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Synthesis and Enzymatic Studies of Bisubstrate Analogues for Farnesyl Diphosphate Synthase

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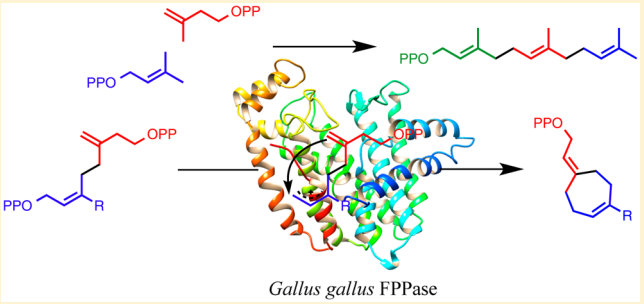
Supporting Information

ABSTRACT: Farnesyl diphosphate synthase catalyzes the sequential chain elongation reactions between isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) to form geranyl diphosphate (GPP) and between IPP and GPP to give farnesyl diphosphate (FPP). Bisubstrate analogues containing the allylic and homoallylic substrates were synthesized by joining fragments for IPP and the allylic diphosphates with a C−C bond between the methyl group at C3 in IPP and the *Z*-methyl group at C3 in DMAPP (**3-OPP**) and GPP (**4-OPP**), respectively. These constructs placed substantial limits on the conformational space available to the analogues relative to the two substrates. The key features of the synthesis of bisubstrate analogues **3-OPP** and **4-OPP** are a regioselective *C*-alkylation of the dianion of 3-methyl-3-buten-1-ol (**5**), a *Z*-selective cuprate addition of alkyl groups to an *α,β*-alkynyl ester intermediate, and differential activation of allylic and homoallylic alcohols in the analogues, followed by a simultaneous displacement of the leaving groups with tris(tetra-*n*-butylammonium) hydrogen diphosphate to give the corresponding bisdiphosphate analogues. The bisubstrate analogues were substrates for FPP synthase, giving novel seven-membered ring analogues of GPP and FPP. The catalytic efficiencies for cyclization of **3-OPP** and **4-OPP** were similar to those for chain elongation with IPP and DMAPP.



Gallus gallus FPPase

INTRODUCTION

Isoprenoid compounds are synthesized by nature's most diverse biosynthetic pathway, which is responsible for the production of a wide variety of compounds with different carbon skeletons and substitution patterns. Over 63 000 isoprenoid natural products have been identified to date.[1] These molecules have numerous and diverse biological functions and perform essential functions in all living systems.[2]

Prenyltransfer reactions, which attach the hydrocarbon chains of electrophilic allylic diphosphates to the carbon−carbon double bond in isopentenyl diphosphate (IPP), form the core of the isoprenoid biosynthetic pathway.[3] Beginning with dimethylallyl diphosphate (DMAPP, C_5), a series of linear allylic diphosphates are synthesized, which, in turn, are substrates for biosynthesis of monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sterols and hopanoids (C_{30}), carotenoids (C_{40}), and larger molecules.[4]

Farnesyl diphosphate synthase (FPPase) catalyzes the sequential chain elongation reactions between IPP and DMAPP to form geranyl diphosphate (GPP) and between IPP and GPP to give farnesyl diphosphate (FPP).[5,6] The enzyme belongs to the *E*-double bond chain elongation enzymes in Pfam PF00348[7] within the larger isoprene synthase clan[8] that includes chain elongation enzymes,[9] squalene[10] and phytoene synthases,[11] and terpene cyclases.[12] FPPase is widely distributed in all three kingdoms of life and is most likely essential.[6] Thus far, only one isoform of FPPase, a homodimeric Mg^{2+}-dependent enzyme composed of *α*-helices with two signature aspartate-rich regions that bind the allylic substrate, is known.[5,13] A large body of evidence suggests the reaction, which couples IPP with an allylic substrate, is a dissociative electrophilic alkylation (Scheme 1).[14] Although FPPase is highly selective for synthesis of GPP and FPP, small changes in the structure of the enzyme through site-directed mutagenesis or the structure of the substrate increase the promiscuity of the enzyme, giving products typical of carbocationic reactions.[9] This phenomenon is also observed for terpene cyclases[15] and squalene synthase.[16] Thus, the precise topology of the enzyme−substrate complex appears to be crucial for maintaining strict regiocontrol during catalysis by isoprenoid synthases.

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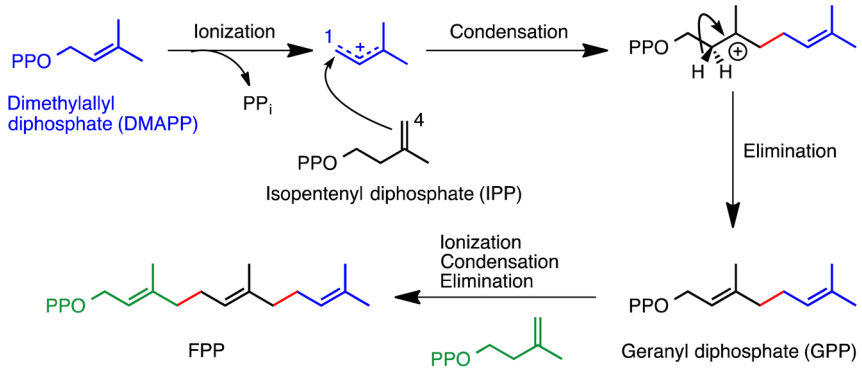
Scheme 1. Mechanism for Chain Elongation by FPP Synthase



A substantial body of work with simple alkyl analogues of IPP and DMAPP indicates that FPPase accepts a large variety of alternate substrates to give products consistent with chain elongation.[17] However, the chemoselectivity of the enzyme was compromised by bisubstrate analogues **1-OPP** and **2-OPP**, where the IPP and DMAPP fragments were joined by a methylene bridge between the *Z*-methyl group at C3 in DMAPP and the *E*- or *Z*-locus at C4 in IPP to limit the number of conformations accessible to IPP and DMAPP in the active site (Figure 1).[18] Both analogues were alternate substrates with catalytic efficiencies (k_{cat}/K_M) that were ~10-fold lower than those for IPP and DMAPP. However, the analogues were promiscuous, giving a mixture of double bond and rearranged isomers, which resulted from binding and catalysis of multiple conformers of the analogues, indicating that they did not adopt a single low energy conformation when bound in the active site.[18]

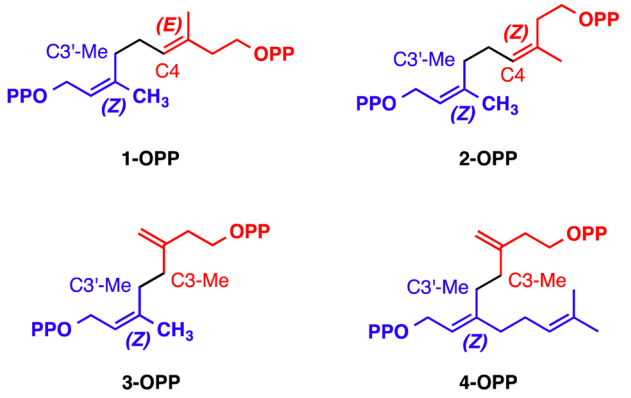


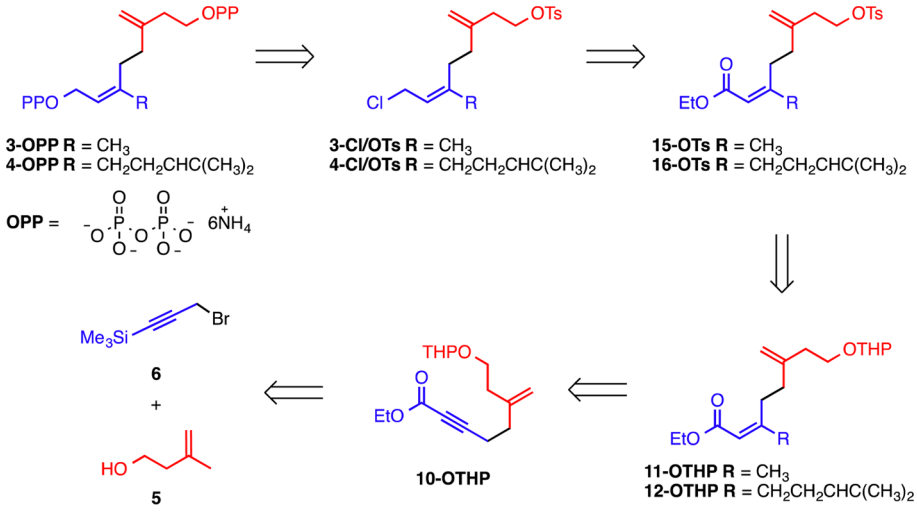
Figure 1. Structures of the bisubstrate analogues.

Inspection of models indicated that a bisubstrate analogue where the IPP and DMAPP fragments were joined by a bond between the *Z*-methyl group in DMAPP and the methyl group in IPP could fold to a position in which C4 in the IPP fragment is in a plane above C1 in the DMAPP fragment, resulting in an orientation compatible with electrophilic alkylation of the "C3–C4" double bond in the IPP fragment (Figure 1). We synthesized analogues **3-OPP** and **4-OPP** as bisubstrate mimics of IPP/DMAPP and IPP/GPP and now report kinetic and product studies that confirm their compatibility with the active site of avian FPPase.

