Stereochemistry of eudesmane cation formation during catalysis by aristolochene synthase from *Penicillium roqueforti*†

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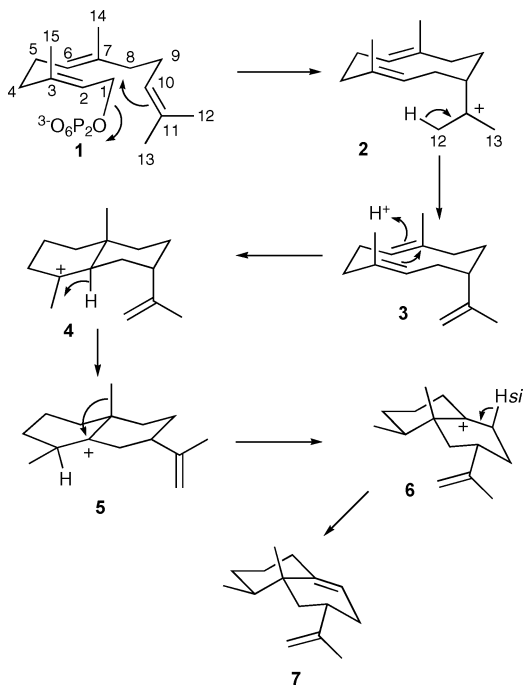
The aristolochene synthase catalysed cyclisation of farnesyl diphosphate (**1**) has been postulated to proceed through (*S*)-germacrene A (**3**). However, the active site acid that reprotonates this neutral intermediate has so far proved difficult to identify and, based on high level *ab initio* molecular orbital and density functional theory calculations, a proton transfer mechanism has recently been proposed, in which proton transfer from C12 of germacryl cation to the C6,C7-double bond of germacryl cation (**2**) proceeds either directly or *via* a tightly bound water molecule. In this work, the stereochemistry of the elimination and protonation reactions was investigated by the analysis of the reaction products from incubation of **1** and of [12,12,12,13,13,13-$^{2}H_6$]-farnesyl diphosphate (**15**) with aristolochene synthase from *Penicillium roqueforti* (PR-AS) in H_2O and D_2O. The results reveal proton loss from C12 during the reaction and incorporation of another proton from the solvent. Incubation of **1** with PR-AS in D_2O led to the production of (6*R*)-[6-^{2}H] aristolochene, indicating that protonation occurs from the face of the 10-membered germacrene ring opposite the isopropylidene group. Hence these results firmly exclude proton transfer from C12 to C6 of germacryl cation. We propose here Lys 206 as the general acid/base during PR-AS catalysis. This residue is part of a conserved network of hydrogen bonds, along which protons could be delivered from the solvent to the active site.

Introduction

Tens of thousands of terpene natural products have been described to date, all of which derive from only a small number of linear precursors. The 15-carbon isoprenoid, farnesyl diphosphate (FPP, **1**) is the biogenetic precursor of more than 300 different sesquiterpene hydrocarbon scaffolds in plants, bacteria and fungi.[1–6] Cyclisation of **1** (and other isoprenyl diphosphates) is catalysed by terpene synthases that rely on a common mainly α-helical structure known as the class I terpene synthase fold to promote one of the arguably most complex biochemical reactions.[4,5] Unlike most other biochemical reactions, the chemistry of terpene cyclases is mainly dependent on the generation and stabilisation of carbocationic intermediates. A common mechanistic feature of all class I terpene synthases is the initial, metal ion-dependent cleavage of the alkyl diphosphate bond generating a putative carbocation intermediate prior to cyclisation. In the absence of the diphosphate group, a strong acid would be required to activate the olefinic substrate.

Aristolochene synthase from *Penicillium roqueforti* (PR-AS) chaperones its linear achiral substrate FPP along a complex reaction pathway to produce (+)-aristolochene in a cyclisation cascade that leads to the generation of two 6-membered rings, three chiral centres, and two double bonds with high regio- and stereospecificity (Scheme 1). Concurrent to diphosphate expulsion PR-AS facilitates attack of C1 in FPP (**1**) by the C10, C11 double bond to produce germacryl cation (**2**). Proton loss from C12 leads



Scheme 1

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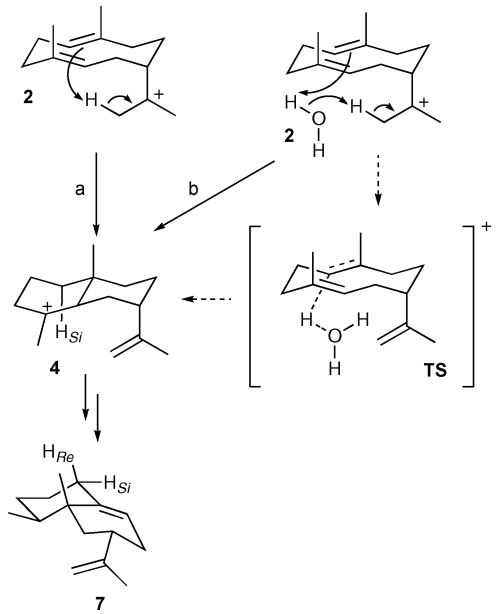
to the production of (*S*)-germacrene A (**3**),[7,8] which was then postulated to undergo reprotonation of the C6, C7 double bond and a further cyclisation to form the bicyclic eudesmane cation (**4**). Successive 1,2 hydride shift and methyl migration followed by loss of H_{Si} on C8 completes the generation of (+)-aristolochene (**7**).[7]

A difficulty with the above mechanism through germacrene A appears to be the reprotonation of the neutral intermediate germacrene A, a step that requires strong acid to generate eudesmane cation (**4**), which has been shown to be stabilised through interactions with the π-system of the indole ring of Trp 334.[9] Several candidates have been proposed for the acid that reprotonates germacrene A (**3**), including a proton shuttle from the solvent to Tyr 92 in the active site by way of Arg 200, Asp 203, and Lys 206,[8] an unprecedented active site oxonium ion,[10] or the diphosphate itself,[11] but it has so far proved difficult to find conclusive experimental support for any of these proposals. We have recently published results from density functional theory (DFT), semi-empirical and high level molecular orbital theory calculations in the gas phase to study the cyclisation of FPP along with molecular docking and modeling studies in the active site of PR-AS, employing combined quantum mechanical and molecular mechanical (QM/MM) methods. This has led to the proposal of an alternative reaction pathway, in which protonation of the C6,C7-double bond is accomplished by intramolecular proton transfer from C12 of **2** (Scheme 2, pathway a) rather than through the involvement of a general acid thereby bypassing the neutral intermediate germacrene A (Scheme 1).[12] In this mechanism the direct intramolecular proton transfer had a computed barrier of about 22 kcal mol^{-1}, which was further lowered to 16–20 kcal mol^{-1} by PR-AS. We now report results from experimental and computational studies to test this intramolecular proton transfer mechanism as well as an alternative pathway through a proton shuttle from the isopropyl group of germacryl cation (**2**) to the C6,C7-double bond *via* an enzyme bound water molecule (Scheme 2, pathway b).



