Supplementary Information for:

Mechanistic studies of an unprecedented enzyme-catalyzed 1,2-phosphono migration reaction

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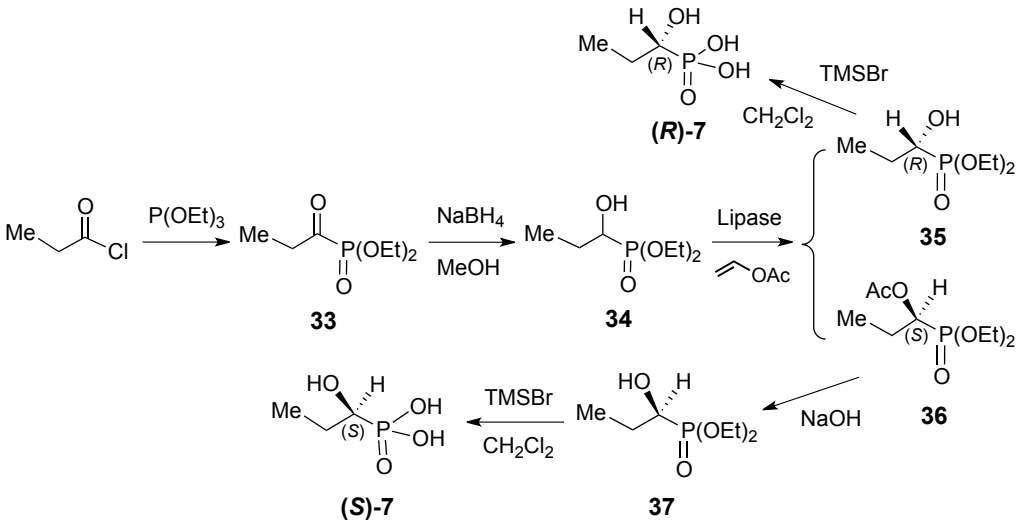
CONTENTS

S0. GENERAL

NMR spectra were recorded on a Varian 400, 500 or 600 MHz spectrometer at the Nuclear Magnetic Resonance Facility of the Department of Chemistry and Biochemistry, The University of Texas at Austin. Chemical shifts (δ in ppm) are given relative to that of solvent ($CDCl_3$, DMSO, or D_2O), with coupling constants reported in Hertz (Hz). Analytical thin layer chromatography (TLC) was carried out on pre-coated TLC aluminum plate (silica gel, grade 60, F_{254}, 0.25 mm layer thickness) acquired from EMD Chemicals. Flash column chromatography was performed using silica gel (230-400 mesh, grade 60) obtained from Sorbent Technologies. Mass analysis was conducted at the Mass Spectrometry and Proteomics Facility of the Department of Chemistry and Biochemistry, The University of Texas at Austin. Iron(II) ammonium sulfate ($Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$) and other reagents were purchased from Sigma-Aldrich (St. Louis, MO). All reagents were used directly as obtained from commercial sources unless otherwise noted. HppE was purified as previously described (*1*). Protein concentrations were determined by Bradford assay (*2*) using bovine serum albumin as the standard. The relative molecular mass and purity of enzyme samples were determined using SDS-polyacrylamide gel electrophoresis.

S1. PREPARATION OF 1-HYDROXYPROPYLPHOSPHONIC ACIDS

S1.1. Preparation of (S)-1-Hydroxypropylphosphonic Acid ((S)-7) and (R)-1-Hydroxypropylphosphonic Acid ((R)-7)



*Diethyl Propionylphosphonate (***33***)* (*3, 4*). Triethyl phosphite (16.6 g, 100 mmol) was slowly added to propionyl chloride (23.1 g, 250 mmol) at room temperature using an additional funnel. After addition, the reaction was stirred for 24 hr. Excess propionyl chloride was removed *in vacuo*, and the crude product (80%) was used in the next step without further purification. The ^{1}H and ^{31}P NMR spectra of **33** are consistent with those reported in the literature (*5*).

