands of complex natural products.[2]



Scheme 1. Reaction schemes for the AS catalysed conversion of FPP (**1a**) to aristolochene (**6**) and of 2F-FPP (**1b**) to 2-fluorogermacrene A (**4b**).

Aristolochene synthase (AS) from *Penicillium roqueforti* catalyses the Mg^{2+}-dependent conversion of **1a** to (+)-aristolochene (**6**)—the biochemical precursor of several fungal toxins, which include the potentially lethal PR toxin (Scheme 1).[3] Based on mechanistic studies with labelled substrates and through the analysis of the reaction products obtained with enzyme mutants a reaction mechanism was proposed in which the initial cleavage of the alkyl diphosphate bond leads to farnesyl cation (**2a**) prior to cyclisation. Attack by the C10=C11 double bond to produce germacryl cation (**3a**) followed by proton loss from C12 has been proposed to lead to germacrene A (**4a**). This uncharged intermediate was then postulated to undergo protonation of the C6=C7 double bond and a further cyclisation to form the bicyclic eudesmane cation (**5**). Successive 1,2-hydride shift and methyl migration followed by loss of H_{Si} on C8 results in the generation of (+)-aristolochene (**6**).[4]

The intermediacy of germacrene A is tentatively supported by the observation that **4a** is a minor by product of AS catalysis.[5] In addition, results obtained with some AS mutants can be interpreted to support a mechanism that involves germa-

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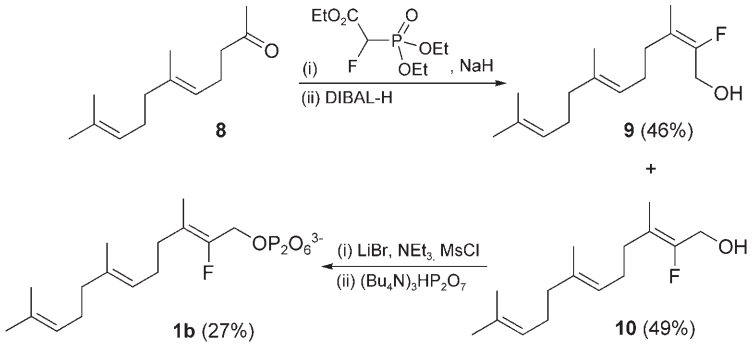
crene A as an intermediate. Replacement of Trp334 with alanine, which based on the 2.5 Å X-ray crystal structure of apo-AS[6] has been postulated to be involved in the stabilization of the positive charge in eudesmane cation, led to the exclusive production of germacrene A,[7] while replacement of Tyr92 with phenylalanine led to increased production of **4a**. While these observations are largely in agreement with the above reaction mechanism, other experiments suggest that germacrene A might not be an intermediate in the predominant mechanism that leads to the formation of aristolochene, but is rather an off-pathway product that is formed in a side reaction. In particular, **4a** does not appear to act as a substrate of AS, as would be expected if germacrene A was an intermediate in the predominant mechanism that leads to the formation of aristolochene.[5] In addition, the active site acid that protonates germacrene A has so far been difficult to identify, even though several candidates have been proposed, which include a proton shuttle from the solvent to Tyr92 in the active site by way of Arg200, Asp203 and Lys206, an unprecedented active site oxonium ion,[8] or the pyrophosphate group.[9]

Rather than analyzing the effects of structural changes of the enzyme on product distribution, substrate analogues can be used to study the reaction mechanism. The turnover of the 6,7-dihydro analogue of FPP by AS generates dihydro germacrene A.[10] However, X-ray crystallographic studies with sesquiterpene cyclases have suggested that the conformation of FPP in the active site is central to the selectivity of the cyclisation reaction. Hence, the reduction of the C6=C7 double bond of FPP might change the reaction mechanism by altering the conformation of both substrate and intermediate. The aim of this work was to address the intermediacy of germacrene A by intercepting the AS-catalysed ring closure to eudesmane cation through the use of a suitable substrate analogue, and to identify the structure of the putative germacrene A analogue. Fluorinated substrates have proved useful in the elucidation of mechanistic details of terpenoid biosynthesis due to the special properties of fluorine substituents which do not greatly affect binding affinities by altering size and shape. At the same time fluorine substituents 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.

We report here the synthesis of 2-fluorofarnesyl diphosphate (**1b**) and the characterization of the reaction products obtained during AS catalysis. The results provide strong support for a reaction mechanism in which an initial cyclisation of FPP to germacryl cation is followed by proton loss from C12 to generate **4a**.[4]

Results and Discussion

We considered that the introduction of a fluorine atom on C2 of FPP should lead to a destabilisation of eudesmane cation during AS catalysis, and hence to the accumulation of 2-fluorogermacrene A (**4b**). We have therefore studied the AS-catalyzed reaction of 2-fluorofarnesyldiphosphate (**1b**), which was prepared by using a modification of a published procedure.[11] Geranyl acetone was treated with triethyl 2-fluoro-2-phosphonoacetate and sodium hydride, followed by DIBAL-H reduction of the resulting ester mixture to give **9** and **10** in a ratio of approximately 1:1 (Scheme 2). Bromination of **10** followed by diphosphorylation[12] and purification by reversed-phase HPLC gave **1b** in 36% yield over these two steps.



