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Synthesis and Enzymatic Studies of Isoprenoid Thiolo Bisubstrate Analogues

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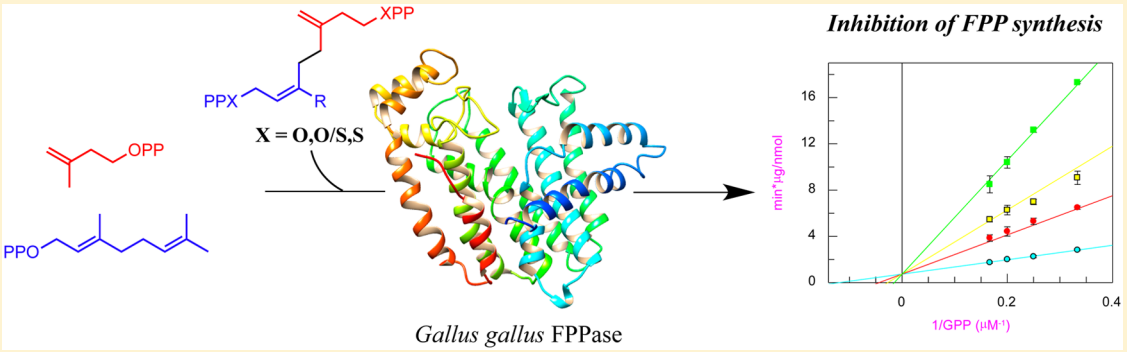
Supporting Information



ABSTRACT: Chain elongation prenyltransferases catalyze the addition of the hydrocarbon moiety of allylic isoprenoid diphosphates to the carbon−carbon double bond in isopentenyl diphosphate (IPP) in the primary building reactions in the isoprenoid biosynthetic pathway. Bis-*O*-diphosphate analogues **3-OPP/OPP**, **4-OPP/OPP**, and **5-OPP/OPP** and bis-thiolodiphosphate bisubstrate analogues **3-SPP/SPP**, **4-SPP/SPP**, and **5-SPP/SPP** were synthesized. The analogues **4-OPP/OPP**, **5-OPP/OPP**, **4-SPP/SPP**, and **5-SPP/SPP** were excellent competitive inhibitors of avian farnesyl diphosphate synthase with K_I = 1.0 ± 0.12 μM, K_I = 0.5 ± 0.2 μM, K_I = 0.7 ± 0.3 μM, and K_I = 2.9 ± 0.27 μM, respectively, whereas, analogues **3-OPP/OPP** and **3-SPP/SPP** displayed mixed type inhibition with K_I = 1.4 μM and K_I = 5.5 μM, respectively.

INTRODUCTION

Prenyltransferases catalyze electrophilic alkylation of electron-rich double bonds or electronegative atoms by isoprenoid allylic diphosphate esters to create new carbon−carbon or carbon−heteroatom bonds.[1] The enzymes alkylate a broad spectrum of nucleophilic substrates, including di- and trisubstituted double bonds (chain elongation and cyclopropanation prenyltransferases),[2] aromatic rings (aromatic prenyltransferases), and heteroatoms in amino acids (tyrosine, serine, etc.),[3] peptides (ComQ-related bacterial aggregation pheromones), proteins (protein prenyltransferases), and nucleic acids (nitrogen atoms of *t*RNA and AMP).[4,5] To date, over 67000 isoprenoid metabolites, originating from prenyltransfer reactions, have been identified according the dictionary of natural products,[6] which are important for a variety of cellular functions.

Chain-elongation polyprenyltransferases catalyze the stepwise electrophilic alkylation of the carbon−carbon double bond in isopentenyl diphosphate (IPP) by isoprenoid allylic diphosphates to give longer chain polyprenyl diphosphate compounds.[7] The most studied chain elongation prenyltransferase is farnesyl diphosphate synthase (FPPase), a critical enzyme found in all kingdoms of life. FPPase catalyzes the sequential addition of two molecules IPP, first to dimethylallyl diphosphate (DMAPP) to give geranyl diphosphate (GPP) and then to GPP to give farnesyl diphosphate (FPP).[8] These reactions are dissociative electrophilic alkylations with the stepwise addition of dimethylallyl and geranyl allylic cations to the IPP double bond.[9] FPPase synthesizes the substrates for branch point enzymes for the biosynthesis of sterols,[10] hopanoids,[11] sesquiterpenes,[12] heme A,[13] and longer chain polyisoprenoid diphosphates.[8] The enzyme has become a target for anti-infective, antitumor, malignant bone disease, antiparasitic, and cholesterol-lowering drug discovery.[14,15]

Several groups have reported novel inhibitors of FPPase, including fluoro- and alkyl-modified analogues of the allylic diphosphates,[16] ammonium analogues (carbocation mimics),[17] phosphonophosphonates,[18] phosphino-phosphinates,[19] bis-phosphonates, and nitrogen-containing bis-phosphonates.[20] While bis-phosphonate inhibitors are used to treat diseases related to FPPase, including different bone disorders such as metabolic bone disease, osteoporosis, hypercalcimia, Paget's disease, and tumor bone metastases, they often suffer from poor binding abilities and undesirable side effects.[21] Isoprenoid allylic thiolodiphosphate analogues of DMAPP and GPP, which are substantially less reactive than their diphosphate counterparts, are potent inhibitors of FPPase.[22] We found that bisubstrate analogues, where IPP was tethered to DMAPP or GPP, were alternative substrates for the enzyme with kinetic constants similar to those of the natural substrates.[23] These observations

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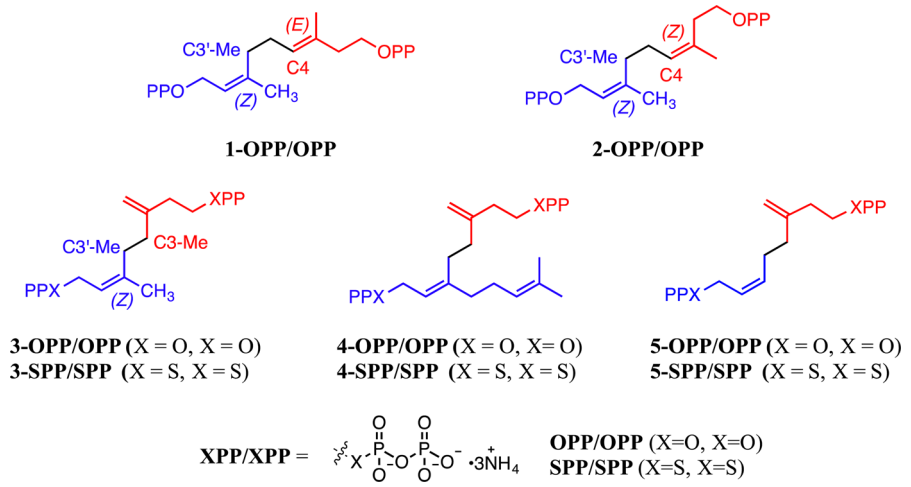


Figure 1. Structures of bis-diphosphate and bis-thiolodiphosphate analogues.

led us to design the new class of thiolo bisubstrate inhibitors of prenyltransfer reactions, which may also be useful for X-ray studies of the enzyme−analogue complexes. We now describe the synthesis of thiolo bisubstrate analogues of IPP linked to *E*-2-butenyl diphosphate, DMAPP, and GPP and inhibition studies with FPPase.