*Diethyl 1-Hydroxypropylphosphonate (***34***)*. $NaBH_4$ (380 mg, 10 mmol) was added to a solution of **33** (1.94 g, 10 mmol) in 30 mL of MeOH at room temperature, and the solution was stirred for 2 hr. The reaction mixture was quenched by the addition of acetone (5 mL) and extracted using ethyl acetate (30 mL × 3) and water (20 mL). The combined organic layers were washed with brine (40 mL), dried over $MgSO_4$, and concentrated under reduced pressure to afford **34** as a colorless oil (84%). ^{1}H NMR ($CDCl_3$) δ 4.16 (q, *J* = 7.0 Hz, 4 H), 3.76 (m, 1 H), 3.40 (br, 1 H, exchangable), 1.81 (m, 1 H), 1.70 (m, 1 H), 1.33 (t, *J* = 7.0 Hz, 6 H), 1.06 (t, *J* = 7.4 Hz, 3 H); ^{31}P NMR ($CDCl_3$) δ 26.3; ^{13}C NMR ($CDCl_3$) δ 68.7 (d, *J* = 160.0 Hz), 62.1 (d, *J* = 6.9 Hz), 62.0 (d, *J* = 7.2 Hz), 24.4 (d, *J* = 2.0 Hz), 16.1 (d, *J* = 1.5 Hz), 10.1 (d, *J* = 13.7 Hz).

*Diethyl (R)-1-Hydroxypropylphosphonate (***35***) and Diethyl (S)-1-Acetoxypropylphosphonate (***36***)*. Lipase AK (4.0 g) (purchased from Amano Enzyme Inc.) and vinyl acetate (50 mL) were added to a solution of **34** (3.92 g, 20 mmol) in 50 mL diisopropyl ether. The reaction was stirred under room temperature and monitored by ^{1}H and ^{31}P NMR. Once the reaction reached roughly 50% conversion (~ 96 hr), the reaction mixture was filtered through a celite pad. Concentration under reduced pressure gave an oily residue that was purified by flash chromatography on silica gel with hexanes/ethyl acetate/Et_3N (20:20:1) as the eluting solvent to give **35** (45%) and **36** (45%) as colorless oil. The enantiomeric excess (e.e.)

of **35** was determined to be 90% (Fig. S1) (*6, 7*) and its ^{1}H NMR and ^{31}P NMR spectra are identical to those of **34**. Compound **36**: ^{1}H NMR ($CDCl_3$) δ 5.00 (m, 1 H), 3.98 (m, 4 H), 1.95 (s, 3 H), 1.77 (m, 1 H), 1.64 (m, 1 H), 1.16 (t, *J* = 7.2 Hz, 3 H), 1.15 (t, *J* = 7.2 Hz, 3 H), 0.80 (t, *J* = 7.3 Hz, 3 H). ^{31}P NMR ($CDCl_3$) δ 20.6. ^{13}C NMR ($CDCl_3$) δ 169.4 (d, *J* = 6.0 Hz), 68.8 (d, *J* = 166.8 Hz), 62.3 (d, *J* = 7.1 Hz), 62.1 (d, *J* = 6.8 Hz), 22.5, 20.3, 16.8 (d, *J* = 5.6 Hz), 16.0 (d, *J* = 5.9 Hz), 9.8 (d, *J* = 12.3 Hz).

*Diethyl (S)-1-Hydroxypropylphosphonate (***37***).* NaOH (1 M, 10 mL) was added to a solution of **36** (1.19 g, 5 mmol) in 10 mL of water at 0 °C. After 48 hr, the reaction was quenched by the addition of 10 mL of 1 M K_2HPO_4 and partitioned between ethyl acetate (15 mL × 3) and water. The organic layers were collected, dried over $MgSO_4$, and concentrated under reduced pressure to afford **37** as a colorless oil (90%). The ^{1}H, ^{31}P, and ^{13}C NMR spectra of **37** are identical to those of **34**. The enantiomeric excess (e.e.) of **37** was determined to be 88% (Fig. S1) (*6, 7*).

