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Synthesis and Evaluation of Chlorinated Substrate Analogues for Farnesyl Diphosphate Synthase

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Abstract

Substrate analogues for isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), where the C3 methyl groups were replaced by chlorine, were synthesized and evaluated as substrates for avian farnesyl diphosphate synthase (FPPase). The IPP analogue (**3-CIIPP**) was a co-substrate when incubated with dimethylallyl diphosphate (DMAPP) or geranyl diphosphate (GPP) to give the corresponding chlorinated analogues of geranyl diphosphate (**3-CIGPP**) and farnesyl diphosphate (**3-CIFPP**), respectively. No products were detected in incubations of **3-CIIPP** with **3-CIDMAPP**. Incubation of IPP with **3-CIDMAPP** gave **11-CIFPP** as the sole product. Values of $K_M^{3-CIIPP}$ (with DMAPP) and $K_M^{3-CIDMAPP}$ (with IPP) were similar to those for IPP and DMAPP, however values of k_{cat} for both analogues were substantially lower. These results are consistent with a dissociative electrophilic alkylation mechanism where the rate-limiting step changes from heterolytic cleavage of the carbon-oxygen bond in the allylic substrate to alkylation of the double bond of the homoallylic substrate.

Introduction

Farnesyl diphosphate (FPP) is a key precursor for synthesis of isoprenoid metabolites containing three or more isoprene units. (1) FPP is synthesized in two steps by addition of dimethylallyl diphosphate (DMAPP) to isopentenyl diphosphate (IPP) to give geranyl diphosphate (GPP), followed by addition of GPP to a second molecule of IPP to provide FPP. (2) The reactions are catalyzed by farnesyl diphosphate synthase (FPPase), a short chain elongation enzyme that contains a distinctive α -helical fold characteristic of members of the isoprenoid synthase superfamily (1,3–4). This group includes *E*-selective short and long chain elongation enzymes; the cyclopropanation enzymes squalene synthase, phytoene synthase, and chrysanthemyl diphosphate synthase; and mono-, sesqui-, and diterpene cyclases. In FPPase the hydrocarbon tail of the allylic substrate is located in a hydrophobic pocket that dictates the ultimate chain length of the product. (1) The diphosphate moiety is bound to active-site arginine and lysine residues and to three Mg^{2+} ions stabilized by two aspartate-rich DDXX(XX)D motifs. (5) The hydrocarbon moiety in IPP lies above the allylic substrate and its diphosphate moiety is stabilized by a combination of arginine and lysine residues and tightly bound waters. (5)

Mechanistic studies with fluorinated (2,6) and bisubstrate (7–8) analogues indicate that the chain elongation reaction is a dissociative electrophilic alkylation that involves cleavage of

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Supporting Information Available: General methods; ¹H, ¹³C, ³¹P, ¹H NOE-difference, COSY NMR spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

the carbon-oxygen bond in the allylic substrate to form a resonance stabilized carbocation, alkylation of the double bond in IPP to generate a tertiary carbocation, and elimination of the *pro-R* proton at C2 to generate an *E* double bond between C2 and C3 in the product. (9–10) FPPase is tolerant to modifications of the C3 methyl groups of IPP, DMAPP, and GPP. (11–15) These analogues have been used for *in vitro* enzymatic synthesis of a variety of biological molecules. (15–17) We now report the synthesis and evaluation of analogues of IPP and DMAPP where the C3 methyl groups in both are replaced by chlorine.

Results

Synthesis

The synthesis of the C3 chloro analogue of IPP (**3-CIIPP**) is shown in Scheme 1. 3-Buten-1-ol (**1-OH**) was converted to the corresponding tosylate (**1-OTs**) followed by treatment with sulfuryl chloride and phenyl selenyl chloride. (18) The resulting selenoxide was heated with aqueous sodium bicarbonate and treated with hydrogen peroxide to give (**2-OTs**). (18) The tosylate was treated with tris-tetrabutylammonium hydrogen pyrophosphate in acetonitrile (19) to give **3-CIIPP** in an overall yield of 45%.

Chlorinated DMAPP (**3-CIDMAPP**) was synthesized in 3 steps starting from but-2-yn-1-ol (**3-OH**) as shown in Scheme 2. Alcohol **3-OH** was converted to **4-OH** by treatment with Red-Al and NCS (20) followed by NCS and DMS to give dichloride **4-Cl**. (19) The allylic chloride was then converted to **3-CIDMAPP** using tris-tetrabutylammonium hydrogen pyrophosphate (19) in an overall yield of 9 % from **3-OH**.

Product Studies

In preliminary experiments, **3-CIIPP** and DMAPP, GPP, or **3-CIDMAPP** and **3-CIDMAPP** and IPP were incubated with FPPase in 35 mM HEPES buffer, pH 7.4, followed by incubation with alkaline phosphatase to hydrolyze the diphosphate esters to the corresponding alcohols. The mixtures were then analyzed by GCMS to determine if the chlorinated substrates were substrates for chain elongation. As seen in Table 1, peaks with characteristic masses for chain elongation products were seen for incubations of **3-CIIPP** with DMAPP and GPP and of **3-CIDMAPP** with IPP. In each case mass spectra of the putative products had an M+2 peak whose intensity was 30% of M, as expected for the isotopic pattern of chlorine. Thus, incubation of **3-CIIPP** and GPP with FPPase gave a mono-chlorinated derivative of FPP and incubation with DMAPP gave a mono-chlorinated derivative of GPP. A di-chlorinated FPP derivative was not detected. Incubation of **3-CIDMAPP** and IPP with FPPase resulted in the exclusive production of a mono-chlorinated FPP derivative.