RESULTS

Retrosynthesis of the Bisubstrate Analogues. The overall strategy for synthesis of the bisubstrate analogues relied on a simultaneous introduction of the sensitive allylic and homoallylic diphosphates using tris(tetra-*n*-butylammonium) hydrogen diphosphate. The retrosynthesis of **3-OPP** and **4-OPP** is outlined in Scheme 2. The diphosphate residues were introduced by displacing the homoallylic tosylates and allylic

Scheme 2. Retrosynthetic Analysis of the Bisubstrate Analogues



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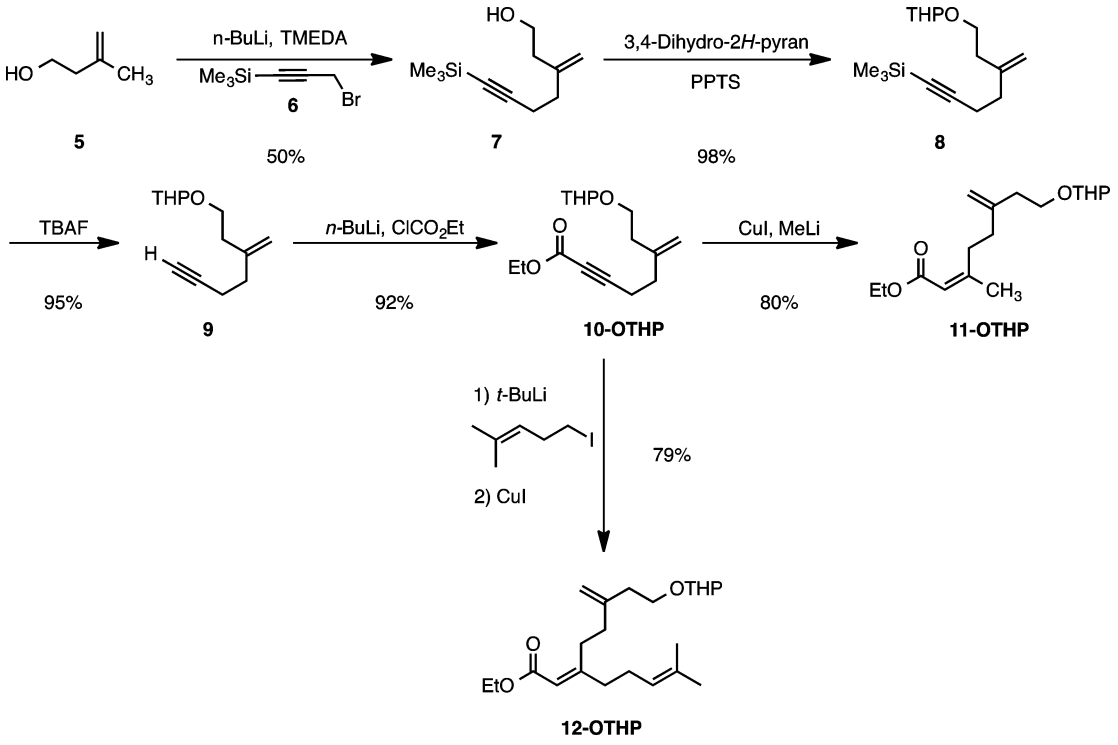
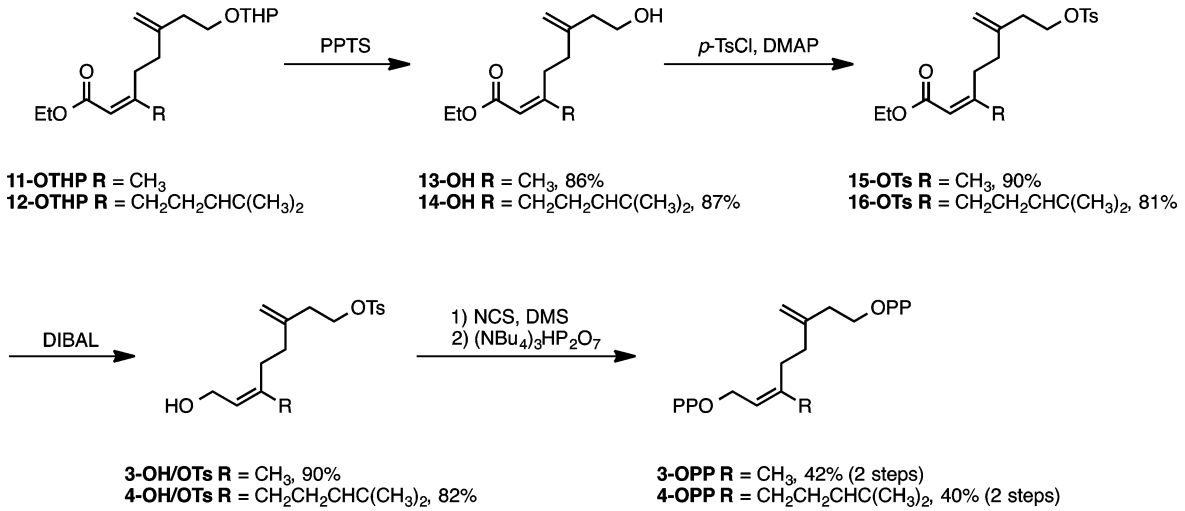
J. Org. Chem. 2015, 80, 3902–3913

Scheme 3. Synthesis of the α,β-Unsaturated Esters 11-OTHP and 12-OTHP



Scheme 4. Synthesis of Bisdiphosphates 3-OPP and 4-OPP



halides in **3-OTs/Cl** and **4-OTs/Cl** by inorganic pyrophosphate at the same time. The carbon skeletons of the two analogues were constructed by a regioselective *C*-alkylation of propargylic bromide **6** by the dianion of isopentenyl alcohol **5**, followed by a *Z*-selective conjugate addition methyl or homodimethylallyl cuprate to α,β-acetylenic ester **10-OTHP**.

Synthesis of the Bisubstrate Analogues 3-OPP and 4-OPP. As outlined in Scheme 3, the dianion of 3-methyl-3-butene-1-ol (**5**), generated in tetrahydrofuran with *n*-butyl lithium and TMEDA, was trapped with 3-bromo-(1-trimethylsilyl)-1-propyne (**6**) to give **7** in 50% yield.[19] The resulting alcohol **7** was protected as a THP ether and treated with tetrabutylammonium fluoride in tetrahydrofuran to afford the terminal alkyne **9**. Lithiation of **9** with *n*-BuLi, followed by the trapping of the lithium acetylide with ethyl chloroformate, gave α,β-acetylenic ester **10-OTHP** in 92% yield. The carbon skeletons for the IPP/DMAPP and IPP/GPP analogues were constructed by a *Z*-selective conjugate addition of the appropriate Gilman reagents, prepared from freshly crystallized copper(I) iodide and the corresponding alkyl lithium reagents. Alkylation of alkynyl ester **10-OTHP** using the methyl Gilman reagent in tetrahydrofuran provided *Z*-methyl olefin **11-OTHP** in excellent yield (80%).[20] To synthesize the compound **12-OTHP**, 5-iodo-2-methyl-2-pentene was treated with *t*-BuLi in diethyl ether[21] and transferred to a slurry of copper(I) iodide in tetrahydrofuran via a cannula, followed by addition of alkynyl ester **10-OTHP** in tetrahydrofuran, which resulted in *Z*-olefin **12-OTHP** in 79%.[20]

The conversions of α,β-unsaturated esters **11-OTHP** and **12-OTHP** were carried out using procedures similar to those

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J. Org. Chem. 2015, 80, 3902−3913

described for synthesis of **1-OPP** and **2-OPP** (Scheme 4).[18] The THP groups in **11-OTHP** and **12-OTHP** were removed by treatment with PPTS to give the corresponding homoallylic alcohols **13-OH** and **14-OH**. The alcohols were converted to the corresponding tosylates with *p*-toluenesulfonyl chloride and *N*,*N*′-dimethylaminopyridine to provide **15-OTs** and **16-OTs**.[22] The *α*,*β*-unsaturated esters were reduced to allylic alcohols **3-OH/OTs** and **4-OH/OTs**, respectively, with DIBAL and then treated with *N*-chlorosuccinimide and dimethylsulfide to give **3-Cl/OTs** and **4-Cl/OTs**.[23] The allylic chlorides decomposed during chromatography and, without purification, were treated with tris(tetra-*n*-butylammonium) pyrophosphate. Chromatography, first on DOWEX AG 50W-X8 (ammonium form) and then on cellulose, gave **3-OPP** and **4-OPP**.[18,24]

The installation of both diphosphate moieties in **3-OPP** and **4-OPP** was confirmed by their NMR and mass spectra. The ^{1}H-decoupled ^{31}P NMR spectrum of **3-OPP** showed a doublet at −6.56 ppm ($J_{P\text{-}P}$ = 21.2 Hz) for P2 and an overlapping pair of doublets at −9.80 ppm ($J_{P\text{-}P}$ = 20.1 Hz) and −9.86 ($J_{P\text{-}P}$ = 20.7 Hz) for P1 in the diphosphate moieties (Figure 2). The ^{1}H NMR spectrum of **3-OPP** had a doublet of doublets at 4.42 ppm ($J_{H\text{-}H}$ = 6.6 Hz, $J_{H\text{-}P}$ = 6.6 Hz) for the allylic methylene attached to the diphosphate moiety and a doublet of triplets at 4.01 ppm ($J_{H\text{-}H}$ = 6.6 Hz, $J_{H\text{-}P}$ = 6.6 Hz) for the homoallylic methylene. The ^{31}P spectrum of **4-OPP**, with resonances at −6.49 ppm ($J_{P\text{-}P}$ = 20.1 Hz) for P2 and −9.58 ppm ($J_{P\text{-}P}$ = 17.1 Hz) and −9.76 ppm ($J_{P\text{-}P}$ = 17.1 Hz) for P1, was similar to the pattern seen for **3-OPP**. The ^{1}H NMR spectrum of **4-OPP** was similar to that of **3-OPP**, with a doublet of doublets at 4.45 ppm ($J_{H\text{-}H}$ = 6.6 Hz, $J_{H\text{-}P}$ = 6.6 Hz) for the allylic methylene attached to the diphosphate moiety and a doublet of triplets at 4.02 ppm ($J_{H\text{-}H}$ = 6.9 Hz, $J_{H\text{-}P}$ = 6.9 Hz) for the homoallylic methylene (Figure 2). High-resolution mass spectra for **3-OPP** and **4-OPP** had molecular ions at 488.98759 and 559.0676, respectively, corresponding to the molecular formulas $C_{10}H_{21}O_{14}P_4$ $(M - H)^-$ and $C_{15}H_{31}O_{14}P_4$ $(M + H)^+$.