RESULTS AND DISCUSSION

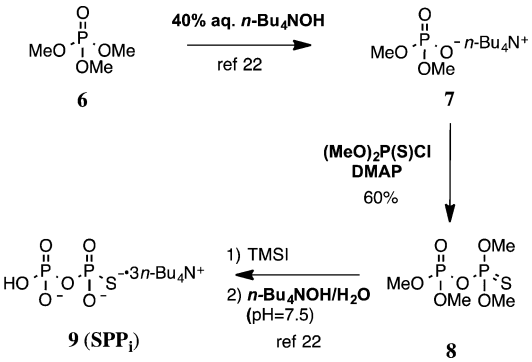
Our interest in the substrate selectivity of FPPase led us to synthesize two types of bisubstrate analogues constructed from IPP and allylic diphosphate fragments. Compounds **1-OPP/OPP** and **2-OPP/OPP** (Figure 1) are double bond isomers where C4 of IPP is linked to the *E*- or *Z*-methyl group in DMAPP by a one-carbon bridge.[24] These analogues are substantially less reactive than IPP and DMAPP and give a mixture of products that suggests more than one conformer of the substrates are bound in the enzyme−substrate complex. In contrast, another class of bisubstrate analogues represented by compounds **3-OPP/OPP** and **4-OPP/OPP** tether the C3-methyl group in IPP directly to the *Z*-methyl group at C3 in DMAPP and GPP, respectively. These bis-diphosphates are excellent substrates for FPPase that give novel seven-membered cyclic products with kinetic constants similar to those for IPP and DMAPP. The bisubstrate analogues are apparently bound in a single conformation in the FPPase·**3-OPP/OPP** and FPPase·**4-OPP/OPP** complexes.[23]

We used two approaches for designing inhibitors for FPPase based on the structures of **3-OPP/OPP** and **4-OPP/OPP** but are less reactive. The first is based on the observation that 2-butenyl analogues, where a methyl group at C-3 in DMAPP is replaced with hydrogen, are 10^2−10^3 are less reactive than DMAPP.[25] The second approach is based on our work with thiolodiphosphate analogues of DMAPP and GPP, which are substantially less reactive than the corresponding diphosphates and are excellent competitive inhibitors of FPPase.[22] Accordingly, we synthesized the diphosphate and thiolodiphosphate bisubstrate analogues lacking a methyl group in the DMAPP fragment (**5-OPP/OPP** and **5-SPP/SPP**) and where the oxygens between C1 and phosphorus in the allylic DMAPP and GPP fragments are replaced by sulfur (**3-SPP/SPP** and **4-SPP/SPP**).

Synthesis of Tris(tetra-*n*-butylammonium) Thiopyrophosphate. The synthesis of thiolo bisubstrate analogues was based on our previous synthesis of isopentenyl thiolodiphosphate, dimethylallyl thiolodiphosphate, geranyl thiolodiphosphate, farnesyl thiolodiphosphate, and geranylgeranyl thiolodiphosphate. This strategy relied on the installation of suitable leaving groups in the allylic and homoallylic precursors and regioselective displacement by the nucleophilic sulfur in tris(tetra-*n*-butylammonium) thiopyrophosphate (**9**).[22]

Thiopyrophosphate **9** was prepared by modifying the procedure described earlier (Scheme 1).[22] In the original

Scheme 1. Synthesis of Tris(tetra-*n*-butylammonium) Thiopyrophosphate



synthesis, treatment of salt **7** with dimethyl chlorothiophosphate gave a 30% yield of anhydride **8**. We found that addition of a catalytic amount of *N*,*N*′-dimethylaminopyridine (DMAP) increased the yield of anhydride **8** to 60%. This modification was used to synthesize multigram quantities of **9**.

Synthesis of Diphosphate and Thiolodiphosphate Analogues. Synthesis of the isopentenyl/*Z*-2-butenyl bisubstrate carbon skeleton (Scheme 2) began with the selective mono THP-protection of commercially available *cis*-but-2-en-1,4-diol (**10-OH/OH**). A Corey−Kim allylic bromination of **10-OH/OTHP** at −30 °C gave bromide **10-Br/OTHP** in good yield.[26] The bromide was used for the C-selective alkylation of the dianion of 3-methyl-3-butene-1-ol to give a 60% yield of **5-OTHP/OH**.[27] Removal of the THP group with PPTS gave a 77% yield of **5-OH/OH**.

Syntheses of the carbon skeletons for the IPP/DMAPP and IPP/GPP bisubstrate analogues are shown in Scheme 3. *α*,*β*-Unsaturated esters **11-OTHP** and **12-OTHP** were synthesized as previously described[23] and treated with DIBAL to the give

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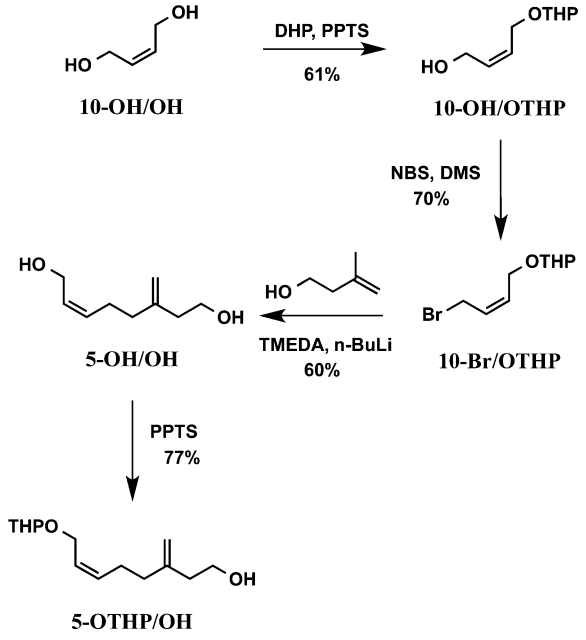
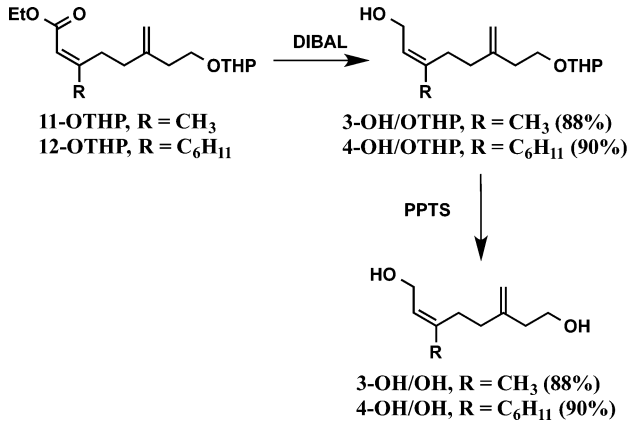
Scheme 2. Synthesis of Diol 5-OH/OH



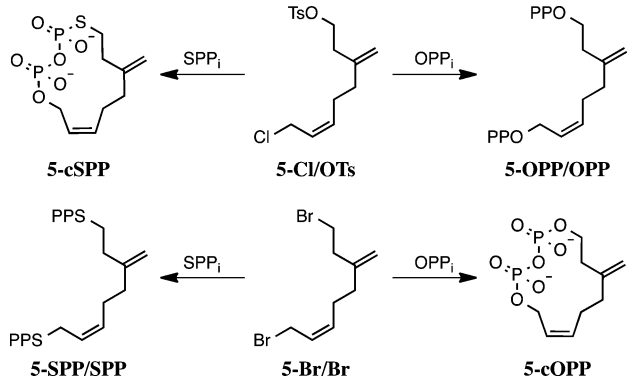
Scheme 3. Synthesis of Diols 3-OH/OH and 4-OH/OH



corresponding monoprotected diols **3-OH/OTHP** and **4-OH/OTHP**. The THP protecting group was removed with PPTS to give diols **3-OH/OH** and **4-OH/OH**, respectively.