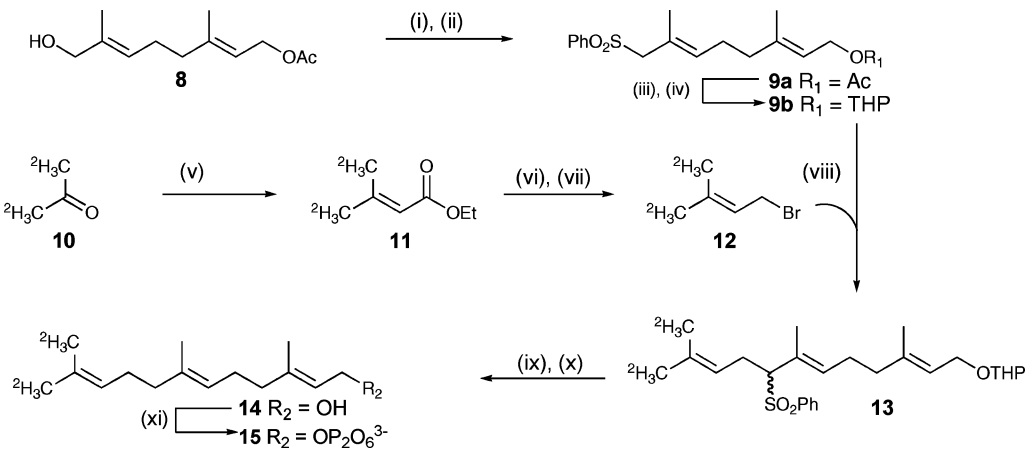
Scheme 2

Results and discussion

Direct proton transfer mechanism

Our computational analysis revealed that intramolecular proton transfer from C12 in germacryl cation was a thermodynamically feasible reaction for the generation of eudesmane cation during PR-AS catalysis.[12] To test whether the enzymatic reaction followed this pathway, [12,12,12,13,13,13-$^2H_6$]-farnesyl diphosphate (**15**) required preparation. This was achieved as outlined in Scheme 3.

The procedure was a modification of that used to prepare [12,12,12-$^2H_3$]-FPP.[7] 8-Hydroxygeranyl acetate **8** was brominated



Scheme 3 *Reagents and conditions:* (i) PBr_3, CH_2Cl_2, 5 °C; (ii) $PhSO_2Na$, DMF, 70% 2 steps; (iii) NaOH, MeOH–H_2O, 91%; (iv) 3,4-DHP, *p*-TsOH, CH_2Cl_2, 90%; (v) $(EtO)_2P(O)CH_2CO_2Et$, NaH, 57%; (vi) $LiAlH_4$, Et_2O, 47%; (vii) MsCl, NEt_3, LiBr, THF, −45 °C; (viii) n-BuLi, THF, HMPA, −78 °C, 36%; (xi) $LiBHEt_3$, $PdCl_2$dppf, THF, 58%; (x) PPTS, EtOH, 50 °C, 65%; (xi) MsCl, NEt_3, LiBr, CH_2Cl_2, −45 °C then $(Bu_4N)_3HP_2O_7$, CH_3CN followed by NH_4^+ cation exchange, 53%.

with PBr_3 in CH_2Cl_2 and then transformed into phenylsulfone **9a** by treatment with sodium benzenesulfinate in DMF. It was then necessary to remove the acetate functionality by saponification with methanolic sodium hydroxide and then to reprotect the alcohol with a THP group to give **9b** in 82% yield over these two steps. In parallel to this d6-acetone **10** was transformed into hexadeuterated ester **11** by treatment with triethyl phosphonoacetate and sodium hydride in anhydrous THF. Reduction of the ester with DIBAL-H[13] was followed by transformation into bromide **12** by treatment with MsCl and NEt_3 at −45 °C in THF followed by addition of LiBr. All these steps proceeded in moderate to good yields. Coupling of bromide **12** and sulfone **9b** was achieved by treatment of a mixture of the two in anhydrous THF containing 10% HMPA with n-BuLi at −78 °C. Sulfone **13** was isolated in 36% yield after chromatography. Final synthesis of **15** was achieved by reductive desulfonylation of **13** in 58% yield using $LiBHEt_3$ solution as reductant and $PdCl_2$dppf as catalyst followed by acid catalysed removal of the THP group in ethanol and then diphosphorylation of the resulting hexadeuterofarnesol **14** using conditions previously reported.[14,15]

Incubation of **15** with PR-AS in the presence of 5 mM Mg^{2+} was followed by GC-analysis of the hexane-extractable reaction products. This indicated the same distribution that has been observed in incubations with unlabelled FPP. In addition to the main product aristolochene, approximately 8% of germacrene A was observed (Fig. 1). Direct proton transfer from C12 to the C6,C7-double bond of **2** would generate $^2H_6$-aristolochene and a molecular ion peak *m/z* of 210 in the mass spectrum of the product. However, the mass spectrum of the main reaction product (and also the germacrene A by-product) was characterised by a molecular ion peak *m/z* of 209 (Fig. 1) indicating that conversion of germacryl cation to aristolochene occurred with the replacement of one deuteron with a proton. This finding is in agreement with an earlier report[7] in which the reaction products from an incubation of [12,12,12-$^2H_3$]-FPP with AS from *Aspergillus terreus* were analysed by ^{2}H-NMR spectroscopy and a single olefinic peak at δ_D 4.71 ppm was identified.

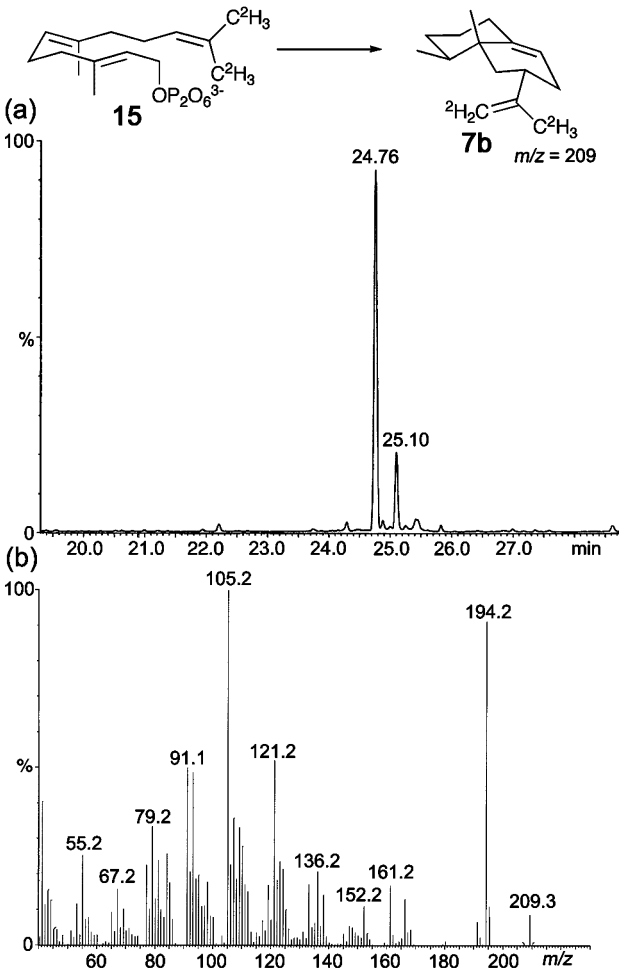


Fig. 1 (a) Total ion chromatogram of the hexane-extractable products arising from incubation of [12,12,12,13,13,13-$^2H_6$]-FPP **15** with PR-AS and (b) the mass spectrum of the aristolochene product at 24.76 min.