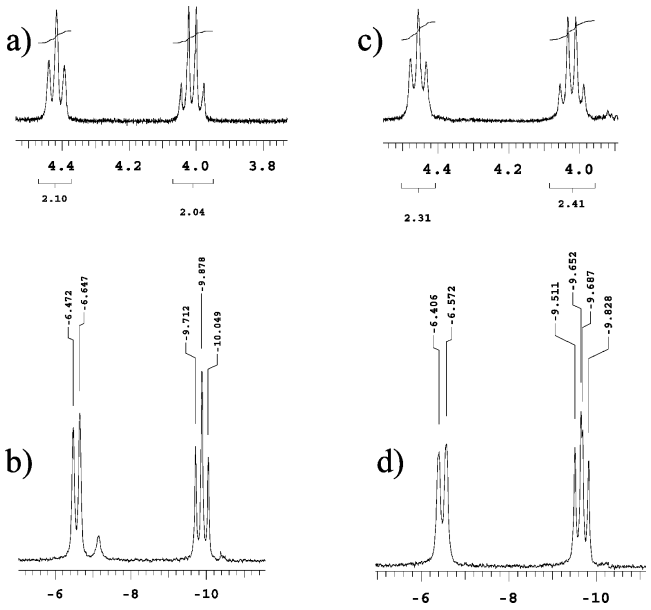

Figure 2. ^{31}P and ^{1}H NMR spectra of **3-OPP** and **4-OPP**. (a) ^{1}H NMR spectrum of the $-CH_2O-$ resonances for **3-OPP** in D_2O. (b) ^{31}P NMR spectrum of P1 and P2 for **3-OPP** in D_2O. (c) ^{1}H NMR spectrum of the $-CH_2O-$ resonances for **4-OPP** in D_2O. (d) ^{31}P NMR spectrum of P1 and P2 for **4-OPP** in D_2O.

[**1-^{3}H**]**3-OPP** and [**1-^{3}H**]**4-OPP** were synthesized from **3-OH/OTs** and **3-OH/OTs** using procedures similar to those for introducing tritium into **1-OPP** and **2-OPP**.[18] Alcohols **3-OH/OTs** and **4-OH/OTs** were oxidized with activated MnO_2, and the corresponding aldehydes, without purification, were reduced with $NaB^{3}H_4$ in methanol and THF. The reactions were monitored by TLC, and upon completion, the radiolabeled alcohols were purified by silica gel chromatography. The allylic hydroxyl groups were converted to the corresponding allylic chlorides as described for the unlabeled compounds and phosphorylated with tris(tetra-*n*-butylammonium) hydrogen pyrophosphate. The diphosphates were purified as previously described to give [**1-^{3}H**]**3-OPP** and [**1-^{3}H**]**4-OPP**.

Enzymatic Studies. *Product Studies.* In separate experiments, **3-OPP** and **4-OPP** were incubated with *Gallus gallus* FPPase, and the reactions were monitored by ^{31}P NMR spectroscopy. Figure 3 shows ^{31}P NMR spectra for **4-OPP** (similar results were seen for **3-OPP**). Upon completion (12 h), a broad singlet (∼ −7 ppm) and doublet (∼ −10 ppm) in a 3:1 ratio were observed. The broad singlet resulted from overlap of the singlet from inorganic pyrophosphate with one of phosphorus signals from the product (part a). The diphosphate moiety and inorganic pyrophosphate were hydrolyzed to inorganic phosphate by treatment with alkaline phosphatase (part b). These experiments are consistent with formation of cyclic diphosphates upon incubation of bisubstrate analogues **3-OPP**and **4-OPP** with FPPase (Scheme 5).

The hydrolysis mixtures were extracted with ethyl acetate, and TLC analysis gave a single spot for each analogue. The products were purified by silica gel chromatography to give pure samples of alcohols **17-OH** and **18-OH**. The ^{1}H NMR spectrum of **17-OH** has a two-proton doublet at 4.15 ppm with a cross-peak to the carbon at 58.8 ppm in the HMBC spectrum and was assigned to the methylene protons at C1 (Table 1, Figure 4). The methyl protons at C10 gave a three-proton singlet at 1.71 ppm. The C2 vinyl proton at 5.41 ppm appeared as a triplet (J = 6.6 Hz) and gave a strong COSY cross-peak with the C1 methylene protons at 4.15 ppm. The chemical

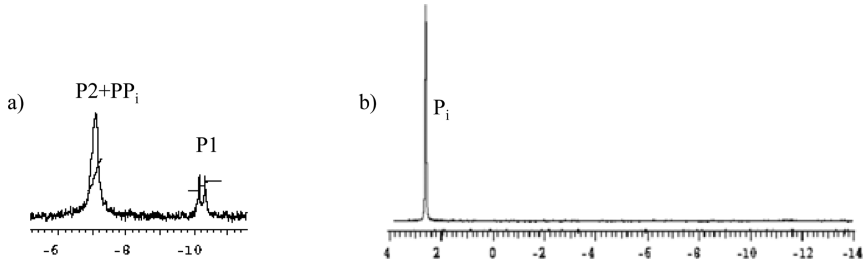

Figure 3. ^{31}P NMR spectra: (a) FPPase-catalyzed reaction of **4-OPP** after overnight incubation; (b) incubation with alkaline phosphatase for 12 h.

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J. Org. Chem. 2015, 80, 3902−3913

Scheme 5. FPP Synthase-Catalyzed Reactions of 3-OPP and 4-OPP

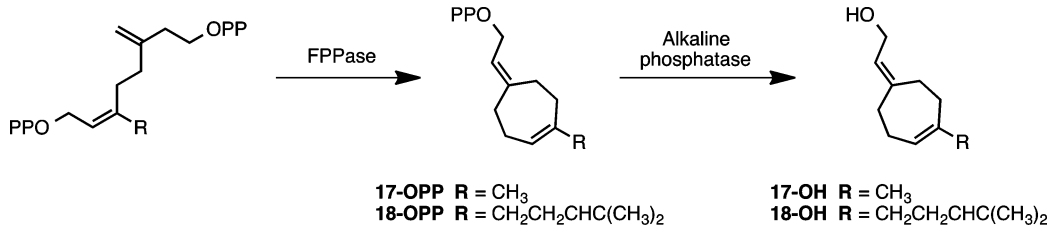

Table 1. ^{1}H, ^{13}C, HMBC, and HSQC NMR Data of 17-OH

position	δ_H (600 MHz)	multiplicity (J Hz)	δ_C (125 MHz)	HMBC (C-H)
1	4.15	2H, d (6.6)	59.03	H2
2	5.41	1H, t (6.6)	122.66	H1, 4, 9
3			146.72	H1, 4, 5, 9
4	2.36−2.34	2H, m	28.95	H2, 5, 9
5	2.13−2.11	2H, m	32.99	H4, 7, 10
6			138.91	H4, 5, 10
7	5.49	1H, t (6.0)	124.82	H5, 9, 10
8	2.13−2.11	2H, m	28.43	H7, 9
9	2.29−2.27	2H, m	37.50	H2, 4, 7, 8, 10
10	1.72	3H, s	26.65	H5, 7

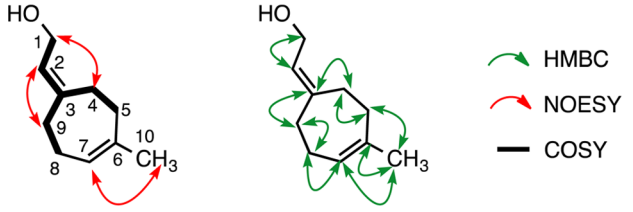

Figure 4. Important COSY, HMBC, and NOESY correlations for **17-OH**.

shifts for the methylene protons at C4 and C9 were assigned from the NOESY spectrum of **17-OH**. A strong cross-peak was observed between the methylene protons at 4.15 ppm (C1) and 2.35 ppm, while another strong cross-peak was seen between the vinyl proton at 5.41 ppm (C2) and the methylene protons at 2.28 ppm. Thus, the protons at 2.35 ppm were attached to C4, while those at 2.28 ppm were attached to C9 (Table 2, Figure 4). The C5 and C8 methylene protons gave overlapping patterns between 2.11 and 2.13 ppm but could be distinguished from HSQC cross-peaks to ^{13}C resonances at 32.99 and 28.43 ppm. HMBC correlations established connectivity patterns for C4−C5−C6−C10 and C7−C8−C9 bonding networks, establishing assignments for the cycloheptenyl carbon and hydrogen resonances and a *Z*-stereochemistry of the C2−C3 double bond. Finally, chemical shifts for the C3 and C6 quaternary carbons in **17-OH** were assigned from HMBC correlations. C6 (138.7 ppm) gave cross-peaks to the proton signals at 1.72 ppm (C10 methyl) and 2.12 ppm (C5 methylene), while the resonance at 146.5 ppm (C3) gave cross-peaks to the proton signals at 2.36 ppm (C4 methylene) and 2.28 ppm (C9 methylene).

Table 2. COSY and NOESY NMR Data of 17-OH

position	δ_H (600 MHz)	COSY (H-H)	NOESY (H-H)
1	4.15	2, 4, 9	4
2	5.41	1, 4, 9	9
3			
4	2.36−2.34	1, 2, 5	1
5	2.13−2.11	4, 10	
6			
7	5.49	5, 10	
8	2.13−2.11	7, 9	
9	2.29−2.27	1, 2	2
10	1.72	5, 7	7

The one- and two-dimensional ^{1}H and ^{13}C spectra for **18-OH** had similar chemical shifts, coupling patterns, and cross-peak correlations for nuclei at positions C1−C9 (Tables 3 and 4, Figure 5), establishing the substructure and stereochemistry of C1−C9. The proton signals for the C10 methylene group appeared as a multiplet at 1.98−2.00 ppm with an HSQC cross-peak to a carbon signal at 40.67 ppm. C10 was part of an HMBC network that connected into the cycloheptene ring and into the C11−C15 isoprenoid side chain.

The initial departure of the pyrophosphate from the substrates **3-OPP** and **4-OPP** produced the allylic carbocation, and a subsequent attack of the carbocation by the electron-rich double bond produced the stable tertiary carbocation. Upon β-elimination, an exocyclic double bond with *Z*-stereochemistry was generated as a single product in each case (**17-OPP** and **18-OPP**).

Kinetic Studies. The acid lability assay[25] was used to measure rates for the enzymatic reactions with FPPase. In this case, the allylic diphosphate products were solvolyzed in acid to give a mixture of hydrocarbon-soluble materials. While the allylic unit in the substrate analogues also solvolyzed, the homoallylic diphosphate was resistant, giving hydrocarbon-insoluble materials. The rate of formation of products was measured by determining the radioactivity of ligroin extracts following treatment of the incubation mixtures with acid.