On the basis of our previous experience with isoprenoid diphosphates[28] and thiolodiphosphates,[22,23] we selected allylic chloride/bromide and homoallylic tosylate leaving groups for the phosphorylation reactions required to make bis-thiolodiphosphates. To this end, **5-OTHP/OH** was converted to **5-Cl/OTs** by treatment with *p*-TsCl/DMAP, removal of the THP protecting group with PPTS, and treatment of the resulting allylic alcohol with NCS/DMS to give **5-Cl/OTs**. In preliminary experiments, **5-Cl/OTs**, when treated with inorganic thiopyrophosphate **9**, unexpectedly gave cyclic thiolodiphosphate **5-cSPP**, as determined from its NMR and mass spectra (Scheme 4). The ^{1}H-decoupled ^{31}P NMR spectrum of the crude cyclic thiolodiphosphate showed an AX quartet with resonances at 8.0 ppm and −7.0 ppm for P(S) and P(O), respectively. In the ^{1}H spectrum of the crude mixture, the proton at C8 appeared as a quartet at 3.4 ppm as the result of an overlapping doublet of triplets and the two protons at C7, while the protons at C1 appeared as a triplet at 4.3 ppm as a result of overlapping double of doublet patterns

Scheme 4. Phosphorylation/Thiolophosphorylation Reactions of Chloride/Tosylate and Dibromide Derivatives of 5-OH/OH



from couplings to ^{31}P(O) and the proton at C2. In addition, the negative ion electrospray (ES) mass spectrum of **5-cSPP** gave a peak at m/z 313.3 $[M - H^+]^-$ ($C_9H_{15}P_2O_6S$). The structure of **5-cSPP** indicates that the tosylate in **5-Cl(Br)/OTs** was displaced first by the more nucleophilic sulfur, followed by intramolecular displacement of halogen by the oxygen nucleophile. In contrast, treatment of **5-Cl/OTs** with tris(tetra-*n*-butylammonium) pyrophosphate (OPP_i) gave **5-OPP/OPP**.[23,24]

After some experimentation, we discovered that dibromide **5-Br/Br**, obtained by treatment of **5-OH/OH** with *N*-bromosuccinimide and PPh_3, gave linear bis-thiolodiphosphate **5-SPP/SPP** in 39% yield. Formation of the bis-thiolodiphosphate depended on the slow addition of **5-Br/Br** to an excess of **9** in acetonitrile. A ^{1}H-decoupled ^{31}P NMR spectrum (Figure 2b) of **5-SPP/SPP** has two overlapping AX quartets for the P(S) and P(O) phosphorus atoms in the thiolodiphosphate moieties. The coupling constants for between phosphorus atoms in both of the quartets are identical (J_{P-P} = 29.2 Hz). The ^{31}P signals for the thiolophosphate moieties are clearly resolved and resonate at 8.36 and 7.89 ppm, while the signals for the phosphate moieties give overlapping signals at −6.76 and −6.83 ppm (Figure 2b). The ^{1}H NMR spectrum for **5-SPP/SPP** (Figure 2a) has an overlapping doublet of triplets (J_{P-H} = 9.0 Hz and J_{H-H} = 6.0 Hz) at 2.93 ppm for the C8 protons and a doublet of doublets (J_{P-H} = 9.0 Hz and J_{H-H} = 6.0 Hz) at 3.49 ppm for the C1 protons. A high-resolution mass spectrum of **5-SPP/SPP** had a molecular ion peak at m/z = 530.9244, corresponding a molecular formula $C_9H_{20}O_{12}NaP_4S_2$ $(M + Na)^+$.

However, dibromide **5-Br/Br** was not suitable for synthesis of the corresponding bis-diphosphate under similar conditions but instead gave cyclic diphosphate **5-cOPP**! The structure of **5-cOPP** was deduced from its NMR and mass spectra. The ^{1}H-decoupled ^{31}P NMR spectrum of crude **5-cOPP** had an AX quartet at 7.0 ppm and −10.0 ppm. The ^{1}H-coupled ^{31}P spectrum was used to assign the resonance at 7.0 ppm to the phosphate attached to C1 and the downfield resonance to the phosphate attached to C8. In the ^{1}H NMR spectrum, the C8 protons gave an apparent quartet at 4.0 ppm, while the protons at C1 at 4.3 ppm gave an apparent doublet of triplets. The negative ion ES mass spectrum of **5-cOPP** had a peak at m/z 297.3 $[M - H^+]^-$ ($C_9H_{15}P_2O_7$), which confirmed the presence of **5-cOPP**. At this point, we do not have a satisfactory explanation for why the combined effects of differences in

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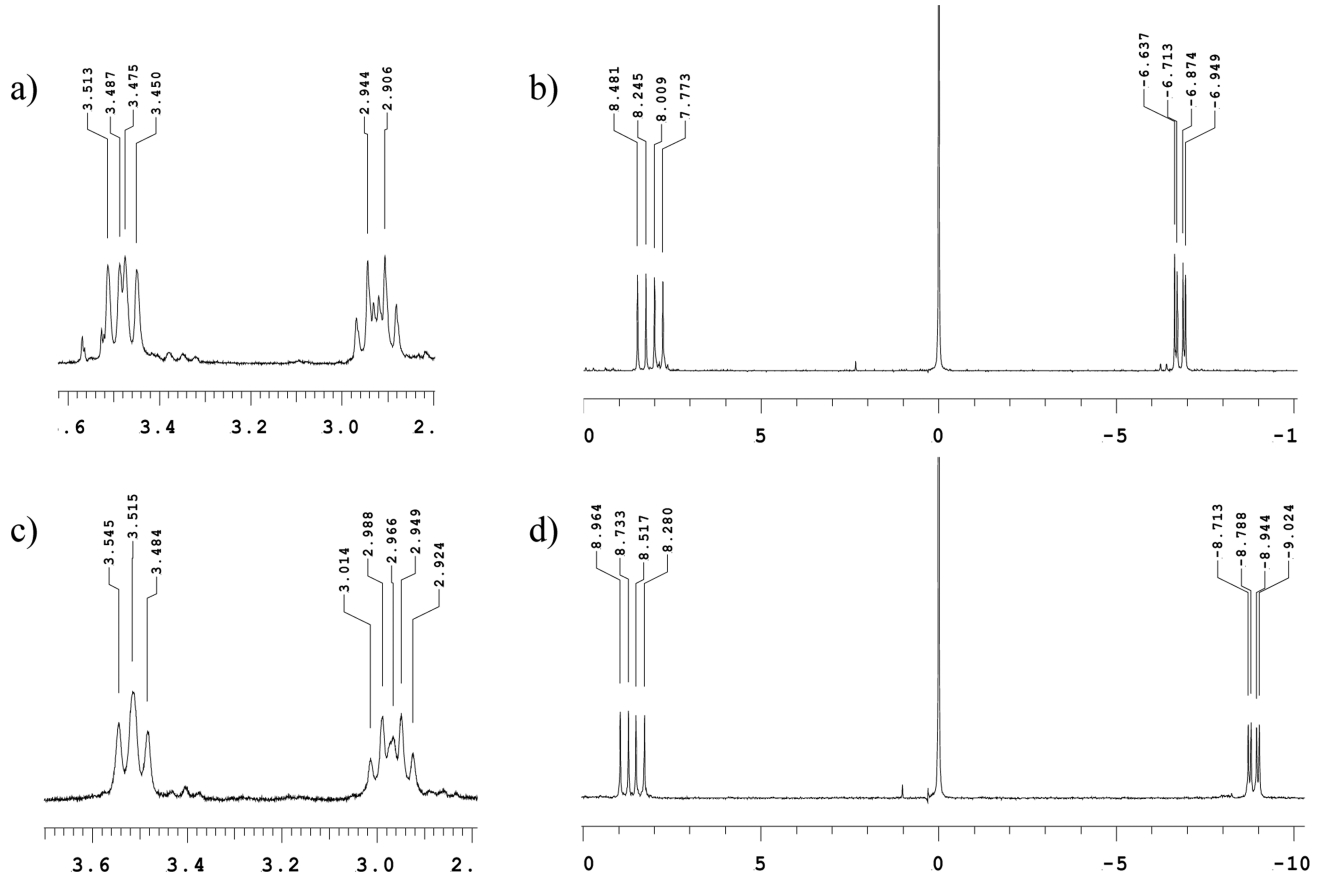

