Supplementary Material (ESI) for Chemical Communications

Supporting Information for

Discovery of Potent Inhibitors for Farnesyl Pyrophosphate Synthase in Mevalonate Pathway

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Materials

A rat liver Quick-Clone cDNA library was purchased from Clontech (Palo Alto, CA). A HiTrap chelating metal affinity column was purchased from Amersham Pharmacia Biotech. *Taq* DNA polymerase, HB101 competent cells, *E. coli* strain BL21(DE3) competent cells, agarose, the Plasmid Mini kit and synthesized oligonucleotides came from Invitrogen Life Technologies. A gel extraction kit, T4 DNA ligase, and restriction enzymes came from MBI Fermentas. All other reagents were of research grade or better and were obtained from commercial sources.

Cloning of Rat FPPS Gene

To clone rat FPPS gene, rat cDNA library was used as a template for PCR. The sequence of the forward primer was 5' CGTGAC GGATCC GAAGGAGGAATTTAAA ATGAGAGGATCG CATCACCATCACCATCAC AATGGGGACCAGAAACTGGATGTTC 3', containing a *BamHI* site (GGATCC), a ribosome binding site (AGGAGGA), codons for the amino acid sequence MRGSHHHHHH (start codon and hexahistag), and codons for amino acids 2–9 of rat FPPS. The sequence of the reverse primer was 5' CTGCAG GTCGAC TTACTTTCTCCGCTTGTAGATCTTG 3', containing a *SalI* site (GTCGAC), a stop anticodon (TTA) and anticodons for the last seven amino acids of rat FPPS. PCR amplification was performed with the addition of 10% volume of DMSO and the sample was subjected to 25 cycles of 1 min of denaturation at 95 ^{o}C, 1 min of annealing at 60 ^{o}C, and 2 min of elongation at 72 ^{o}C. The amplification product was analyzed with 0.9% agarose gel electrophoresis. The resulting PCR product was gel purified, double digested using BamHI and SalI, and ligated into the pLM1 expression vector digested with the same restriction enzymes, resulting in the plasmid pLM1::RFPPS for rat FPPS. The constructed plasmid was transformed into HB101 competent cells. The positive colony with the expected gene was identified by single and double restriction digestion of the plasmid, followed by agarose gel analysis, and confirmed further by DNA sequencing to be the same as that previously deposited in Gene Bank. The identified positive colony was grown in an LB medium containing ampicillin (50 mg/L) and the plasmid was isolated from harvested cells using a plasmid extraction kit, and then transformed into *E. coli* strain BL21::DE3 competent cells for protein expression.

Expression and Purification of Rat FPPS

PCR techniques were used to add six continuous histidine residues to the *N*-terminus of rat FPPS, which greatly accelerated the protein purification process. The expression of the enzyme was carried out with the induction of 0.5 mM IPTG at 37 ^{o}C for 5-6 hours, and the soluble protein was obtained. The

expression of the functional rat FPPS was verified by SDS-PAGE where ~ 39kDa band for rat FPPS was observed. Nickel metal-affinity resin column was used for a single-step purification of the His-tagged enzyme. The protein was purified to apparent homogeneity as shown in Figure S1. The eluted protein fractions were dialyzed against 20 mM Tris-HCl buffer, pH 7.4, 5% glycerol, and 5 mM β-mercaptoethanol as soon as possible after the purification. The purified enzyme then can be stored at –80 ^{o}C for future use. The optimal pH value for rat FPPS was found to be 7.5 in 50 mM Tris-HCl buffer.

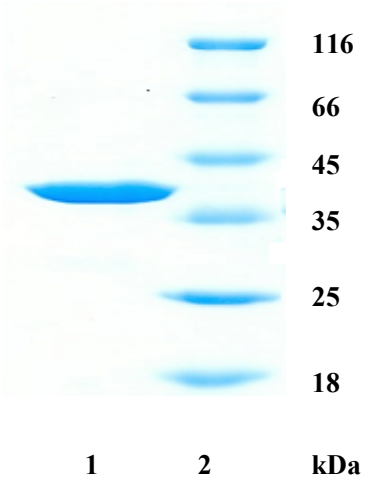


Figure S1. SDS-PAGE of purified rat FPPS. Lane 1, rat FPPS; Lane 2, standard protein molecular weight marker.

Kinetic Studies of Rat FPPS with Radioactive Assay Method

For the kinetic characterization of rat FPPS, reaction rates were measured at five concentrations of one substrate when the concentration of another substrate was fixed, and averages of two assays were used for kinetic analysis. Since [1-^{14}C]IPP was used to determine the kinetic parameters in all the assay procedure, the initial experiment was carried out to determine its kinetic constants with 40 μM GPP so that an appropriate concentration of [1-^{14}C]IPP was chosen to determine kinetic parameters of GPP and DMAPP. The results of kinetic studies, obtained by nonlinear curve fitting using the SigmaPlot 8.0 program, are summarized in Table S1. The kinetic parameters obtained in this study were similar to those published previously [1,2]. The K_M value of DMAPP for rat FPPS was similar to that of avian FPPS [3].

These results indicate that rat FPPS has expected enzyme activity, which facilitates the further study of the interactions between substrate analogs and the enzyme.

Table S1. Kinetic parameters of substrates for rat FPPS

Substrate	Kinetic Parameter	
IPP	K_M (μM)	5.6 ± 1.8
	V_{max} (μmol/mg/min)	1.21 ± 0.21
	k_{cat} (S^{-1})	0.78 ± 0.14
	k_{cat}/K_M ($S^{-1} M^{-1}$)	1.39×10^5
GPP	K_M (μM)	0.82 ± 0.12
	V_{max} (μmol/mg/min)	0.90 ± 0.03
	k_{cat} (S^{-1})	0.58 ± 0.02
	k_{cat}/K_M ($S^{-1} M^{-1}$)	7.07×10^5
DMAPP	K_M (μM)	1.09 ± 0.22
	V_{max} (μmol/mg/min)	0.61 ± 0.03
	k_{cat} (S^{-1})	0.39 ± 0.02
	k_{cat}/K_M ($S^{-1} M^{-1}$)	3.58×10^5

General Procedures for Organic Synthesis

^{1}H NMR spectra were recorded on a Varian Mercury 300 MHz NMR spectrometer at room temperature. Chemical shifts are reported in ppm on the δ scale relative to the internal standard TMS. High-resolution mass spectra were recorded on a Bruker microOTOF-Q II spectrometer. Flash chromatography was performed in columns of various diameters with silica gel by elution with appropriate solvents. Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel 60 G254 plates (25 mm) and developed with appropriate solvents and was visualized either with UV light or by dipping into a staining solution of potassium permanganate and then heating.

Organic Syntheses and Characterization of Synthetic Intermediates and Products

(*E*)-Diethyl 4,8-dimethylnona-3,7-dienylphosphonate (1). To a solution of *n*-BuLi (1.5 g, 14.4 mmol) in THF (20 mL) was added dropwise a solution of diethyl methylphosphonate (12.0 mmol) in THF (10 mL) under N_2 at –78 °C within 10 min. The pale yellow solution was stirred at –78 °C for 30 min, and geranyl bromide (10.8 mmol), dissolved in THF (10 mL), was added over a period of 10 min. The solution was maintained at –78 °C for 12 h, and the reaction was quenched with acetic acid (1.2 mL) dissolved in ether (16 mL). The mixture was allowed to warm to room temperature and was partitioned between ether and 10% HCl. The ether layer was washed with water and saturated $NaHCO_3$. The combined aqueous layer was extracted with dichloromethane (2 x 40 mL). The combined organic phase was dried (Na_2SO_4) and evaporated to provide an orange colored oil, which was subjected to flash chromatography to give compound **1** as a yellow liquid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.20-5.05 (m, 2H), 4.15-4.03 (m, 4H), 2.15-1.95 (m, 6H), 1.8-1.6 (m, 11H), 1.4-1.2 (t, 6H). MS (ESI): m/z 288.

(*E*)-Ethyl hydrogen 4, 8-dimethylnona-3,7-dienylphosphonate (2). A solution of diester **1** (9.0 mmol) in methanol (40 mL) was treated with 1 M aqueous potassium hydroxide (40 mL) and heated at 65 ^{o}C. The reaction course was monitored by ^{31}P NMR. After 36 h, the pH was adjusted to 6.5, and methanol was evaporated. The aqueous solution was acidified with 10% hydrochloric acid and extracted with ethyl acetate (3 x 50 mL). The organic layer was washed in turn with water and brine, dried ($MgSO_4$), and evaporated to provide the monoacid (2.4 g) as a pale yellow liquid. MS (ESI): m/z 260.