Scheme 2. Synthesis of 2-fluorofarnesyl-diphosphate (**1b**).

Enzymatic cyclisation of 2-fluorofarnesyl diphosphate

Preparative scale incubation of **1b** with recombinant AS (pH 7.5, 25 °C, 48 h) followed by GC mass spectral analysis of the pentane-extractable material revealed a single product (Figure 1), which showed the molecular mass expected for a monofluorinated sesquiterpenoid (*m/z* 222; Figure 2).

The ^{1}H NMR spectroscopic analysis of compound **4b** at room temperature provided a rather poorly resolved spectrum, which was similar to the unassigned literature reference spectrum of germacrene A.[13] The ^{19}F NMR spectrum of **4b** indicated two resonances ($\delta_F = -88.8$ and -90.9 ppm) at room temperature. Because of the known flexibility of cyclodecadienes a variable temperature NMR study was carried out in [D_8]toluene between -80 and 90 °C, at 10 °C intervals. The compound proved to be stable over the whole temperature range as evidenced by the reversibility of the temperature-dependent spectral changes. Significant line sharpening was observed both on lowering and increasing the temperature from 25 °C (Figure 3). Compound **4b** existed as two resolvable conformers at temperatures below 0 °C, while at elevated temperatures the position of the sharp resonance signals indicated a fast equilibrium between the two conformers, which resulted in a weighted average of the NMR-resonances. A coalescence temperature of approximately 30 °C was observed.

Variable temperature NMR spectroscopic analysis of 4b

The COSY and HSQC NMR spectra were obtained both at -60 and 90 °C; this made it possible to assign all the ^{1}H resonances of **4b** (Table 1). Together with the room temperature HMBC spectrum these data also allowed us to assign the ^{13}C NMR

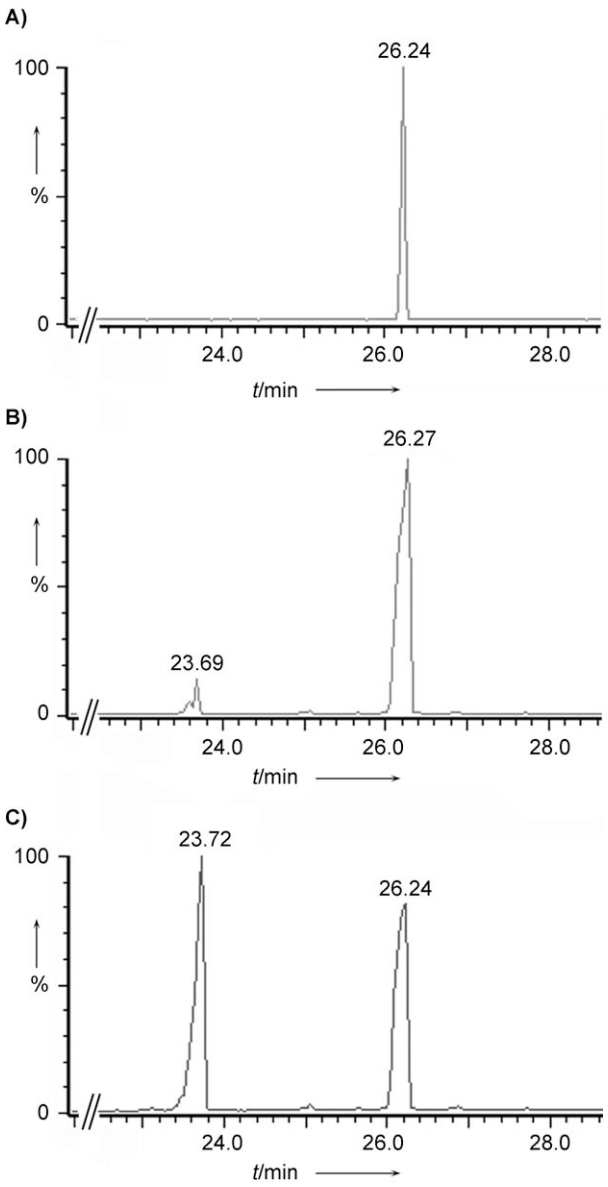


Figure 1. Analysis of the temperature-induced reaction of the pentane extractable products of 2-fluoroFPP (**1 b**) utilisation by AS. A) Total ion chromatogram (TIC) from the GC analysis of the compound produced by AS from **1 b** at an injection port temperature of 50 °C. B) TIC from the GC analysis of the same compound at an injection port temperature of 250 °C. C) TIC from the GC analysis of the same compound at an injector port temperature of 300 °C.