Large scale incubations were then conducted with avian FPPase in 35 mM sodium phosphate buffer pH 7.4, to facilitate purification of the diphosphate products for analysis by NMR. Following the incubations, FPPase was removed by ultrafiltration. The diphosphate products were purified by chromatography on cellulose and analyzed by ¹H, ¹³C, and ³¹P NMR spectroscopy. In addition ¹H-¹H COSY and 1D NOE spectra were used to determine the stereochemistry of the double bonds in the hydrocarbon chain. High resolution mass spectra showed an M+2 isotope peak due to the presence of chlorine.

Incubations with **3-CIIPP** and GPP gave a chlorinated analogue of FPP (**3-CIFPP**) where the methyl at C3 was replaced by chlorine (see Scheme 3). Incubations with **3-CIIPP** and DMAPP gave a chlorinated analogue of GPP (**3-CIGPP**) with a similar replacement. In this case chain elongation terminated with the formation of **3-CIGPP**, and longer incubation times and increased substrate and enzyme concentrations did not give detectable amounts of a doubly chlorinated FPP derivative. Incubation of **3-CIDMAPP** and IPP gave a chlorinated

FPP derivative (**11-CIFPP**) where the methyl group at C11 was replaced by chlorine as the sole product.

The stereochemistry of the double bonds in the chlorine-containing isoprene units of **3-CIGPP**, **3-CIFPP**, and **11-CIFPP** was established from ^1H NOE difference spectra, where NOEs were recorded as positive peaks and the irradiation frequency appeared as a negative peak. The difference spectrum for **3-CIGPP** had a strong NOE between the resonances from 2.41 to 2.44 ppm for the methylene protons at C4 and the resonance at 5.84 ppm for the vinyl proton at C2, indicating a *Z* C2-C3 double bond. A similar NOE was seen between the resonances from 2.40 to 2.44 ppm and the resonance at 5.86 ppm in the spectrum for **3-CIFPP**. The stereochemistry of the C10-C11 double bond of **11-CIFPP** was also determined to be *Z* due to the presence of a strong NOE between the methyl group at 2.1 ppm and the vinyl proton at 5.6 ppm.

Kinetic Studie

Steady-state kinetic constants were measured for chain elongation with $[1-^{14}\text{C}]\text{IPP}$ and **3-CIDMAPP** and with **3-CIDMAPP** and $[1-^{14}\text{C}]\text{DMAPP}$ (see Table 2). The standard acid lability assay was used to determine rates for chain elongation with $[1-^{14}\text{C}]\text{IPP}$. (21) Rates for chain elongation with $[1-^{14}\text{C}]\text{DMAPP}$ were determined by analyzing assay mixtures by TLC using autoradiography to quantify radioactivity in substrates and products. $K_{\text{M}}^{\text{3-CIDMAPP}} = 2.1 \mu\text{M}$ is similar to the value reported for addition of DMAPP to IPP, while $k_{\text{cat}} = 0.005 \text{ s}^{-1}$ is a 100-fold lower (22). The $K_{\text{M}}^{\text{3-CIIPP}}$ of $6.1 \mu\text{M}$ is only slightly higher than the value reported for IPP, (23) while $k_{\text{cat}} = 0.006 \text{ s}^{-1}$ is substantially lower.

Discussion

FPPase can accept a rather wide variety of IPP and DMAPP analogues as substrates. Numerous studies have shown that the enzyme is more tolerant of modifications in the allylic substrate than the homoallylic substrate. The enzyme can utilize DMAPP analogues with the *E* methyl group replaced by unbranched alkyl chains of up to C9. (12,24) In contrast, it is incapable of utilizing IPP analogues where the C3 methyl group has been replaced by a carbon chain longer than *n*-propyl. (14) The ability of FPPase to accept analogues of both DMAPP and IPP has been utilized for the stereospecific synthesis of naturally occurring isoprenoid compounds. (16–17)

3-CIIPP and **3-CIDMAPP** are alternate substrates for chain elongation with their respective natural co-substrates. Both of the analogues are ~200-fold less active (k_{cat}) than their natural counterparts and have somewhat elevated K_{Ms} – 10-fold for **3-CIIPP** and 1.5-fold for **3-CIDMAPP**. Incubation of **3-CIIPP** and DMAPP or GPP with FPPase gave the corresponding 3-chloro derivatives of GPP and FPP, while incubation of **3-CIDMAPP** with IPP gave **11-CIFPP**. In each case the stereochemistry of the newly formed double bonds was the same as seen for the natural substrates. Turnover was not detected for incubations with **3-CIIPP** and **3-CIDMAPP**. We estimate that chain elongation with both chlorinated analogues is at least 2000 times slower than for IPP and DMAPP. **3-CIIPP** and **3-CIDMAPP** can be used for the enzyme-catalyzed synthesis of **3-CIGPP**, **3-CIFPP**, **11-CIFPP**, and presumably 7-CIFPP by incubating **3-CIGPP** with IPP. A similar strategy with a combination of FPPase and GGPPase would permit synthesis of 3-chloro, 7-chloro, 11-chloro, and 15-chloro analogues of GGPP. These molecules should be valuable tools for probing the mechanisms of sesquiterpene and diterpene cyclases and for structural studies of enzymes in the isoprenoid biosynthetic pathway by X-ray crystallography.