Figure 2. ^{1}H and ^{31}P NMR spectra of **4-SPP/SPP** and **5-SPP/SPP**. **5-SPP/SPP**: (a) ^{1}H NMR spectrum of the C1 and C8 methylene protons; (b) ^{31}P NMR spectrum showing resonances for P(S) and P(O). **4-SPP/SPP**: (c) ^{1}H NMR spectrum of the C1 and C8 methylene protons. (d) ^{31}P NMR spectrum showing resonances for P(S) and P(O).

nucleophilicity for sulfur and oxygen and in the leaving group reactivities for tosylate and the halides lead to formation of the various combination of linear and cyclic products shown in Scheme 4. Because the binding pockets for the diphosphate moieties in IPP and the allylic substrate are separated by over 6 Å in the active site of FPPase, the cyclic diphosphates cannot mimic the binding interactions seen in the FPPase−substrate complex. As a result, we purified the materials sufficiently to obtain ^{31}P and high resolution mass spectra but did not optimize yields or obtain highly purified samples.

Bis-thiolodiphosphates **3-SPP/SPP** and **4-SPP/SPP** were synthesized from diols **3-OH/OH** and **4-OH/OH** as described for **5-SPP/SPP**. Cyclic products were not seen when the dibromides were slowly added to an excess of **9** in acetonitrile. Conversely, a substantial amount of cyclic product was observed when **9** was added to an acetonitrile solution of the dibromides. While we typically purify diphosphate and bis-diphosphate esters by chromatography on cellulose,[23,24,28] the procedure was not suitable for the purification of bis-thiolodiphosphates. Several attempts using 0.1 M NH_4HCO_3 in combination with either 2-propanol−acetonitrile or 1-butanol−THF were unsuccessful. Ultimately, pure samples of **3-SPP/SPP**, **4-SPP/SPP**, and **5-SPP/SPP** were obtained by chromatography on DEAE Sephacel (a weak anion exchanger). The ^{1}H and ^{31}P NMR spectra of **3-SPP/SPP** (Supporting Information (SI) Figures S16 and S18) and **4-SPP/SPP** (Figure 2c and 2d) were similar to those of **5-SPP/SPP**.

Kinetic Studies. Inhibition studies with avian FPPase were conducted for bisubstrate analogues **3-OPP/OPP**, **3-SPP/SPP**, **4-OPP/OPP**, **4-SPP/SPP**, **5-OPP/OPP**, and **5-SPP/SPP**. Initial velocities were measured by the standard acid-lability assay.[29] Although the C_9 and C_{15} analogues were competitive inhibitors against GPP for avian FPPase (Figures S35 and S36), with inhibition constants in the low micromolar/high nanomolar range (Table 1), the C_{10} bisubstrate analogues showed a mixed type inhibition behavior (Figures S33 and S34).

Table 1. Inhibition Constants (K_I) of Thiolo Substrates and Bisubstrates

no.	inhibitors	inhibition constant K_I (μM)
1.	GSPP	24.8 ± 1.94
2.	**3-OPP/OPP**	1.4
3.	**3-SPP/SPP**	5.5
4.	**4-OPP/OPP**	1.0 ± 0.12
5.	**4-SPP/SPP**	0.7 ± 0.3
6.	**5-OPP/OPP**	0.5 ± 0.2
7.	**5-SPP/SPP**	2.9 ± 0.27

These values are slightly lower than the related K_M values for chain elongation of GPP and GSPP to FPP measured under same conditions.[22] The small difference between binding of the normal substrates and the bisubstrate analogues of IPP and DMAPP (or GPP) are most likely the result of slight difference in the topology of the two classes of molecules in the active site. The normal substrates are bound in the active site of FPPase through strong electrostatic interactions between three tightly bound Mg^{2+} ions with highly conserved aspartate residues and

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strong hydrogen bonds to lysine and arginine residues, while the methyl (or homogeranyl) moieties are inserted into a hydrophobic pocket.[8a] Previously, we reported that diphosphate residues of **3-OPP/OPP** and **4-OPP/OPP** are bound in the same locations as the normal substrates; however, their conformations and the conformations of the carbon atoms are distorted to accommodate the linkage between the isopentenyl and allylic units. These distortions would be expected to have a negative impact on binding affinity. Replacement of the oxygen between carbon and phosphorus with sulfur appears to have little impact on the binding while reducing the reactivity of the allylic electrophile by $>10^6$-fold.[22]

CONCLUSION

Unreactive bis-thiolodiphosphate bisubstrate analogues were constructed where the isopentenyl moiety in IPP was fused to allylic *Z*-2-butenyl, dimethylallyl, and geranyl moieties. The general strategy was based on a one-pot displacement of leaving groups attached to terminal methylene carbons in the fused isopentenyl (homoallylic) and allylic units. A homoallylic tosylate/allylic chloride combination gave a linear bis-diphosphate when treated with inorganic tris(tetra-*n*-butylammonium) pyrophosphate and a cyclic thiolodiphosphate treated with tris(tetra-*n*-butylammonium) thiopyrophosphate. In contrast, when both leaving groups were bromide, treatment with inorganic tris(tetra-*n*-butylammonium) pyrophosphate gave a cyclic diphosphate and tris(tetra-*n*-butylammonium) thiopyrophosphate gave a linear bis-thiolodiphosphate. The bisubstrate analogues were strong competitive inhibitors against GPP for avian FPPase. The analogues are excellent candidates for cocrystallization of chain elongation prenyltransferases with occupancy in the IPP and allylic regions of the active site. The ability to synthesize linear bis-diphosphate esters by the proper choice of leaving groups provides a practical approach to this class of biologically important molecules.