(*E*)-Ethyl 4,8-dimethylnona-3,7-dienylphosphonochloridate (3). Thionyl chloride (1.04 mmol) was added to compound **2** (0.443 mmol) suspended in CH_2Cl_2 (8 mL) at room temperature. This mixture was stirred for 4 hours. Then the reaction was concentrated in vacuum, diluted with CH_2Cl_2 and reconcentrated to remove all volatile contaminants. The phosphonochloridate **3** was immediately reacted with next compound as described below.

(*E*)-Diethyl[(4,8-dimethylnona-3,7-dienyl)(ethoxy)phosphoryl] methyl-phosphonate (4). To a solution of *n*-BuLi (1.5 g, 14.4 mmol) in THF (20 mL) was added dropwise a solution of diethyl

methylphosphonate (12.0 mmol) in THF (10 mL) under N_2 at –78 °C within 10 min. The pale yellow solution was stirred at –78 °C for 30 min, and phosphonochloridate **3** (10.8 mmol), dissolved in THF (10 mL), was added over a period of 10 min. The solution was maintained at –78 °C for 12 hours, and the reaction was quenched with acetic acid (1.2 mL) dissolved in ether (16 mL). The mixture was allowed to warm to room temperature and was partitioned between ether and 10% HCl. The ether layer was washed with water and saturated $NaHCO_3$. The combined aqueous layer was extracted with dichloromethane (2 x 40 mL). The combined organic phase was dried (Na_2SO_4) and evaporated to provide an orange colored oil, which was subjected to flash chromatography to give compound **4** as a yellow liquid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.15-5.05 (m, 2H), 4.41-4.08 (m, 6H), 2.52-1.68 (m, 13H), 1.6 (s, 6H) 1.4-1.2 (m, 9H). MS (ESI): m/z 395 $(M+H)^+$.

(*E*)-Diethyl 1-((4,8-dimethylnona-3,7-dienyl)(ethoxy)phosphoryl)vinyl-phosphonate (6). Paraformaldehyde (3.47 mmol) and diethylamine (0.69 mmol) were combined in 50 ml methanol, and the mixture was warmed until clear. The heat was removed and compound **4** (0.69 mmol) was added. The mixture was heated under reflux for 48 hours, and then an additional 20 ml methanol was added. The solution was concentrated under vacuum at 35 ^{o}C. Toluene (20 ml) was added, and the solution was again concentrated. This last step was repeated to ensure complete removal of methanol from the product, which was obtained as a clear liquid.

p-Toluenesulfonic acid monohydrate (5 mg) was added and the mixture was heated under reflux. Methanol was removed from the reaction mixture either by collection in a Dean-Stark trap or by adsorption into 4Å molecular sieves contained in a Soxhlet extractor. After 12 h, the solution was concentrated. The crude product was diluted with 200 ml of chloroform and washed with water (2 X 150 mL). The chloroform solution was dried over $MgSO_4$ and concentrated to afford compound **6**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.2-6.8 (m, 2H), 5.2-5.0 (m, 2H), 4.32-4.00 (m, 6H), 2.2-1.7 (m, 11H), 1.6 (s, 6H), 1.4-1.2 (m, 9H). MS (ESI): m/z 407 $(M+H)^+$.

(*E*)-Diethyl 1-((4,8-dimethylnona-3,7-dienyl)(ethoxy)phosphoryl)-2-(pyridin-2-ylamino)ethyl phosphonate (7). Compound **6** (5 mmol) was added to amino pyridine (5.5 mmol) in toluene solution at

50 ^{o}C for 5h. Then the organic layers were concentrated in vacuum. The resulting yellow oil was purified by flash chromatography producing a pale yellow oil compound **7** in 87% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.15-7.9 (m, 2H), 7.45-7.35 (m, 1H), 6.75-6.4 (m, 1H), 5.2-5.0 (m, 2H), 4.35-3.95 (m, 6H), 3.20-3.01 (m, 1H), 2.22-1.45 (m, 19H), 1.41-1.22 (m, 9H). MS (ESI): m/z 501 $(M+H)^{+}$.

(*E*)-1-((4,8-Dimethylnona-3,7-dienyl)oxidophosphoryl)-2-(pyridin-2-yl amino)ethylphosphonate (23). To a solution of compound **7** (0.54 mmol) in dry dichloromethane (3 mL) under N_2 were added iodotrimethylsilane (0.50 mL, 3.81 mmol) at 0 °C dropwise over 5 min. The reaction was stirred at 0 °C for 3 hours. The solvent was evaporated, and the residue was dried under vacuum. Dichloromethane (2 mL) was added, followed with ether (20 mL). A yellow solid emerged in the solution, which was filtered and dried to produce compound **23** in a yield of 80%. ^{1}H NMR (300 MHz, D_2O): δ 8.19-7.91 (m, 2H), 7.43-7.32 (m, 1H), 6.75-6.41 (m, 1H), 5.22-4.95 (m, 2H), 3.22-2.95 (m, 1H), 2.15-1.15 (m, 19H). MS (ESI): m/z 417 $(M+H)^{+}$. HRMS (m/z): Calcd for $(C_{18}H_{30}N_2O_5P_2+H)^{+}$ 417.1703, found 417.1708.

(*E*)-Methyl 6-(2-(diethoxyphosphoryl)-2-((4,8-dimethylnona-3,7-dienyl) (ethoxy)phosphoryl)-ethylamino)nicotinate (8). The product was obtained in a yield of 85%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.25-7.8 (2H), 6.95-6.8 (1H), 5.16-5.01 (m, 2H), 4.32-3.96 (m, 6H), 3.89 (s, 3H), 3.22-3.01 (m, 1H), 2.22-1.43 (m, 19H), 1.42-1.05 (m, 9H). MS (ESI): m/z 559 $(M+H)^{+}$.

(*E*)-6-(2-((4,8-Dimethylnona-3,7-dienyl)oxidophosphoryl)-2-phosphonato ethyl amino)-nicotinate (24). To a solution of compound **8** (0.54 mmol) in dry dichloromethane (3 mL) under N_2 were added iodotrimethylsilane (0.50 mL, 3.81 mmol) at 0 °C dropwise over 5 min. The reaction was stirred at 0 °C for 3 hours, and the volatiles were removed in vacuo to afford a yellow solid that was dissolved in aqueous NaOH (1 N, 5 mL) at room temperature. After 12 hours, the mixture was lyophilized to give a gray solid. This solid was dissolved in a buffer solution (1:49 v/v isopropyl alcohol : 25 mM aqueous NH_4HCO_3), passed through an ion-exchange column using Dowex resin (50WX8-200) hydrogen form, washed with ammonium hydroxide solution (1 N), and then allowed to equilibrate with ion-exchange buffer solution. The sample was eluted with the buffer solution and the eluant was lyophilized to give bisphosphonate salt as a white solid in a yield of 74%. ^{1}H NMR (300 MHz, D_2O): δ 8.12-7.82 (2H),

6.85-6.75 (1H), 5.18-5.02 (m, 2H), 3.25-3.01 (m, 1H), 2.21-1.1 (m, 19H). MS (ESI): m/z 461 $(M+H)^+$. HRMS (m/z): Calcd for $(C_{19}H_{30}N_2O_7P_2+H)^+$ 461.1601, found 461.1606.

(*E*)-Methyl 2-(2-(diethoxyphosphoryl)-2-((4,8-dimethylnona-3,7-dienyl) (ethoxy)phosphoryl)-ethylamino)benzoate (9). The product was obtained in a yield of 95%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.85-6.8 (4H), 5.21-5.01 (m, 2H), 4.22-3.99 (m, 6H), 3.89 (s, 3H), 3.25-3.01 (m, 1H), 2.23-1.31 (m, 28H). MS (ESI): m/z 558 $(M+H)^+$.

(*E*)-2-(2-((4,8-Dimethylnona-3,7-dienyl)oxidophosphoryl)-2-phosphonato ethylamino)benzoate (25). The product was obtained in a yield of 65% using the same procedure for making compound **24**. ^{1}H NMR (300 MHz, D_2O): δ 7.9-6.5 (4H), 5.21-5.11 (m, 2H), 3.26-3.01 (m, 1H), 2.21-1.15 (m, 19H). MS (ESI): m/z 460 $(M+H)^+$. HRMS (m/z): Calcd for $(C_{20}H_{31}NO_7P_2+H)^+$ 460.1649, found 460.1642.

Diethyl 1-(((*E*)-4,8-dimethylnona-3,7-dienyl)(ethoxy)phosphoryl)-2-((2*R*,3*R*,4*S*,5*R*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl amino) ethylphosphonate (10). The product was obtained in a yield of 48%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.41-5.05 (2H), 4.42-3.99 (m, 6H), 3.89-3.21 (m, 11H), 2.71-2.41 (m, 1H), 2.22-1.22 (m, 16H), 1.21-0.92 (m, 12H).