Proton shuttle mechanism—gas phase calculations

The above observations ruled out the direct intramolecular proton transfer from C12 to the C6,C7-double bond (Scheme 2a) previously suggested based on computation.[12] However, the potential absence of deuterium incorporation at C6 does not exclude a transfer of the label from C12 to the C6,C7-double bond, but may simply suggest an indirect mechanism in which the transfer occurs by way of an active site general acid such as a bound water molecule[12] or the pyrophosphate group.[11] A water molecule that could serve as a proton relay had been identified in the model of the transition state in the active site of PR-AS (Scheme 2, pathway b) between the isopropyl group and the double bond. Such a water molecule seemed ideally placed to deliver a proton to C6 of germacryl cation from the *Si*-face.

To investigate this shuttle mechanism a set of semi-empirical molecular orbital theory and DFT calculations were performed. The starting point for the calculations was based on the previously identified minimum energy structure for germacryl cation (**2**).[12] Using Molden,[16] a hydroxonium ion was placed in close proximity to the lower face of the ten-membered ring and a proton was removed from C12 to provide a possible model for the structure of the transition state **TS** (Scheme 2). A relaxed potential energy surface scan was performed using Gaussian03, revision B.03[17] and AM1[18] with the distance between the hydroxonium proton closest to the C6,C7 π-bond varied and all other geometric parameters allowed to adjust freely resulting in an approximate transition state geometry. As had been observed for the direct proton transfer,[12] there was broad agreement between the results obtained using DFT (mPW1PW[19] and MPWB1K[20]) and semi-empirical methods (AM1[18] and PM3[21]). Conversion of **2** to eudesmane cation (**4**) *via* water mediated proton transfer was exergonic for all methods (Table 1). The free energy of the transition state **TS** is between 15 and 18 kcal mol^{-1} higher than that of germacryl cation (**2**) at the DFT level of theory, while semiempirical methods yielded somewhat higher barriers. Compared to the results obtained for direct proton transfer, the energy barrier was lowered by 7.4 and 11 kcal mol^{-1} for mPW1PW and MPWB1K (Table 1). The semi-empirical methods, on the other hand, showed a slight increase in the activation free energy (0.4 and 2.2 kcal mol^{-1} for AM1 and PM3 respectively) but this may be due to the inadequacies in the semi-empirical method.

Table 1 Gibbs energy changes ΔG°_{298} of reaction intermediates and transition state for the conversion of germacryl cation (2) to eudesmane cation (4) *via* the potential proton shuttle mechanism[a]

	ΔG°_{298}/kcal mol^{-1}		
	2	TS	**4**
AM1	2.6	31.0	−16.2
PM3	3.5	24.5	−14.7
mPW1PW	3.8	21.6	−25.2
MPWB1K	1.1	16.1	−38.6

[a] For comparison to previous calculations, all energies are reported relative to that of the calculated free energy of farnesyl cation (set to zero kcal mol^{-1}).[12] A water molecule was also included in these calculations. The Pople 6-31+G(d,p) basis set was used for the DFT calculations (mPW1PW and MPWB1K).

Stereochemistry of eudesmane cation (4) generation

These computational results suggest that proton transfer from C12 to the C6,C7-double bond *via* a shuttle involving a water molecule may be energetically more favourable than direct intramolecular transfer. The incubations of PR-AS with [12,12,12,13,13,13-$^2H_6$]-FPP described above are in agreement with such a water shuttle mechanism since the expected kinetic isotope effect and potential exchange with bulk solvent might result in the loss of the label.

To test whether proton incorporation at C6 was observed during PR-AS catalysis, preparative scale incubation of FPP with PR-AS in buffer made up in D_2O (the pH was adjusted to 7.1 instead of 7.5 because of the 0.4 difference between pD and the measured pH for solutions containing a high percentage of D_2O) resulted in the isolation of aristolochene characterised by a molecular ion peak of 205 according to GC-MS analysis (Fig. 2) suggesting deuteron uptake from the solvent. ^{1}H-NMR spectroscopic analysis of the product indicated the absence of the multiplet at $\delta_H = 2.02$–2.10 ppm (Fig. 3), which is one of the signals observed for the two protons on C6 of aristolochene (Fig. 3a; Table 2). ^{2}H-NMR analysis of the same material showed a resonance at 2.1 ppm (ESI†) indicating that a proton is delivered to C6 ultimately from the solvent during the cyclisation reaction. Steady-state measurements of the turnover of FPP by PR-AS in H_2O and D_2O showed a kinetic solvent isotope effect of 1 indicating that the rate-limiting step does not involve proton transfer. It is worthy of note that the ^{2}H-NMR spectrum of this material also showed a resonance at 5.3 ppm (ESI†). This may correspond to the C8-proton of aristolochene possibly suggesting that the elimination of H_{Si}[7] from C8 in the final step of PR-AS catalysis is a reversible process under the reaction conditions used here. However, the appearance of this peak is not matched by the disappearance of the corresponding proton resonance in the ^{1}H-NMR spectrum although there is a small reduction in the integration of this peak relative to that of the C12 resonance. The ^{2}H-NMR spectrum is also noisy due to the low level of material coupled with the inherent insensitivity of ^{2}H-NMR spectroscopy and so the integration of these two peaks may be unreliable. Furthermore there is not a significant quantity of doubly deuterated aristolochene ($m/z = 206$) observed in the mass spectrum (Fig. 2) so it seems most likely that deuterium incorporation into C8 is happening at a low level at best.

Solvent proton uptake to C6 is in agreement with both proton transfer from C12 *via* a bound water molecule to the

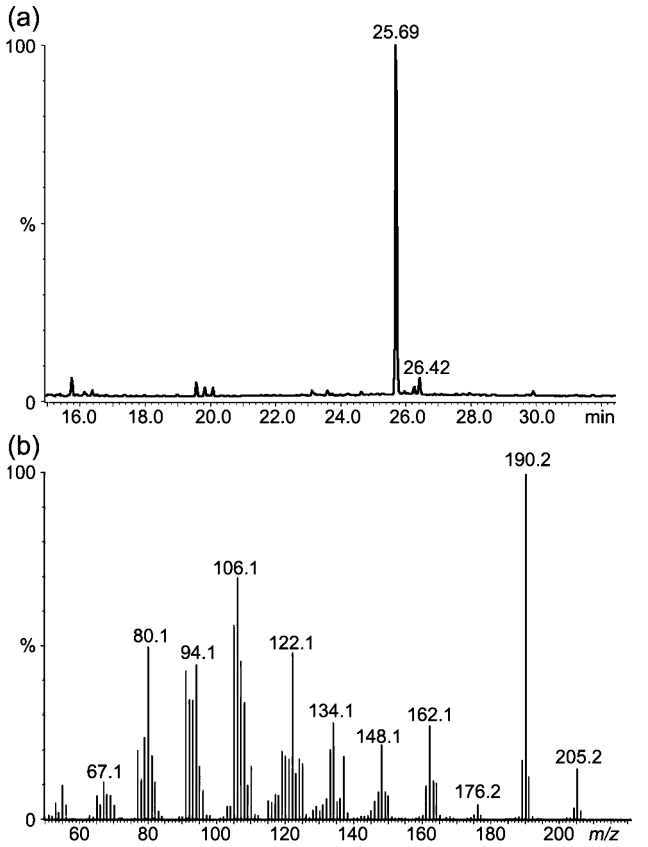


Fig. 2 (a) Total ion chromatogram of hexane extractable products generated from incubation of PR-AS, FPP and Mg^{2+} in buffered ^2H_2O and (b) EI^+ mass spectrum of aristolochene eluting at 25.69 min.