EXPERIMENTAL SECTION

General Methods and Materials. All the air- and moisture-sensitive reactions were performed using anhydrous solvents and under nitrogen atmosphere in oven-dried glassware (100 °C). The solvents Et_2O, THF, CH_3CN, and CH_2Cl_2 were dried by passing through a column of activated alumina. The organic compounds were purified by silica gel flash column chromatography (230−400 mesh, 60 Å), and the glass-backed TLC plates (silica gel 60 Å F254) were used for the thin-layer chromatographic analysis. The spots on the TLC plates were visualized by using phosphomolybdic acid stain. Dowex AG 50W-X8 cation-exchange resin was purchased as hydrogen form and exchanged to the active ammonium form by washing with 3 M ammonium hydroxide solution prior to use. All the bis-thiolodiphosphates were purified by cellulose flash column chromatography using DEAE Sephacel (weak anion exchanger). The thin-layer chromatographic analysis of bis-thiolodiphosphates was performed on the glass-backed cellulose TLC plates, and the spots were visualized using 5-sulfosalicylic acid−ferric chloride spray. All the ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded at room temperature, and chemical shifts were reported in δ ppm (parts per million) values. ^{1}H NMR spectra were recorded at 300 MHz and were referenced using the residual $CHCl_3$ singlet at 7.26 ppm or HDO singlet at 4.80 ppm from the deuterated NMR solvents. ^{13}C NMR spectra were recorded at 75 and 125 MHz and were referenced using the residual $CHCl_3$ triplet at 77.23 ppm from the deuterated NMR solvents. ^{13}C NMR spectra recorded in D_2O were unreferenced. ^{31}P NMR spectra were recorded at 121.5 MHz, and the ^{31}P NMR chemical shifts were referenced to 85% H_3PO_4 as an external reference. HRMS-ESI data were recorded on an LC-TOF mass spectrometer.

Radioactivity was measured in Cytoscint scintillation cocktail. Concentrations of diphosphate compounds were analyzed by the phosphate analysis method[30] before using in the enzyme assays. [^{14}C]-IPP was purchased from Amersham (now GE Healthcare) and PerkinElmer.

*Tetramethyl Thiodiphosphate (**9**).* In a flame-dried flask, a solution of 12.8 g of tetra-*n*-butylammonium dimethyl phosphate (**7**) (34.5 mmol) and 0.4 g of *N*,*N*-dimethyl-4-aminopyridine (3.48 mmol) in 15 mL of acetonitrile was cooled to −35 °C before dropwise addition of 4.5 mL of dimethyl chlorothiophosphate (5.9 g, 36.7 mmol). The mixture was stirred at −35 °C for 30 min, warmed to 0 °C over 30 min, immediately loaded onto a silica gel column, and run with two column volumes of CH_2Cl_2 before the product was eluted with 50% EtOAc−CH_2Cl_2. Solvents were removed under reduced pressure to give 5.5 g (60%) of a clear oil.

*(Z)-4-((Tetrahydro-2H-pyran-2-yl)oxy)but-2-en-1-ol (**10-OH/OTHP**).* To a solution of *cis*-but-2-ene-1,4-diol **10-OH/OH** (1.2 g, 13.64 mmol) and 3,4-dihydro-2*H*-pyran (1.09 g, 12.95 mmol) in CH_2Cl_2 (30 mL) was added 35 mg of PPTS (0.136 mmol). The mixture was stirred for 16 h at rt, diluted with 20 mL of CH_2Cl_2, washed with saturated aqueous $NaHCO_3$, water, and brine, and dried over anhydrous Na_2SO_4. CH_2Cl_2 was removed at reduced pressure, and the resulting residue was chromatographed by flash column chromatography on silica gel, eluting with 30% Et_2O/hexanes to give 1.45 g (61%) of a colorless oil; TLC (R_f 0.5, 50% EtOAc/hexanes); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 5.84−5.80 (m, 1H), 5.73−5.65 (m, 1H), 4.67 (t, 1H, J = 3.0 Hz), 4.29−4.09 (m, 4H), 3.81−3.48 (m, 1H), 3.56−3.48 (m, 1H), 2.39 (bs, 1H), 1.85−1.49 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 132.6, 128.3, 97.7, 62.6, 62.3, 58.5, 30.6, 25.5, 19.4; HRMS (ESI+) calculated for $C_9H_{16}O_3Na$ $(M + Na)^+$ m/z = 195.0997, found: 195.0999.

*(Z)-2-((4-Bromobut-2-en-1-yl)oxy)tetrahydro-2H-pyran (**10-Br/THP**).* A solution of 1.24 g (6.97 mmol) of *N*-bromosuccinimide in 20 mL of CH_2Cl_2 was chilled to 0 °C, after which 0.43 g (6.97 mmol) of dimethyl sulfide was added slowly. The resulting white slurry was stirred at 0 °C for 10 min before the temperature was lowered to −30 °C. A solution of 1.0 g (5.81 mmol) of **10-OH/OTHP** in 10 mL of CH_2Cl_2 was added slowly over a period of 10 min. The mixture was diluted with 10 mL of CH_2Cl_2, washed water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by silica gel flash chromatography, eluting with 10% Et_2O/hexanes to afford 0.96 g (70%) of a colorless oil; TLC (R_f 0.75, 20% EtOAc/hexane); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 5.94−5.84 (m, 1H), 5.76−5.68 (m, 1H), 4.64 (t, 1H, J = 3.0 Hz), 4.36−4.29 (m, 1H), 4.19−4.13 (m, 1H), 4.03 (d, 2H, J = 8.1 Hz), 3.90−3.83 (m, 1H), 3.57−3.50 (m, 1H), 1.85−1.50 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 131.1, 128.5, 98.2, 62.5, 62.1, 30.7, 26.8, 25.6, 19.6; HRMS (ESI +) calculated for $C_9H_{15}O_2NaBr$ $(M + Na)^+$ m/z = 257.0153, found: 257.0156.

*(Z)-3-Methylene-8-((tetrahydro-2H-pyran-2-yl)oxy)oct-6-en-1-ol (**5-OH/OTHP**).* A solution of 3.9 mL (9.79 mmol) of *n*-butyllithium (2.5 M in hexane) in 10 mL of Et_2O was cooled to 0 °C while 1.8 mL (11.7 mmol) of tetramethylethylenediamine (TMEDA) was added slowly over a 5 min period. The mixture was stirred 0 °C for 1 h before 0.34 g (3.92 mmol) of 3-methyl-3-buten-1-ol in 2 mL of Et_2O was added slowly at 0 °C over a period of 10 min. The resulting solution was stirred at 0 °C for 1 h, allowed to warm to room temperature, and vigorously stirred for 6 h. The yellow mixture was then cooled to −78 °C, and a solution of 0.92 g (3.91 mmol) of **10-Br/OTHP** in Et_2O was slowly added over a period of 10 min. The reaction mixture was stirred at −78 °C for 2 h and then allowed to warm to room temperature with stirring for an additional 16 h. The dark brown mixture was quenched by a slow addition ammonium chloride at 0 °C followed by the addition of water, and the layers were separated. The aqueous layer was extracted with diethyl ether, and the combined organic layers were washed with water and brine, dried over Na_2SO_4, and concentrated at reduced pressure. The residue was purified by silica gel flash chromatography eluted using 30% EtOAc/hexanes to afford 0.67 g (60%) of a light brown oil; TLC (R_f 0.45, 50% EtOAc/hexanes); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 5.62−5.52 (m, 2H),

