Supplementary Material for Organic & Biomolecular Chemistry

Electronic Supplementary Information for

Competitive Inhibition of Aristolochene Synthase by Phenyl-Substituted Farnesyl Diphosphates: Evidence of Active Site Plasticity

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General procedures

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Tetrahydrofuran (THF), dimethoxyethane (DME) and diethyl ether were distilled from sodium/benzophenone ketyl under nitrogen. Acetonitrile, dichloromethane, toluene and triethylamine were distilled from calcium hydride under nitrogen. 9-BBN was used as the commercially available crystalline form, solutions were not found to work adequately in hydroboration reactions employed in this study. Ecoscint scintillation fluid was purchased from National Diagnostics. All other chemicals were of analytical 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. ^{13}C NMR spectra were measured on a Bruker Avance 500 NMR spectrometer and are reported as chemical shift downfield from tetramethylsilane, coupling constant where appropriate and assignment. Assignments are made to the limitations of COSY, DEPT 90/135, gradient HSQC and gradient HMBC spectra. ^{31}P NMR spectra were recorded on a Jeol Eclipse +300 NMR spectrometer and are reported in chemical shift downfield from 85% H_3PO_4 followed by multiplicity and coupling constant (to the nearest 0.5 Hz). 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; CI^+ and ES^- mass spectra were provided by the UK EPSRC mass spectrometry service, Swansea.

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.[3] Reverse phase HPLC was performed on a system comprising of a Dionex P680 pump and a Dionex UVD170U detector unit

Expression in *E. coli* and purification of AS

AS was produced in *E. coli* BL21(DE3) cells harbouring a cDNA for AS under the control of the T7 promoter. 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-thiogalactopypyranoside, 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 the protocol described previously.[1,2] AS was pure as judged by SDS-gel electrophoresis.

(2*E*,6*E*,10*E*)-3,7-Dimethyl-11-phenyldodeca-2,6,10-trien-1-yl diphosphate tris-ammonium salt 19[4, 5]

To a stirred solution of **18** (222 mg, 0.782 mmol) and triethylamine (218 mm^3, 1.56 mmol) in anhydrous THF (5 cm^3) at –45 °C under N_2 was added methanesulfonyl chloride (72 mm^3, 0.94 mmol). The resulting milky mixture was stirred at – 45 °C for 45 min then a solution of lithium bromide (0.27 g, 3.12 mmol) in THF (5 cm^3) was added *via* a cannula. The resulting suspension was allowed to warm to 0 °C and stirred for an additional 1 h. Cold water (10 cm^3) and hexane (10 cm^3) were added and the two layers were separated. The aqueous layer was extracted with hexane (2 × 10 cm^3) and the combined organic layers were washed with saturated $NaHCO_3$ solution (10 cm^3) and brine (10 cm^3) then dried over Na_2SO_4 and filtered. The solvent was removed under reduced pressure to give the intermediate bromide as a light-yellow oil which was used without further purification. To a stirred solution of this material in anhydrous acetonitrile (10 cm^3) under N_2 was added freshly recrystallized tris (tetra-n-butylammonium) hydrogenpyrophosphate[24] (1.40 g, 1.56 mmol). The complete reaction mixture was stirred for 2 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 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 **19** as a white solid (0.12 g, 31%); HPLC t_R 39.28 min; HRMS (ES^-, $[M - H]^-$) found 443.1410, $C_{20}H_{29}O_7P_2$ requires 443.1389; υ_{max}(KBr disc)/cm^{-1} 2922.8, 2190.6, 1668.4, 1493.0, 1444.1, 1381.1, 1201.3, 1092.4, 1024.6, 906.9, 798.2, 757.3, 722.5 and 696.3; δ_H (500 MHz; ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) 1.46 (3 H, s, C*H*$_3$C=CH), 1.59 (3 H, s, C*H*$_3$C=CHCH$_2$O), 1.76 (3 H, s, PhCC*H*$_3$), 1.89-2.10 (8 H, m, 2 x C*H*$_2$C*H*$_2$), 4.37 (2 H, t, *J* 5.5, CHC*H*$_2$O), 5.03 (1 H, t, *J* 6.5, CH$_3$C=C*H*), 5.33 (1 H, t, *J* 7.0, C=C*H*CH$_2$O), 5.68 (1 H, t, *J* 6.5, PhC=C*H*) and 6.98-7.16 (5 H, m, Ar-H); δ_C (125 MHz; ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) 15.1 (PhC*C*H$_3$), 15.4 (*C*H$_3$C=CH), and 15.8 (*C*H$_3$C=CHCH$_2$O), 26.0, 26.9, 38.9 and 39.1 (2 × *C*H$_2$*C*H$_2$), 62.4 (d, J_{CP} 5.0, CH$_2$O), 119.7 (d, J_{CP} 7.5, *C*HCH$_2$O), 124.4 (CH$_3$C=*C*H), 128.0 (PhC=*C*H), 125.3, 126.6 and 128.2 (Ar-CH) and 134.6, 135.5, 142.4 and 143.3 (quaternary C); δ_P (122 MHz; ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) –6.58 (1 P, d, J_{PP} 22.0) and –10.34 (1 P, d, J_{PP} 22.0); *m/z* (ES^-) 443.1 (100%, $[M - H]^-$).