Our kinetic results are consistent with a dissociative electrophilic alkylation mechanism for chain elongation (see Scheme 4) (25) where heterolytic cleavage of the C-O bond in

DMAPP gives an intimate ion pair with the allylic dimethylallyl cation sandwiched between IPP and PP_i. In this scenario irreversible alkylation to form the tertiary cation would compete with internal return to DMAPP. $K_M^{3\text{-CIDMAPP}}$ is only slight larger than K_M^{DMAPP} . The van der Waals radii of chlorine and methyl groups are similar (26) and apparently the larger bond dipole for the C-Cl bond (27) does not substantially alter binding. However, $k_{\text{cat}}^{3\text{-CIDMAPP}}$ is ~200-fold smaller than $k_{\text{cat}}^{\text{DMAPP}}$, presumably because of a reduction in k_1 , the step for carbon-oxygen heterolysis in **3-CIDMAPP**. If one assumes that k_1 is rate-limiting for **3-CIDMAPP**, and at least partially rate-limiting for DMAPP, the changes in k_{cat} are consistent with an electronic effect when the methyl group ($\sigma_p^+ = -0.311$) at C3 in DMAPP is replaced with chlorine ($\sigma_p^+ = 0.114$) in **3-CIDMAPP**, (28) and related decreases k_{cat} seen for H, CH₂F, CHF₂, and CF₃ substituents at C3 of DMAPP, (29) Interestingly, **3-CIIPP** shows similar behavior in both K_M and k_{cat} . It is likely that the substantially lower value of k_{cat} results from an electronic effect for the homoallylic substrate as well. Although FPPase tolerates a rather broad range of homoallylic substrate analogues consistent with the similar values for K_M^{IPP} and $K_M^{3\text{-CIIPP}}$, one cannot exclude the possibility that the carbon-chlorine bond dipole causes a change in alignment of **3-CIIPP** and DMAPP that places the C3-C4 double bond in **3-CIIPP** in an unfavorable orientation for alkylation and thus lowers the rate of the reaction. We favor a change in the rate-limiting step from k_1 to k_2 , alkylation of the double bond in **3-CIIPP**, caused by the chlorine substituent. Attempts to directly detect internal return (k_{-1}) in FPPase by positional isotope exchange or internal trapping experiments have not been successful, (30–31) presumably because the conformation of inorganic pyrophosphate in the ion pair is locked in the active site through coordination with magnesium ions. (5) Related experiments were successful in detecting internal return for dimethylallyltryptophan synthase, a prenyl transfer enzyme that does not bind the magnesium form of DMAPP. (32)

In conclusion, we describe the synthesis of analogues of IPP and DMAPP where the methyl at C3 is replaced by chlorine. These molecules are alternative substrates for FPPase, giving novel linear chlorinated geranyl and farnesyl analogues. The kinetic parameters for the analogues are consistent with a dissociative electrophilic alkylation mechanism for the chain elongation reaction where the rate-limiting step changes from carbon-oxygen heterolysis in the allylic substrate for **3-CIDMAPP** to addition of the allylic cationic intermediate to the double bond in **3-CIIPP**.

Experimental Section

3-Buten-1-yl tosylate (1-OTs)

To a solution containing 5.3 g (27.7 mmol) of p-toluenesulfonyl chloride and 6.8 g (55.5 mmol) of N,N-dimethylaminopyridine in 25 mL of CH₂Cl₂ was added 2.0 g (27.7 mmol) of 3-buten-1-ol. The reaction mixture was allowed to stir at room temperature under N₂ for 2 h. Solvent was removed by rotary evaporation and the resulting white solid was chromatographed on silica using 4:1 (v:v) hexane:ethyl acetate to give 5.8 g (89%) of a colorless oil; ¹H NMR (CDCl₃) 2.37–2.44 (m, 2H), 2.46 (s, 3H), 4.07 (t, 2H, $J = 6.6$ Hz), 5.05–5.06 (m, 1H), 5.08–5.12 (m, 1H), 5.61–5.75 (m, 1H), 7.33–7.38 (m, 2H), 7.78–7.82 (m, 2H) ppm; ¹³C NMR (CDCl₃) 21.9, 33.3, 69.6, 118.4, 128.1, 130.0, 132.6, 133.3, 145.0 ppm; HRMS (EI) calcd for C₁₁H₁₅O₃S (M+H) 227.0736, found 227.0735.