1-(((*E*)-4,8-Dimethylnona-3,7-dienyl)oxidophosphoryl)-2-((2*R*,3*R*,4*S*,5*R*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-ylamino) ethylphosphonate (26). The product was obtained in a yield of 56% using the same procedure for making compound **24**. ^{1}H NMR (300 MHz, D_2O): δ 5.35-5.05 (m, 2H), 3.89-3.21 (m, 11H), 2.7-1.32 (m, 20H).

(2*S*)-2-(2-(((*E*)-4,8-Dimethylnona-3,7-dienyl)oxidophosphoryl)-2-phosphonatoethylamino)-3-methylbutanoate (27). The product was obtained in a yield of 57% using the same procedure for making compound **24**. ^{1}H NMR (300 MHz, D_2O): δ 5.31-5.0 (2H), 3.5-3.3 (1H), 2.89-2.11 (m, 3H), 2.12-1.32 (m, 18H), 1.0-0.95 (6H). MS (ESI): m/z 440 $(M+H)^+$. HRMS (m/z): Calcd for $(C_{18}H_{35}NO_7P_2+H)^+$ 440.1962, found 440.1967.

Compound 12. The product was obtained in a yield of 84%**.** ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.25-5.10 (2H), 4.41-3.39 (m, 8H), 3.51-3.28 (1H), 2.75-2.45 (m, 4H), 2.21-1.55 (m, 24H), 1.42-1.2 (m, 11H). MS (ESI): m/z 584 $(M+H)^+$.

(2*S*)-2-(2-(((*E*)-4,8-Dimethylnona-3,7-dienyl)oxidophosphoryl)-2-phosphonatoethylamino)-4-(methylthio)butanoate (28). The product was obtained in a yield of 72% using the same procedure for making compound **24**. ^{1}H NMR (300 MHz, D_2O): δ 5.22-5.10 (2H), 3.51-3.28 (1H), 2.72-2.42 (m, 4H), 2.2-1.45 (m, 23H).

(2*S*,3*S*)-Ethyl 2-(2-(diethoxyphosphoryl)-2-(((*E*)-4,8-dimethylnona-3,7-dienyl) (ethoxy)-phosphoryl)ethylamino)-3-methylpentanoate (13). The product was obtained in a yield of 92%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.25-5.05 (2H), 4.25-4.05 (m, 8H), 3.81-3.62 (1H), 3.25-2.65 (m, 2H), 2.21-1.15 (m, 36H), 1.0-0.8 (t, 3H).

(2*S*,3*S*)-2-(2-(((*E*)-4,8-Dimethylnona-3,7-dienyl)oxidophosphoryl)-2-phosphonatoethylamino)-3-methylpentanoate (29). The product was obtained in a yield of 72% using the same procedure for making compound **24**. ^{1}H NMR (300 MHz, D_2O): δ 5.25-5.05 (2H), 3.75-3.62 (1H), 3.25-2.65 (m, 2H), 2.05-1.05 (m, 24H), 1.01-0.82 (t, 3H). MS (ESI): m/z 454 $(M+H)^+$. HRMS (m/z): Calcd for $(C_{19}H_{37}NO_7P_2+H)^+$ 454.2118, found 454.2123.

(2*S*)-Ethyl 2-(2-(diethoxyphosphoryl)-2-(((*E*)-4,8-dimethylnona-3,7-dienyl) (ethoxy)-phosphoryl)ethylamino)-3-hydroxypropanoate (14). The product was obtained in a yield of 81%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.42-5.02 (2H), 4.28-3.85 (m, 10H), 3.8-3.6 (1H), 3.41-2.95 (m, 2H), 2.15-1.25 (m, 30H).

(2*S*)-2-(2-(((*E*)-4,8-Dimethylnona-3,7-dienyl)oxidophosphoryl)-2-phosphonatoethylamino)-3-hydroxypropanoate (30). The product was obtained in a yield of 75% using the same procedure for making compound **24**. ^{1}H NMR (300 MHz, D_2O): δ 5.45-5.05 (2H), 4.29-3.51 (m, 4H), 2.16-1.15 (m, 19H).

(2*S*,3*R*)-Ethyl 2-(2-(diethoxyphosphoryl)-2-(((*E*)-4,8-dimethylnona-3,7-dienyl) (ethoxy)-phosphoryl)ethylamino)-3-hydroxybutanoate (15). The product was obtained in a yield of 78%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.46-5.03 (2H), 4.28-3.65 (m, 11H), 3.4-2.95 (m, 2H), 2.15-1.20 (m, 32H).

(2*S*,3*R*)-2-(2-(((*E*)-4,8-Dimethylnona-3,7-dienyl)oxidophosphoryl)-2-phosphonatoethylamino)-3-hydroxybutanoate (31). The product was obtained in a yield of 47% using the same procedure for

making compound **24**. ^{1}H NMR (300 MHz, D_2O): δ 5.23-5.03 (2H), 4.29-3.51 (m, 4H), 2.15-1.15 (m, 21H).

(2*S*)-Ethyl 1-(2-(diethoxyphosphoryl)-2-(((*E*)-4,8-dimethylnona-3,7-dienyl) (ethoxy)-phosphoryl)ethyl)pyrrolidine-2-carboxylate (16). The product was obtained in a yield of 86%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.43-5.03 (2H), 4.25-4.05 (m, 8H), 3.61-2.60 (m, 3H), 2.05-1.45 (m, 24H), 1.42-1.15 (m, 12H). MS (ESI): m/z 550 $(M+H)^+$.

(2*S*)-1-(2-(((*E*)-4,8-Dimethylnona-3,7-dienyl)oxidophosphoryl)-2-phosphonatoethyl)-pyrrolidine-2-carboxylate (32). The product was obtained in a yield of 46% using the same procedure for making compound **24**. ^{1}H NMR (300 MHz, D_2O): δ 5.43-5.03 (2H), 3.2-1.15 (m, 27H). MS (ESI): m/z 438 $(M+H)^+$. HRMS (m/z): Calcd for $(C_{18}H_{33}NO_7P_2+H)^+$ 438.1805, found 438.1807.

(2*S*)-Ethyl 2-(2-(diethoxyphosphoryl)-2-(((*E*)-4,8-dimethylnona-3,7-dienyl) (ethoxy)-phosphoryl)ethylamino)-3-phenylpropanoate (17). The product was obtained in a yield of 93%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.4-7.1 (m, 5H), 5.23-5.03 (2H), 4.25-3.65 (m, 9H), 3.2-2.55 (m, 4H), 2.2-1.49 (m, 18H), 1.42-1.05 (m, 12H). MS (ESI): m/z 560 $(M+H)^+$.

(2*S*)-2-(2-(((*E*)-4,8-Dimethylnona-3,7-dienyl)oxidophosphoryl)-2-phosphonatoethylamino)-3-phenylpropanoate (33). The product was obtained in a yield of 53% using the same procedure for making compound **24**. ^{1}H NMR (300 MHz, D_2O): δ 7.4-7.1 (m, 5H), 5.43-5.01 (2H), 4.2-2.45 (m, 5H), 3.2-1.15 (m, 18H). MS (ESI): m/z 488 $(M+H)^+$. HRMS (m/z): Calcd for $(C_{22}H_{35}NO_7P_2+H)^+$ 488.1962, found 488.1965.

(2*S*)-Ethyl 2-(2-(diethoxyphosphoryl)-2-(((*E*)-4,8-dimethylnona-3,7-dienyl) (ethoxy) phosphoryl)ethylamino)-3-(4-hydroxyphenyl)propanoate (18). The product was obtained in a yield of 95%**.** ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.4-6.8 (m, 4H), 5.43-5.03 (3H), 4.25-3.65 (m, 9H), 3.21-2.45 (m, 4H), 2.2-1.48 (m, 18H), 1.41-1.12 (m, 12H). MS (ESI): m/z 616 $(M+H)^+$.

(2*S*)-2-(2-(((*E*)-4,8-Dimethylnona-3,7-dienyl)oxidophosphoryl)-2-phosphonatoethylamino)-3-(4-hydroxyphenyl)propanoate (34). The product was obtained in a yield of 45% using the same procedure

for making compound **24**. ^{1}H NMR (300 MHz, D_2O): δ 7.5-6.8 (m, 4H), 5.45-5.02 (3H), 4.15-2.43 (m, 5H), 3.21-1.25 (m, 18H).

Compound 19. The product was obtained in a yield of 65%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.45-5.01 (2H), 4.25-3.65 (m, 7H), 3.31-2.41 (m, 4H), 2.23-1.05 (m, 31H).

(2*S*)-2-(2-(((*E*)-4,8-Dimethylnona-3,7-dienyl)oxidophosphoryl)-2-(hydrogen-phosphonato)-ethylamino)-5-guanidinopentanoate (35). The product was obtained in a yield of 40% using the same procedure for making compound **24**. ^{1}H NMR (300 MHz, D_2O): δ 5.55-5.05 (2H), 4.35-2.55 (m, 6H), 2.22-1.05 (m, 22H).