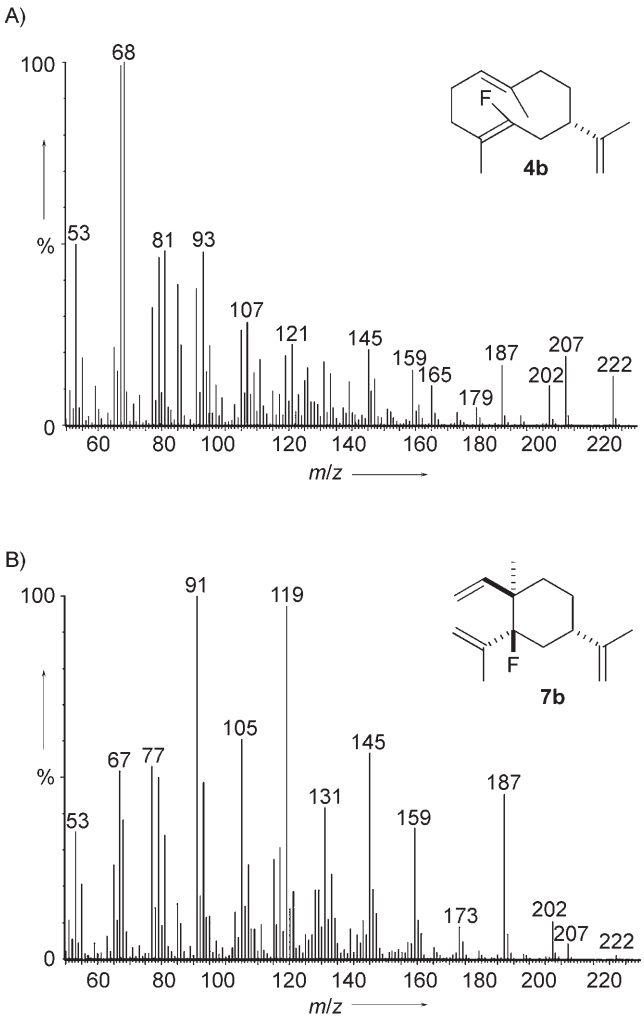


Figure 2. Mass spectral analysis of the two isomers produced by thermal rearrangement in the GC injector. A) EI^+ Mass spectrum of the material eluted at 26.24 min, which is the presumed 2-fluoro-germacrene A derivative **4 b**; B) EI^+ mass spectrum of the material eluted at 23.72 min, which is the presumed β-elemene analogue **7 b**.

spectrum of this compound. Based on these assignments the spectrum of **4 b** at room temperature could also be assigned. The appearance of signals for the olefinic protons on C12 ($\delta_H =$ 4.68 and 4.73 ppm at 25 °C), which resolved into an AB system at low temperature, together with the disappearance of the signals for the C12 methyl group and the C1 methylene protons at 4.42 ppm in FPP are consistent with bond formation between C1 and C10. The double bond was assigned to be between C12 and C11 as opposed to C13 and C11 based on the known stereochemistry of germacrene A formation by AS from FPP.[4] While all ^{1}H NMR signals of **4 b** were dependent on the temperature, the existence of two conformers was most obvious from the resonances for the protons on C4, C6 and C14 (Figure 3). For instance, H6 gave rise to two well-resolved signals at 5.05 and 5.36 ppm at −60 °C, while at high temperature only a poorly resolved double doublet was observed at 5.18 ppm. At low temperature the splitting patterns for the respective C6 protons were different in the two conformers; in the predominant conformer a relatively broad doublet was observed while in the minor conformer coupling to both protons on C5 was evident. Similarly, the patterns of the H4 resonances changed with temperature. One of the two protons leads to a complex multiplet at 90 °C; this proton was well resolved at low temperature with clear coupling to the geminal proton, the vicinal protons on C5 and the fluoro substituent.

Conformational isomerism of the germacrene family of natural products, in particular the (*E*,*E*)-germacranes, has been studied previously both by NMR spectroscopy and computationally.[14] The ten-membered ring can adopt four distinct conformations with up–up, down–down (Figure 3), up–down and down–up orientations of the methyl groups on C3 and C7.[14]