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4.86 (s, 1H), 4.83 (s, 1H), 4.62 (t, 1H, J = 3.0 Hz), 4.25 (dd, 1H, J = 6.0, 12.0 Hz), 4.09−4.03 (m, 1H), 3.90−3.83 (m, 1H), 3.70 (dd, 2H, J = 6.3, 12.0 Hz), 3.54−3.47 (m, 1H), 2.28 (t, 2H, J = 6.6 Hz), 2.25−2.21 (m, 2H), 2.09 (dd, 2H, J = 6.9, 8.1 Hz), 1.88−1.48 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 145.5, 132.9, 126.5, 112.1, 98.1, 62.8, 62.4, 60.5, 39.3, 35.7, 30.8, 25.9, 25.6, 19.7; HRMS (ESI+) calculated for $C_{14}H_{24}O_3Na$ $(M + Na)^+$ m/z = 263.1623, found: 263.1624.

*(Z)-6-Methyleneoct-2-ene-1,8-diol (**5-OH/OH**).* To a solution of 240 mg (0.96 mmol) of **5-OH/OTHP** in 5 mL of EtOH was added 25 mg (0.096 mmol) of PPTS. The mixture was stirred for 16 h at 50 °C. Solvent was removed on rotary evaporator, and the residue was dissolved in EtOAc, washed with sat. $NaHCO_3$, water, and brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was purified by silica gel flash column chromatography eluted with 70% EtOAc/hexane to afford 115 mg (77%) of a light yellow oil; TLC (R_f 0.20, 50% EtOAc/hexanes); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 5.65−5.57 (m, 1H), 5.54−5.45 (m, 1H), 4.85 (s, 1H), 4.84 (s, 1H), 4.16 (d, 2H, J = 6.6 Hz), 3.71 (dd, 2H, J = 6.3, 12.0 Hz), 2.28 (t, 2H, J = 6.6 Hz), 2.25−2.20 (m, 2H), 2.10 (t, 2H, J = 7.2 Hz), 2.01 (bs, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 145.4, 132.1, 129.1, 112.1, 60.5, 58.5, 39.0, 35.8, 25.8; HRMS (ESI+) calculated for $C_9H_{16}O_2Na$ $(M + Na)^+$ m/z = 179.1048, found: 179.1052.

*6-Methyleneoct-2(Z)-enyl-1,8-S,S-bis-thiolodiphosphate (**5-SPP/SPP**).* A solution of 134 mg (0.86 mmol) of **5-OH/OH** and 382 mg (2.15 mmol) *N*-bromosuccinimide in 5 mL of dichloromethane was cooled at 0 °C before 560 mg (2.15 mmol) of PPh_3 was added slowly over a 5 min period. After 30 min, the ice bath was removed, and the solution was stirred at room temperature for 16 h. The mixture was concentrated on a rotary evaporator and then diluted with 20 mL of hexane. The resulting white precipitate was removed by filtration. The solvent was removed in vacuo to give a brown oil. The product was immediately used in the next step without further purification.

The oil was dissolved in 1 mL of acetonitrile and added slowly over a 5 min period to a cold (0 °C) stirred solution of 2.37 g (2.58 mmol) of tris(tetra-*n*-butylammonium)hydrogen thiopyrophosphate **9** in 3 mL of acetonitrile. The solution was stirred at 0 °C for 30 min and at room temperature for 3 h, after which the solvent was removed by a rotatory evaporator. The residue was dissolved in 2 mL of exchange buffer (25 mM ammonium bicarbonate containing 2% (v/v) of 2-propanol) and was passed through a 2 × 30 cm column containing DOWEX AG 50W-X8 cation-exchange resin (NH_4^+ form). The product was eluted with two column volumes of exchange buffer, and the eluent was lyophilized to afford a white solid. The powder was dissolved in a minimal amount of 10 mM NH_4HCO_3 and chromatographed on a DEAE Sephacel (weak anion exchanger) cellulose flash column using a gradient 10−500 mM ammonium bicarbonate. Thiolodiphosphate-containing fractions were visualized by 5-sulfosalicylic acid−ferric chloride spray, pooled, and lyophilized to give 240 mg (45%) of a white powder; TLC (cellulose, R_f 0.65, 5.0:2.5:2.5 (v/v/v) 50 mM NH_4HCO_3/THF/1-propanol); ^{1}H NMR (300 MHz, D_2O) 5.66−5.47 (m, 2H), 4.86 (s, 1H), 4.73 (s, 1H), 3.49 (dd, 2H, J = 6.0 Hz, 9.0 Hz), 2.93 (dt, 2H, J = 6.0 Hz, 9.0 Hz), 2.38 (t, 2H, J = 9.0 Hz), 2.25−2.21 (m, 2H), 2.12−2.07 (m, 2H); ^{13}C NMR (75 MHz, D_2O) 148.4, 132.9, 126.4 (J_{C-P} = 7.5 Hz), 110.6, 36.5 (J_{C-P} = 7.5 Hz), 34.8, 28.3 (J_{C-P} = 3.0 Hz), 26.9 (J_{C-P} = 3.0 Hz), 24.7; ^{31}P NMR (121.5 MHz, D_2O) 8.36 (d, 1P, J_{P-P} = 29.2 Hz), 7.89 (d, 1P, J_{P-P} = 29.2 Hz), − 6.76 (d, 1P, J_{P-P} = 29.2 Hz), − 6.83 (d, 1P, J_{P-P} = 29.2 Hz); HRMS (ESI+) calculated for $C_9H_{20}O_{12}NaP_4S_2$ $(M + Na)^+$ m/z = 530.9244, found: 530.9244.

*(Z)-3-Methyl-6-methylene-8-((tetrahydro-2H-pyran-2-yl)oxy)oct-2-en-1-ol (**3-OH/OTHP**).* To a solution of 250 mg (0.85 mmol) of α,β-unsaturated ester **11-OTHP** in 5 mL of toluene at −78 °C was slowly added 1.4 mL (2.11 mmol) of 1.5 M DIBAL in toluene. The resulting mixture was stirred for 2 h under N_2 before addition of 10 mL of saturated sodium potassium tartrate, followed by water and EtOAc. The mixture was allowed to warm to room temperature and then vigorously stirred until the layers separated (45−60 min). The aqueous layer was extracted with EtOAc, and the combined organic layers were washed with water and brine and dried over anhydrous Na_2SO_4. Solvent was removed under reduced pressure, and the residue was purified by silica gel flash column chromatography, eluting with 50% EtOAc/hexane to give 185 mg (86%) of a light yellow oil; TLC (R_f 0.4, 30% EtOAc/hexanes); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 5.43 (t, 1H, J = 6.6 Hz), 4.80 (s, 1H), 4.79 (s, 1H), 4.59 (dd, 1H, J = 2.7, 4.2 Hz), 4.09 (d, 2H, J = 6.0 Hz), 3.90−3.82 (m, 2H), 3.55−3.47 (m, 2H), 2.33 (t, 2H, J = 6.9 Hz), 2.24−2.18 (m, 2H), 2.14−2.09 (m, 2H), 1.74 (d, 3H, J = 1.2 Hz), 1.83−1.65 (m, 2H), 1.60−1.46 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 146.6, 139.7, 124.8, 111.0, 99.1, 64.5, 62.6, 59.1, 36.1, 35.3, 30.9, 30.7, 25.6, 23.6, 19.8; HRMS (ESI+) calculated for $C_{15}H_{26}O_3Na$ $(M + Na)^+$ m/z = 277.1780, found: 277.1782.