(2*S*)-Ethyl 2-(2-(diethoxyphosphoryl)-2-(((*E*)-4,8-dimethylnona-3,7-dienyl) (ethoxy) phosphoryl)ethylamino)-3-(1H-imidazol-4-yl)propanoate (20). The product was obtained in a yield of 90%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.65-6.61 (2H), 5.42-5.03 (2H), 4.85-3.95 (m, 9H), 3.81-2.25 (m, 4H), 2.25-1.42 (m, 18H), 1.41-1.05 (m, 12H). MS (ESI): m/z 690 $(M+H)^+$.

(2*S*)-2-(2-(((*E*)-4,8-Dimethylnona-3,7-dienyl)oxidophosphoryl)-2-phosphonato ethylamino)-3-(1H-imidazol-4-yl)propanoate (36). The product was obtained in a yield of 47% using the same procedure for making compound **24**. ^{1}H NMR (300 MHz, D_2O): δ 7.63-6.63 (2H), 5.3-5.03 (2H), 4.05-2.45 (m, 4H), 2.22-1.05 (m, 18H).

(2*S*)-Diethyl 2-(2-(diethoxyphosphoryl)-2-(((*E*)-4,8-dimethylnona-3,7-dienyl) (ethoxy)-phosphoryl)ethylamino)succinate (21). The product was obtained in a yield of 80%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.38-5.05 (2H), 4.35-3.61 (m, 11H), 2.85-2.45 (m, 3H), 2.21-1.42 (m, 19H), 1.41-1.15 (m, 15H). MS (ESI): m/z 596 $(M+H)^+$.

(2*S*)-2-(2-(((*E*)-4,8-Dimethylnona-3,7-dienyl)oxidophosphoryl)-2-phosphonato ethylamino)-succinate (37). The product was obtained in a yield of 71% using the same procedure for making compound **24**. ^{1}H NMR (300 MHz, D_2O): δ 5.39-5.07 (2H), 3.65-2.31 (m, 5H), 2.23-1.13 (m, 18H). MS (ESI): m/z 456 $(M+H)^+$. HRMS (m/z): Calcd for $(C_{17}H_{31}NO_9P_2+H)^+$ 456.1547, found 456.1549.

(2*S*)-Diethyl 2-(2-(diethoxyphosphoryl)-2-(((*E*)-4,8-dimethylnona-3,7-dienyl) (ethoxy)-phosphoryl)ethylamino)pentanedioate (22). The product was obtained in a yield of 90%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.35-5.15 (2H), 4.35-4.01 (m, 10H), 3.75-2.25 (m, 7H), 2.20-1.45 (m, 16H), 1.40-1.21 (m, 17H). MS (ESI): m/z 610 $(M+H)^+$.

(2*S*)-2-(2-(((*E*)-4,8-Dimethylnona-3,7-dienyl)oxidophosphoryl)-2-phosphonato ethylamino)-pentanedioate (38). The product was obtained in a yield of 69% using the same procedure for making compound **24**. ^{1}H NMR (300 MHz, D_2O): δ 5.32-5.12 (2H), 3.25-2.25 (m, 5H), 2.20-1.15 (m, 20H). MS (ESI): m/z 470 $(M+H)^+$. HRMS (m/z): Calcd for $(C_{18}H_{33}NO_9P_2+H)^+$ 470.1703, found 470.1706.

Development of Colorimetric FPPS Assay Method

To avoid using radiochemical FPPS assays, the procedures were developed based on the detection of PPi or Pi, the byproduct of FPPS catalyzed reaction. The FPPS activity is indexed with the amount of PPi or Pi. It was found that the PPi or Pi can react rapidly and quantitatively with ammonium heptamolybdate, resulting in the formation of a PPi-molybdate complex or a Pi-molybdate complex ($PMo_{12}MoO_{40}^{3-}$) under acidic conditions. These colorless complexes can be converted to a stable colored molybdate blue complexes by the addition of reducing agents (bisulfate reagent and 2-mercaptoethanol) (Figure S2 and S3).

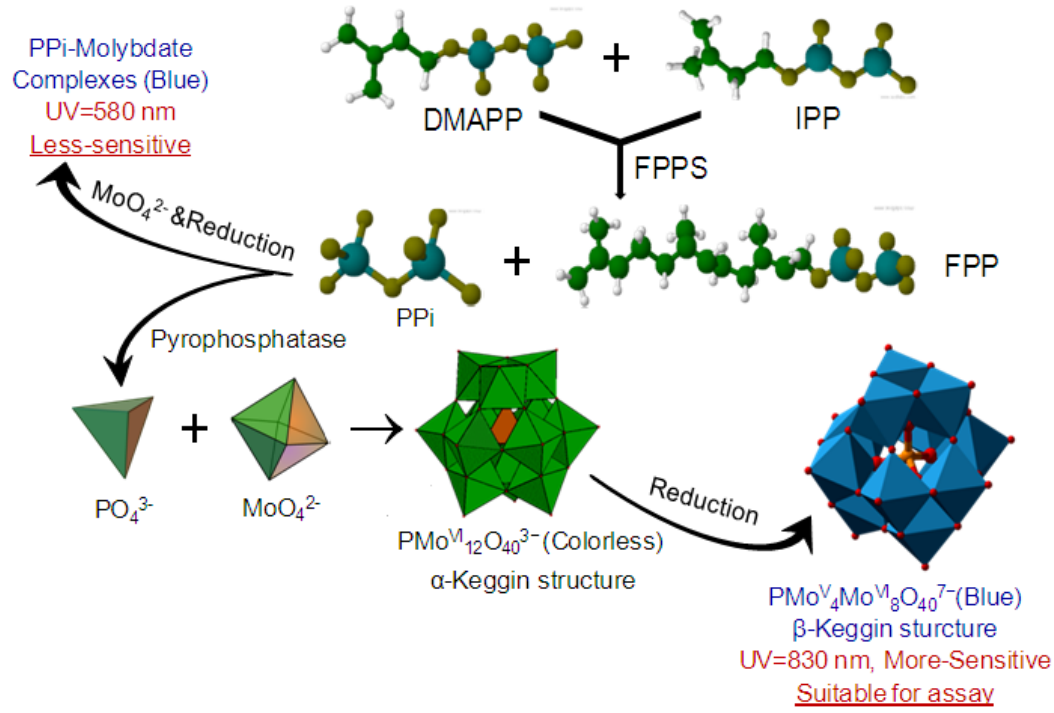


Figure S2. Strategies for assaying FPPS activity through determination of PPi or Pi concentration. PPi or Pi can react rapidly and quantitatively with ammonium heptamolybdate, resulting in the formation of a PPi-molybdate complex or a Pi-molybdate complex ($PMo_{12}MoO_{40}^{3-}$) under acidic conditions. These colorless complexes can be converted to stable colored molybdate blue complexes through addition of reducing agent 2-mercaptoethanol. The colored product can be monitored using spectrophotometer.