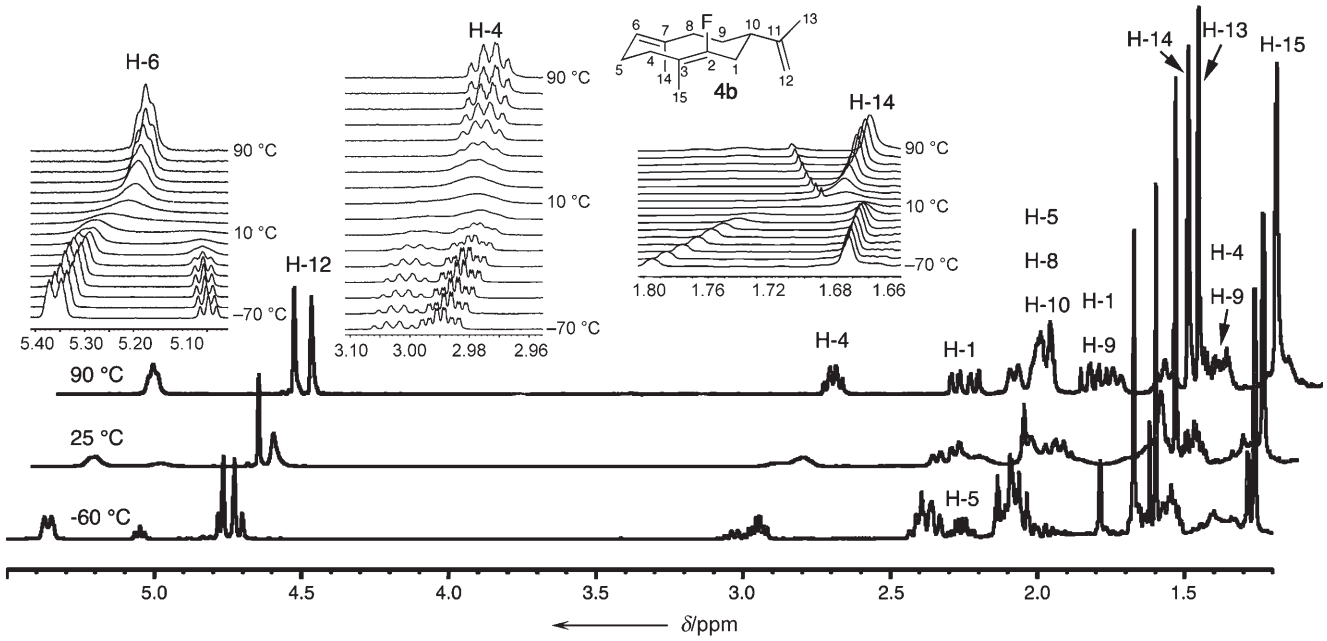


Figure 3. The ^{1}H NMR spectra (500 MHz) of **4b** at −60, 25 and 90 °C with assignments shown. Insets show the temperature dependent change in the resonances for H6, one of the H4 protons and CH_3-14. Clearly two conformational isomers exist in a ratio of approximate 3:1 at the lower temperatures (integrals not shown), and the signals for the two coalesce at approximately 30 °C. The conformation of the down–down isomer of **4b** is also shown.

Table 1. ^{1}H NMR spectra of **4b** at −60, 25 and 90 °C.[a]

	−60 °C major	−60 °C minor	25 °C	90 °C
CH_2-1	1.90–2.07 (m) and	1.90–2.07 (m) and	1.99–2.14 (m) and	1.88–2.02 (m) and
	2.33–2.44 (m)	2.33–2.44 (m)	2.35–2.45 (m)	2.42 CH_2-1 (ddd, J = 3.5, 14.5, 32.5 Hz)
CH_2-4	1.51–1.58 (m)	1.51–1.58 (m)	1.53–1.58 (m) and	1.52–1.60 (m) and
	2.95 (ddt, J = 2, 6, 12.5 Hz)	3.03 (br q, J = 10 Hz)	2.83–3.02 (br m)	2.83–2.90 (dq, J = 1.5, 9.5 Hz)
	3.00–3.06 (m)			
CH_2-5	2.02–2.14 (m) and	2.02–2.14 (m) and	2.11–2.14 (m) and	2.12–2.17 (m) and
	2.25 (ddt, J = 1.5, 5.5, 12 Hz)	2.25 (ddt, J = 1.5, 5.5, 12 Hz)	2.22–2.30 (br m)	2.24–2.26 (m)
CH-6	5.36 (br d, J = 12.5 Hz)	5.05 (t, J = 8 Hz)	5.02–5.10 (br s) and	5.18 (m)
			5.25–5.32 (br m)	
CH_2-8	2.02–2.14 (m)	2.02–2.14 (m)	2.11–2.14 (m)	2.12–2.17 (m)
CH_2-9	1.51–1.58 (m) and	1.51–1.58 (m) and	1.53–1.58 (m) and	1.52–1.60 (m) and
	1.90–2.07 (m)	1.90–2.07 (m)	1.99–2.14 (m)	1.88–2.02 (m)
CH-10	2.02–2.14 (m)	2.02–2.14 (m)	2.11–2.14 (m)	2.12–2.17 (m)
CH_2-12	pseudo AB system	pseudo AB system	4.68 (br s) and	4.64 (s) and 4.70 (s)
	4.70, 4.73, 4.76, 4.78	4.70, 4.73, 4.76, 4.78	4.73 (s)	
CH_3-13	1.67 (s)	1.79 (s)	1.62 (s)	1.62 (s)
CH_3-14	1.60 (s)	1.62 (s)	1.67 (br)	1.66 (s)
CH_3-15	1.26 (d, J_{HF} = 3 Hz)	1.28 (d, J_{HF} = 3 Hz)	1.32 (d, J_{HF} = 3 Hz)	1.36 (d, J_{HF} = 2.5 Hz)

[a] All resonances are δ_H (500 MHz, [D_8]toluene) in ppm. Entries are chemical-shift range followed by multiplicity in parentheses and coupling constants. Some proton resonances overlapped; no distinction was made in the chemical-shift range quoted for these protons.