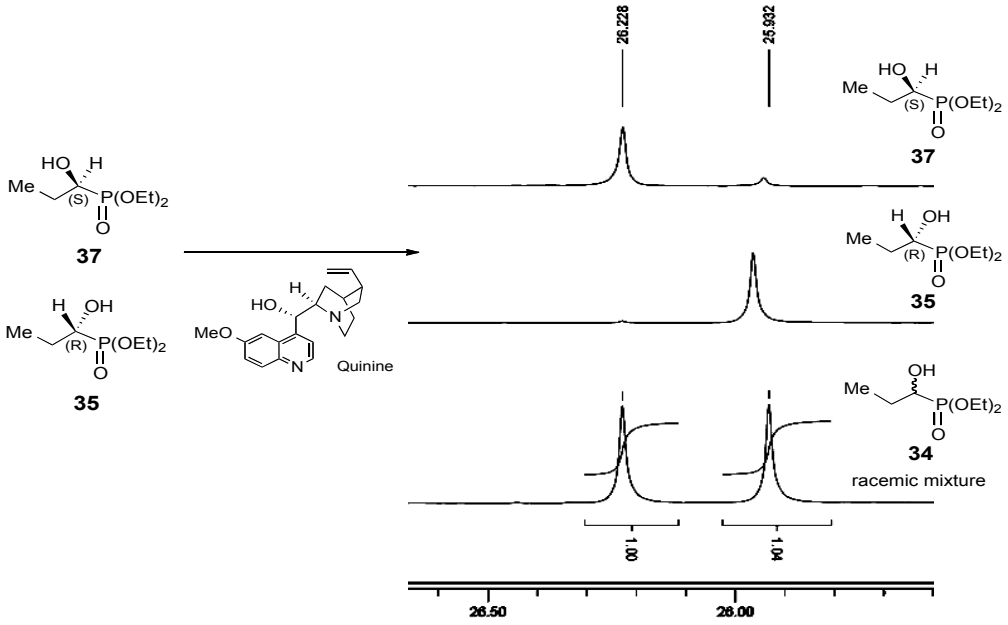


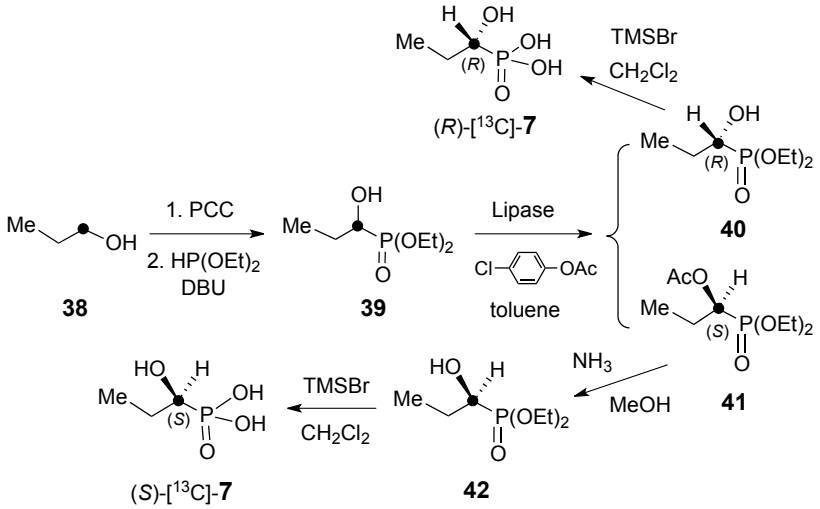
Figure S1. Determination of the enantiomeric excess (e.e.) of diethyl (*R*)-1-hydroxypropylphosphonate (35) and diethyl (*S*)-1-hydroxypropylphosphonate (37). A ^{31}P NMR method utilizing quinine as a chiral solvating agent (*6*, *7*) was used to determine the e.e. values of **35** (90%) and **37** (88%).

*(S)-1-Hydroxypropylphosphonic Acid ((S)-***7***).* TMSBr (1.53 g, 10 mmol) was added to a solution of **37** (392 mg, 2 mmol) in CH_2Cl_2 (30 mL) at room temperature, and the solution was stirred overnight. Solvent was removed under reduced pressure. The residue was dissolved in $CHCl_3$ (20 mL) and extracted with 30 mL of a 0.2 M ammonium acetate aqueous solution. The aqueous layer was collected and lyophilized to afford (*S*)-**7** as a white solid (83%). ^{1}H NMR (D_2O) δ 3.38 (m,

1 H), 1.55 (m, 1 H), 1.30 (m, 1 H), 0.77 (t, J = 7.3 Hz, 3 H). ^{31}P NMR (D_2O) δ 19.6; ^{13}C NMR (D_2O) δ 71.3 (d, J = 155.0 Hz), 25.0 (d, J = 1.8 Hz), 10.6 (d, J = 13.1 Hz).