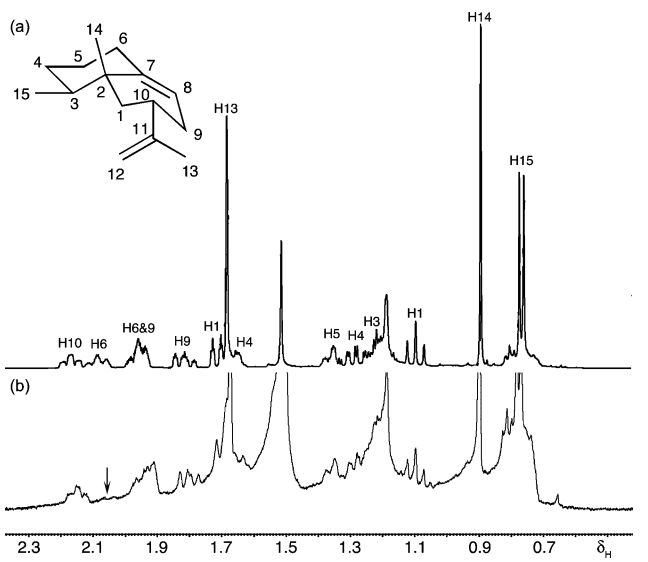


Fig. 3 (a) ^{1}H NMR spectrum (500 MHz) of and assignments for aristolochene (**7**) isolated from incubation of FPP with PR-AS and Mg^{2+}; the vinylic region of the spectrum (4.5–5.5 ppm) is not shown (see ESI†); (b) ^{1}H NMR spectrum (500 MHz) of aristolochene isolated from incubation of FPP with PR-AS and Mg^{2+} in ^2H_2O; the arrow indicates that a proton on C6 is absent from the spectrum in this material. The carbon atoms in aristolochene are numbered as in the parent molecule FPP (Scheme 1).

Table 2 Assignments for the 500 MHz ^{1}H NMR spectrum of aristolochene (**7**)[a]

δ_H/ppm	Integration	Multiplicity	Assignment
0.77	3 H	d, *J* 6.5 Hz	CH_3-15
0.90	3 H	s	CH_3-14
1.10	1 H	t, *J* 13 Hz	H-1 (axial)
1.18–1.21	1 H	m	CH-3
1.24–1.31	1 H	m	H-4
132–1.38	2 H	m	CH_2-5
1.61–1.65	1 H	m	H-4
1.67	3 H	s	CH_3-13
1.69–1.72	1 H	m	H-1 (equatorial)
1.76–1.84	1 H	m	H-9
1.92–1.96	2 H	m	H-9 and H-6 (equatorial)
2.02–2.10	1 H	m	H-6 (axial)
2.11–2.18	1 H	m	H-10
4.64	2 H	m	CH_2-12
5.25	1 H	d, *J* 5.5	CH-8

[a] The compound is numbered according to the numbering of the parent molecule FPP (**1**). Assignments are based on COSY, HSQC, HMBC as well as the observed coupling constants. All resonances are δ_H (500 MHz) in ppm. Entries are chemical-shift range followed by multiplicity in parentheses and coupling constants.

C6,C7-double bond or direct protonation of the double bond from other solvent exchangeable groups such as amino acid side chains, the diphosphate group or an as of yet unidentified water molecule bound on the opposite side of the germacryl ring system. Transfer *via* a bound water molecule however would necessarily lead to a specific stereochemical outcome in that the proton must be delivered from the lower (*Si*) face of the germacryl ring. In this scenario, incubation of FPP with PR-AS in D_2O should lead to (6*S*)-[6-^{2}H] aristolochene (Scheme 2), while protonation of C6 from the *Re*-face would exclude the water mediated proton transfer (Scheme 2).

To distinguish the chemical shifts of the two diastereotopic protons on C6, a sample of (+)-aristolochene generated from a preparative scale incubation of **1** with PR-AS was analysed by ^{1}H difference NOE NMR experiments (Fig. 4). Selective saturation of the resonance for the protons on C14 (δ_H 0.90) resulted in the enhancement of the resonances for protons on C1 (δ_H 1.69–1.72), C4 (δ_H 1.24–1.32), C10 (δ_H 2.11–2.18), C15 (δ_H 0.77) and importantly to the signal at δ_H 2.02–2.10 ppm. Analysis of the conformation of aristolochene, which is determined by the equatorial-like orientation of the isopropylidene substituent (Fig. 3), identified this resonance to be from H_{Re} on C6, since the axial proton is closest to C14 and therefore likely to display a NOE. It is interesting to note that protonation of germacrene A during catalysis by tobacco epi-aristolochene synthase (TEAS) also occurs from the *Re*-face of the double bond at C6.[22] Despite minimal sequence similarity and strikingly different active site contours,[11] TEAS and PR-AS may use similar strategies to achieve the protonation of (*R*)- and (*S*)-germacrene A for the generation of stereochemically different products.

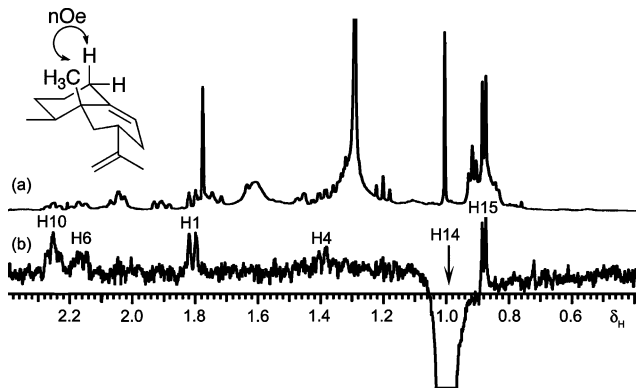


Fig. 4 (a) 600 MHz ^{1}H NMR spectrum of PR-AS generated aristolochene and (b) difference NOE spectrum of the same material with saturation of the CH_3-14 resonance. NOEs are evident to protons on C10 and C15 and one proton each on C6, C1 and C4. The proton showing a NOE to CH_3-14 on C6 is that replaced by deuterium when FPP and PR-AS are incubated in buffered D_2O (Fig. 3).