3-Chloro-3-butenyl tosylate (2-OTs)

A solution containing 0.5 g (2.2 mmol) of **1-OTs** and 0.42 g (2.2 mmol) of phenylselenenyl chloride in 4 mL of dry CHCl₃ was allowed to stir under N₂ at rt overnight. Sulfuryl chloride (202 μL, 2.5 mmol) was added dropwise via syringe and the reaction mixture was allowed to stir for h at rt. The mixture was concentrated by rotary evaporation to give a

yellow oil. Benzene and aqueous NaHCO₃ were added and the mixture was heated at reflux for 4 h. The mixture was allowed to cool and the organic layer was shaken with 30% H₂O₂ until the yellow color disappeared (40 mL for 30 min). The organic layer was dried over MgSO₄, filtered, and concentrated by rotary evaporation. The residue was purified by chromatography on silica using 10:1 (v:v) hexane:ethyl acetate to give 625 mg (54%) of a light yellow oil; ¹H NMR (CDCl₃) 2.47 (s, 3H), 2.68 (t, 2H, *J* = 5.8 Hz), 4.21 (t, 2H, *J* = 6.0 Hz), 5.23 (s, 1H), 5.25 (s, 1H), 7.35–7.38 (m, 2H), 7.79–7.82 (m, 2H) ppm; ¹³C NMR (CDCl₃) 21.8, 38.8, 66.8, 115.8, 128.1, 130.0, 132.8, 136.8, 145.1 ppm; HRMS (FAB) calcd for C₁₁H₁₄SClO₃ (M+H) 261.0353, found 261.0353.

General procedure for preparation of diphosphates

To a stirred mixture of tris(tetra-*n*-butylammonium) hydrogen pyrophosphate (2 equiv) in dry acetonitrile was added via syringe either tosylate or chloride dissolved in a minimal amount of dry acetonitrile. The reaction mixture was allowed to stir under N₂ for 2 h and then concentrated by rotary evaporation. The resulting oil was dissolved in a minimal amount of ion exchange buffer (1:49 (v:v) isopropanol:25 mM ammonium bicarbonate) and loaded on to the ammonium form of Dowex ion exchange resin. The column was eluted with 2 column volumes of exchange buffer. The elutant was flash frozen and dried by lyophilization to yield a white solid. The solid was dissolved in 0.1 M ammonium bicarbonate; 15 mL of 1:1 (v:v) isopropanol:acetonitrile was added; and the mixture was vortexed. The resulting white slurry was cleared by centrifugation; the supernatant was removed; and the process was repeated 3 times. The supernatants were pooled, concentrated and dried by lyophilization to yield a white solid. The solid was chromatographed on cellulose using 70:30 (v:v) isopropanol:0.1 M ammonium bicarbonate. Fractions containing the organic diphosphate were combined, flash frozen and dried by lyophilization to yield a fluffy white solid.

3-Chloro-3-butenyl diphosphate (3-ClIPP)

Following the general procedure, 1.66 g (1.84 mmol) of tris(tetra-*n*-butylammonium) hydrogen pyrophosphate and 0.240 g (0.92 mmol) of **2-OTs** gave 188 mg (78%) of a fluffy white solid; ¹H NMR (D₂O) 2.71 (t, 2H, *J* = 6.3 Hz), 4.11 (dd, 2H, *J* = 6.3, 6.1 Hz), 5.33 (s, 1H), 5.38 (s, 1H) ppm; ¹³C NMR (D₂O) 40.0 (d, *J* = 7.3 Hz), 63.4 (d, *J* = 5.3 Hz), 115.2, 139.2 ppm; ³¹P NMR (D₂O) –6.25 (d, *J* = 19.5 Hz), –10.24 (d, *J* = 20.0 Hz) ppm; HRMS (FAB) calcd for C₄H₈ClO₇P₂ (M-H) 264.9434, found 264.9430.

Z-3-Chloro-2-but-en-1-ol (4-OH)

To a solution of 0.162 g (2.3 mmol) of 2-butyne-1-ol in 10 mL of dry THF was added dropwise via syringe 1.17 mL (3.9 mmol) of Red-Al. The mixture was allowed to stir at rt for 18 h, cooled to –78 °C before 0.59 g (4.4 mmol) of NCS dissolved in 5 mL of THF was added dropwise, and allowed to stir at –78 °C for an additional 1 h. The mixture was stirred at 0 °C for 2 h and was quenched with saturated Rochelle's salt. The aqueous layer was extracted with ether. The organic layers were combined, washed with brine, dried over MgSO₄ and concentrated under vacuum. The residue was purified by chromatography on silica using 3:2 (v:v) hexane:ether to yield 96 mg (39%) of a pale yellow oil; ¹H NMR (CDCl₃) 2.08–2.09 (m, 3H), 3.12 (br s, 1H), 4.19–4.22 (m, 2H), 5.61–5.67 (m, 1H) ppm; ¹³C NMR (CDCl₃) 26.4, 59.9, 125.1, 133.0 ppm; HRMS (EI) calcd for C₄H₇OCl (M+) 106.0180, found 106.0174.