*(R)-1-Hydroxypropylphosphonic Acid ((R)-**7**).* (*R*)-**7** was obtained from **35** in 85% yield following the same procedure for the synthesis of (*S*)-**7**. The ^{1}H, ^{31}P and ^{13}C NMR spectra of (*R*)-**7** are identical to those of (*S*)-**7**.

S1.2. Preparation of (R)-[1-^{13}C]-1-Hydroxypropylphosphonic Acid ((R)-[^{13}C]-7) and (S)-[1-^{13}C]-1-Hydroxypropylphosphonic Acid ((S)-[^{13}C]-7)



*Diethyl [1-^{13}C]-1-Hydroxypropylphosphonate (**39**).* To a solution of [1-^{13}C]-1-propanol (**38**, 0.2 g, 3.3 mmol,) in dry CH_2Cl_2 (30 mL) was added pyridinium chlorochromate (PCC, 0.75 g, 3.5 mmol). The resulting mixture was stirred for 2 hr at room temperature, and then distilled at 60 °C. The collected distillate was treated with diethyl phosphite (0.48 g, 3.5 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (0.53 g, 3.5 mmol), followed by stirring for 3 hr at room temperature. The solution was diluted with CH_2Cl_2 (10 mL), washed with 1 N HCl, dried, and concentrated under reduced pressure. The residue was purified by flash chromatography (hexanes/ethyl acetate/Et_3N (20:20:1)) to afford **39** as a colorless oil in 53% yield. ^{1}H NMR ($CDCl_3$) δ 4.69 (br, 1 H, exchangable), 4.08 (q, J = 7.0 Hz, 4 H), 3.68 (m, 1 H), 1.72 (m, 2 H), 1.25 (t, J = 7.0 Hz, 6 H), 0.98 (t, J = 7.4 Hz, 3 H); ^{31}P NMR (D_2O) δ 26.6; ^{13}C NMR (D_2O) δ 68.7 (d, J = 160.0 Hz), 62.1 (d, J = 7.6 Hz), 24.4 (d, J = 2.0 Hz), 16.1 (d, J = 1.5 Hz), 10.1 (d, J = 13.7 Hz).

*Diethyl (R)-[1-^{13}C]-1-Hydroxypropylphosphonate (**40**) and Diethyl (S)-[1-^{13}C]-1-Acetoxypropylphosphonate (**41**).* A mixture of **39** (0.3 g, 1.5 mmol), *p*-chlorophenyl acetate (1.7 g, 10 mmol), lipase PS (0.5 g) in toluene (50 mL) was shaken for 10 days at 30 °C (*8*). The mixture was filtered through a short Celite column, and the filtrate was concentrated under reduced pressure. The residue was flash chromatographed on silica gel to give **40** and **41** as colorless oil in 42 and 45%

yield, respectively. The ^{1}H, ^{31}P and ^{13}C NMR spectra of **40** are identical to those of **39**. Compound **41**: ^{1}H NMR ($CDCl_3$) δ 5.09 (m, 1 H), 4.06 (q, *J* = 7.2 Hz, 4 H), 1.95 (s, 3 H), 1.81 (m, 2 H), 1.15 (t, *J* = 7.2 Hz, 6 H), 0.89 (t, *J* = 7.2 Hz, 3 H); ^{31}P NMR (D_2O) δ 21.9; ^{13}C NMR (D_2O) δ 169.8, 68.9 (d, *J* = 160.0 Hz), 62.5 (d, *J* = 7.6 Hz), 22.6, 20.5, 16.3 (d, *J* = 7.2 Hz), 10.0 (d, *J* = 12.7 Hz).

*(R)-[1-^{13}C]-1-Hydroxypropylphosphonic Acid ((R)-[1-^{13}C]-**7**).* A solution of compound **40** (0.14 g, 0.7 mmol), TMSBr (0.40 g, 2.6 mmol) and allyl trimethylsilane (0.20 g, 1.7 mmol) in 4 mL of CH_2Cl_2 was stirred for 25 hr at room temperature. The solution was concentrated under reduced pressure, and the residue was vigorously stirred with 5 mL of water for 10 min. The aqueous solution was neutralized with ammonium bicarbonate, washed with $CHCl_3$, and lyophilized to give (*R*)-[1-^{13}C]-**7** as a white powder in 92% yield. ^{1}H NMR ($CDCl_3$) δ 3.16 (dm, *J* = 137.6 Hz, 1 H), 1.40-1.50 (m, 2 H), 0.75 (t, *J* = 7.3 Hz, 3 H); ^{31}P NMR (D_2O) δ 19.2; ^{13}C NMR (D_2O) δ 72.5 (d, *J* = 155.0 Hz), 25.3 (d, *J* = 1.8 Hz), 10.9 (d, *J* = 13.1 Hz); High resolution CIMS (NH_3) calcd for $C_2{}^{13}C_1H_{10}O_4P$ $(M+H)^+$ 142.0350, found 142.0347.