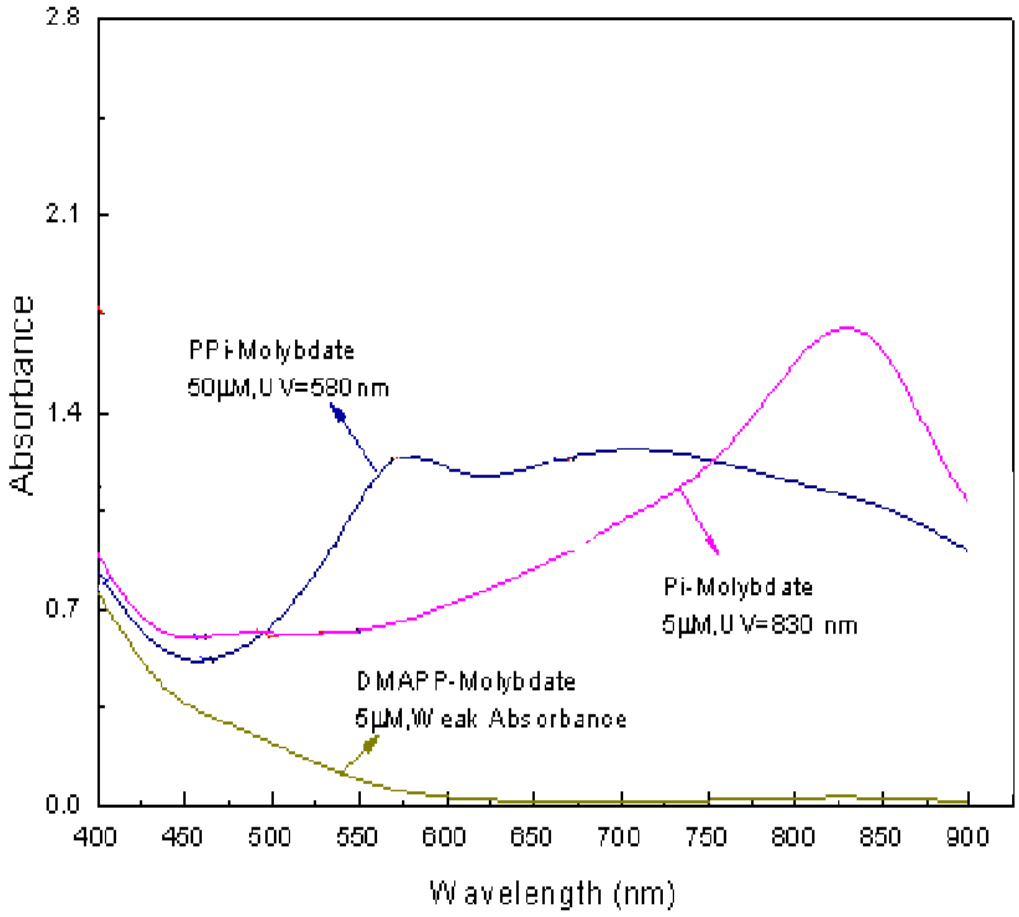


Figure S3. Absorbance spectra of the complexes formed by Pi, PPi and DMAPP reacting with molybdate reagent A: 5 μM of DMAPP was reacted with molybdate reagent followed with reduction to form DMAPP-molybdenum blue; B: 50 μM PPi was reacted with molybdate reagent followed with reduction to form PPi-molybdenum blue; C: 5 μM Pi was reacted with molybdate reagent followed with reduction to form Pi-molybdenum blue.

The colored product can be monitored using UV/Vis spectrophotometer. The amount of PPi or Pi generated in the reaction can be determined by constructing standard curves, which reflects the activity of FPPS. The standard curves were constructed using Na_2HPO_4 as the source of Pi and $Na_2P_2O_7$ as the source of PPi (Figure S4). The PPi forms a colored PPi-molybdate complex that is reduced with bisulfate salts and 2-mercaptoethanol to give the peak absorption at 580 nm (Figure S3).

Another method is based on the use of the enzyme inorganic pyrophosphatase to hydrolyze PPi to Pi. Then Pi and molybdate also react in strong acidic condition to form Pi-molybdate ($PMo_{12}MoO_{40}^{3-}$), which is then reduced with bisulfate salts and 2-mercaptoethanol to give molybdenum blue ($PMo_{12}MoO_{40}^{7-}$) with maximum absorption at 830 nm (Figure S3). Based on the slopes of the standard

curves in Figure S4, comparison of the PPi-molybdate (absorbance at 580 nm) and Pi-molybdate complex (absorbance at 830) shows that the detection of one molecule of Pi is 8 times more sensitive than the detection of one molecule of PPi (Figure S3), producing very low background and having the best color stability without the affection of the substrate DMAPP (Figure 3A). Therefore, the Pi-molybdate assay has advantage over the other colorimetric phosphate assay methods.

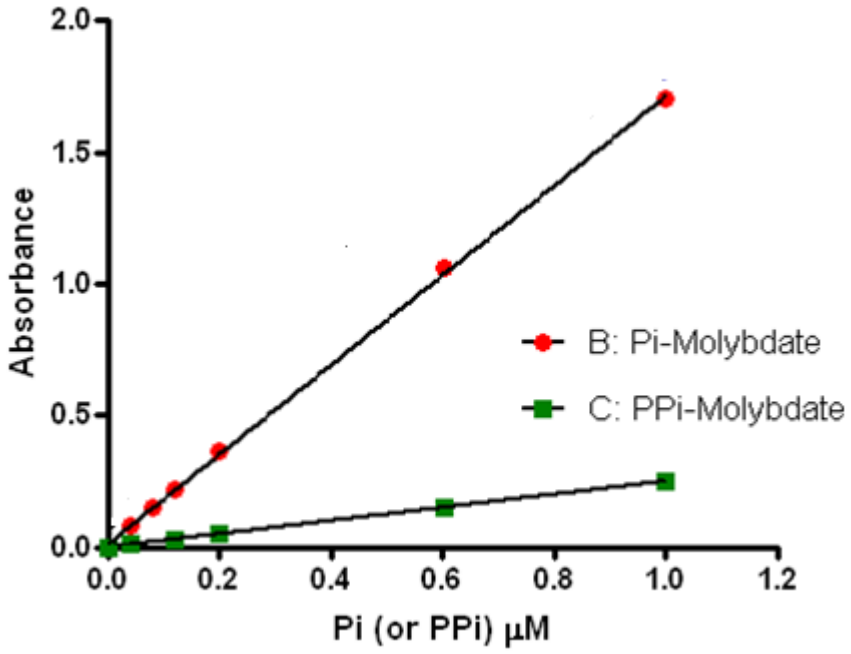


Figure S4. Calibration curve for the determination of PPi or Pi. Comparison of the sensitivities of analysis methods based on the formation of Pi-molybdate, and PPi-molybdate complexes. Absorbance was measured 830 nm for the Pi-molybdate reduction method to form molybdenum blue (B), and 580 nm for the PPi-molybdate reduction method (C).

Then, the Pi-molybdate assay was used to determine kinetic parameters of FPPS by measuring phosphate concentration after hydrolyzing pyrophosphate by inorganic pyrophosphatase. The rate of FPPS was measured in the presence of DMAPP (4.4 µM) and varying IPP concentration between 2 and 15 µM, the absorbance spectra of Pi-molybdate complex formed at different IPP concentration was shown in Figure S5. K_M and V_{max} values were obtained by nonlinear regression fit of the data to Michaelis-Menten equation. The K_M value of 5.1±0.8 µM and the V_{max} of 12±1 units/mg were obtained through calculation (Figure S5, Table S2). When the concentration of IPP was kept at 8.8 µM and the

DMAPP concentration was varied between 2.2 and 18 μM, the K_M value was determined to be 7.8±0.5 μM and the V_{max} was determined to be 5.5±0.9 units/mg (Table S2).

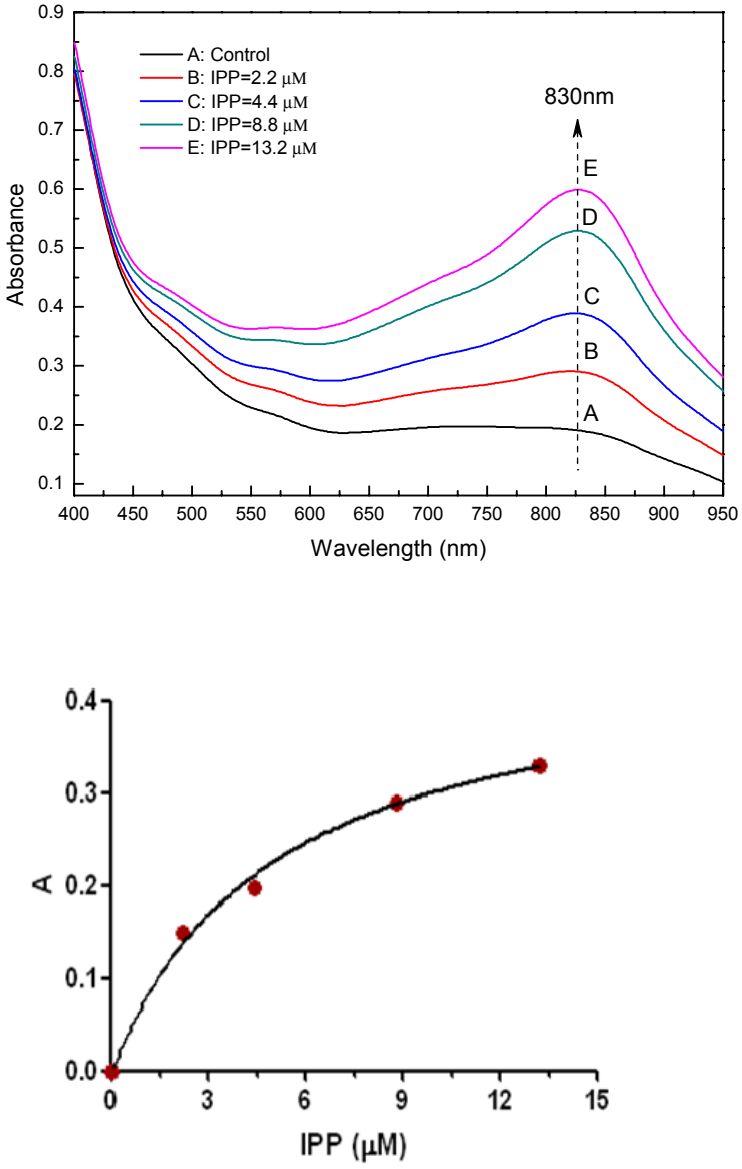


Figure S5. Absorbance spectra of Pi-molybdate complex formed in the presence of different concentration of IPP. The FPPS activity was measured as described, and the reaction rate is defined as micromoles of pyrophosphate formed per minute and per milligram of FPPS.