Steady-state kinetic constants are given in Table 5. FPPase is a highly efficient enzyme, with $k_{cat}/K_M \sim 10^7$ s^{-1} M^{-1} for its normal substrates. The catalytic efficiency only drops slightly for bisubstrate analogues of IPP and DMAPP joined by a methylene bridge between C4 of IPP and the *Z*-methyl in DMAPP (**1-OPP** and **2-OPP**) or directly by a carbon−carbon bond between the methyl group in IPP and the *Z*-methyl in

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Table 3. ^{1}H, ^{13}C, HMBC, and HSQC NMR Data for 18-OH

position	δ_H (600 MHz)	multiplicity (J Hz)	δ_C (125 MHz)	HMBC (C-H)
1	4.16	2H, d (7.2)	59.01	H2
2	5.41	1H, t (6.6)	122.62	H1, 4, 9
3			147.00	H1, 4, 5, 8, 9
4	2.33−2.32	2H, m	29.48	H2, 5, 8, 9
5	2.16−2.12	2H, m	31.38	H4, 7, 10
6			142.94	H4, 5, 8, 10, 11
7	5.51	1H, t (6.0)	124.92	H5, 8, 9
8	2.16−2.12	2H, m	28.75	H7, 9
9	2.27−2.25	2H, m	37.55	H2, 4, 7, 8
10	2.00−1.98	2H, m	40.67	H5, 7, 11, 15
11	2.07−2.04	2H, m	27.10	H10
12	5.10	1H, t (6.6)	124.42	H10, 11, 14, 15
13			131.66	H11, 14, 15
14	1.60	3H, s	17.93	H12, 15
15	1.68	3H, s	25.91	H12, 14

Table 4. COSY and NOESY NMR Data for 18-OH

position	δ_H (600 MHz)	COSY (H-H)	NOESY (H-H)
1	4.16	2, 9	4, 5
2	5.41	1	9
3			
4	2.33−2.32	1, 2, 5	1
5	2.16−2.12	4	1, 14, 15
6			
7	5.51	8, 10	10, 11
8	2.16−2.12	7, 9	2
9	2.27−2.25	1, 2	2, 7
10	2.00−1.98	7, 11	7, 14
11	2.07−2.04	12, 14, 15	7, 14
12	5.10	11, 14, 15	15
13			
14	1.60	11, 12	
15	1.68	11, 12	12

Table 5. Steady-State Kinetic Constants for IPP/DMAPP and Bisubstrate Analogues with Avian FPP Synthase Catalysis

substrate	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
IPP	0.13	1.60	1.2×10^7
DMAPP	0.50	1.60	3.2×10^6
GPP	0.50	1.60	3.2×10^6
1-OPP[a]	0.082	0.160	2.0×10^6
2-OPP[a]	0.046	0.145	3.2×10^6
3-OPP	0.20	1.10	5.5×10^6
4-OPP	0.64	1.56	2.4×10^6

[a]Reported kinetic constants from ref 14.

DMAPP (**3-OPP** and **4-OPP**). The kinetic constants for the bisubstrate analogues are within a factor of 5 of those for IPP and DMAPP.

Homology Modeling and Docking Studies. The active site of FPPase is optimized to catalyze the electrophilic alkylation and elimination reactions required for chain elongation while excluding other common reactions of carbocationic intermediates. Alterations in the active site by mutagenesis or in the structures of substrates can result in promiscuous behavior.[26,18] This was the case for the IPP/DMAPP bisubstrate analogues **1-OPP** and **2-OPP**, which were efficiently processed by avian FPPase but whose products suggested that the substrates were bound in more than one conformation. In contrast, **3-OPP** and **4-OPP** each gave a single product whose structure is the result of an intramolecular "chain elongation" reaction. The conformation of the analogues in the active site of the enzyme was predicted by docking studies. ApoFPPase exists in an open state that undergoes a large conformational change to a closed form upon binding IPP and DMAPP.[5,13b] Since the crystal structures reported for avian FPPase show the enzyme in the open inactive conformation (PDB codes: 1UBY, 1FPS),[9] we constructed a homology model of the closed form based on the structure of closely related human enzyme in the catalytically active closed form (PDB code: 4KQS).[27] **3-OPP** and **4-OPP** along with three essential Mg^{2+} cations were docked in the active site of the modeled structure. The analogues bound in a single pose with the allylic diphosphate moiety coordinated to magnesium in the region of the active site containing the highly conserved aspartate-rich motifs required for binding the allylic substrate and catalysis. The hydrocarbon moieties of the analogues adopted similar conformations with the allylic methyl in **3-OPP** and the homodimethylallyl chain in **4-OPP** located in the hydrophobic pocket defined by Phe124, Leu125, Thr192, Gln196, Lys225, and Tyr229, which accommodates the

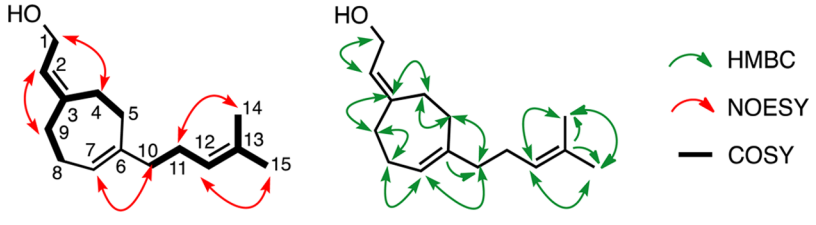

Figure 5. Important COSY, HMBC, and NOESY correlations for **18-OH**.

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J. Org. Chem. 2015, 80, 3902−3913

growing isoprenoid chain during chain elongation (Figure S43, Supporting Information).

DISCUSSION

3-OPP and **4-OPP** are excellent substitutes for IPP and the allylic substrates DMAPP or GPP in incubations with avian FPP synthase. The exclusive products of each reaction are cycloheptene derivatives **17-OPP** and **18-OPP**, whose structures are the logical consequence of an intramolecular condensation between the IPP and DMAPP/GPP units of the bisubstrate analogues (Figure 6). The stereochemistry of the

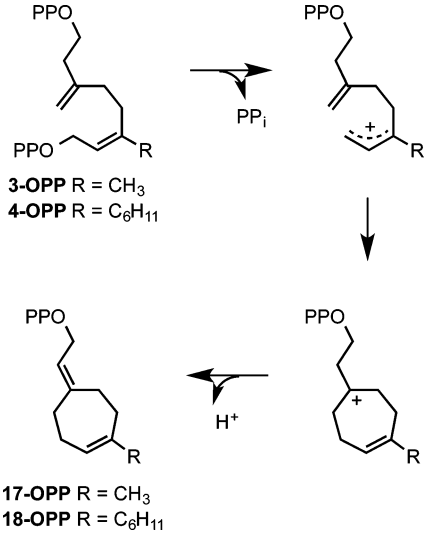

Figure 6. Mechanism for formation of **17-OPP** and **18-OPP**.

exocyclic double bonds in **17-OPP** and **18-OPP** is consistent with the *E*-selective chain elongation reaction normally catalyzed by the enzyme. The catalytic efficiencies for processing the bisubstrate analogues are only slightly lower than those for the natural substrates.

Inspection of models for bisubstrate analogues **3-OPP** and **4-OPP** suggests that they can fold into conformations where the allylic moiety is in a plane parallel to the plane of the IPP double bond in orientations that permit an intramolecular alkylation of the "IPP" double bond to produce **17-OPP** and **18-OPP**, respectively. However, the conformations of hydrocarbon chains in IPP and thioDMAPP, an unreactive substrate analogue of DMAPP,[28] for the *E. coli* FPPase complex are substantially different.[13b] As a result, the carbon atoms that join the IPP and allylic fragments in the bisubstrate analogues are located ~5.9 Å apart in the *E. coli* structure. Thus, although **3-OPP** and **4-OPP** are excellent substrates for FPPase and give a single product, they cannot bind in conformations where their hydrocarbon chains can be superimposed on those of the hydrocarbon moieties in IPP and DMAPP.

To resolve this discrepancy, we conducted docking studies for **3-OPP** and **4-OPP** with a homology model of avian FPPase constructed from a crystal structure of the closed (reactive) form of the human enzyme.[27] These structures were compared to the crystal structure of *E. coli* complexed with Mg^{2+}, IPP, and thioDMAPP[28] (an unreactive substrate analogue of DMAPP)[13b] in the closed conformation in order to explore the differences in substrate binding between the **3-OPP** and **4-OPP** and IPP/DMAPP. The active sites of FPPases have a constellation of eight aspartate residues whose spatial orientations are highly conserved, even among enzymes with modest homologies. These aspartates (D1−D8) are shown in Figure 7 for the *E. coli* (blue) and avian (yellow) FPPases along with the bound ligands. Six of the aspartates (D1−D3 and D4−D6) belong to highly conserved DDxx(xx)D motifs that anchor the diphosphate moiety of the allylic substrate by three Mg^{+} atoms. Despite only 25% (BLAST E-value 1e−09) sequence identity between the amino acid sequences of the *E. coli* and avian enzymes, the topologies of the aspartate-rich:Mg^{2+} regions of their active sites are virtually superimposable in the crystal and homology modeled/docked structures (Figure 7, part a). In *E. coli* FPPase, the first apartate-rich motif (D1−D3) binds two Mg^{2+}'s, one of which forms a bridge between the P1

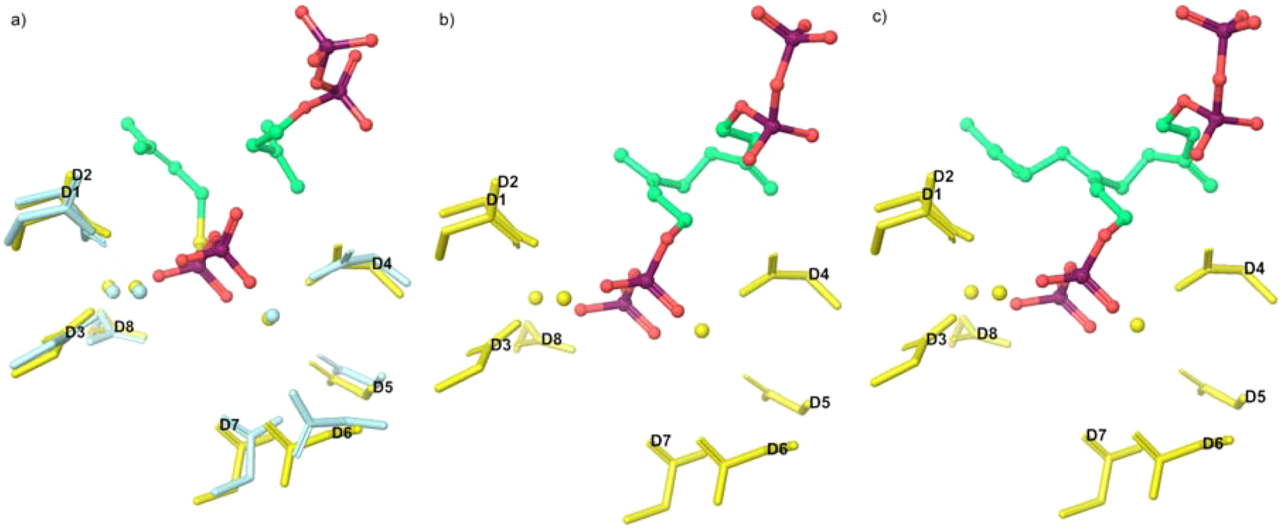

Figure 7. Conserved aspartate residues in the X-ray structure of *E. coli* FPPase·IPP·thioDMAPP (a) and homology modeled/docked structures for avian FPPase·**3-OPP** (b) and avian FPPase·**4-OPP** (c). In part (a), the aspartates in avian FPPase (yellow) are superimposed on those in the *E. coli* enzyme (blue). D1−D8 correspond to the following amino acids in *E. coli* FPPase, D1:D105, D2:D106, D3:D111, D4:D244, D5:D245, D6:D248, D7:D263, D8:D182, and in avian FPPase, D1:D117, D2:D118, D3:D121, D4:D257, D5:D258, D6:D261, D7:D275, D8:D188. Mg^{2+} is shown as a small sphere.