*Diethyl (S)-[1-^{13}C]-1-Hydroxypropylphosphonate (**42**).* Compound **41** (0.15 g, 0.6 mmol) was dissolved in 30 mL of 2 M NH_3 in methanol (60 mmol) and the mixture was stirred for 24 hr at room temperature. The solvent was removed under reduced pressure, and the residue was purified by flash chromatography using hexanes/ethyl acetate = 1/1 with 5% Et_3N as the eluent to give **42** as a colorless oil in 87% yield. Its spectral characteristics are identical to those of compound **40**.

*(S)-[1-^{13}C]-1-Hydroxypropylphosphonic Acid ((S)-[1-^{13}C]-**7**).* Preparation of this compound followed the same procedure used for the synthesis of (*R*)-[1-^{13}C]-**7** to afford (*S*)-[1-^{13}C]-**7** in 94% yield. The ^{1}H, ^{31}P and ^{13}C NMR spectra of (*R*)-[1-^{13}C]-**7** are identical to those of (*S*)-[1-^{13}C]-**7**. High resolution CIMS (NH_3) calcd for $C_2{}^{13}C_1H_{10}O_4P$ $(M+1)^+$ 142.0350, found 142.0345.

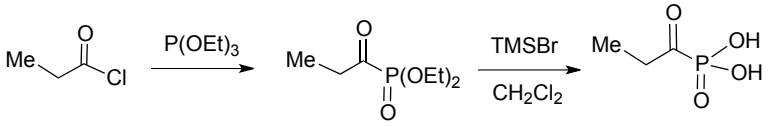
S2. Studies of Reactions of HppE with 1-Hydroxylpropylphosphonic Acids

S2.1. Incubation of (S)-[1-^{13}C]-1-Hydroxypropylphosphonic Acid ((S)-[^{13}C]-7) with HppE

The reaction mixture containing 19 mM of (*S*)-[^{13}C]-**7**, 0.2 mM of HppE, 0.2 mM of $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$, 37.5 mM NADH and 7.5 mM FMN in 5 mL of Tris·HCl buffer (pH 7.5) was shaken at room temperature for 1 hr. The enzymes were removed by filtration (PM 10 membrane, Amicon) and the filtrate was chromatographed on a P2 column using water as the eluent. Fractions containing compound **8** were combined and lyophilized. ^{1}H NMR (D_2O) δ 2.65 (dq, *J* = 72.1, 7.5 Hz, 2H), 0.81 (t, *J* = 7.0 Hz, 3H). The identity of **8** was confirmed spectroscopically by comparison to the standard, which was synthesized as described below.

S2.2. Preparation of 1-Oxo-propylphosphonic Acid Standard (8) (4, 9-11)

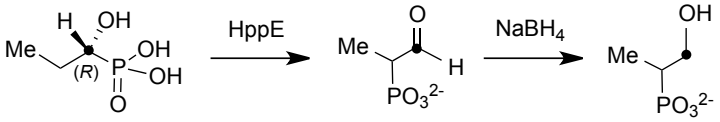
Triethylphosphite (0.8 g, 5 mmol) was added dropwise to a stirred neat propionylchloride solution (0.46 g, 5 mmol) over 10 min at room temperature. The reaction was kept at room temperature in a water bath for 4 hr. The reaction was diluted with water and extracted with CH_2Cl_2. The organic solvents were removed under reduced pressure and the residue was purified by flash chromatography using hexanes/ethyl acetate/Et_3N (20:10:1) to give **33** as colorless oil in 80% yield. ^{1}H NMR ($CDCl_3$) δ 4.14 (m, 4 H), 2.79 (q, *J* = 7.2 Hz, 2 H), 1.29 (m, 6 H), 1.02 (t, *J* = 7.2 Hz, 3 H); ^{31}P NMR ($CDCl_3$) δ -1.7; ^{13}C NMR ($CDCl_3$) δ 211.2 (d, *J* = 164.9 Hz), 63.5 (d, *J* = 7.0 Hz), 36.5 (d, *J* = 55.0 Hz), 16.1 (d, *J* = 5.4 Hz), 6.11 (d, *J* = 3.8 Hz).