These conformations are interconvertible by rotation of each of the double bonds through the ring and inversion of the C9–C10 unit, the conformation of which is largely determined by the exocyclic subsistent on C10, which favours a pseudo-equatorial orientation. For (+)-hedycaryol ((10*R*)-11-hydroxy-germacrene A) the up–up configuration predominated at all temperatures according to NMR studies (75% at −30 °C).[15] It is reasonable to suggest that the down–down orientation of the methyl groups is the dominant orientation in **4b** since AS produces (−)-germacrene A;[16] hence, the isopropylidene group will predominantly adopt the pseudoequatorial configuration. Analysis of the relative intensities of the resonances for the proton on C6 in the −60 °C ^{1}H NMR spectrum indicates that the ratio between the major and minor conformer of **4b** was 3.22:1; this is similar to what had been observed for (+)-hedycaryol.

Thermal rearrangement of 4b

Germacrene A is known to undergo a Cope rearrangement to generate β-elemene (**7a**).[13] It was partly because of the temperature induced Cope rearrangement in GC-MS experiments that **4a** was first identified as a minor product of FPP turnover by AS.[5] To further characterize compound **4b**, GC-MS analysis was performed with varying injector temperatures but under otherwise identical conditions (Figure 1). Germacrene A is known to undergo significant Cope rearrangement when the injector temperature is set to 200 °C.[5] In contrast, thermal rearrangement of **4b** was not observed at this temperature. However, at 250 °C a small amount of rearranged product was observed and at 300 °C thermal rearrangement of **4b** was dominant (Figure 1); furthermore, high-resolution mass spectrometry indicated that the two compounds had an identical elemental composition ($C_{15}H_{23}F$). As had been observed for **4b** the mass to charge ratio for the parent ion of **7b** was 222 (Figure 2). The fragmentation patterns of both compounds were indicative of a loss of CH_3 (m/z −15) and HF (m/z −20) as well as loss of both CH_3 and HF (m/z −35). The parent molecular ion of **4b** was more intense than that of the presumed fluoro-β-elemene (**7b**); this was most likely as a consequence of facilitated loss of hydrogen fluoride from **7b** to generate a conjugated diene compared to elimination of HF from **4b**.

The presence of the fluoro-substituent in **4b** appears to slow the Cope rearrangement compared to **4a**. The effects of fluorine substituents on six electron electrocylic processes is unclear since steric and electronic effects often compete, which leads to unpredictable effects on the reaction kinetics;[17] hence a rationalization of the reduced reactivity of **4b** is difficult.

Conclusions

In summary, the work described here shows that 2-fluorofarnesyl-diphosphate (**1b**) is a substrate of aristolochene synthase and is converted exclusively to 2-fluorogermacrene A (**4b**). This result provides strong support that germacrene A is generated as an on-path reaction product of the conversion of **1a** to aristolochene (**6**). If germacrene A was produced in a minor, but separate pathway by AS, more than one product would be expected from the incubation with 2-fluoro-FPP. The observation that germacrene A does not act as a substrate for AS is most likely the consequence of its very poor solubility in aqueous solvents.

The effectiveness of the fluoro analogue of germacrene A in blocking the second cyclisation step is most likely a consequence of the inductive effect of this highly electronegative group on the C2=C3 double bond and the destabilising effect on the positive charge on C3 in eudesmane cation. This effect leads to an increase in the height of the energy barrier of the step following **4b** formation. It has been shown previously that vinyl-fluoro groups reduce the rate of solvolysis of fluoro terpenoid methanesulfonates.[18–20] Interestingly, the 2-fluoro substituent of FPP did not prevent the first cyclisation step that leads to 2-fluorogermacrene A in the present case despite evidence from mutational studies that this process occurs in a stepwise fashion through farnesyl cation.[21–23] Either the allylic stabilisation in farnesyl cation is sufficient to push the reaction to the decadiene-ring system or the results obtained with the mutants do not apply to the wild-type enzyme and the cyclisation of FPP to germacrene A is indeed a concerted process in which cyclisation and diphosphate expulsion occur concomitantly. This would be in agreement with the observation that cyclisation of FPP occurs with inversion of configuration on C1.[24]

Clearly, vinyl fluoro analogues of farnesyldiphosphate are powerful reagents for studying the mechanism of enzyme-catalysed cyclisations of sesquiterpenoids, which are reactions that are inherently difficult to study spectrometrically. A similar approach has been used to study the taxadiene synthase-catalysed reaction of geranyl-geranyl-diphosphate.[25] Chemical interception could provide a unique opportunity to interrupt these multistep polyene cylisation reactions to decipher the mechanistic principles used by terpene cyclases to control the chemistry of carbocationic processes with exquisite specificity.

Experimental Section

Expression and purification of AS in *E. coli*: AS was produced in *E. coli* BL21(DE3) cells that harboured a cDNA for AS under the control of the T7 promoter. Cells were grown at 37 °C in Luria–Bertani (LB) medium with ampicillin (0.3 mM) until they reached an A_{600} of 0.5. They were induced with isopropyl-β-D-1-thiogalactopyranoside (0.5 mM), incubated for a further 3 h and harvested by centrifugation at 8000 *g* for 10 min. Protein was then extracted from the inclusion bodies and purified by following the protocol described previously.[5] The AS sample was pure as judged by SDS gel electrophoresis.