*(Z)-3-Methyl-6-methyleneoct-2-ene-1,8-diol (**3-OH/OH**).* To a solution of 160 mg (0.63 mmol) of **3-OH/OTHP** in 3 mL of EtOH was added 16 mg (0.063 mmol) of PPTS. The resulting mixture was stirred at rt under nitrogen for 16 h and then concentrated by rotary evaporation. The residue was dissolved in EtOAc, washed with saturated $NaHCO_3$, water, and brine, and dried over anhydrous Na_2SO_4. Solvent was removed at reduced pressure, and the residue was purified by silica gel flash chromatography eluted using 70% EtOAc/hexanes to afford 91 mg (88%) of a light yellow oil; TLC (R_f 0.3, 50% EtOAc/hexanes); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 5.43 (t, 1H, J = 6.0 Hz), 4.86 (s, 1H), 4.84 (s, 1H), 4.08 (d, 2H, J = 6.0 Hz), 3.72 (dd, 2H, J = 6.0, 9.0 Hz), 2.29 (t, 2H, J = 6.0 Hz), 2.22−2.19 (m, 2H), 2.13−2.08 (m, 2H), 2.02 (bs, 1H), 1.74 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 145.9, 139.6, 124.9, 111.9, 60.5, 59.0, 39.1, 34.6, 30.6, 23.5; HRMS (ESI+) calculated for $C_{10}H_{18}O_2Na$ $(M + Na)^+$ m/z = 193.1204, found: 193.1205.

*(Z)-3-Methyl-6-methyleneoct-2-ene-1,8-bis-thiolodiphosphate (**3-SPP/SPP**).* The procedure described for **5-SPP/SPP** was used to synthesize **3-SPP/SPP** from 40 mg (0.24 mmol) of **3-OH/OH** to give 55 mg (39%) of a white powder; TLC (cellulose, R_f 0.54, 5:2.5:2.5 (v/v/v) 50 mM NH_4HCO_3/THF/1-propanol); ^{1}H NMR (300 MHz, D_2O) 5.45 (t, 1H, J = 6.0 Hz), 4.89 (s, 1H), 4.87 (s, 1H), 3.49 (dd, 2H, J = 9.0 Hz, J = 9.0 Hz), 2.97 (dt, 2H, J = 6.0 Hz, J = 6.0 Hz), 2.44 (t, 2H, J = 6.0 Hz), 2.28−2.11 (m, 4H), 1.72 (s, 3H); ^{13}C NMR (125 MHz, D_2O) 148.7, 140.1, 121.2 (J_{C-P} = 7.5 Hz), 110.1, 36.3 (J_{C-P} = 7.5 Hz), 33.4, 29.4, 28.1 (J_{C-P} = 3.8 Hz), 27.4 (J_{C-P} = 3.8 Hz), 22.4; ^{31}P NMR (121.5 MHz, D_2O) 8.35 (d, 1P, J_{P-P} = 29.2 Hz), 8.0 (d, 1P, J_{P-P} = 29.2 Hz), − 6.72 (d, 1P, J_{P-P} = 29.2 Hz), − 6.77 (d, 1P, J_{P-P} = 29.2 Hz); HRMS (ESI+) calculated for $C_{10}H_{21}O_{12}P_4S_2$ $(M - H)^-$ m/z = 520.9425, found: 520.9428.

*(Z)-7-Methyl-3-(3-methylene-5-((tetrahydro-2H-pyran-2-yl)oxy)-pentyl)octa-2,6-dien-1-ol (**4-OH/OTHP**).* Alcohol **4-OH/OTHP** was prepared from 420 mg (1.15 mmol) of **12-OTHP** and 1.9 mL (2.89 mmol) of 1.5 M DIBAL in toluene using the procedure described for **3-OH/OTHP** to give 335 g (90%) of a colorless oil; TLC (R_f 0.45, 50% EtOAc/hexanes); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 5.43 (t, 1H, J = 7.2 Hz), 5.10−5.07 (m, 1H), 4.80 (s, 1H), 4.79 (s, 1H), 4.59 (dd, 1H, J = 2.1, 4.2 Hz), 4.13 (d, 2H, J = 7.2 Hz), 3.90−3.82 (m, 2H), 3.55−3.47 (m, 2H), 2.33 (t, 2H, J = 6.0 Hz), 2.23−2.18 (m, 2H), 2.12−2.04 (m, 6H), 1.82−1.70 (m, 2H), 1.68 (s, 3H), 1.60 (s, 3H), 1.58−1.48 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 146.7, 143.3, 132.0, 124.2, 124.1, 111.9, 99.1, 66.5, 62.6, 59.2, 36.9, 36.2, 35.8, 30.9, 29.3, 26.8, 25.9, 25.6, 19.8, 17.9; HRMS (ESI+) calculated for $C_{20}H_{34}O_3Na$ $(M + Na)^+$ m/z = 345.2406, found: 345.2414.

*(Z)-6-Methylene-3-(4-methylpent-3-en-1-yl)oct-2-ene-1,8-diol (**4-OH/OH**).* The procedure described for **3-OH/OH** was used to convert 335 mg (1.04 mmol) of **4-OH/OTHP** into 223 mg (90%) of a colorless oil; TLC (R_f 0.4, 50% EtOAc/hexanes); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 5.44 (t, 1H, J = 7.2 Hz), 5.14−5.06 (m, 1H), 4.87 (s, 1H), 4.85 (s, 1H), 4.12 (d, 2H, J = 7.2 Hz), 3.73 (t, 2H, J = 6.0 Hz), 2.30 (t, 2H, J = 6.0 Hz), 2.22−2.18 (m, 2H), 2.12−2.03 (m, 6H), 1.76 (bs, 2H), 1.68 (s, 3H), 1.60 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 146.0, 143.2, 132.1, 124.3, 124.0, 112.0, 60.5, 59.1, 39.1, 36.8, 35.1, 29.2, 26.8, 25.9, 17.9; HRMS (ESI+) calculated for $C_{15}H_{26}O_2Na$ $(M + Na)^+$ m/z = 261.1831, found: 261.1831.