Z-1,3-Dichloro-2-butene (4-Cl)

To a solution containing 0.451 g (3.38 mmol) N-chlorosuccinimide in 20 mL of dry CH₂Cl₂ at –30 °C was added 2.1 mL (3.38 mmol) of DMS. The mixture was briefly allowed

to warm to 0 °C and cooled to −40 °C before a solution of 0.3 g (2.82 mmol) of **4-OH** in 2 mL of CH₂Cl₂ was added. The reaction mixture was allowed to warm to 0 °C over 1 h and was stirred at this temperature for an additional 1 h. The mixture was warmed to room temperature and stirred for 15 min. The mixture was added to 15 mL of brine and extracted with pentane. The combined organic layers were washed with brine, dried over MgSO₄, filtered and concentrated to give 185 mg (53%) of a yellow oil. The residue was used in the subsequent phosphorylation reaction without further purification.

Z-3-Chloro-2-butenyl diphosphate (3-CIDMAPP)

Following the general procedure, 2.67 g (2.96 mmol) of *tris*(tetra-*n*-butylammonium) hydrogen pyrophosphate and 0.185g (1.48 mmol) of **4-Cl** gave 174 mg (45%) of a white solid; ¹H NMR (D₂O) 2.14 (s, 3H), 4.56 (t, 2H, *J* = 6.9 Hz), 5.83 (t, 1H, *J* = 5.5 Hz) ppm; ¹³C NMR (D₂O) 26.1, 63.6(d, *J* = 5.1 Hz), 122.7(d, *J* = 7.6 Hz), 134.7 ppm; ³¹P NMR (D₂O) −6.75 (d, *J* = 21.7 Hz), −10.29 (d, *J* = 21. Hz) ppm; HRMS (FAB) calcd for C₄H₈ClO₇P₂ (M-H), 264.9434 found 264.9434.

Protein expression and purification

The plasmid for avian FPPase was transformed into *E. coli* XA90 cells and plated onto LB ampicillin plates. Overnight liquid cultures were grown and 15 mL was used to inoculate 1.5 L cultures. Cultures were grown @ 37 °C to an OD₆₀₀ of 0.5 and were induced by adding IPTG (1 mM). Cultures were allowed to grow an additional 4–5 h at 37 °C and cells were collected by centrifugation at 6000 rpm for 10 min. Cell paste was kept at −80 °C until further use. Frozen cell paste was thawed on ice and suspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole). Cells were lysed by sonication and cellular debris was cleared by centrifugation at 6000 rpm for 15 min. The supernatant was added to 10 mL of Ni-NTA resin and shaken for 2 h at 4 °C. The resulting slurry was poured into a fritted glass column and allowed to settle. The column was washed with 2 column volumes of lysis buffer, followed by 50 mL of wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0). Protein was eluted with 100 mL of elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0), and precipitated with 33% (w/v) ammonium sulfate. This solution was centrifuged at 7000 rpm for 20 min, and the resulting pellet was dissolved in 5–10 mL of dialysis buffer (20 mM Tris-HCl, 4 mM DTT @ pH 8.0). The solution was transferred to a dialysis cassette (10,000 MWCO) and dialyzed for 3 h in dialysis buffer. The protein was then dialyzed overnight against this same dialysis buffer, and then dialyzed an additional 4 h in storage buffer (20 mM Tris-HCl pH 8.0, 4 mM DTT, 20% glycerol). The protein was stored at −80 °C in 100 µL aliquots until use.

Product studies

GC and GC/MS analysis—Incubations were in 35 mM HEPES buffer, pH 7.4, containing 10 mM MgCl₂, 5 mM β-ME, and 200 µM of each substrate in a total volume of 400 µL at 30 °C. After 2 h, 36 µL of 0.5 M glycine buffer, pH 10.6, containing 5 mM ZnCl₂ followed by 40 U of alkaline phosphatase was added and incubation was continued for 1 h at 37 °C. Solid NaCl was added and the mixture was extracted with tert-butyl methyl ether. Samples were concentrated to 5–10 µL with a gentle stream of N₂ and analyzed by GC and GC-MS on a DB5 capillary column using the following program: 55 °C for 3 min, followed by a linear gradient (1 °C/min) to 65 °C, then (2 °C/min) to 120 °C, followed by (10 °C/min) to 230 °C.

Kinetic studies

Initial velocities were measured using the acid lability assay for reactions with [1-¹⁴C]IPP and Cl-DMAPP. Incubations were in 35 mM HEPES buffer, pH 7.4, containing 10 µM

[¹⁴C]IPP (30 μCi/μmol), 0.5–50 μM **3-CIDMAPP**, 10 mM MgCl₂, 10 mM β-ME, and 1 mg/mL BSA in a total volume of 100 μL. Reactions were initiated by the addition of enzyme (10 μL) and were incubated at 37 °C for 10 min. The reaction was quenched by the addition of 200 μL of 4:1 MeOH:HCl, and incubated for an additional 10 min at 37 °C. One mL of ligroine (b.p. 95–110 °C) was added, the mixture was vortexed, and a 0.5 mL sample of the ligroine layer was counted by liquid scintillation spectrometry. For incubations with [¹⁴C]DMAPP and **3-CIIPP**, reactions were run in 35 mM HEPES buffer, pH 7.4, containing 50 μM [¹⁴C]DMAPP (20 μCi/μmol), 0.5 μM–50 μM **3-CIIPP**, 10 mM MgCl₂, 10 mM β-ME, and 1 mg/mL BSA in a total volume of 50 μL. Reactions were initiated by the addition of enzyme (10 μL) and incubated at 37 °C for 10 min. The assay mixtures were quenched by the addition of 50 μL of MeOH. The resulting mixture was spotted onto a silica gel TLC plate and developed with chloroform:pyridine:formic acid:water (30:70:16:10). The plate was allowed to dry overnight and was visualized by phosphorimaging. Radioactivity was quantified using Imagequant. Kinetic constants were determined from plots of initial velocity versus substrate concentration using Grafit 5.