(2*Z*,6*E*)-2-Fluoro-3,7,11-trimethyldodeca-2,6,10-trien-1-ol (10) and (2*E*,6*E*)-2-fluoro-3,7,11-trimethyldodeca-2,6,10-trien-1-ol (9): Triethyl 2-fluoro-2-phosphonoacetate (0.84 cm^3, 4.13 mmol) and a solution of geranyl acetone (1.02 cm^3, 4.54 mmol) in anhydrous THF (5 cm^3) were sequentially added dropwise to a stirred suspension of sodium hydride (60% dispersion in mineral oil, 0.10 g, 4.13 mol) in anhydrous THF (10 cm^3), at 0 °C under an N_2 atmosphere. The complete reaction mixture was stirred for 14 h under N_2. Water (20 cm^3) and diethyl ether (20 cm^3) were added and the organic layer was separated. The aqueous layer was extracted with diethyl ether (2×15 cm^3). The combined ethereal extracts were washed with water (2×10 cm^3) and saturated NaCl solution (1×10 cm^3), dried over anhydrous $MgSO_4$, filtered and then concentrated under reduced pressure. The resulting ester was a 1:1 mixture of *E* and *Z* isomers and was reduced without further purification. Diisobutylaluminum hydride (1.0 M solution in hexanes, 10.33 cm^3, 10.33 mmol) was added dropwise to a stirred solution of the crude ester in anhydrous THF (30 cm^3) at −78 °C, (over 3 min). The reaction mixture was allowed to stir at −78 °C for 1 h, then 0 °C for 1 h and was then quenched with saturated sodium potassium tartrate solution (30 cm^3). The resulting mixture was then extracted with diethyl ether (3×20 cm^3). The combined organic extracts were dried over anhydrous $MgSO_4$, filtered and then concentrated under reduced pressure. Purification of the residue by flash-column chromatography on silica gel with hexane and ethyl acetate (4:1) as eluent gave the *Z* isomer (0.48 g, 49%) as a colourless oil followed by the *E* isomer (0.46 g, 46%) as a colourless oil.

The *Z* isomer (**10**): R_f 0.28 (4:1 hexane-ethyl acetate); HRMS (EI^+, M^+): found 240.1890, $C_{15}H_{25}OF$ requires 240.1889; ν_{max} (thin film): 3358.1, 2920.4, 1703.7, 1444.7, 1380.2, 1266.9, 1148.9, 1105.3, 1012.5 cm^{-1}; δ_H (500 MHz, C^2HCl_3): 1.62 (6H, s, 2×CH_3), 1.70 (3H, d, $^4J_{HH}$ = 1.0, CH_3), 1.71 (3H, d, $^4J_{HF}$ = 3.48, CH_3C=CF), 1.79 (br s, 1H; OH), 1.99–2.14 (8H, m, 2×CH_2CH_2), 4.19 (2H, dd, $^3J_{HF}$ = 23.0, $^3J_{HH}$ = 6.0, CH_2OH), 5.09 (2H, m, 2×C=CH); δ_C (125 MHz, C^2HCl_3): 13.48 (d, $^3J_{CF}$ = 9.0, CH_3C=CF), 15.99 (CH_3), 17.67 (CH_3), 25.67 (CH_3), 26.36 (d, $^4J_{CF}$ = 4.0, CH_2CH_2C=CF), 26.62 (CH_2), 31.71 (d, $^3J_{CF}$ = 5.00, CH_2CH_2C=CF), 39.67 (CH_2), 57.67 (d, $^2J_{CF}$ = 31.0, C=CFCH_2OH), 115.74 (d, $^2J_{CF}$ = 14, CH_3C=CFCH_2), 123.00 (C=CH), 124.14 (C=CH), 131.52 (C=CH), 136.52 (C=CH), 152.57 (d, $^1J_{CF}$ = 242.50, CH_3C=CF); δ_F (283 MHz, C^2HCl_3): −119.5 (t, $^3J_{HF}$ = 23.0); *m/z* (EI^+) 240.2 (2%, $[M]^+$), 69.1 (100%), 81.1 (20%).

The *E* isomer (**9**): R_f 0.20 (4:1 hexane-ethyl acetate); HRMS (EI^+, M^+): found 240.1897, $C_{15}H_{25}OF$ requires 240.1889; ν_{max} (thin film): 3349.0, 2931.5, 2361.3, 1703.1, 1667.2, 1450.6, 1380.5, 1270.8, 1238.0, 1158.9, 1105.5, 1012.8, 910.9 cm^{-1}; δ_H (500 MHz, C^2HCl_3): 1.62 (6H, s, 2×CH_3), 1.70 (3H, d, $^4J_{HF}$ = 2.5, CH_3C=CF), 1.70 (3H, s, CH_3), 1.82 (1H, b, OH), 1.98–2.15 (8H, m, 2×CH_2CH_2), 4.23 (2H, dd, $^3J_{HF}$ = 22.5, $^3J_{HH}$ = 5.5, CH_2OH), 5.11 (2H, m, 2×C=CH); δ_C (125 MHz, C^2HCl_3): 15.37 (d, ^3J_C = 5.0, CH_3C=CF), 16.0 (CH_3), 17.7 (CH_3), 25.7 (CH_3), 25.9 (d, $^4J_{CF}$ = 1.25, CH_2CH_2C=CF), 26.7 (CH_2), 29.8 (d, $^3J_{CF}$ = 6.25, CH_2CH_2C=CF), 39.7 (CH_2), 58.0 (d, $^2J_{CF}$ = 32.5, CH_2OH), 116.0 (d, $^2J_{CF}$ = 16.25, CH_3CCFCH_2), 123.5 (C=CH), 124.3 (C=CH), 131.4 (C=CH), 135.7 (C=CH), 151.79 (d, $^1J_{CF}$ = 240, CH_3C=CF); δ_F (283 MHz, C^2HCl_3): 121.13 (t, $^3J_{HF}$ = 22.5); *m/z* (EI^+) 240.2 (4%, $[M]^+$), 69.1 (100%), 81.1 (35%).