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J. Org. Chem. 2015, 80, 3902−3913

and P2 phosphates of the allylic diphosphate, while the second Mg^{2+} is coordinated to P1. The second motif (D4−D6) binds the third Mg^{2+} in a P1-P2 bridge on the opposite face of the diphosphate moiety. In contrast to the strong ionic interactions between the enzyme and the allylic diphosphate through bridging Mg^{2+} atoms, the IPP binding site with water molecules surrounding the diphosphate moiety, appears to be more flexible IPP.[13b]

Our docking studies suggest that the bisubstrate analogues bind with the allylic diphosphate anchored by three magnesium atoms in similar bridged structures. Also, the *E*-alkyl groups in the DMAPP/GPP fragments are located in a hydrophobic pocket that accommodates the growing hydrocarbon chain during chain elongation.[5] However, the locations of P1 and P2 for **3-OPP** and **4-OPP** (Figure 7b,c) are reversed relative to IPP in the *E. coli* structure (Figure 7a), resulting in a large change in the conformations of the atoms between P1 of the allylic phosphate and P1 of the homoallylic phosphate in **3-OPP** and **4-OPP** relative to the locations of the corresponding atoms in IPP and thioDMAPP in the *E. coli* structure. The flip-flop of the allylic diphosphate is necessary for the analogues to bind in a conformation that allows the intramolecular electrophilic alkylation between the IPP and allylic fragments to proceed.

The Mg^{2+}-aspartate-rich motifs in FPPase are essential for catalysis and are thought to trigger the electrophilic alkylation by activating the diphosphate moiety as a leaving group in the stepwise mechanism for the alkylation via an allylic carbocation−PP_i ion pair. Preservation of the Mg^{2+}-aspartate structure in the FPPase·bisubstrate analogue complex should facilitate heterolytic rupture of the allylic C−O bond similar to catalysis of chain elongation with IPP and DMAPP/GPP. Although the distance between the reacting centers in the *E. coli* FPPase·IPP·thioDMAPP complex (3.3 Å) is substantially shorter than that for **3-OPP** (4.1 Å) or **4-OPP** (4.2 Å) in the avian homology model, the bisubstrate allylic carbocations should be able to move toward the double bond in the IPP unit within the ion pair following rupture of the C(1)−O bonds.

In conclusion, bisubstrate analogues **3-OPP** and **4-OPP** are excellent substrates for avian FPPase to produce cycloheptenyl products whose structures are those expected for an intramolecular "chain elongation" reaction. However, analysis of the structures of *E. coli* FPPase·IPP·thioDMAPP and the avian FPPase·bisubstrate complexes indicates that the highly conserved Mg^{2+}-aspartate-rich motifs bind the allylic diphosphate moieties of the analogues in a reversed orientation from that of DMAPP. In spite of this difference, the enzyme retains its ability to catalyze the electrophilic alkylation reaction for the analogues.

EXPERIMENTAL SECTION

General Methods and Materials. The reactions that required anhydrous conditions were performed using anhydrous solvents under a nitrogen atmosphere in oven-dried glassware (100 °C) unless otherwise mentioned. Anhydrous Et_2O, THF, CH_3CN, and CH_2Cl_2 were obtained by passing through a column of activated alumina.[29] Purification of the organic compounds was carried out using the silica gel flash column chromatography with silica gel (230−400 mesh, 60 Å). Merck Silica Gel 60 Å F254 glass-backed TLC plates were for the thin-layer chromatographic analysis, and the spots were visualized using phosphomolybdic acid stain. Cellulose (fibers, medium) for flash column chromatographic purification of bisdiphosphates was purchased from Sigma-Aldrich and used according to the procedure described earlier.[24] Dowex AG 50W-X8 cation-exchange resin (hydrogen form, 100−20 mesh) was exchanged to the ammonium form by washing with 3 M ammonium hydroxide solution before use. Glass-backed cellulose TLC plates were used for the thin-layer chromatographic analysis of bisdiphosphates, and the spots were visualized by 5-sulfosalisylic acid-ferric chloride. All the ^1H, ^{13}C, and ^{31}P NMR spectra were recorded at 25 °C, and chemical shifts were reported in δ ppm (parts per million) values. ^1H NMR spectra were recorded at 300 and 600 MHz and were referenced using the residual $CHCl_3$ singlet at 7.26 ppm or the 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 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 LC-TOF and LTQ-FTMS mass spectrometers.

Synthesis of Bisubstrate Analogues. *3-Methylene-7-(trimethylsilyl)hept-6-yn-1-ol (**7**).* In a flame-dried flask, containing a stirred solution of 4.05 g (5.2 mL, 34.88 mmol) of TMEDA in anhydrous Et_2O (40 mL) under a N_2 atmosphere was added slowly at 0 °C 11.6 mL (29.07 mmol, 2.5 M in hexanes) of *n*-BuLi. The resulting solution was allowed to stir at rt for an additional hour. The reaction was then cooled to 0 °C, and 1.0 g (1.2 mL, 11.63 mmol) of 3-methyl-3-butene-1-ol (**5**) was added slowly. The resulting mixture was stirred at 0 °C for 1 h, allowed to warm to rt, and then stirred for 6 h. The heterogeneous reaction mixture was cooled to −78 °C, and a solution of 2.2 g (1.9 mL, 11.39 mmol) of 3-(trimethylsilyl)propargyl bromide (**6**) in Et_2O (20 mL) was added. The mixture was vigorously stirred at −78 °C for 2 h, slowly allowed to warm to rt, and stirred for an additional 16 h. The reaction mixture was cooled to 0 °C and quenched with saturated aqueous NH_4Cl (20 mL), followed by the addition of water to separate the layers. The aqueous layer was extracted with Et_2O, 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 flash column chromatography on silica gel (0−20% Et_2O−hexanes) to give 1.15 g (50%) of a light brown liquid; TLC (R_f 0.3, 20% ethyl acetate−hexanes); ^1H NMR (300 MHz, $CDCl_3$) δ ppm 4.90 (s, 2H), 3.71 (t, 2H, J = 6 Hz), 2.40−2.23 (m, 6H), 1.61 (s, 1H), 0.13 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 144.4, 113.0, 106.8, 85.3, 60.5, 39.3, 34.8, 19.0, 0.28; HRMS (ESI+) calculated for $C_{11}H_{20}OSiNa$ $(M + Na)^+$ m/z = 219.1181, found: 219.1187.

*Trimethyl(5-methylene-7-((tetrahydro-2H-pyran-2-yl)oxy)hept-1-yn-1-yl)silane (**8**).* To a solution of 21.0 g of **7** (10.7 mmol) and 270 mg (1.08 mmol) PPTS in CH_2Cl_2 (50 mL) was added 1.8 g (1.95 mL, 21.43 mmol) of 3,4-dihydro-2*H*-pyran, and the resulting solution was allowed to stir at rt for 16 h. The reaction was diluted with CH_2Cl_2 (10 mL), washed with saturated aqueous $NaHCO_3$, water, and brine, and dried over anhydrous Na_2SO_4. Solvent was removed under reduced pressure, and the residue was purified by flash column chromatography on silica gel (0−10% Et_2O−hexanes) to give 2.95 g (98%) of a colorless oil; TLC (R_f 0.7, 20% ethyl acetate−hexanes); ^1H NMR (300 MHz, $CDCl_3$) δ ppm 4.81 (s, 2H), 4.58 (dd, 1H, J = 2.4, 4.2 Hz), 3.88−3.79 (m, 2H), 3.52−3.44 (m, 2H), 2.37−2.23 (m, 6H), 1.84−1.46 (m, 6H), 0.12 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 145.2, 111.6, 107.1, 99.0, 84.9, 66.4, 62.5, 36.1, 35.7, 30.9, 25.6, 19.7, 19.0, 0.30; HRMS (ESI+) calculated for $C_{16}H_{28}O_2SiNa$ $(M + Na)^+$ m/z = 303.1756, found: 303.1762.

*2-((3-Methylenehept-6-yn-1-yl)oxy)tetrahydro-2H-pyran (**9**).* A solution of tetrabutylammonium fluoride (TBAF) in THF (21 mL, 21.2 mmol, 1 M in THF) was added slowly to a cooled (0 °C) solution of **8** (2.7 g, 9.64 mmol) in THF (30 mL), and the homogeneous brown solution was allowed to stir at rt for 16 h. The reaction was diluted with Et_2O (75 mL) and washed successively with saturated aqueous NH_4Cl, water, and brine, and dried over anhydrous Na_2SO_4. Solvent was removed under reduced pressure, and the residue was purified by flash column chromatography on silica gel (0−10% Et_2O−hexanes) to provide 2.05 g (95%) of a clear oil; TLC (R_f 0.4, 10% diethyl ether−hexanes); ^1H NMR (300 MHz, $CDCl_3$) δ ppm 4.85 (s, 2H), 4.59 (dd, 1H, J = 2.4, 4.2 Hz), 3.88−3.80 (m, 2H), 3.53−

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J. Org. Chem. 2015, 80, 3902−3913

3.46 (m, 2H), 2.37−2.25 (m, 6H), 1.95 (t, 1H, J = 2.7 Hz), 1.84−1.46 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 145.1, 111.6, 99.0, 84.2, 68.7, 66.4, 62.5, 36.1, 35.4, 30.9, 25.6, 19.8, 17.4; HRMS (ESI+) calculated for $C_{13}H_{20}O_2Na$ $(M + Na)^+$ m/z = 231.1361, found: 231.1359.