Table S2. Kinetic parameters for rat FPPS using colorimetric assay

Substrate	Kinetic Parameter	
IPP	K_M (μM)	5.1 ± 0.8
	V_{max} (μmol/mg/min)	12 ± 1
	k_{cat} (S^{-1})	7.2 ± 0.7
	k_{cat}/K_M (S^{-1} M^{-1})	1.4×10^6
DMAPP	K_M (μM)	7.8 ± 0.5
	V_{max} (μmol/mg/min)	5.5 ± 0.9
	k_{cat} (S^{-1})	3.3 ± 0.5
	k_{cat}/K_M (S^{-1} M^{-1})	4.2×10^5

High-Throughput Screening Assay of FPPS

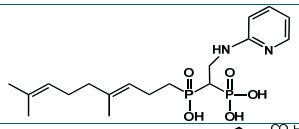
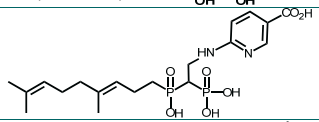
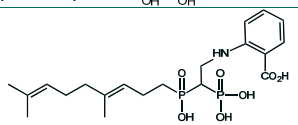
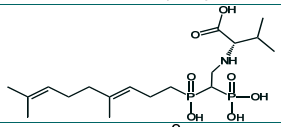
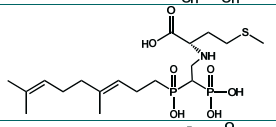
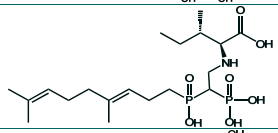
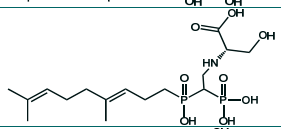
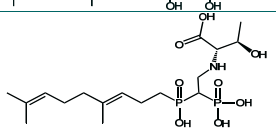
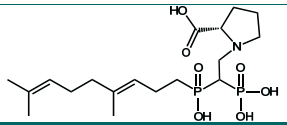
Assays were performed in flat bottom, 96-well plates. For kinetic analysis, 100 ng of pure FPPS was assayed in a final volume of 100 µl buffer, and 50 mM Tris pH 7.5, 2 mM $MgCl_2$, 1 mM DTT, 5 µg/ml BSA, and 100 µU/µl of inorganic pyrophosphatase were added to each well. The reaction was initiated by the addition of FPPS and allowed to proceed for an appropriate period at 37 °C. The enzyme was incubated with inhibitor for 10 min, and then the substrates were added to start the reaction. Assays were allowed to proceed for 5 min and terminated by the addition of 10 µl of 2.5% ammonium molybdate reagent (in 5 N H_2SO_4), 10 µl of 0.5 M 2-mercaptoethanol and 5 µl of Eikonogen reagent (0.25 g of sodium sulfite and 14.7 g of meta-bisulfite were dissolved in 100 ml water). The plates were incubated with gentle mixing on a plate shaker for 20 min. The absorbance was measured at 830 nm using a Microplate Reader. The control experiment was carried out with incubation mixture in the absence of substrate or FPPS for background deduction.

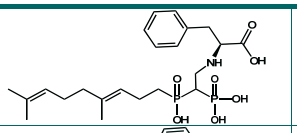
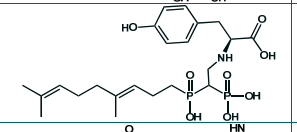
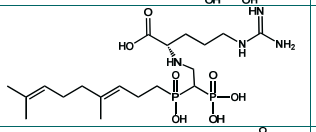
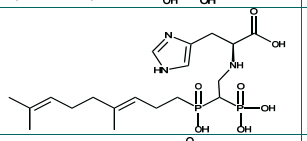
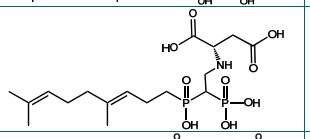
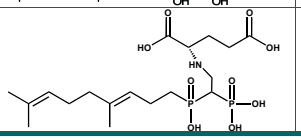
Molecular Docking

Ligand–protein docking was performed with the molecular docking algorithm MolDock [4] using the Molegro Virtual Docker version 3.0.0 software (Molegro ApS, Aarhus, Denmark,

http://www.molegro.com) according to its instruction manual. The nitrogen-containing bisphosphonate analogs (N-BPs) and the human FPPS-zoledronate-IPP complex (PDB ID: 2F8Z)) were imported into the docking program. All the bonds, hybridization and explicit hydrogens were assigned if missing, and charges and flexible torsions were made compatible with the Molegro Virtual Docker software. The molecular structure of imported ligand was manually checked before docking. Water molecules in the protein structure were excluded from the docking experiments, but metal and salt ions were retained [5,6]. The predicted binding affinity ($E_{binding}$) of the enzyme with the bisphosphonates are shown in Table S3.

Table S3. Predicted binding energy for bisphosphonates against human FPPS.[a]

Substrate analogs	*h*FPPS $E_{binding}$ (kcal/mol)
	-219.48
	-153.89
	-236.53
	-225.97
	-251.20
	-245.23
	-237.06
	-200.16
	-213.79

	-120.63
	-193.17
	-95.59
	-121.86
	-149.29
	-230.60

[a]. MolDock scoring function was used to precompute score grids for rapid dock evaluation. Potential binding sites (cavities) were detected using the grid-based cavity prediction algorithm.

Abbreviations

Abbreviations used are as follows: BP, bisphosphonate; DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl pyrophosphate; FPPS, farnesyl pyrophosphate synthase; GPP, geranyl pyrophosphate; IPP, isopentenyl pyrophosphate; IPTG, isopropyl-β-D-thiogalactopyranoside; PAGE, polyacryl amide gel electrophoresis; PCR, polymerase chain reaction; Pi, monophosphate; PPi, pyrophosphate; SDS, sodium dodecylsulfate; UV/Vis, ultraviolet-visible spectroscopy.

References

[1] Marrero, P. F.; Poulter, C. D.; Edwards, P. A. Effects of site-directed mutagenesis of the highly conserved aspartate residues in domain II of farnesyl diphosphate synthase activity. *J. Biol. Chem.* **1992**, *267*, 21873-21878.

[2] Joly, A.; Edwards, P. A. Effect of site-directed mutagenesis of conserved aspartate and arginine residues upon farnesyl diphosphate synthase activity. *J Biol Chem.* **1993**, *268*, 26983-26989.

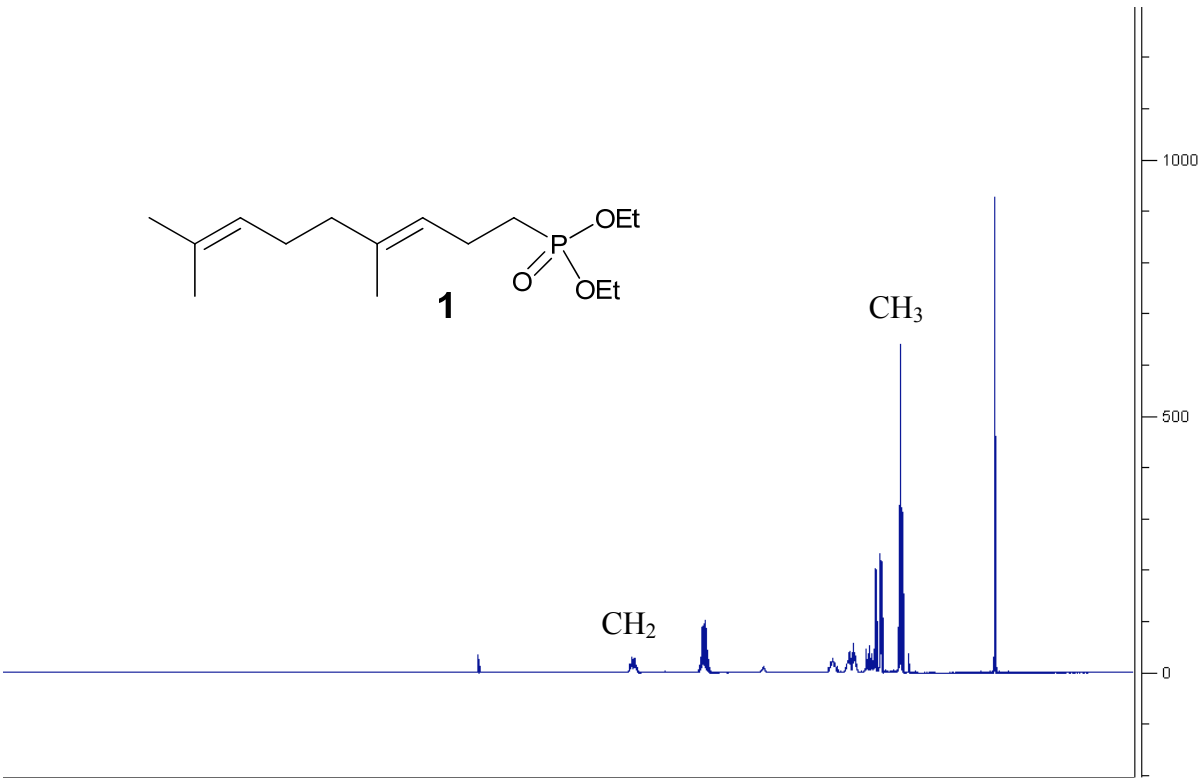
[3] Fernandez, S. M. S.; Kellogg, B. A.; Poulter, C. D. Farnesyl diphosphate synthase. Altering the catalytic site to select for geranyl diphosphate activity. *Biochemistry* **2000**, *39*, 15316-15321.