Tris-ammonium-(2*Z*,6*E*)-2-fluoro-3,7,11-trimethyldodeca-2,6,10-trien-1-yl diphosphate (1b): A stirred solution of alcohol **10** (0.22 g, 0.92 mmol) and triethylamine (0.26 cm^3, 1.84 mmol) in anhydrous THF (10 cm^3) was cooled to −45 °C; MsCl (0.09 cm^3, 1.20 mmol) was then added. The resulting milky mixture was stirred at −45 °C for 45 min, then a solution of LiBr (0.35 g, 3.68 mmol) in THF (5 cm^3) was added at −45 °C, by using a needle. The suspension was allowed to warm to 0 °C and stirred for 1 h, after which time the reaction was judged complete by TLC analysis. Cold water (10 cm^3) and hexane (10 cm^3) were added. The two layers were separated, and the aqueous layer was extracted with hexane (2×10 cm^3). The pooled organic layers were washed with saturated $NaHCO_3$ solution (10 cm^3) and saturated NaCl solution (10 cm^3), and then dried over anhydrous $NaSO_4$ and filtered. Concentration of the solvent under reduced pressure gave the required bromide as a light yellow oil that was used without further purification. Freshly recrystallized tris(tetra-*n*-butylammonium) hydrogen diphosphate (1.80 g, 2.00 mmol), which was prepared by the method of Poulter, was added to a stirred solution of the bromide in anhydrous acetonitrile (10 cm^3) under N_2.[12] The complete reaction mixture was stirred for 2 h. Solvent was then removed under reduced pressure and the resulting residue was dissolved in 1:49 (*v/v*) isopropyl alcohol and 25 mM ammonium hydrogencarbonate solution (2 cm^3; ion-exchange buffer). The pale yellow solution was slowly passed through a column that contained 30 equiv of DOWEX 50W-X8 (100–200 mesh) cation-exchange resin that had been pre-equilibrated with two-column volumes of ion-exchange buffer. The column was eluted with two-column volumes of ion-exchange buffer at a flow rate of one-column volume per 15 min. The clear, light yellow eluent was freeze dried to yield a fluffy yellow solid, which was purified by reverse phase HPLC (150× 21.2 mm Phenomenex Luna C-18 column, eluted under isocratic conditions with 10% B for 20 min, a linear gradient to 60% B over 25 min, then a linear gradient to 100% B over 5 min and finally with 100% B for 10 min; solvent B: CH_3CN; solvent A: 25 mM NH_4HCO_3 in water, flow rate 5.0 $cm^3 min^{-1}$, detection at 220 nm) to give title compound as a white solid (0.15 g, 36%); HPLC t_R 36.99 min; purity 96.33% by analytical HPLC; HRMS (EI^-, $[M-H]^-$): found 399.1135, $C_{15}H_{26}O_7FP_2$ requires 399.1143; ν_{max} (KBr disc): 2963.4, 1701.8, 1494.4, 1455.4, 1411.8, 1204.0, 1164.6, 1122.1, 1091.9, 1034.7, 908.7, 825.9, 723.3, 595.4, 552.7 cm^{-1}; δ_H (500 MHz, ^2H_2O): 1.47 (3H, s, CH_3), 1.48 (3H, s, CH_3), 1.54 (3H, s, CH_3), 1.59 (3H, d, $^4J_{HF}$ = 2.5, CH_3C=CF), 1.86–2.01 (8H, m, 2CH_2CH_2), 4.42 (2H, dd, $^3J_{HF}$ = 24.0, $^3J_{HP}$ = 6.0, C=CFCH_2OP), 5.02 (1H, t, $^3J_{HH}$ = 7.0, C=CH), 5.06 (1H, m, C=CH); δ_C (125 MHz, ^2H_2O): 4.82 (d, $^3J_{CF}$ = 5.0, CH_3C=CF), 15.33 (CH_3), 17.06 (CH_3), 24.98 (CH_3), 25.33 (CH_2), 26.04 (CH_2), 29.38 (d, $^3J_{CF}$ = 6.0, CH_2CH_2C=CF), 39.04 (CH_2), 60.54 (dd, $^2J_{CF}$ = 31.25, $^2J_{CP}$ = 5.00, C=CFCH_2OP), 119.33 (d, $^2J_{CF}$ = 14.0, CH_3C=CFCH_2), 123.92 (C=CH), 124.44 (C=CH), 132.82 (C=CH), 136.62 (C=CH), 149.25 (dd, $^1J_{CF}$ = 237.5, $^4J_{CP}$ = 9.0, CH_3C=CFCH$_2$OP); δ_F (283 MHz, ^2H_2O): −120.23 (t, $^3J_{HF}$ = 24.0); δ_P (121 MHz, ^2H_2O): −6.29 (d, $^3J_{PP}$ = 21.0), 10.50 (d, $^3J_{PP}$ = 1.0); MS (ES^-) *m/z* 399.2 (15%, $[M-H^+]^-$), 74.9 (100%,), 198.8 (40%).