NMR Analysis—Avian FPPase was dialyzed against 35 mM Na₂HPO₄ buffer, pH 7.4, for 24 h to remove HEPES, glycerol, and DTT from the storage buffer. The resulting enzyme was concentrated by centrifugation (Centricon, 10,000 MWCO). Incubations were in 35 mM Na₂HPO₄ buffer, pH 7.4, containing 10 mM MgCl₂, 10 mg of avian FPPase, and the indicated concentrations of substrates at 30 °C. The mixture was incubated for 4 h, after which an additional 2.5 mg of enzyme was added, followed by another 4 h of incubation. A final 2.5 mg portion of enzyme was added and the incubation was continued overnight. The mixture was filtered by centrifugation (Centricon, 10,000 MWCO) to remove enzyme and precipitated salts. The precipitate was washed several times with 0.1 M NH₄HCO₃ and filtered as before. The filtrates were combined, flash frozen and dried by lyophilization to give a white powder. The solid precipitate stirred overnight with 400 mg of Chelex in 10 mL of 0.1 M NH₄HCO₃. The remaining precipitate was removed by filtration as described above. The filtrates were flash frozen and dried by lyophilization to give a white powder. The lyophilized solid was and purified by chromatography on cellulose. The column was eluted with 3 column volumes of 100 % isopropanol, followed by 4:1 (v/v) isopropanol:0.1 M NH₄HCO₃. Fractions were analyzed by silica TLC using p-anisaldehyde. Those containing diphosphate products were pooled, concentrated at reduced pressure to remove isopropanol, flash frozen and dried by lyophilization to give a white powder. Purified products were characterized by NMR and mass spectrometry.

***E,E,Z*-11-Chloro-3,7-dimethyldodeca-2,6,10-triene (11-CIFPP)**

Following the general procedure for synthesis of chlorinated metabolites, 11 mM **3-CIIPP** and 12.1 mM GPP were incubated with FPPase to give a white solid; ¹H NMR (D₂O) 1.63 (s, 3H), 1.72 (s, 3H), 2.10–2.17 (m, 9H), 2.23–2.30 (m, 2H), 4.47 (t, 2H, *J* = 6.1 Hz), 5.23 (t, 1H, *J* = 6.4 Hz), 5.47 (t, 1H, *J* = 6.6 Hz), 5.53 (t, 1H, *J* = 6.9 Hz) ppm; ¹³C NMR (D₂O) 15.7, 16.2, 25.8, 26.2, 27.1, 38.1, 39.4, 63.0 (d, *J* = 4.7 Hz), 120.5 (d, *J* = 7.3 Hz), 125.2, 126.3, 130.6, 136.6, 143.2 ppm; ³¹P NMR (D₂O) –4.92 and –9.05 ppm; HRMS (FTMS) calcd for C₁₄H₂₄ClO₇P₂ (M-H) 401.0691, found 401.0690.

***Z,E*-3-Chloro-7,11-dimethyldodeca-2,6,10-triene (3-CIFPP)**

Following the general procedure for synthesis of chlorinated metabolites, 22 mM IPP and 12 mM **3-CIDMAPP** were incubated with FPPase to give a white solid; ¹H NMR (D₂O) 1.62 (s, 3H), 1.63 (s, 3H), 1.69 (s, 3H), 2.02–2.04 (m, 2H), 2.09–2.14 (m, 2H), 2.27–2.31 (m, 2H), 2.40–2.44 (m, 2H), 4.59 (t, 2H, *J* = 6.5 Hz), 5.17–5.22 (m, 2H), 5.86 (t, 1H, *J* = 6.4 Hz) ppm; ¹³C NMR (D₂O) 16.0, 17.6, 25.5, 26.0, 26.5, 39.3, 39.4, 63.4 (d, *J* = 4.5 Hz), 123.0 (d,

$J = 7.7$ Hz), 123.5, 125.1, 134.2, 138.2, 138.3 ppm; ^{31}P NMR (D_2O) -5.91 and -10.04 ppm; HRMS (FTMS) calcd for $\text{C}_{14}\text{H}_{24}\text{ClO}_7\text{P}_2$ (M-H) 401.0691, found 401.0691.