*Ethyl 6-Methylene-8-[(tetrahydro-2H-pyran-2-yl)oxy]oct-2-ynoate (**10-OTHP**).* To a flame-dried flask containing a stirred solution of 2.0 g (9.90 mmol) of alkyne **9** in THF (30 mL) under a N_2 atmosphere was slowly added 5.2 mL (12.9 mmol, 2.5 M in hexanes) of *n*-BuLi at −78 °C. The mixture was stirred for 1 h at −78 °C before a solution 1.61 g (1.4 mL, 14.86 mmol) of ethyl chloroformate in THF (10 mL) was added dropwise at −78 °C. The mixture was allowed to warm to rt and allowed to stir for another 5 h. The reaction was quenched with saturated aqueous NH_4Cl (20 mL), diluted with Et_2O (75 mL), washed with water and brine, and dried over anhydrous Na_2SO_4. Solvent was removed under reduced pressure, and the residue was then purified by flash column chromatography on silica gel (0−10% Et_2O−hexanes) to afford 2.2 g (92%) of a clear oil; TLC (R_f 0.4, 20% ethyl acetate−hexanes); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 4.86 (s, 1H), 4.84 (s, 1H), 4.57 (dd, 1H, J = 2.4, 4.2 Hz), 4.19 (q, 2H, J = 7.2 Hz), 3.87−3.79 (m, 2H), 3.52−3.44 (m, 2H), 2.50−2.45 (m, 2H), 2.35−2.29 (m, 2H), 1.86−1.46 (m, 6H), 1.28 (t, 3H, J = 7.2 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 154.0, 144.7, 112.1, 99.0, 88.8, 73.6, 66.4, 62.5, 62.0, 36.1, 34.4, 30.9, 25.6, 19.76, 17.6, 14.2; HRMS (ESI+) calculated for $C_{16}H_{24}O_4Na$ $(M + Na)^+$ m/z = 303.1572, found: 303.1573.

*Ethyl (Z)-3-Methyl-6-methylene-8-((tetrahydro-2H-pyran-2-yl)-oxy)oct-2-enoate (**11-OTHP**).* In a flame-dried flask under N_2, recrystallized CuI (329 mg, 1.72 mmol) was suspended in anhydrous THF (5 mL) and cooled to −40 °C. CH_3Li (2.1 mL, 3.30 mmol, 1.6 M in Et_2O) was slowly added, and resulting heterogeneous mixture was allowed to warm to −20 °C over 45 min, during which the suspension cleared. The solution was cooled to −78 °C, and 400 mg (1.43 mmol) of a solution of **10-OTHP** in THF (2 mL) was added slowly. The mixture was allowed to stir at −78 °C for 2 h, 3 mL of EtOH was added dropwise, and the mixture was allowed to warm to rt while stirred vigorously. Saturated aqueous NH_4Cl (10 mL) was added, followed by Et_2O (20 mL). The mixture was stirred vigorously until the layers were separated (∼1 h). The aqueous layer was extracted with Et_2O, and the combined organic layers were washed with saturated aqueous NH_4Cl, water, and brine, and dried over anhydrous Na_2SO_4. Solvent was removed under reduced pressure, and the residue was purified by flash column chromatography on silica gel (0−10% Et_2O−hexanes) to afford 340 mg (80%) of a clear oil; TLC (R_f 0.5, 20% ethyl acetate−hexanes); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 5.65 (s, 1H), 4.82 (s, 1H), 4.79 (s, 1H), 4.59 (dd, 1H, J = 2.7, 4.2 Hz), 4.12 (q, 2H, J = 7.2 Hz), 3.89−3.81 (m, 2H), 3.55−3.45 (m, 2H), 2.77−2.72 (m, 2H), 2.36 (t, 2H, J = 7.2 Hz), 2.19 (t, 2H, J = 7.4 Hz), 1.87 (d, 3H, J = 1.2 Hz), 1.83−1.46 (m, 6H), 1.25 (t, 3H, J = 7.2 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 166.4, 160.0, 146.4, 116.6, 110.9, 99.0, 66.5, 62.5, 59.6, 36.1, 35.0, 32.1, 30.9, 25.7, 25.4, 19.8, 14.5; HRMS (FTMS/ESI+) calculated for $C_{17}H_{29}O_4$ $(M + H)^+$ m/z = 297.20604, found: 297.20623; calculated for $C_{17}H_{28}O_4Na$ $(M + Na)^+$ m/z = 319.18798, found: 319.18815.

*Ethyl (Z)-8-Hydroxy-3-methyl-6-methyleneoct-2-enoate (**13-OH**).* To a stirred solution of **11-OTHP** (250 mg, 0.85 mmol) in EtOH (5 mL) was added 21 mg (0.085 mmol) of PPTS, and the resulting solution was allowed to stir at 50 °C for 16 h. EtOH was removed at reduced pressure, and the residue was dissolved in EtOAc (10 mL), washed with saturated aqueous $NaHCO_3$, water, and brine, and dried over anhydrous Na_2SO_4. Solvent was removed under reduced pressure, and the residue was purified by flash column chromatography on silica gel (20−50% Et_2O−hexanes) to give 154 mg (86%) of a clear oil; TLC (R_f 0.3, 30% ethyl acetate−hexanes); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 5.68 (s, 1H), 4.91 (s, 1H), 4.85 (s, 1H), 4.13 (q, 2H, J = 7.2 Hz), 3.74 (t, 2H, J = 6.3 Hz), 2.79−2.73 (m, 2H), 2.36 (t, 2H, J = 6.3 Hz), 2.20 (t, 2H, J = 7.4 Hz), 1.90 (d, 3H, J = 1.2 Hz), 1.26 (t, 3H, J = 7.2 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 166.6, 160.0, 145.8, 116.0, 112.3, 60.7, 59.8, 36.2, 34.3, 32.2, 25.4, 14.5; HRMS (FTMS/ESI+) calculated for $C_{12}H_{21}O_3$ $(M + H)^+$ m/z = 213.14852, found: 213.14864.

*Ethyl (Z)-3-Methyl-6-methylene-8-(tosyloxy)oct-2-enoate (**15-OTs**).* To a stirred solution of 190 mg (0.71 mmol) of **13-OH** and 164 mg (1.34 mmol) of DMAP in CH_2Cl_2 (5 mL) was added 222 mg (1.17 mmol) of *p*-toluenesulfonyl chloride at rt. The mixture was allowed to stir for 16 h rt, was diluted with Et_2O (10 mL), and was washed with 5% citric acid solution, saturated aqueous $NaHCO_3$, water, and brine, and dried over anhydrous Na_2SO_4. Solvent was removed under reduced pressure, and the residue was purified by flash column chromatography on silica gel (5−20% Et_2O−hexanes) to give 300 mg (90%) of a clear oil; TLC (R_f 0.5, 20% ethyl acetate−hexanes); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 7.78 (d, 2H, 8.4 Hz), 7.33 (d, 2H, 8.1 Hz), 5.64 (s, 1H), 4.84 (s, 1H), 4.72 (s, 1H), 4.12 (q, 2H, J = 7.2 Hz), 2.70−2.64 (m, 2H), 2.45 (s, 3H), 2.41 (t, 2H, J = 6.9 Hz), 2.07 (t, 2H, 7.5 Hz), 1.84 (d, 3H, J = 1.2 Hz), 1.25 (t, 3H, J = 7.2 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 166.3, 159.6, 144.9, 143.7, 133.3,130.0, 128.1, 116.7, 112.6, 68.89, 59.7, 35.0, 34.4, 31.8, 25.3, 21.8, 14.5; HRMS (FTMS/ESI+) calculated for $C_{19}H_{27}O_5S$ $(M + H)^+$ m/z = 367.15737, found: 367.15768; calculated for $C_{19}H_{26}O_5SNa$ $(M + Na)^+$ m/z = 389.13932, found: 389.13971.

*(Z)-8-Hydroxy-6-methyl-3-methyleneoct-6-en-1-yl 4-Methylbenzenesulfonate (**3-OH/OTs**).* A solution of 300 mg (0.82 mmol) of **15-OTs** in toluene (10 mL) was cooled to −78 °C, and 1.4 mL (2.05 mmol, 1.5 M in toluene) of DIBAL was added slowly under a N_2 atmosphere. The reaction was allowed to stir for 2 h at −78 °C before a saturated solution of sodium potassium tartrate (5 mL) was added at −78 °C. EtOAc (15 mL) was added to the slurry, and the mixture was allowed to stir until the layers separated (45−60 min). The aqueous layer was extracted with EtOAc, the combined organic layers were washed with water and brine, and dried over anhydrous Na_2SO_4. Solvent was removed at reduced pressure, and the residue was purified by flash column chromatography on silica gel (30−60% Et_2O−hexanes) to give 250 mg (94%) of a clear oil; TLC (R_f 0.2, 30% ethyl acetate−hexanes); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 7.78 (d, 2H, 8.7 Hz), 7.34 (d, 2H, 8.7 Hz), 5.42 (t, 1H, J = 6.9 Hz), 4.81 (s, 1H), 4.73 (s, 1H), 4.14−4.05 (m, 4H), 2.44 (s, 3H), 2.37 (t, 2H, J = 6.9 Hz), 2.17−2.10 (m, 2H), 2.05−1.98 (m, 2H), 1.70 (d, 3H, J = 1.2 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 145.0, 144.0, 139.4, 133.2, 130.0, 128.1, 124.9, 112.5, 68.9, 59.1, 35.3, 34.7, 30.5, 23.5, 21.8; HRMS (FTMS/ESI+) calculated for $C_{17}H_{25}O_4S$ $(M + H)^+$ m/z = 325.14681, found: 325.14693; calculated for $C_{17}H_{24}O_4SNa$ $(M + Na)^+$ m/z = 347.12875, found: 347.12894.