(2*E*,6*E*,10*Z*)-3,7-Dimethyl-11-phenyldodeca-2,6,10-trien-1-yl diphosphate tris- ammonium salt (29)

This compound was prepared from **28** and purified in a manner identical to that for the diphosphate **19** to give **29** as a white solid (63.8 mg, 36%); HPLC t_R = 38.82 min; HRMS (ES^-, $[M - H]^-$) found 443.1374, $C_{20}H_{29}O_7P_2$ requires 443.1389; υ_{max} cm^{-1} (film) 3292.3, 1494.2, 1457.3, 1409.4, 1202.2, 1122.0, 1089.8, 1024.0, 910.5, 757.0, 723.3 and 697.3; δ_H (500 MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) 1.34 (3 H, s, C*H*$_3$C=CH), 1.59 (3 H, s, C*H*$_3$C=CH), 1.85 (3 H, s, C*H*$_3$C=CH), 1.88-2.03 (8 H, m, 2 x C*H*$_2$C*H*$_2$), 4.39 (2 H, b, C*H*$_2$OH), 4.98 (1 H, b, C=CH), 5.33 (2 H, bd, 2 × C=CH) and 7.06-7.18 (5 H, m, Ar-CH); δ_C (125 MHz; ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) 15.3, 15.7 and 24.9 (3 × CH_3), 25.9, 27.1, 39.0 and 39.3 (2 × *C*H$_2$*C*H$_2$), 62.6 (*C*H$_2$OH), 119.6, 124.2 and 127.3 (3 × C=*C*H), 126.5, 127.8 and 128.0 (Ar-CH) and 135.4, 136.3, 141.8 and 142.6 (quaternary C); δ_P (122 MHz; ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) –6.51 (1 P, d, J_{PP} 21.0) and –10.33 (1 P, d, J_{PP} 21.0); *m/z* (ES^-) 443.1 (100%, $[M - H]^-$).

(2*Z*,6*E*)-7,11-dimethyl-3-phenyldodeca-2,6,10-trien-1-yl diphosphate tris-ammonium salt (36)

This compound was prepared from **35** and purified in a manner identical to that for the diphosphate **19** to give **36** as a white solid (53.9 mg, 31%); HPLC t_R 39.02 min; HRMS (ES^-, $[M - H]^-$) found 443.1375, $C_{20}H_{29}O_7P_2$ requires 443.1389; υ_{max} (KBr disc)/cm^{-1} 2924.0, 1442.5, 1200.4, 1128.9, 1101.1, 1023.2, 971.5, 927.5, 812.5 and 706.3; δ_H (500 MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) 1.16 (3 H, s, CH_3), 1.23 (3 H, s, CH_3), 1.31 (3 H, s, CH_3), 1.61 (6 H, m, $(CH_3)_2$C=CHC*H*$_2$C*H*$_2$ and C*H*$_2$CH$_2$CPh), 2.24 (2 H, t, J 7.5, CH$_2$C*H*$_2$CPh), 4.32 (2 H, t, *J* 6.5, C*H*$_2$O), 4.77 (1 H, t, *J* 6.5, C=C*H*), 4.84 (1 H, t, J 6.5, C=C*H*), 5.67 (1 H, t, J 7.0, C=C*H*CH$_2$O) and 7.05 (5 H, m, Ar-H); δ_C (125 MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) 15.5 (CH_3), 17.1 (CH_3), 25.1 (CH_3), 26.3, 26.5, 38.7 and 39.3 (2 × *C*H$_2$*C*H$_2$), 63.9 (CH$_2$O), 122.4 (C=C*H*CH$_2$O), 123.7 and 124.3 (2 × C=C*H*), 127.5, 128.3 and 128.4 (Ar-CH) and 131.2, 135.5, 139.4 and 146.0 (quaternary C); δ_P (122 MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) –10.18 (d, J_{PP} 21.0), –10.82 (d, J_{PP} 21.0); *m/z* (ES^-) 443.1 (100%, $[M - H]^-$).

Characterisation of products from incubation of FPP analogues with aristolochene synthase

Purified AS (50 μM) was incubated with each FPP analogue (200 μM) in 10 mM Tris, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol and 15% glycerol (pH 7.5) in a final volume of 500 mm^3 overlayed with pentane (200 mm^3) at 30 °C for 1-7 days. Reactions were terminated by addition of EDTA (100 mM, 100 mm^3) and the products were extracted by vortexing against pentane (3 x 3 cm^3). The pooled extracts were vortexed with 1.5 g of silica then decanted and concentrated under reduced pressure on ice.