Z-3-Chloro-7-methylocta-2,6-diene (3-CIGPP)

Following the general procedure for synthesis of chlorinated metabolites, 16.6 mM DMAPP and 15 mM **3-CIIPP**; were incubated with FPPase to give a white solid; ^1H NMR (D_2O) 1.63 (s, 3H), 1.69 (s, 3H), 2.26–2.30 (m, 2H), 2.41–2.44 (m, 2H), 4.60 (t, 2H, $J = 6.4$ Hz), 5.17–5.20 (m, 1H), 5.84 (t, 1H, $J = 5.8$ Hz) ppm; ^{13}C NMR (D_2O) 17.7, 25.5, 25.9, 39.3, 63.8 (d, $J = 3.1$ Hz), 122.7 (d, $J = 7.3$ Hz), 123.2, 135.3, 138.4 ppm; ^{31}P NMR (D_2O) -8.43 and -10.51 ; HRMS (FTMS) calcd for $\text{C}_9\text{H}_{16}\text{ClO}_7\text{P}_2$ (M-H) 333.0065, found 333.0065.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Tarshis LC, Yan M, Poulter CD, Sacchettini JC. Crystal structure of recombinant farnesyl diphosphate synthase at 2.6-Å resolution. *Biochemistry* 1994;33:10871–10877. [PubMed: 8086404]
2. Poulter CD, Rilling HC. Prenyltransferase: the mechanism of the reaction. *Biochemistry* 1976;15:1079–1083. [PubMed: 175829]
3. Thulasiram HV, Erickson HK, Poulter CD. Chimeras of Two Isoprenoid Synthases Catalyze All Four Coupling Reactions in Isoprenoid Biosynthesis. *Science* 2007;316:73–76. [PubMed: 17412950]
4. Christianson DW. Structural Biology and Chemistry of the Terpenoid Cyclases. *Chem Rev* 2006;106:3412–3442. [PubMed: 16895335]
5. Hosfield DJ, Zhang Y, Dougan DR, Broun A, Tari LW, Swanson RV, JF. Structural basis for bisphosphonate-mediated inhibition of isoprenoid biosynthesis. *J Biol Chem* 2004;279:8526–8529. [PubMed: 14672944]
6. Poulter CD, Argyle JC, Mash EA. Farnesyl pyrophosphate synthetase. *J Biol Chem* 1978;253:7227–7233. [PubMed: 701246]
7. Davisson VJN, TR, Poulter CD. Farnesyl-diphosphate synthase, catalysis of an intramolecular prenyl transfer with bisubstrate analogs. *J Am Chem Soc* 1993;115:1235–1245.
8. Davisson VJP, CD. Farnesyl-diphosphate synthase, interplay between substrate topology, stereochemistry, and regiochemistry in electrophilic alkylations. *J Am Chem Soc* 1993;115:1245–1260.
9. Ogura K, Koyama T. Enzymatic aspects of isoprenoid chain elongation. *Chem Rev* 1998;98:1263–1276. [PubMed: 11848932]
10. Popjak G, Cornforth JW. *Biochem J* 1966;101:553–568. [PubMed: 16742426]
11. Popjak G, Rabinowitz JL, Baron JM. Artificial Substrates for Prenyltransferases. *Biochem J* 1969;113:861–868. [PubMed: 4309597]
12. Ogura K, Nishino T, Koyama T, Seto S. Enzymatic Condensation of 3-Methyl-2-Alkenyl Pyrophosphates with Isopentenyl Pyrophosphate. *J Am Chem Soc* 1970;92:6036–6041. [PubMed: 4318869]
13. Nagaki M, Kannari H, Ishibashi J, Maki Y, Nishino T, Ogura K, Koyama T. Substrate specificity of thermostable farnesyl diphosphate synthase with alkyl group homologs of isopentenyl diphosphate. *Bioorg Med Chem Lett* 1998;8:2549–2554. [PubMed: 9873578]