*(Z)-6-Methylene-3-(4-methylpent-3-en-1-yl)oct-2-ene-1,8-bis-thiolodiphosphate (**4-SPP/SPP**).* Using the procedure described for **3-SPP/SPP**, 83 mg (0.35 mM) of **4-OH/OH** was converted into 110 mg (41%) of a white powder; TLC (cellulose, R_f 0.34, 5:2.5:2.5 (v/v/

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v) 50 mM NH_4HCO_3/THF/1-propanol); ^{1}H NMR (300 MHz, D_2O) 5.42 (t, 1H, J = 6.0 Hz), 5.22−5.14 (m, 1H), 4.90 (s, 1H), 4.87 (s, 1H), 3.52 (dd, 2H, J = 9.0 Hz, J = 12.0 Hz), 2.98 (dt, 2H, J = 9.0 Hz, J = 12.0 Hz), 2.44 (t, 2H, J = 6.0 Hz), 2.28−2.23 (m, 2H), 2.15−2.08 (m, 6H), 1.66 (s, 3H), 1.60 (s, 3H); ^{13}C NMR (75 MHz, D_2O) 148.8, 143.9, 133.8, 124.3, 121.0 (J_{C-P} = 7.5 Hz), 110.4, 36.4 (J_{C-P} = 7.5 Hz), 36.0, 34.1, 28.5 (J_{C-P} = 3.8 Hz), 27.9 (J_{C-P} = 3.0 Hz), 27.8, 25.9, 24.9, 17.1; ^{31}P NMR (121.5 MHz, D_2O) 8.85 (d, 1P, J_{P-P} = 27.9 Hz), 8.40 (d, 1P, J_{P-P} = 27.9 Hz), − 8.83 (d, 1P, J_{P-P} = 29.2 Hz), − 8.91 (d, 1P, J_{P-P} = 29.2 Hz); HRMS (ESI+) calculated for $C_{15}H_{30}O_{12}NaP_4S_2$ $(M + Na)^+$ m/z = 613.0027, found: 613.0027.

*(Z)-3-Methylene-8-((tetrahydro-2H-pyran-2-yl)oxy)oct-6-en-1-yl 4-Methylbenzenesulfonate (**5-OTHP/OTs**).* Using a previously described procedure,[23] 26 mg (0.11 mmol) of **5-OTHP/OH** was converted into 34 mg (79%) of a colorless oil; TLC (R_f 0.3, 20% EtOAc−hexanes); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 7.77 (d, 2H, J = 9.0 Hz), 7.33 (d, 2H, J = 9.0 Hz), 5.59−5.44 (m, 2H), 4.80 (s, 1H), 4.72 (s, 1H), 4.61 (t, 2H, J = 6.0 Hz), 4.23 (dd, 2H, J = 6.0 and J = 12.0 Hz), 4.10 (t, 2H, J = 9.0 Hz), 4.07−4.00 (m, 1H), 3.92−3.80 (m, 1H), 3.54−3.44 (m, 1H), 2.44 (s, 3H), 2.34 (t, 2H, J = 9.0 Hz), 2.16 (dd, 2H, J = 6.0 and J = 12.0 Hz), 1.98 (dd, 2H, J = 6.0 and J = 9.0 Hz), 1.82−1.70 (m, 2H), 1.60−1.45 (m, 4H) ; ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 144.9, 143.4, 133.3, 132.5, 130.0 (2C), 128.1 (2C), 126.7, 112.5, 98.1, 68.8, 62.8, 62.4, 35.8, 35.3, 30.8, 25.7, 25.6, 21.8, 19.7; HRMS (ESI+) calculated for $C_{21}H_{30}O_5SNa$ $(M + Na)^+$ m/z = 417.1712, found: 417.1711.

*(Z)-8-Hydroxy-3-methyleneoct-6-en-1-yl 4-Methylbenzenesulfonate (**5-OH/OTs**).* Using a previously described procedure,[23] 40 mg (0.068 mmol) of **5-OTHP/OTs** was converted into 20 mg (63%) of a colorless oil. TLC (R_f 0.4, 50% v/v EtOAc/hexanes); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 7.79 (d, 2H, J = 9.0 Hz), 7.35 (d, 2H, J = 9.0 Hz), 5.65−5.57 (m, 1H), 5.50−5.41 (m, 1H), 4.81 (s, 1H), 4.74 (s, 1H), 4.16 (d, 2H, J = 6.0 Hz), 4.12 (t, 2H, J = 6.0 Hz), 2.45 (s, 3H), 2.36 (t, 2H, J = 6.0 Hz), 2.17 (dd, 2H, J = 9.0 and J = 15.0 Hz), 2.01 (dd, 2H, J = 9.0 and J = 15.0 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 145.0, 143.5, 133.3, 131.9, 130.0 (2C), 129.3, 128.1(2C), 112.7, 68.9, 58.7, 36.0, 35.3 25.7, 21.9; HRMS (ESI+) calculated for $C_{16}H_{22}O_4SNa$ $(M + Na)^+$ m/z = 333.1137, found: 333.1138.

*6-Methyleneoct-2(Z)-enyl-1,8-bis-diphosphate (**5-OPP/OPP**).* Using a previously described procedure,[23] 100 mg (0.32 mmol) of **5-OH/OTs** was converted into 103 mg (55%) of a hygroscopic white solid; TLC (cellulose, R_f 0.5, 2.5:2.5:5 (v/v/v) THF/1-propanol/0.1 M NH_4HCO_3); ^{1}H NMR (300 MHz, D_2O) δ ppm 5.74−5.59 (m, 2H), 4.92 (s, 1H), 4.91 (s, 1H), 4.54 (t, 2H, J = 6.0 Hz), 4.06 (q, 2H, J = 6.0 Hz), 2.41 (t, 2H, J = 6.0 Hz), 2.37−2.26 (m, 2H), 2.22−2.13 (m, 2H); ^{13}C NMR (75 MHz, D_2O) δ ppm 146.7, 134.1, 125.8 (d, J_{C-P} = 7.5 Hz), 111.0, 64.3 (d, J_{C-P} = 7.5 Hz), 61.8 (d, J_{C-P} = 5.5 Hz), 36.3 (d, J_{C-P} = 7.5 Hz), 35.2, 25.1; ^{31}P NMR (121 MHz, D_2O) δ ppm −5.95 (d, 1P, J_{P-P} = 20.7 Hz), − 5.91 (d, 1P, J_{P-P} = 21.9 Hz), − 9.88 (d, 1P, J_{P-P} = 21.9 Hz), − 10.1 (d, 1P, J_{P-P} = 21.9 Hz); HRMS (FTMS/ESI+) calculated for $C_9H_{20}O_{14}P_4Na$ $(M + Na)^+$ m/z = 498.9701, found: 498.9695.

Kinetic Studies. The acid lability assay was used to determine kinetic constants for avian FPPase.[29] Each assay contained 70 ng (C9 and C15 bisubstrates) or 20 ng (C10 bisubstrates) of purified enzyme, 10 μM [^{14}C]IPP (10 μCi/μmol), and varied concentrations of GPP and bis-diphosphates (**3-OPP/OPP**, **4-OPP/OPP**, or **5-OPP/OPP**) or thiolodiphosphates (**3-SPP/SPP**, **4-SPP/SPP**, or **5-SPP/SPP**), in 40 mM BHDA buffer, pH 7.3, containing 20 mM BME, 2 mM $MgCl_2$, and 2 mg/mL BSA in a total volume of 200 μL. The assay mixture was incubated at 37 °C for 10 min and then quenched with 200 μL of methanol/HCl (4:1, v/v), followed by incubation for an additional 10 min before extraction with 1 mL of ligroin. The radioactivity in a 0.5 mL sample of the organic layer was measured by liquid scintillation spectrometry.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.6b00664.

^{1}H, ^{13}C, and ^{31}P NMR spectra of all the new compounds and inhibition data of bisubstrate analogues (PDF)

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Notes

The authors declare no competing financial interests.

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