Incubation of 2-fluoro farnesyl pyrophosphate (1b) with AS: Aristolochene synthase solution (3.60 cm^3, 638 μM) was diluted with buffer consisting of 20 mM tris-HCl, 5 mM β-mercaptoethanol, 5 mM $MgCl_2$ and 15% (*v/v*) glycerol, pH 7.5 (to 33.5 cm^3). The assay solution was gently mixed as **1b** (30.0 mg, 0.067 mmol) and pentane (2 cm^3) were added sequentially. After incubation for 48 h at 25 °C, the olefin products were extracted with pentane (3×80 cm^3). The pooled pentane extracts were concentrated under a nitrogen stream until about 5 cm^3 of solvent remained. This solution was passed through a short pad of silica gel overlayed with anhydrous $MgSO_4$. The pentane was concentrated under reduced pressure to yield a single product as judged by GC-MS analysis (4.0 mg, 27%); GC-MS t_R 26.24 min. GC-MS was performed by using a Hewlett Packard 6890 GC fitted with a J&W scientific DB-5MS column (30 m×0.25 mm internal diameter) and a Micromass GCT Premiere that detected in the range *m/z* 50–800 in EI^+ mode by scanning once a second with a scan time of 0.9 s. Injections were performed in split mode (split ratio 5:1) at 50 °C. Chromatograms were begun with an oven temperature of 50 °C, this was raised at 4 °C min^{-1} for 25 min (up to 150 °C) and then at 20 °C min^{-1} for 5 min (250 °C final temperature).

HRMS (EI^+): found 222.1785, $C_{15}H_{23}F$ requires 222.1784. Compound **4b** displayed conformational isomerism which complicated the interpretation of its NMR spectra. Consequently, variable temperature NMR experiments were performed. The ^1H NMR data are reported in Table 1. The ^{13}C NMR spectrum could not be observed directly due to the small quantity of material isolated and reduction of line intensity due to the conformational isomerism, but could be indirectly observed and interpreted through the cross peaks observed in the various 2D spectra. δ_C (125 MHz, [D_8]toluene, 25 °C): 14.7 (C-14), 19.5 (C-13 and C-15), 25.1 (C-5), 27.5 (C-4), 33.3 (C-9), 33.8 (C-8), 34.1 (C-1), 45.4 (C-10), 108.9 (C-12), 112.0 (C-3), 128.2 (C-7), 137.1 (C-6), 149.6 (C-11), 154.5, 156.7 (d, C-2); δ_F (283 MHz, [D_8]toluene, −60 °C) −89.2 (major conformer) and −91.8 (minor); δ_F (283 MHz, [D_8]toluene, 25 °C) −88.8 (major conformer) and −91.3 (minor); *m/z* (EI^+) 222.2 (12%, $[M]^+$), 207.2 (19%, $[M-CH_3]^+$), 202.2 (10%, $[M-HF]^+$), 187.2 (18%, $[M-HF-CH_3]^+$), 179.1 (5%), 165.1 (11%), 159.1 (18%), 145.1 (22%), 131.0 (20%), 121.1 (21%), 107.1 (29%), 93.1 (50%), 79.1 (48%), 68.1 (100%), 65.0 (21%), 53.0 (50%).

Thermal rearrangement of 4b: The pentane extractable products obtained from incubation of **1b** with AS were diluted to approximately 1 mg mL^{-1} in hexane and 2 mm^3 of this solution was injected onto the GC-MS column by using the conditions described above, except the injector temperature was varied from 50 to

300 °C in 50 °C steps for each run. The peak at t_R 26.24 min remained unchanged. At temperatures 250–300 °C a second peak appeared with t_R 23.72 min (Figure 1); HRMS (EI^+): found 222.1792, $C_{15}H_{23}F$ requires 222.1784; *m/z* (EI^+) 222.2 (2%, $[M]^+$), 207.2 (5%, $[M-CH_3]^+$), 202.2 (10%, $[M-HF]^+$), 187.2 (50%, $[M-HF\text{-}CH_3]^+$), 173.1 (10%), 159.1 (38%), 145.1 (60%), 133.1 (25%), 131.1 (40%), 119.1 (98%), 105.1 (60%), 91.1 (100%), 77.0 (55%), 67.1 (50%), 53.0 (35%).

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