The hexane extractable products were analysed by GCMS. This was performed on a system comprising of a Hewlett Packard 6890 GC fitted with a J&W scientific DB-5MS column (30 m x 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 unless otherwise stated and used helium as the carrier gas. 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).

None of the compounds made in this study produced any terpenoid products as determined by this method.

Kinetic characterisation of FPP analogues as inhibitors of AS

Assays (250 mm^3 final volume) were initiated by addition of purified AS solution (1 μM, 25 mm^3, final concentration 100 nM). Assays contained 0.1-5 μM [1-^{3}H]-farnesyl diphosphate (240000 dpm/nmol), 0-3 μM inhibitor, 10 mM Tris, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol and 15% glycerol and were prewarmed to 30 °C prior to addition of enzyme solution. After incubation for 4 min. each assay was stopped by addition of 100 mM EDTA 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 x 500 mm^3). The pooled hexane extracts were vortexed with silica (50 mg) the sample was centrifuged at 13000 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 $K_{M(app)}$ 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.‡ Mode of action of the inhibitors was determined by examination of double reciprocal plots of 1/v versus 1/[S]. K_i values were determined using plots of [I] versus $K_{M(app)}$ once each inhibitor was observed to be competitive.

Notes and references

‡ Data were fitted using Systat Sigmaplot 10.0, 2007. Sigmaplot for Windows Version 10.0,, Build 10.0.0.54, 2006, Systat Software Inc. 1735, Technology Drive, Ste 430, San Jose, CA 95110, USA.

1. M. J. Calvert, P. R. Ashton, R. K. Allemann, *J. Am. Chem. Soc.*, 2002, **124**, 11636.
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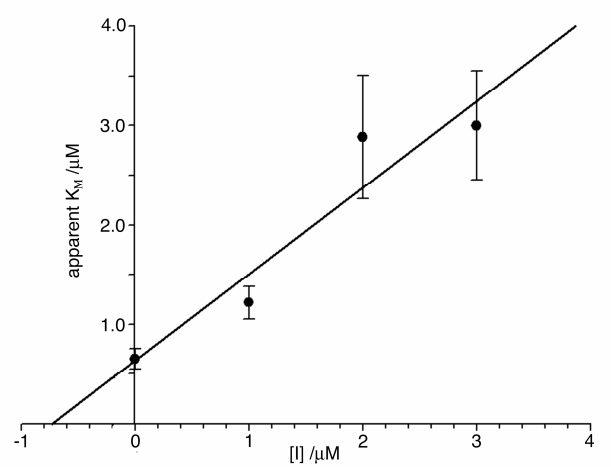

Figure S1 Graph of [I] *versus* apparent K_M for FPP turnover by AS in the presence of *E*-11-phenyl FPP **19**. The x-axis intersection indicates a K_I of 0.8 μM.

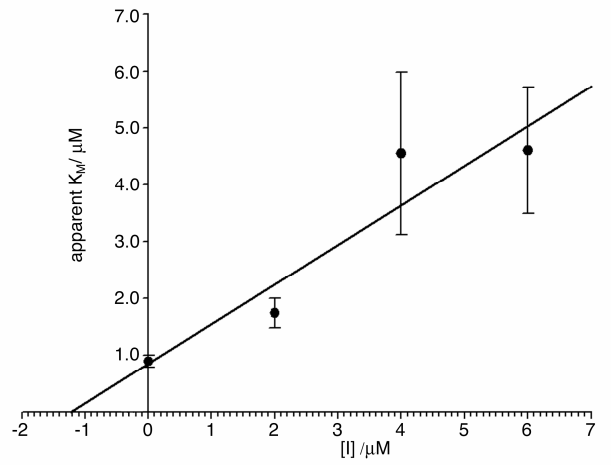

Figure S2 Graph of [I] *versus* apparent K_M for FPP turnover by AS in the presence of *Z*-11-phenyl FPP **29**. The x-axis intersection indicates a K_I of 1.2 μM.

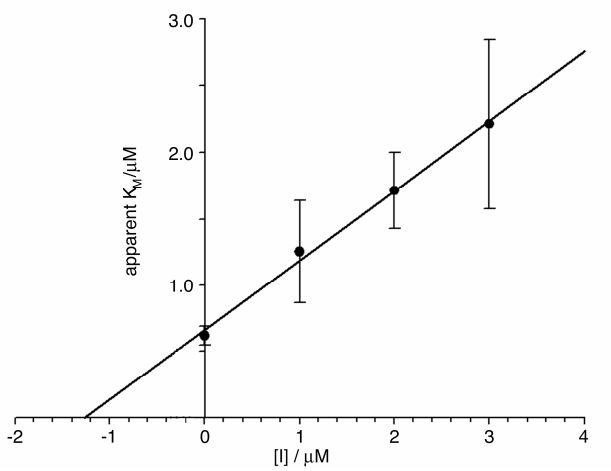

Figure S3 Graph of [I] *versus* apparent K_M for FPP turnover by AS in the presence of 3-phenyl FPP **36** The x-axis intersection indicates a K_I of 1.2 μM.