*(Z)-3-Methyl-6-methyleneoct-2-ene-1,8-diyl Bisdiphosphate (**3-OPP**).* To a stirred solution of 147 mg (1.10 mmol) of *N*-chlorosuccinimide in CH_2Cl_2 (7 mL) was added 84 mg (100 μL, 1.36 mmol) of dimethyl sulfide dropwise at 0 °C, and the cloudy mixture was stirred for an additional 10 min. The reaction was then cooled to −30 °C, and a solution of 275 mg (0.85 mmol) of **3-OH/OTs** in CH_2Cl_2 (3 mL) was added slowly. The mixture was allowed to stir at −30 °C for 1 h and then at room temperature for 5 h, diluted with CH_2Cl_2 (5 mL), washed with water and brine, and dried over anhydrous Na_2SO_4. Solvent was removed under reduced pressure, and the residue (TLC (R_f 0.6 20% ethyl acetate−hexanes)) was used in the next step without purification.

To a solution of 3.05 mg (3.39 mmol) of tris(tetra-*n*-butylammonium)hydrogen pyrophosphate trihydrate in CH_3CN (3 mL) at 0 °C was added slowly over 5 min a solution of 290 mg (0.85 mmol) of **3-OTs/Cl** in 0.5 mL of CH_3CN. The resulting mixture was allowed to stir for 1 h at 0 °C and for 5 h at room temperature. CH_3CN was removed at reduced pressure. The residue was dissolved in 5 mL of 25 mM NH_4HCO_3 containing 2% (v/v) isopropanol and chromatographed on a 2 × 14 cm column of DOWEX AG 50W-X8 cation-exchange resin (NH_4^+ form). The mixture was eluted with 2 column volumes of 25 mM NH_4HCO_3 containing 2% (v/v) isopropanol and lyophilized to yield a white crusty solid. The solid was dissolved in a minimum volume of 3.5:3.5:3 (v/v/v) THF/1-propanol/10 mM NH_4HCO_3 purified by medium-pressure chromatography on cellulose (3 × 30 cm) at a flow rate of 2.0 mL/min with the same solvent. Fractions containing the product were identified by TLC (R_f 0.5,

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J. Org. Chem. 2015, 80, 3902−3913

2.5:2.5:5 (v/v/v) THF/1-propanol/0.1 M NH_4HCO_3) and were combined. Organic solvents were removed at reduced pressure, and the aqueous residue was lyophilized to give 210 mg (42%) of a foamy 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 5.43 (t, 1H, J = 6.9 Hz), 4.87 (s, 2H), 4.42 (t, 2H, J = 7.2 Hz), 4.01 (q, 2H, J = 6.6 Hz), 2.38 (t, 2H, J = 6.9 Hz), 2.29−2.23 (m, 2H), 2.18−2.11 (m, 2H), 1.73 (s, 3H); ^{13}C NMR (75 MHz, D_2O) δ ppm 146.9, 142.7, 120.8 (d, $J_{C\text{-}P}$ = 8.0 Hz), 110.9, 64.3 (d, $J_{C\text{-}P}$ = 5.5 Hz), 62.3 (d, $J_{C\text{-}P}$ = 5.0 Hz), 36.2 (d, $J_{C\text{-}P}$ = 7.5 Hz), 34.0, 29.9, 22.6; ^{31}P NMR (121 MHz, D_2O) δ ppm −6.56 (d, 2P, $J_{P\text{-}P}$ = 20.7 Hz), −9.88 (t, 2P, $J_{P\text{-}P}$ = 20.1 Hz); HRMS (FTMS/ESI−) calculated for $C_{10}H_{21}O_{14}P_4$ $(M - H)^-$ m/z = 488.98872, found: 488.98759.

*Ethyl (Z)-7-Methyl-3-(3-methylene-5-((tetrahydro-2H-pyran-2-yl)oxy)pentyl)octa-2,6-dienoate (**12-OTHP**).* In a flame-dried flask under nitrogen, to a stirred solution of 450 mg (2.14 mmol) of 5-iodo-2-methylpent-2-ene in Et_2O (5 mL) was slowly added 2.8 mL (4.71 mmol, 1.7 M in pentane) of *t*-BuLi at −78 °C. The mixture was allowed to stir for 15 min at −78 °C, allowed to warm to rt, and allowed to stir for an additional 1 h. The resulting solution was transferred under nitrogen by a syringe to a flame-dried flask containing a suspension of 266 mg (1.39 mmol) of CuI in THF (5 mL) at −50 °C under nitrogen. The resulting dark gray suspension was allowed to stir at −50 °C for 45 min and cooled to −78 °C before a solution of 300 mg (1.07 mmol) of **10-OTHP** in THF (5 mL) was added dropwise at −78 °C. The dark gray mixture was stirred for 2 h at −78 °C. EtOH (5 mL) was added to the reaction mixture, and the mixture was allowed to warm to rt. Vigorous stirring was continued for 15 min before the mixture was poured into a solution of saturated aqueous NH_4Cl (20 mL), diluted with Et_2O (20 mL), and allowed to stir until the blue aqueous and clear organic layers separated. The aqueous layer was extracted with Et_2O, and the combined organic layers were washed with saturated aqueous NH_4Cl, water, and brine and dried over anhydrous Na_2SO_4. Solvents were removed, and the residue was purified by flash column chromatography on silica gel (0−10% Et_2O−hexanes) to give 310 mg (79%) of a clear oil; TLC (R_f 0.6, 20% ethyl acetate−hexanes); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 5.64 (s, 1H), 5.12−5.06 (m, 1H), 4.84 (s, 1H), 4.80 (s, 1H), 4.60 (dd, 1H, J = 2.7, 4.2 Hz), 4.14 (q, 2H, J = 7.2 Hz), 3.90−3.82 (m, 2H), 3.56−3.46 (m, 2H), 2.76−2.71 (m, 2H), 2.37 (t, 2H, J = 7.2 Hz), 2.21−2.16 (m, 6H), 1.86−1.46 (m, 6H), 1.68 (s, 3H), 1.60 (s, 3H), 1.27 (t, 3H, J = 7.2 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 166.6, 163.6, 146.5, 132.7, 123.2, 115.9, 110.9, 99.0, 66.5, 62.5, 59.7, 38.6, 36.2, 35.5, 31.1, 30.9, 26.4, 25.9, 25.7, 19.8, 17.9, 14.5; HRMS (FTMS/ESI+) calculated for $C_{22}H_{37}O_4$ $(M + H)^+$ m/z = 365.26864, found: 365.26934.

*Ethyl (Z)-3-(5-Hydroxy-3-methylenepentyl)-7-methylocta-2,6-dienoate (**14-OH**).* To a stirred solution of 300 mg (0.82 mmol) of **12-OTHP** in EtOH (5 mL) was added 21 mg (0.082 mmol) of PPTS, and the resulting solution was allowed to stir at 50 °C for 16 h. EtOH was removed at reduced pressure, the residue was dissolved in EtOAc (10 mL) and washed with saturated aqueous $NaHCO_3$, water, and brine, and was dried over anhydrous Na_2SO_4. Solvent was removed at reduced pressure, and the residue was purified by flash column chromatography on silica gel (20−50% Et_2O−hexanes) to 200 mg (87%) of a clear oil; TLC (R_f 0.3, 30% ethyl acetate−hexanes); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 5.65 (s, 1H), 5.12−5.04 (m, 1H), 4.90 (s, 1H), 4.84 (s, 1H), 4.13 (q, 2H, J = 7.2 Hz), 3.73 (t, 2H, J = 6.3 Hz), 2.75−2.70 (m, 2H), 2.35 (t, 2H, J = 6.3 Hz), 2.20−2.15 (m, 6H), 1.68 (s, 3H), 1.60 (s, 3H), 1.26 (t, 3H, J = 7.2 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 166.7, 163.6, 146.1, 132.8, 123.2, 115.9, 112.1, 60.7, 59.8, 39.3, 38.6, 34.8, 31.13, 26.4, 25.9, 17.9, 14.5; HRMS (FTMS/ESI+) calculated for $C_{17}H_{29}O_3$ $(M + H)^+$ m/z = 281.21112, found: 281.21152.

*Ethyl (Z)-7-Methyl-3-(3-methylene-5-(tosyloxy)pentyl)octa-2,6-dienoate (**16-OTs**).* To a solution of 200 mg (0.71 mmol) of **14-OH** and 131 mg (1.07 mmol) of DMAP in CH_2Cl_2 (5 mL), was added 177 mg (0.93 mmol) of *p*-toluenesulfonyl chloride at rt, and the resulting mixture was allowed to stir for 16 h at rt. The mixture was diluted with Et_2O (15 mL) and washed with 5% citric acid solution, saturated aqueous $NaHCO_3$, water, and brine. The organic layer was dried over anhydrous Na_2SO_4, solvent was removed at reduced pressure, and the residue was purified by flash column chromatography on silica gel (5−20% Et_2O−hexanes) to yield 250 mg (81%) of a clear oil; TLC (R_f 0.6, 20% ethyl acetate−hexanes); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 7.79 (d, 2H, J = 8.4 Hz), 7.32 (d, 2H, J = 8.1 Hz), 5.63 (s, 1H), 5.10−5.04 (m, 1H), 4.84 (s, 1H), 4.72 (s, 1H), 4.17−4.08 (m, 4H), 2.67−2.62 (m, 2H), 2.43 (s, 3H), 2.42 (t, 2H, J = 6.6 Hz), 2.13−2.04 (m, 6H), 1.68 (s, 3H), 1.60 (s, 3H), 1.26 (t, 3H, J = 7.2 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 166.5, 163.2, 144.9, 143.8, 133.3, 132.9, 130.0, 128.1, 123.1, 116.0, 112.5, 68.9, 59.7, 38.6, 35.0, 35.0, 30.9, 26.3, 25.9, 21.8, 17.9, 14.5; HRMS (FTMS/ESI+) calculated for $C_{24}H_{35}O_5S$ $(M + H)^+$ m/z = 435.21997, found: 435.22064.