Conclusions

The results described here indicate that protonation of the C6,C7-double bond in (*S*)-germacrene A occurs from the *Re*-face at C6 and hence rule out proton transfer from C12 to C6 either by direct proton transfer or by a mechanism involving a mediating water molecule. This observation is also in agreement with a large body of experimental work that suggests that germacrene A is an intermediate during AS catalysis.[8,23,24]

The identity of the active site acid remains, therefore, elusive. Tyr 92 had initially been proposed as the active site general acid responsible for the protonation of germacrene A. However, site directed mutagenesis experiments, in which Tyr 92 was replaced with Phe, revealed that the mutant still produced substantial amounts of aristolochene thereby ruling out Tyr 92 as the obligatory active site acid.[8,10] The elucidation of the X-ray crystal structure of AS from *Aspergillus terreus* in which a diphosphate ion was found bound to one of the subunits of the tetrameric enzyme, together with comparisons with trichodiene synthase and the monoterpene cyclase bornyl-diphosphate synthase, led to the proposal that the diphosphate anion might be positioned to function as a possible general acid/base during AS catalysis.[11] In the absence of evidence for this proposal, other than the proximity of the diphosphate group to the C6,C7-double bond, we propose here Lys 206 as the potential general acid/base during PR-AS catalysis. Preliminary results from an analysis of the effects of replacing Lys 206 are in agreement with this proposal. Substitution with glutamine led to a reduction of the catalytic efficiency of approximately 3 orders of magnitude, while only a 4-fold reduction was observed when Lys 206 was replaced by arginine.[25]

Based on the X-ray structure of PR-AS, a hydrogen-bonding network from the side chain of Lys 206 to Asp 203 and Arg 200 was identified.[26] The hydrogen bonding network might provide a proton shuttle from Arg 200 at the solvent exposed top of the active site to the double bond at C6,C7 of germacrene A.[8] Since the ε-amino group of Lys 206 is approximately equidistant to carbons 6 and 8 of germacrene A and eudesmane cation, it could not only serve as the active site general acid but also abstract a proton from **6** to produce **7**. It is worthy of note that despite only 61% sequence identity between the two fungal aristolochene synthases from *P. roqueforti* and *A. terreus*, this network of hydrogen bonds is conserved with Lys 181, Asp 178 and Arg 175 of AT-AS taking the positions of Lys 206, Asp 203 and Arg 200 in PR-AS. A full

study of the effects of replacing the residues in the network of amino acids in PR-AS is currently under way.

Experimental

General procedures

All chemicals were purchased from Sigma-Aldrich. Tetrahydrofuran (THF) and diethyl ether were distilled from sodium–benzophenone ketyl under nitrogen. Acetonitrile, dichloromethane, toluene and triethylamine were distilled from calcium hydride under nitrogen. Ecoscint scintillation fluid was purchased from National Diagnostics. All other chemicals were of analar quality or better and used as received unless otherwise stated. Reactions were stirred at room temperature in air unless otherwise stated. All glassware was clean and dry before use.

^{1}H NMR spectra were measured on a Bruker Avance 500 NMR spectrometer or a Bruker Avance DPX400 NMR spectrometer and are reported as chemical shifts in parts per million downfield from tetramethylsilane, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant (to the nearest 0.5 Hz) and assignment respectively. ^{31}P and ^{13}C NMR spectra were measured on a Bruker Avance 500 NMR spectrometer and are reported as chemical shift downfield from 85% H_3PO_4 and tetramethylsilane respectively, coupling constants in Hertz where appropriate and assignment. Assignments are made to the limitations of COSY, DEPT 90/135, gradient HSQC and gradient HMBC spectra. ^{2}H NMR spectra were recorded on a Jeol Eclipse +300 NMR spectrometer. NOE difference spectra were recorded on an Inova 600 MHz spectrometer using a selective excitation pulsed field gradient 1D-NOESY pulse sequence. IR spectra were recorded on a Perkin ELMER 1600 series FTIR spectrometer and samples were prepared as thin films of neat liquid on sodium chloride discs for oils and as KBr disks for solids. EI mass spectra were measured on a Micromass LCT premiere XE mass spectrometer. ES mass spectra were recorded on a Micromass Q-Tof micro mass spectrometer.

Thin layer chromatography was performed on pre-coated aluminium plates of silica G/UV_{254} (Fluka). Flash chromatography was performed according to the method of Still.[27] Reverse phase HPLC was performed on a system comprising of a Dionex P680 pump and a Dionex UVD170U detector unit.

Expression and purification of AS and AS-F112A in *E. coli*

Enzymes were produced in *E. coli* BL21(DE3) harbouring a cDNA for AS under the control of the T7 promoter as previously described.[28] Cells were grown at 37 °C in LB medium with 0.3 mM ampicillin until they reached an A_{600} of 0.5. They were induced with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside, incubated for a further 3 h and harvested by centrifugation at 8000*g* for 10 min. Proteins were then extracted from the inclusion bodies and purified following our established protocols.[8,28] Each enzyme was pure as judged by SDS-gel electrophoresis.

(*E*,*E*) *O*-Acetyl 3,7-dimethyl-8-phenylsulfonyl-octa-2,6-diene-1-ol (9a)

To a stirred solution of **8**[29] (5.00 g, 23.3 mmol) in anhydrous CH_2Cl_2 at 5 °C (ice–water bath) under N_2 was added PBr_3 (2.41 cm^3, 25.6 mmol) in a dropwise fashion and stirring was continued for 1 h. The solution was concentrated under reduced pressure and the residue was partitioned between diethyl ether (100 cm^3) and a saturated sodium hydrogencarbonate solution (100 cm^3). The separated ethereal layer was successively washed with water (100 cm^3) and brine (100 cm^3) then dried over anhydrous $MgSO_4$ and filtered under reduced pressure. After concentration under reduced pressure, the crude intermediate bromide was dissolved in anhydrous dimethylformamide (50 cm^3) and to this stirred solution under nitrogen was added benzenesulfinic acid sodium salt (3.82 g, 23.3 mmol). Stirring was continued for 16 h then the solution was diluted with diethyl ether (200 cm^3). The organic solution was successively washed with water (3 × 200 cm^3) and brine (200 cm^3) then dried over anhydrous $MgSO_4$. After filtration and concentration under reduced pressure the oily product was chromatographed on silica gel (eluting with 1 : 1 hexane–ethyl acetate) to give **9a** as a colourless oil (5.46 g, 70%); ν_{max}/cm^{-1} (thin film) 3060 (w), 2971 (m), 2920 (m), 1732 (vs), 1669 (w), 1447 (s), 1385 (m), 1367 (m), 1307 (vs), 1234 (vs), 1134 (vs), 1086 (s), 1024 (m), 954 (m), 884 (w); δ_H (500 MHz, C^2HCl_3) 1.66 and 1.72 (2 × 3 H, 2 × s, 2 × CH_3C=CH), 1.88–1.91 and 2.03–2.15 (4 H, m, CH_2CH_2), 2.02 (3 H, s, CH_3CO), 2.18 (2 H, s, O_2SCH_2), 4.57 (2 H, d, *J* 7.5, OCH_2), 5.05 (1 H, t, *J* 6.5, C=CH), 5.26 (1 H, tq, *J* 7.5 and 1, C=CH), 7.54–7.57, 7.64–7.68 and 7.85–7.87 (5 H, m, ArCH); δ_C (125 MHz, C^2HCl_3) 16.4 and 16.7 (2 × *C*H_3C=CH), 21.0 (*C*H_3CO), 26.5 and 38.4 (CH_2CH_2), 61.2 and 66.2 (SCH_2 and OCH_2), 118.8, 128.5, 128.9, 133.5 and 135.4 (ArCH and 2 × C=*C*H), 123.8, 138.5 and 141.2 (quaternary ArC and 2 × *C*=CH), 171.1 (C=O); *m*/*z* (ES$^+$) 337 (100%, [M + H]$^+$).