14. Nagaki M, Yamamoto H, Takahashi A, Maki Y, Ishibashi J, Nishino T, Koyama T. Substrate Specificity of Thermostable Farnesyl Diphosphate Synthase with Respect to 4-Alkyl Group Homologs of Isopentenyl Diphosphate. *J Mol Cat* 2002;17:81–89.
15. Koyama T, Saito A, Ogura K, Seto S. Substrate Specificity of Farnesylpyrophosphate Synthase. Application to Asymmetric Synthesis. *J Am Chem Soc* 1980;102:3614–3618.
16. Koyama T, Ogura K. Synthesis and Absolute Configuration of 4-Methyl Juvenile Hormone I (4-MeJH) by a biogenetic Approach: A Combination of Enzymatic Synthesis and Biotransformation. *J Am Chem Soc* 1987;109:2853–2854.
17. Kobayashi M, Koyama T, Ogura K, Seto S. Bioorganic Synthesis and Absolute Configuration of Faranal. *J Am Chem Soc* 1980;102:6602–6604.
18. Engman L. Hydrolytic selenoxide elimination reaction for the preparation of 2-chloro-1-olefins. *Tet Lett* 1987;28:1463–1466.
19. Davisson VJ, Woodside AB, Neal TR, Stremmer KE, Muehlbacher M, Poulter CD. Phosphorylation of isoprenoid alcohols. *J Org Chem* 1986;51:4768–4779.
20. Marshall JA, DeHoff BS. The Synthesis of Cembranolide Precursors via Addition of Allylstannanes to Conjugated Aldehydes. *J Org Chem* 1986;51:863–872.
21. Satterwhite DM. Isopentenyl diphosphate Isomerase. *Methods Enzymol* 1985;110:92–99. [PubMed: 4021823]
22. Stanley Fernandez SMK, BA, Poulter CD. Farnesyl diphosphate synthase. Altering the catalytic site to select for geranyl diphosphate activity. *Biochemistry* 2000;39:15316–15321. [PubMed: 11112517]
23. Tarshis LC, Proteau PJ, Kellog BA, Sacchettini JC, Poulter CD. Regulation of product chain length by isoprenyl diphosphate synthases. *Proc Nat Acad Sci USA* 1996;93:15018–15023. [PubMed: 8986756]
24. Nishino T, Kyoze O, Seto S. Substrate Specificity of Farnesyl Pyrophosphate Synthetase. *J Am Chem Soc* 1972;94:6849–6853. [PubMed: 4340921]
25. Poulter CD. Bioorganic Chemistry. A Natural Reunion of the Physical and Life Sciences. *J Org Chem* 2009;74:2631–2645. [PubMed: 19323569]
26. Carey, FA.; Sundberg, RJ. *Advanced Organic Chemistry Part A: Structure and Mechanisms*. Vol. A. Plenum; New York: 2000.
27. McClellan, AL. *Tables of Experimental Dipole Moments*. Vol. I. W.H. Freeman and Company; San Francisco: 1963.
28. Brown HC, Okamoto Y. Electrophilic Substituent Constants. *J Am Chem Soc* 1958;80:4979–4987.
29. Poulter CD, Wiggins PL, Le AT. Farnesylpyrophosphate Synthetase. A Stepwise Mechanism for the 1'-4 Condensation Reaction. *J Am Chem Soc* 1981;103:3926–3927.
30. Poulter CD, Mautz DS. Solvolysis of Allylic Isoprene Phosphorothioate esters. A Mechanistic Study of the Thiono to Thiolo Rearrangement. *J Am Chem Soc* 1991;113:4895–4903.
31. Poulter CD, Mash EA, Argyle JC, Muscio OJ, Rilling HC. Farnesyl Pyrophosphate Synthase. Mechanistic Studies of the 1'-4 Coupling Reaction in the Terpene Biosynthetic Pathway. *J Am Chem Soc* 1979;101:6761–6763.
32. Luk LYP, Tanner ME. Mechanism of Dimethylallyltryptophan Synthase: Evidence for a Dimethylallyl Cation Intermediate in an Aromatic Prenyltransferase Reaction. *J Am Chem Soc* 2009;131:13932–13933. [PubMed: 19743851]

Scheme 1.
Synthesis of **3-ClIPP**.

Scheme 2.
Synthesis of **3-CIDMAPP**.

Scheme 3.

Structures of chlorinated metabolites resulting from incubations of **3-ClIPP** and **3-CIDMAPP** with IPP, DMAPP, or GPP.^a

^aIncubations were run in 35 mM sodium phosphate buffer, pH 7.4, containing 10 mM MgCl₂, substrate, and 10 mg of avian FPPase in a total volume of 2 mL at 30 °C for 24 h.

Scheme 4.

Dissociative electrophilic alkylation mechanism for chain elongation.

Table 1GCMS analysis of alcohols obtained by hydrolysis of diphosphates produced by FPPase.^a

Homoallylic Substrate	Allylic Substrate	Products	GC r.t. (min)	<i>m/z</i>
3-CIIPP	GPP	3-CIFOH	39.5	242
3-CIIPP	DMAPP	3-CIGOH	26.7	156 (M-H ₂ O)
IPP	3-CIDMAPP	11-CIFOH	39.8	224 (M-H ₂ O)
3-CIIPP	3-CIDMAPP	No Reaction	–	–

^aFPPase incubations: 35 mM HEPES buffer, pH 7.4, containing 10 mM MgCl₂, 5.0 mM β-mercaptoethanol, 200 μM allylic substrate and 200 μM homoallylic substrate, 200 μg of avian FPPase in a final volume of 400 μL at 30 °C for 2 h. Phosphatase incubations: 80 μL of 500 mM glycine buffer, pH 10.5, containing 5 mM ZnCl₂ and 40 units of calf mucosa alkaline phosphatase were added and the mixtures were incubated an additional 1 h at 37 °C.

Table 2Steady-State Kinetic Constants of Chlorinated Analogues with avian FPPase^a

Varied Substrate	Fixed Substrate	k_{cat} (s ⁻¹)	K_{M} (μM)	$k_{\text{cat}}/K_{\text{M}}$ M ⁻¹ s ⁻¹
IPP	DMAPP	1.1 ^b	0.6 ^b	1.8×10^6
DMAPP	IPP	1.1	1.6 ^c	6.0×10^5
3-ClIPP	DMAPP	0.006 ± 0.002	6.1 ± 0.1	1.0×10^3
3-CIDMAPP	IPP	0.005 ± 0.001	2.1 ± 0.2	2.4×10^3

^aMichaelis constants were determined by plotting initial velocity versus substrate concentration using GRAFIT (Sigma). K_{M} for **3-ClIPP** and **3-CIDMAPP** are apparent values determined with saturating concentrations of the fixed substrate.

^bRef. 18.

^cRef. 19