[4] Thomsen, R.; Christensen, M. H. MolDock: a new technique for high-accuracy molecular docking. *J Med Chem* **2006**, *49*, 3315-3321.

[5] Mao, J.; Mukherjee, S.; Zhang, Y.; Cao, R.; Sanders, J. M. et al. Solid-state NMR, crystallographic, and computational investigation of bisphosphonates and farnesyl diphosphate synthase-bisphosphonate complexes. *J Am Chem Soc* **2006**, *128*, 14485-14497.

[6] Celik, L.; Sinning, S.; Severinsen, K.; Hansen, C. G.; Moller, M. S. et al. Binding of serotonin to the human serotonin transporter. Molecular modeling and experimental validation. *J Am Chem Soc* **2008**, *130*, 3853-3865.

NMR Spectra of some compounds:



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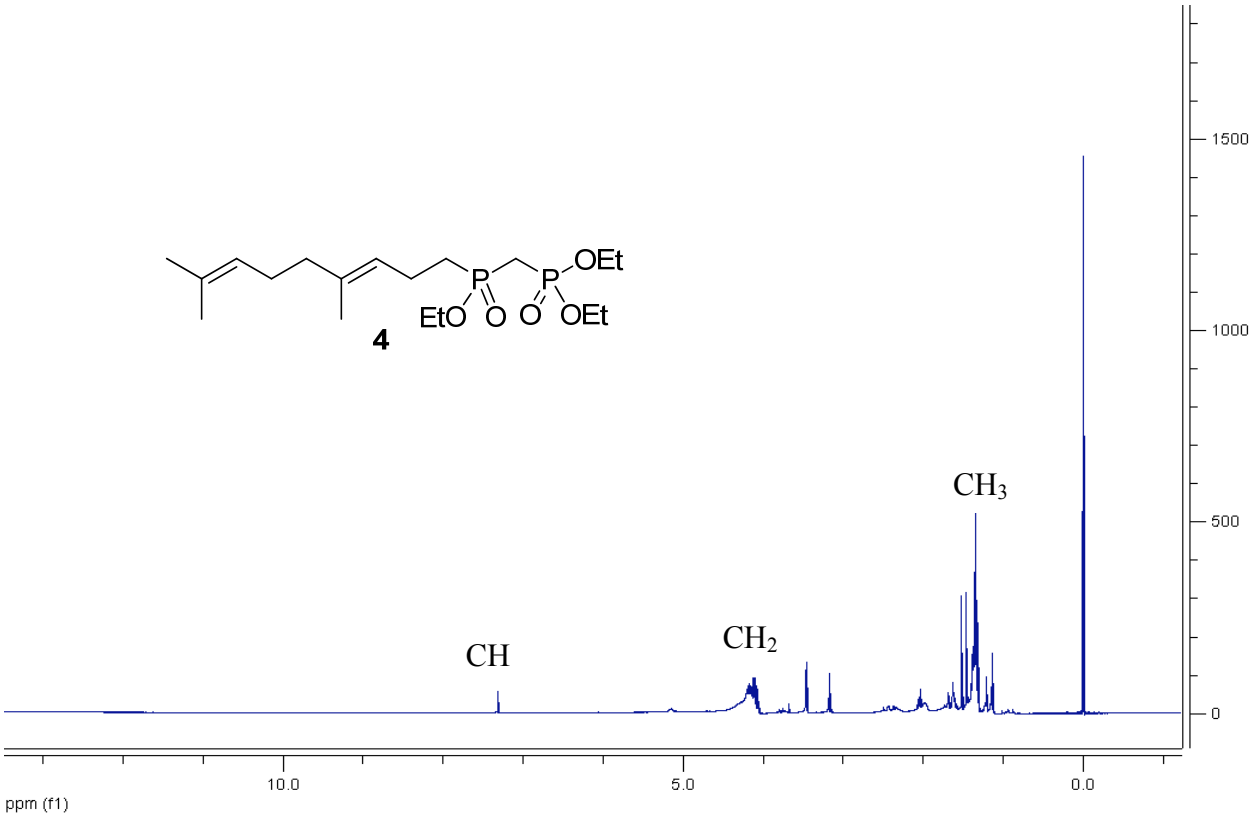