(*E*,*E*) 8-Benzenesulfonyl-3,7-dimethyl-1-(tetrahydro-2-pyranyloxy)-octa-2,6-diene (9b)

A solution of **9a** (5.27 g, 14.4 mmol) in a 10% solution of NaOH in 9 : 1 methanol–water (50 cm^3) was stirred for 16 h. The ethanol was removed under reduced pressure and the residue was partitioned between ethyl acetate (50 cm^3) and water (50 cm^3). The separated aqueous layer was extracted with ethyl acetate (2 × 50 cm^3) and the pooled organics were washed with brine (150 cm^3) then dried over anhydrous $MgSO_4$. After concentration under reduced pressure the residual oil was chromatographed on silica gel (eluting with 1 : 1 hexane–ethyl acetate) to give the intermediate alcohol as a colourless oil (3.87 g, 91%).

To a stirred solution of the crude alcohol (3.69 g, 12.5 mmol) in dichloromethane (50 cm^3) was added 3,4-dihydropyran (2.29 cm^3, 25 mmol) followed by *p*-toluene sulfonic acid (238 mg, 1.25 mmol). Stirring was continued for 24 h then the solution was concentrated under reduced pressure and the residue was dissolved in ethyl acetate (50 cm^3). This solution was washed successively with saturated sodium hydrogencarbonate solution (50 cm^3), water (50 cm^3) and brine (50 cm^3). After drying over anhydrous $MgSO_4$, the solution was filtered and concentrated under reduced pressure. The oily residue was chromatographed on silica gel (eluting with 3 : 1 hexane–ethyl acetate) to give **9b** as a colourless oil (4.27 g, 90%); ν_{max}/cm^{-1} (thin film) 2939 (s), 2866 (s), 1732 (w), 1667 (w), 1586 (w), 1446 (s), 1386 (m), 1355 (m), 1307 (s), 1260 (m), 1200 (m), 1134 (s), 1086 (s), 1023 (s), 904 (m), 869 (m), 814 (m), 742 (m), 726 (m), 690 (s); δ_H (500 MHz, C^2HCl_3) 1.54 and 1.69 (2 × 3 H, 2 × s, 2 × CH_3C=CH), 1.44–1.52, 1.63–1.67, 1.73–1.80 and

1.97–2.01 (10 H, m, $OCH_2(CH_2)_3$ and $C{=}CHCH_2CH_2$), 3.42–3.46 and 3.79–3.84 (2 H, m, $OCH_2(CH_2)_3$), 3.65 (2 H, s, SO_2CH_2), 3.91 (1 H, dd, *J* 7 and 12, $C{=}CHCH_2O$), 4.15 (1 H, dd, *J* 6 and 12, $C{=}CHCH_2O$), 4.53 (1 H, t, *J* 3, OCHO), 4.96 (1 H, t, *J* 7, C=CH), 5.20 (1 H, t, *J* 6.5, C=CH), 7.45–7.78 (5 H, m, ArCH); δ_C (125 MHz, C^2HCl_3) 16.3 and 16.7 ($2 \times CH_3C{=}CH$), 19.7, 25.5, 26.6, 30.7 and 38.5 (CH_2CH_2 and $OCH_2(CH_2)_3$), 62.4, 63.6 and 66.2 (SO_2CH_2 and $2 \times OCH_2$), 98.0 (OCHO), 121.1, 128.5, 128.9, 133.5 and 135.7 ($3 \times$ ArCH and $2 \times C{=}CH$), 123.6, 138.5 and 139.1 (quaternary ArC and $2 \times C{=}CH$); *m/z* (ES^+) 379 (100%, $[M + H^+]$.

Ethyl 3,3,3-[^{2}H,^{2}H,^{2}H]-methyl [4,4,4-^{2}H,^{2}H,^{2}H]-but-2-eneoate (11)

To a stirred solution of triethyl phosphonoacetate (15.24 g, 68.0 mmol) in anhydrous THF (100 cm^3) at 5 °C (ice-water bath) under N_2 was added sodium hydride (80% dispersion in mineral oil, 0.918 g, 30.6 mmol) in portions. After effervescence had ceased, d6-acetone (100 atom% Sigma-Aldrich, 1.5 cm^3, 20.4 mmol) was added and the solution was stirred for 16 h whilst slowly warming to room temperature. Water (100 cm^3) was carefully added and the mixture was extracted with diethyl ether (3×100 cm^3). The pooled ethereal extracts were washed with saturated sodium hydrogencarbonate solution (200 cm^3), water (200 cm^3) and brine (200 cm^3) then dried over anhydrous $MgSO_4$ and filtered under reduced pressure. The title compound was obtained as a colourless liquid by fractional distillation at atmospheric pressure collecting the fraction boiling at 154–156 °C (5.17 g, 57%); ν_{max}/cm^{-1} (thin film) 2982 (m), 2130 (w), 1714 (s), 1634 (m), 1446 (w), 1366 (w), 1218 (s), 1154 (m), 1097 (m), 1055 (m), 788 (w); δ_H (500 MHz, C^2HCl_3) 1.29 (3 H, t, *J* 7, CH_2CH_3), 4.16 (2 H, q, *J* 7, CH_2CH_3), 5.69 (1 H, s, C=CH); δ_C (125 MHz, C^2HCl_3) 14.30 (CH_3), 59.4 (CH_2), 116.2 (C=CH), 156.1 (C=CH), 166.7 (C=O); *m/z* (EI^+) 134 (71%, M^+), 116 (50), 106 (68), 89 (100), 61 (70).

(*E*,*E*) 12,12,12-(^{2}H,^{2}H,^{2}H)-8-Benzenesulfonyl-3,7-dimethyl-11-(^{2}H,^{2}H,^{2}H)-methyl-1-(tetrahydro-2-pyranyloxy)-dodeca-2,6,10-triene (13)[30]

To a stirred solution of **11** (536 mg, 4.00 mmol) in anhydrous CH_2Cl_2 at −78 °C (dry ice–acetone bath) under N_2 was added DIBAL-H solution (1.0 M in hexanes, 12 cm^3, 12 mmol) and the mixture was stirred for 1 h at −78 °C. The reaction was quenched by the addition of celite® and $Na_2SO_4{\cdot}10H_2O$ (4 g each) and then this mixture was stirred for 16 h. The resulting suspension was filtered through a thin pad of celite and the pad was washed with several portions of CH_2Cl_2. After concentration under reduced pressure the intermediate alcohol was isolated as a colourless oil (174 mg, 47% crude yield) and used without further purification in the next step (*m/z* (EI^+) 92.1 (60%, M^+), 74.1 (48, $[M - H_2O]^+$), 63.1 (100).