*(Z)-6-(2-Hydroxyethylidene)-10-methyl-3-methyleneundec-9-en-1-yl-4-methylbenzenesulfonate (**4-OH/OTs**).* A solution of 200 mg (0.46 mmol) of **16-OTs** in toluene was cooled to −78 °C, and 0.8 mL (1.15 mmol, 1.5 M in toluene) of DIBAL was added slowly under a N_2 atmosphere. The reaction was allowed to stir for 2 h at −78 °C, and quenched by a dropwise addition of saturated solution sodium potassium tartrate (10 mL) at −78 °C, followed by 10 mL of EtOAc. The resulting slurry was allowed to stir 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 were dried over anhydrous Na_2SO_4. Solvent was removed at reduced pressure and purified by flash column chromatography on silica gel (30−60% Et_2O−hexanes) to give 148 mg (82%) of a colorless liquid; TLC (R_f 0.2, 30% ethyl acetate−hexanes); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 7.78 (d, 2H, J = 8.4 Hz), 7.34 (d, 2H, J = 8.1 Hz), 5.41 (t, 1H, J = 7.2 Hz), 5.10−5.06 (m, 1H), 4.81 (s, 1H), 4.72 (s, 1H), 4.13−4.07 (m, 4H), 2.44 (s, 3H), 2.37 (t, 2H, J = 6.6 Hz), 2.16−1.97 (m, 8H), 1.68 (s, 3H), 1.59 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 145.0, 144.1, 143., 133.2, 132.1, 130.0, 128.1, 124.3, 124.0, 112.4, 68.9, 59.2, 36.8, 35.3, 35.3, 29.5, 29.1, 26.7, 25.9, 21.8, 17.9; HRMS (FTMS/ESI+) calculated for $C_{22}H_{33}O_4S$ $(M + H)^+$ m/z = 393.20941, found: 393.21074; calculated for $C_{22}H_{31}O_3S$ $(M - OH + H)^+$ m/z = 375.19884, found: 375.19982.

*(Z)-1,1′-((6-Methylene-3-(4-methylpent-3-en-1-yl)oct-2-ene-1,8-diyl)) Bisdiphosphate (**4-OPP**).* To a stirred solution of 74 mg (0.55 mmol) of *N*-chlorosuccinimide in CH_2Cl_2 (3 mL) was added 43 mg (51 μL, 0.69 mmol) of dimethyl sulfide dropwise at 0 °C, and the cloudy mixture was allowed to stir for 10 min. The mixture was cooled to −30 °C, and a solution of 180 mg (0.46 mmol) of **4-OH/OTs** in CH_2Cl_2 (3 mL) was added slowly. The mixture was allowed to stir at −30 °C for 1 h and at room temperature for 5 h. CH_2Cl_2 (5 mL) was added, and the mixture was washed with water and brine and dried over anhydrous Na_2SO_4. Solvent was removed at reduced pressure, and the residue (TLC (R_f 0.6, 20% ethyl acetate−hexanes)) was used without purification.

A solution of the residue (**4-OTs/Cl**) in 1 mL of CH_3CN was slowly added to a stirred solution of 2.0 g (2.20 mmol) of tris(tetra-*n*-butylammonium)hydrogen pyrophosphate trihydrate in 2 mL of CH_3CN at 0 °C over 5 min. Stirring was continued for 1 h at 0 °C and for 5 h at room temperature. CH_3CN was removed at reduced pressure, and the residue was purified as described for **3-OPP** to give 120 mg (40%) 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 5.44 (t, 1H, J = 6.9 Hz), 5.22−5.16 (m, 1H), 4.89 (s, 1H), 4.87 (s, 1H), 4.45 (t, 2H, J = 6.6 Hz), 4.02 (q, 2H, J = 6.9 Hz), 2.38 (t, 2H, J = 6.9 Hz), 2.30−2.21 (m, 2H), 2.18−2.02 (m, 2H), 1.65 (s, 3H), 1.59 (s, 3H); ^{13}C NMR (75 MHz, D_2O) δ ppm 147.0, 145.6, 133.8, 124.3, 120.9 (d, $J_{C\text{-}P}$ = 8.5 Hz), 110.8, 64.3 (d, $J_{C\text{-}P}$ = 5.6 Hz), 62.4 (d, $J_{C\text{-}P}$ = 5.6 Hz), 36.2 (d, $J_{C\text{-}P}$ = 7.5 Hz), 36.1, 34.7, 28.4, 25.9, 24.9, 17.1; ^{31}P NMR (121 MHz, D_2O) δ ppm −6.29 (d, 2P, $J_{P\text{-}P}$ = 21.3 Hz), −9.65 (t, 2P, $J_{P\text{-}P}$ = 19.5 Hz); HRMS (ESI+) calculated for $C_{15}H_{31}O_{14}P_4$ $(M + H)^+$ m/z = 559.0664, found: 559.0676.

Product Studies. *(Z)-2-(4-Methylcyclohept-4-en-1-ylidene)-ethan-1-ol (**17-OH**).* In a 10 mL pear-shaped flask, 200 μL of 400 mM bicyclo[2.2.1]hept-5-ene-2,3-dicarboxylic acid (BHDA) buffer (pH 7.0), 200 μL of 20 mM $MgCl_2$, 200 μL of 50 mM β-mercaptoethanol, 200 μL of BSA (20 mg/mL), and 100 μL of 100

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J. Org. Chem. 2015, 80, 3902−3913

mM (14 mg, 0.024 mmol) **3-OPP** were mixed with 1020 μL of deionized water. The mixture was preincubated at 37 °C for 10 min, followed by the addition of 80 μL (10.5 mg/mL) of avian FPP synthase. The mixture was incubated at 37 °C for 7 h, after which, 2 mL of 200 mM Lysine buffer (pH 10.5) and 20 μL of aqueous bovine alkaline phosphatase (2 mg, 2790 U/mg) were added and incubated at 37 °C for an additional 12 h. The assay mixture was transferred to a 15 mL centrifuge tube, and 8 mL of ethyl acetate was added. The heterogeneous mixture was vortexed vigorously and centrifuged. The ethyl acetate layer was removed, and the extraction procedure was repeated three times. The combined organic layers were dried over anhydrous $MgSO_4$, and solvent was removed at reduced pressure. The residue was purified by flash chromatography on silica gel (10−30% ethyl acetate−hexanes) to give 1.6 mg (44%) of **17-OH** as a colorless oil; TLC (R_f 0.4, 30% ethyl acetate−hexanes); ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 5.49 (t, 1H, J = 6.0 Hz), 5.41 (t, 1H, J = 6.6 Hz), 4.15 (d, 2H, J = 6.6 Hz), 2.36−2.34 (m, 2H), 2.29−2.27 (m, 2H), 2.13−2.11 (m, 4H), 1.72 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ ppm 146.7, 138.9, 124.8, 122.7, 59.0, 37.5, 33.0, 29.0, 28.4, 26.7; HRMS (ESI+) calculated for $C_{10}H_{16}ONa$ $(M + Na)^+$ m/z = 175.1099, found: 175.1109.

*(Z)-2-(4-(4-Methylpent-3-en-1-yl)cyclohept-4-en-1-ylidene)-ethan-1-ol (**18-OH**).* Using the procedure described above, 100 μL of 100 mM (13.2 mg, 0.018 mmol) of **4-OPP** was incubated with 80 μL (10.5 mg/mL) of avian FPPase in BHDA buffer (pH 7.0) at 37 °C for 7 h, followed by addition of 2 mL of 200 mM lysine buffer (pH 10.5) and 20 μL of aqueous bovine alkaline phosphatase solution (2 mg, 2790 U/mg) and incubation at 37 °C for an additional 12 h. Flash chromatography on silica gel (10−30% EtOAc−hexanes) gave 0.8 mg (34%) of **18-OH** as a colorless oil; TLC (R_f 0.4, 30% ethyl acetate−hexanes); ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 5.51 (t, 1H, J = 6.0 Hz), 5.41 (t, 1H, J = 6.6 Hz), 5.10 (t, 1H, J = 6.6 Hz), 4.16 (d, 2H, J = 7.2 Hz), 2.33−2.32 (m, 2H), 2.27−2.25 (m, 2H), 2.16−2.12 (m, 4H), 2.07−2.04 (m, 2H), 2.00−1.98 (m, 2H), 1.68 (s, 3H), 1.60 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ ppm 147.0, 142.9, 131.7, 124.9, 124.4, 122.6, 59.0, 40.7, 37.6, 31.4, 29.5, 28.8, 27.1, 25.9, 17.9; HRMS (ESI+) calculated for $C_{15}H_{24}ONa$ $(M + Na)^+$ m/z = 243.1725, found: 243.1728.

Steady-State Kinetic Studies. Assays were carried out in 100 μL of 40 mM BHDA buffer, pH 7, containing 10 mM DTT, 2 mM $MgCl_2$, 2 mg/mL BSA, and 0.095−9.45 μM [**1-^{3}H**]**3-OPP** or [**1-^{3}H**]**4-OPP**. Samples were preincubated at 37 °C for 10 min, and the reaction was initiated by the addition of 38 ng avian FPPase in assay buffer. After incubation at 37 °C for 10 min, the reaction was quenched with 200 μL of 4:1 methanol:HCl and incubated at 37 °C for 10 min to solvolyze the allylic diphosphates. The assay mixture was extracted with 1 mL of ligroin, a 0.5 mL portion of the extract was mixed with 4 mL of scintillation cocktail, and radioactivity in the sample was quantified by scintillation spectrometry. All assays were run as triplicate along with a blank without avian FPPase. Initial rates measured in the linear phase of the progress curve vs substrate concentration were fit to the hyperbolic form of the Michaelis−Menten equation $V_{max}[S]/(K_M + [S])$, where S is the concentration of the radiolabeled **1-OPP** or **2-OPP**. Values for k_{cat} and k_{cat}/K_M were computed and are shown in Table 1.

Homology Modeling and Docking. Homology models of avian FPPase were created by Schrödinger Prime using the human FPPase crystal structure (PDB code: 4KQS) as the template. The BLAST *E*-value for the two sequences was 1e−177. The sequences were further aligned using PROMALS3D (Figure S44, Supporting Information). The models were processed by constrained minimizations (RMSD tolerance 0.35 Å, in the presence of the co-crystallized ligands **3-OPP** and **4-OPP**) with Schrödinger Protein Preparation Wizard. The quality of the homology models was assessed using the discrete optimized protein energy score (a statistical potential score for evaluating protein models) in MODELLER, and a *Z*-score of −1.73 was obtained, implying that the quality of the models was good. The two substrates were built manually and processed by the Schrödinger Ligprep. The OPLS 2005 force field was used throughout this study. The substrates were docked using Schrödinger Glide, and the Glide SP scoring function was used.

■ ASSOCIATED CONTENT

Supporting Information

^{1}H, ^{13}C, ^{31}P, and 2D NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

M.P.J. is a consultant to Schrodinger LLC, which distributes software used in this study. The authors declare no competing financial interest.

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J. Org. Chem. 2015, 80, 3902−3913

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