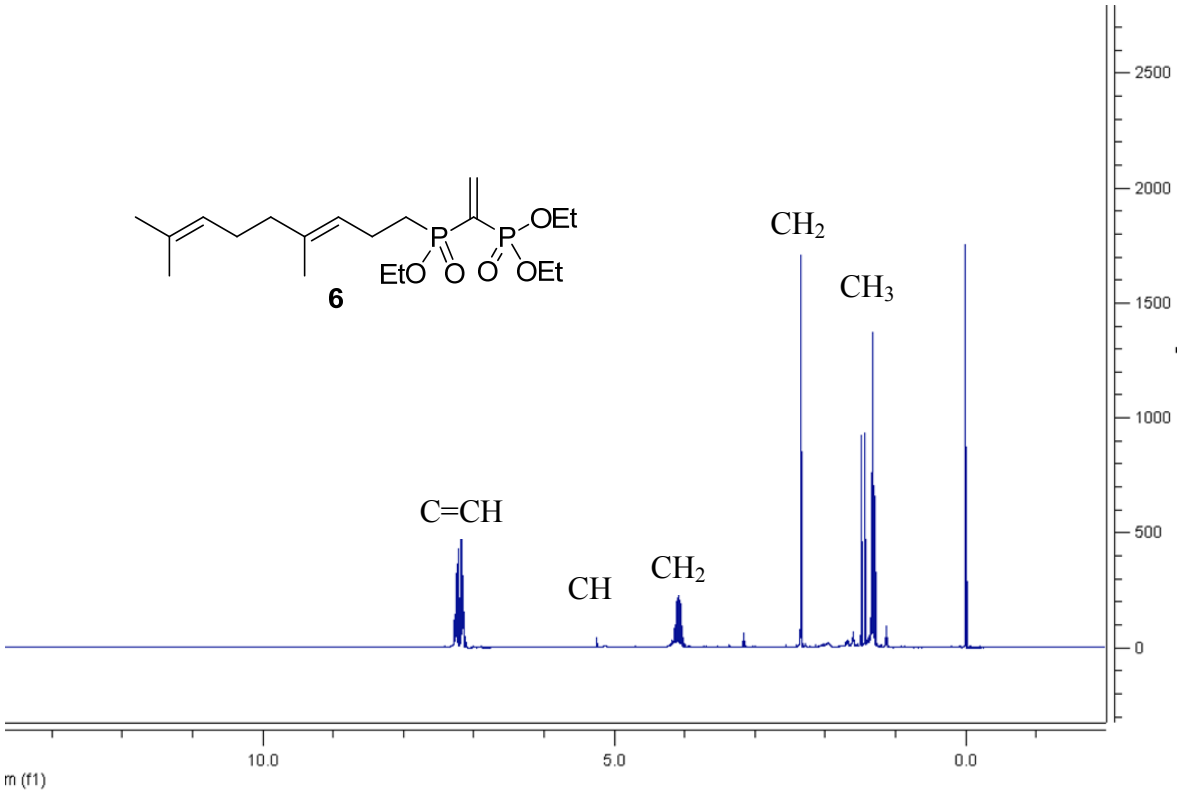
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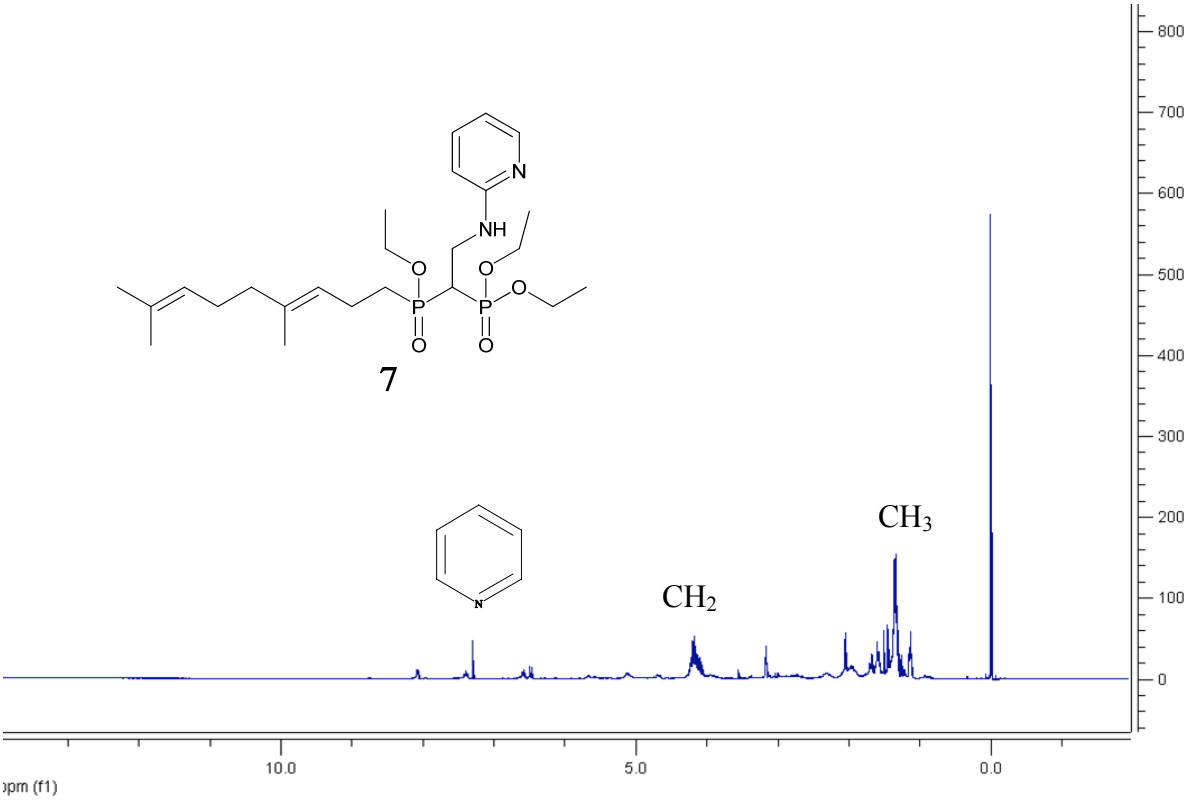
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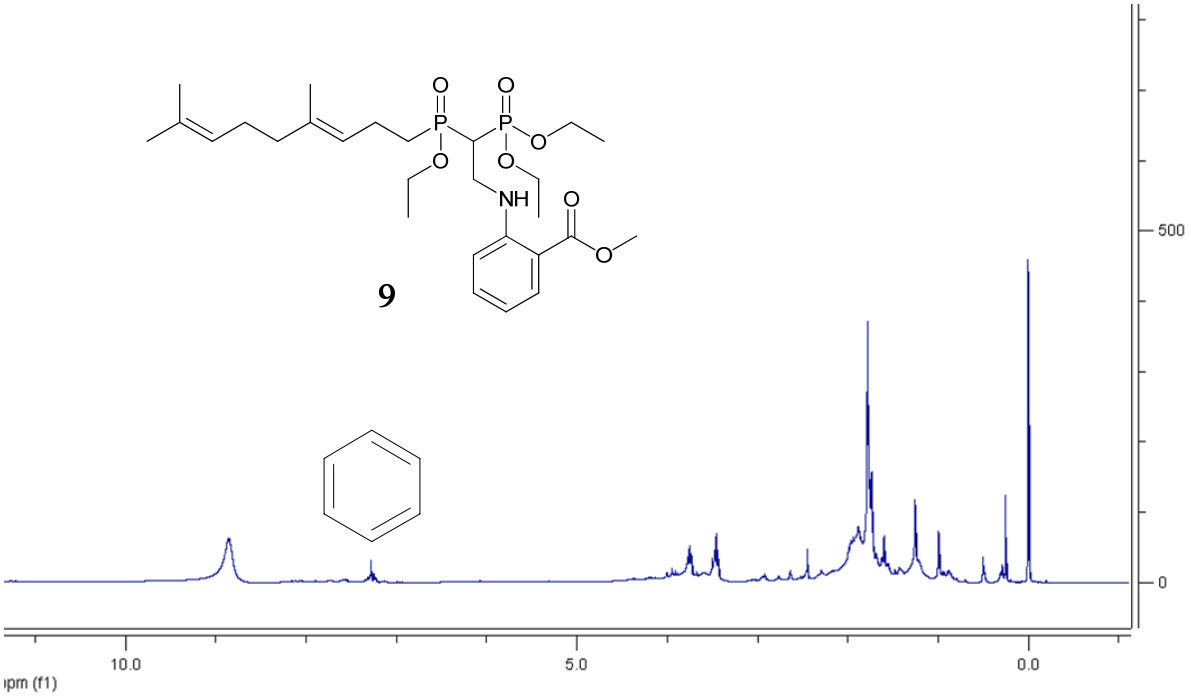
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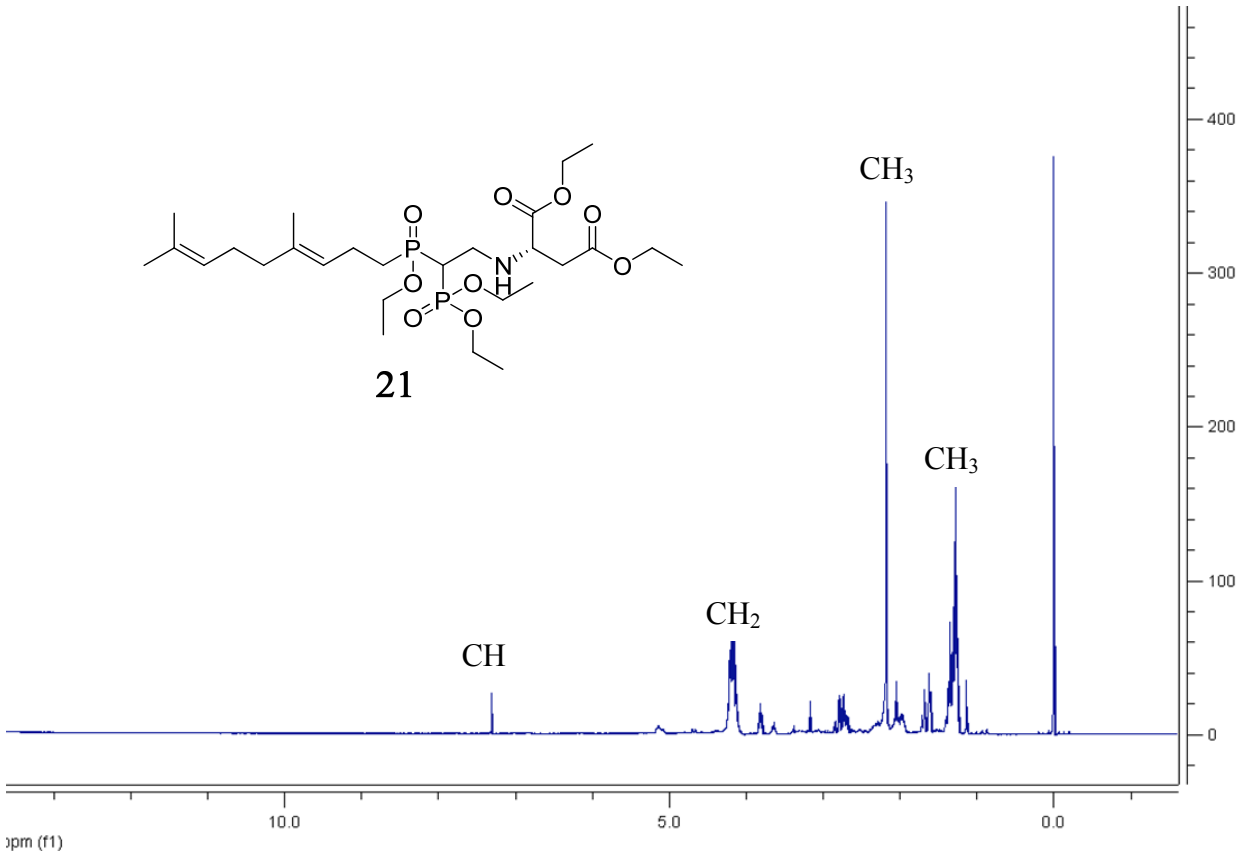
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