To a stirred solution of the crude hexadeuterated alcohol (7.46 mmol) in anhydrous THF (10 cm^3) under N_2 was added anhydrous triethylamine (1.25 cm^3, 8.95 mmol) and the mixture was cooled to −45 °C (dry ice–acetone bath). Methanesulfonyl chloride (754 mm^3, 9.70 mmol) was added and the solution (turning to a white milky suspension) was stirred at −45 °C (dry ice–acetone bath) for 45 min. Lithium bromide (2.59 g, 29.8 mmol) was added as a solution in anhydrous THF (10 cm^3) *via* a cannula and this mixture was stirred at 5 °C (ice–water bath) for 1 h. The reaction was quenched by the addition of cold brine (20 cm^3) and the mixture was extracted with diethyl ether (3×20 cm^3). The pooled ethereal extracts were washed with brine (50 cm^3) then dried over anhydrous $MgSO_4$ and filtered under reduced pressure. The ether was carefully evaporated to give the crude bromide as a pale yellow oil that was immediately dissolved in anhydrous THF (10 cm^3) and added, *via* a cannula, to a solution of **9b** (185 mg, 0.490 mmol) in anhydrous THF (10 cm^3) and HMPA (2 cm^3) under N_2. Freshly activated powdered 4 Å molecular sieves (~200 mg) were added and the solution was cooled to −78 °C (dry ice–acetone bath). To this stirred mixture was slowly added, over 30 min, n-BuLi (2.5 M in hexanes, 196 mm^3, 0.490 mmol) and then the mixture was stirred for 16 h whilst slowly warming to room temperature. The reaction was quenched by the addition of saturated ammonium chloride solution (20 cm^3) and the mixture was extracted with diethyl ether (3×20 cm^3). The pooled ethereal extracts were washed with water (2×50 cm^3) and brine (50 cm^3) then dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel (eluting with 4 : 1 hexane–ethyl acetate) to give **13** as a colourless oil (77 mg, 36%); ν_{max}/cm^{-1} (thin film) 2936 (m), 2869 (m), 2130 (w), 1448 (m), 1302 (s), 1198 (m), 1142 (s), 1083 (s), 1020 (s), 722 (s); δ_H (500 MHz, C^2HCl_3) 1.53 and 1.58 (2×3 H, $2 \times$ s, $2 \times CH_3$), 1.43–1.59, 1.62–1.78 and 1.88–2.02 (10 H, m, $OCH_2(CH_2)_3$ and CH_2CH_2), 2.50 (1 H, ddd, *J* 7, 11.5 and 15, CH_2CHSO_2), 2.74 (1 H, ddd, *J* 4, 7 and 15, CH_2CHSO_2), 3.40 (1 H, dd, *J* 4 and 11.5, CH_2CHSO_2), 3.42–3.45 and 3.79–3.83 (2 H, m, $OCH_2(CH_2)_3$), 3.90 (1 H, dd, *J* 7 and 12, $C{=}CHCH_2$), 4.14 (1 H, dd, *J* 6 and 12, $C{=}CHCH_2$), 4.53 (1 H, dd, *J* 3 and 4, OCHO), 4.79 (1 H, t, *J* 7, C=CH), 4.96 (1 H, t, *J* 7, C=CH), 5.19 (1 H, t, *J* 7, C=CH), 7.43–7.46, 7.53–7.56 and 7.73–7.75 (5 H, m, ArCH); δ_C (125 MHz, C^2HCl_3) 13.7, 16.3 ($2 \times CH_3$), 19.7, 24.1, 25.5, 26.4, 30.7 and 38.5 ($OCH_2(CH_2)_3$ and CH_2CH_2), 62.4 ($OCH_2(CH_2)_3$), 63.7 ($C{=}CHCH_2O$), 74.1 (SO_2CH), 98.0 (OCHO), 118.8, 121.0 and 135.4 ($3 \times C{=}CH$), 128.7, 128.9 and 133.3 (ArCH), 126.9, 134.6, 138.1 and 139.3 (ArC quaternary and $3 \times C{=}CH$); *m/z* (ES^+) 451 (100%, $[M + H]^+$).

(*E*,*E*) 12,12,12-[^{2}H,^{2}H,^{2}H]-3,7-Dimethyl-11-[^{2}H,^{2}H,^{2}H]-methyl-dodeca-2,6,10-triene-1-ol (14)[31,32]

To a stirred solution of **13** (94 mg, 0.216 mmol) in anhydrous THF (10 cm^3) under N_2 was added $PdCl_2$dppf (35 mg, 43.2 mmol) and the solution was stirred under N_2 for 30 min. To this stirred mixture was added, dropwise, lithium triethylborohydride (1.0 M solution in THF, 432 mm^3, 0.432 mmol), the solution immediately turned a deep red colour. The complete mixture was stirred under N_2 for 2 h then water (10 cm^3) was carefully added to quench the reaction. The mixture was extracted with diethyl ether (3×20 cm^3) then the pooled ethereal extracts were washed with water (2×20 cm^3) and brine (20 cm^3). The organic solution was then dried over anhydrous $MgSO_4$, filtered and evaporated to give a pale red oil that was passed through a short column of silica (eluting with 19 : 1 hexane–ethyl acetate) to yield the intermediate THP ether as a colourless oil (39 mg, 58% crude yield). This was used directly in the next step without further purification. HRMS (CI^+, $[M + NH_4]^+$) found 330.3270, $C_{20}H_{32}{}^2H_6NO_2$ requires 330.3279.

The crude THP ether (38 mg, 0.121 mmol) was dissolved in freshly distilled ethanol and to this stirred solution was added pyridinium *p*-toluenesulfonate (2.8 mg, 12 mmol). This stirred mixture was heated at 50 °C for 24 h. After cooling, the ethanol was removed under reduced pressure and the residue was immediately purified by flash chromatography on silica gel (eluting with 3 : 1 hexane–ethyl acetate) to yield the title compound as a colourless oil (18 mg, 65%); HRMS (M^+) found 228.2360, $C_{15}H_{20}{}^2H_6O$ requires 228.2360; ν_{max}/cm^{-1} 3319 (br s), 2927 (s), 2856 (s), 2222 (w), 1661 (w), 1444 (m), 1379 (m), 1010 (s); δ_H (500 MHz, C^2HCl_3) 1.53 (3 H, s, CH_3), 1.62 (3 H, s, CH_3), 1.87–2.07 (8 H, m, 2 × CH_2CH_2), 4.08 (2 H, d, *J* 7, CH_2OH), 5.01–5.06 (2 H, m, 2 × C=CH), 5.35 (1 H, t, *J* 7, C=CH); δ_C (125 MHz, C^2HCl_3) 15.9 and 16.2 (2 × CH_3), 26.0, 26.6, 39.2 and 39.7 (2 × CH_2CH_2), 59.4 (OCH_2), 123.3, 123.8 and 124.3 (3 × C=*C*H), 134.7, 135.4 and 139.8 (3 × *C*=CH); *m*/*z* (EI^+) 228.2 (3%, M^+), 210.2 (18, $[M - H_2O]^+$), 135.1 (35), 93.1 (100).

(*E*,*E*) 12,12,12-[^{2}H,^{2}H,^{2}H]-3,7-Dimethyl-11-[^{2}H,^{2}H,^{2}H]-methyl-dodeca-2,6,10-trien-1-yl diphosphate trisammonium salt (15)[14,15]

The title compound was prepared as previously described for other farnesyl diphosphate analogues.[14,15] To a stirred solution of *N*-chlorosuccinimide (11.6 mg, 86.8 mmol) in anhydrous CH_2Cl_2 (2 cm^3) under N_2 at −30 °C (dry ice–acetone bath) was added dimethyl sulfide (6 mm^3, 86.8 mmol). This solution (that rapidly turned into a milky-white suspension) was stirred for 15 min at −30 °C then briefly warmed to 0 °C in an ice–water bath. After cooling to −40 °C (dry ice–acetone bath), **14** (16.5 mg, 72.4 mmol) was added as a solution in anhydrous CH_2Cl_2 (2 cm^3) *via* a cannula. This solution was stirred for 16 h whilst slowly warming to room temperature. Brine (5 cm^3) was added and the mixture was extracted with hexane (3 × 10 cm^3). The pooled hexane extracts were dried over anhydrous $MgSO_4$ then filtered and concentrated under reduced pressure. The resulting intermediate chloride was used immediately without further purification.

To a stirred solution of this material in anhydrous acetonitrile (2 cm^3) and freshly activated, powdered, 4 Å molecular sieves (100 mg), under N_2, was added tris (tetra-n-butylammonium) hydrogendiphosphate[14,15] (128 mg, 142 mmol). The complete reaction mixture was stirred for 16 h and then solvent was removed under reduced pressure and the resulting opaque residue was dissolved in 2 cm^3 of 1 : 49 (v/v) isopropyl alcohol and 25 mM ammonium hydrogencarbonate solution (ion-exchange buffer). The pale yellow solution was slowly passed through a column containing 30 equiv. of DOWEX 50W-X8 (100–200 mesh) cation-exchange resin that had been equilibrated with two column volumes of ion-exchange buffer. The column was eluted with two column volumes of the same buffer at a flow rate of one column volume per 15 min. The clear light yellow eluent was lyophilized to dryness to give a solid, which was purified by reverse phase HPLC (150 × 21.2 mm Phenomenex Luna column, eluting with 10% B for 20 min, then a linear gradient to 60% B over 25 min and finally a linear gradient to 100% B over 5 min; solvent B: CH_3CN, solvent A: 25 mM NH_4HCO_3 in water, flow rate 5.0 cm^3/min, detecting at 220 nm) to give **7** as a white solid (17 mg, 53%); HPLC t_R 46.2 min; ν_{max}/cm^{-1} (KBr disc) 2848 (br m), 1497 (s), 1453 (s), 1406 (s), 1201 (s), 1120 (s), 1093 (s), 1026 (m), 905 (s), 797 (m); δ_H (500 MHz; ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) 1.50 (3 H, s, CH_3C=CH), 1.60 (3 H, s, CH_3C=CHCH_2O), 1.85–2.03 (8 H, m, 2 × CH_2CH_2), 4.35 (2 H, t, *J* 5.5, CH_2O), 5.09 (2 H, m, 2 × C=CH), 5.33 (1 H, t, *J* 7.0, C=CH); δ_C (125 MHz; ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) 15.3 and 15.7 (2 × CH_3), 25.7, 26.40, 26.9, 38.5 and 38.9 (2 × CH_2CH_2), 62.6 (CH_2O), 120.0, 124.0 and 124.3 (3 × C=*C*H), 136.7, 142.8 and 143.0 (3 × *C*=CH); δ_P (202 MHz; ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) −6.80 (1 P, d, J_{PP} 22.0) and −10.28 (1 P, d, J_{PP} 22.0); *m*/*z* (ES^-) 387.0 (100%, $[M - H]^-$).‡

Incubation of FPP and analogues with aristolochene synthase

Incubations of FPP and deutero analogue **15** with aristolochene synthase were carried out as previously described.[33]

Briefly, enzyme (25 mM) was incubated with FPP (10 mM, 100 mm^3) in a volume of 500 mm^3 containing 25 mM Tris (pH 7.5), 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol and 15% glycerol at 25 °C, overlayed with 100 mm^3 of hexane. The reactions were stopped by addition of 100 mM EDTA solution (100 mm^3), then extracted with hexane (3 × 3 cm^3). The hexane extracts were vortexed with silica (50 mg) then the decanted solvent was removed under a gentle stream of nitrogen. Samples were analysed by GC-MS performed on 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 detecting in the range *m*/*z* 50–800 in EI^+ mode with 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 rising 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).

For incubations carried out in D_2O, the enzyme, substrate and buffers were made up as normal then lyophilised and redissolved in the required volume of 100 atom% D_2O. This was repeated twice and then the buffer was adjusted to pH 7.1 by addition of DCl.

Activity assays

Enzyme assays were carried out essentially as previously described.[15,33] Assays (250 mm^3 final volume) were initiated by addition of purified AS solution (1 mM, 25 mm^3, final concentration 100 nM). Assays contained 0.1–5 mM [1-^{3}H]-farnesyl diphosphate (240 000 dpm $nmol^{-1}$), 20 mM Tris, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol and 15% glycerol and were pre-warmed to 37 °C prior to addition of enzyme solution. After incubation for 4 min, each assay was stopped by addition of 100 mM EDTA (100 mm^3) and overlayed with hexane (500 mm^3). After vortexing for 10 s, the hexane was removed and the sample extracted with hexane in the same way (2 × 500 mm^3). The pooled hexane extracts were vortexed with silica (50 mg), the sample was centrifuged at 13 000 rpm for 5 min and then the hexane was decanted into a scintillation vial containing 15 cm^3 of Ecoscint and analysed for radioactivity. K_M and k_{cat} values were determined by a non-linear fit of the data to the equation $V = V_{max}[S]/(K_M + [S])$ using Sigmaplot for Windows Version 10.0. For assays under normal conditions and in D_2O, K_M (0.8 μM) and k_{cat} (0.02 s^{-1}) were identical within experimental error and so no kinetic solvent isotope effect was measurable.

‡ Note that this compound has recently been prepared using a different methodology.[22]

Gas phase calculations

The starting point for the calculations was based on the previously identified minimum energy structure for the germacryl cation.[12] Using Molden,[16] a hydroxonium ion was placed in close proximity to the lower face of the ten-membered ring and a proton was removed from C12 to provide an approximate model for the structure of the transition state. A relaxed potential energy surface scan was performed using Gaussian03, revision B.03[17] and AM1[18] with the distance between the hydroxonium proton closest to the C6, C7 π-bond varied and all other geometric parameters allowed to adjust freely.

From this simulation, an approximate transition state geometry was obtained. Transition state searches, using the harmonic oscillator approximation, were performed using the AM1,[18] PM3,[21] mPW1PW[19] and MPWB1K[20] methods. To account approximately for anharmonicity, all vibrational modes below 100 cm^{-1} were raised to 100 cm^{-1} in all calculations.

The Gibbs free energy change (ΔG_{298}) for the formation of the TS was defined as the difference in free energy between the TS and the sum of free energies of germacryl cation and an isolated water molecule.

Starting geometries for these calculations are available from the authors on request.

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