33 **8**

A solution of **33** (0.28 g, 1.4 mmol), TMSBr (0.8 g, 5.2 mmol) and allyl trimethylsilane (0.2 g, 1.7 mmol) in 10 mL of CH_2Cl_2 was stirred for 25 hr at room temperature. The solution was concentrated under reduced pressure, and the residue was vigorously stirred with 5 mL of water for 10 min. The aqueous solution was neutralized with ammonium bicarbonate, washed with $CHCl_3$, and lyophilized to give **8** as a white powder in 92% yield. ^{1}H NMR (D_2O) δ 2.65 (q, *J* = 7.0 Hz, 2 H), 0.81 (t, *J* = 7.0 Hz, 3 H); ^{31}P NMR (D_2O) δ 0.4; ^{13}C NMR (D_2O) δ 221.4 (d, *J* = 169.4 Hz), 35.8 (d, *J* = 47.4 Hz), 5.7 (d, *J* = 4.1 Hz).

S2.3. Incubation of (R)-[1-^{13}C]-1-Hydroxypropylphosphonic Acid ((R)-[^{13}C]-7) with HppE



(*R*)-7 **9** **10**

The incubation was carried out under the same conditions as described above for *((S)-[^{13}C]-7)* to give [1-^{13}C]-1-formyl-ethylphosphonic acid (**9**) as the product. ^{1}H NMR (D_2O) of **9** δ 9.55 (s, 1 H), 2.82-2.92 (m, 1 H), 1.03 (dd, *J* = 14.4, 6.6 Hz, 3 H); ^{31}P NMR (D_2O) δ 14.6. This compound is relatively labile and was readily decomposed during purification.

*Conversion of **9** to [1-^{13}C]-1-(Hydroxymethyl)ethylphosphonic Acid (**10**).* The incubation was conducted as described above. The reaction was quenched by the addition of 4 equivalents of $NaBH_4$. The reaction mixture was loaded on a P2 column and the desired product was eluted with water. Fractions containing **10** were collected and lyophilization. The spectroscopic properties of **10** purified from the enzymatic mixture were identical to those of the authentic standard

synthesized using a literature procedure (*11*). ^{1}H NMR (D_2O) δ 3.35 (m, 2 H), 1.73 (m, 1 H), 1.00 (m, 3 H); ^{31}P NMR (D_2O) δ 26.1; ^{13}C NMR (D_2O) δ 63.1 (d, J = 2.7 Hz), 54.8 (d, J = 182.4 Hz), 11.4.

S2.4. Kinetics of HppE-catalyzed Reactions with (R)- and (S)-1-Hydroxypropylphosphonic Acid ((R)-7 and (S)-7)

Kinetic parameters for the HppE-catalyzed reactions with (*R*)- and (*S*)-1-HPP were determined using an HPLC assay previously developed in our laboratory (*12*). Each 50 μL assay mixture contained the following reaction components: 100 μM HppE, 100 μM $Fe(NH_4)_2(SO_4)_2$, 150 μM FMN, 16 mM NADH, and 10 mM substrate in 20 mM Tris-HCl buffer (pH 7.5). With the assumption that the substrate concentration is saturating, the observed rate constants (k_{obs}) for substrate consumption will be equal to k_{cat}. As shown in Table S1, the observed rate constants for both substrates are similar in magnitude to the HppE-catalyzed reaction with the natural substrate, (*S*)-2-HPP (*12*).

Table S1. Observed rate constants for HppE-catalyzed reactions with (*R*)- and (*S*)-isomers of 1- and 2-HPP.

Substrate	k_{obs} (min^{-1})
(*R*)-1-HPP	1.30 ± 0.03
(*S*)-1-HPP	2.08 ± 0.03
(*R*)-2-HPP[a]	1.12 ± 0.04
(*S*)-2-HPP[a]	0.46 ± 0.10

[a]Values obtained from reference 12.

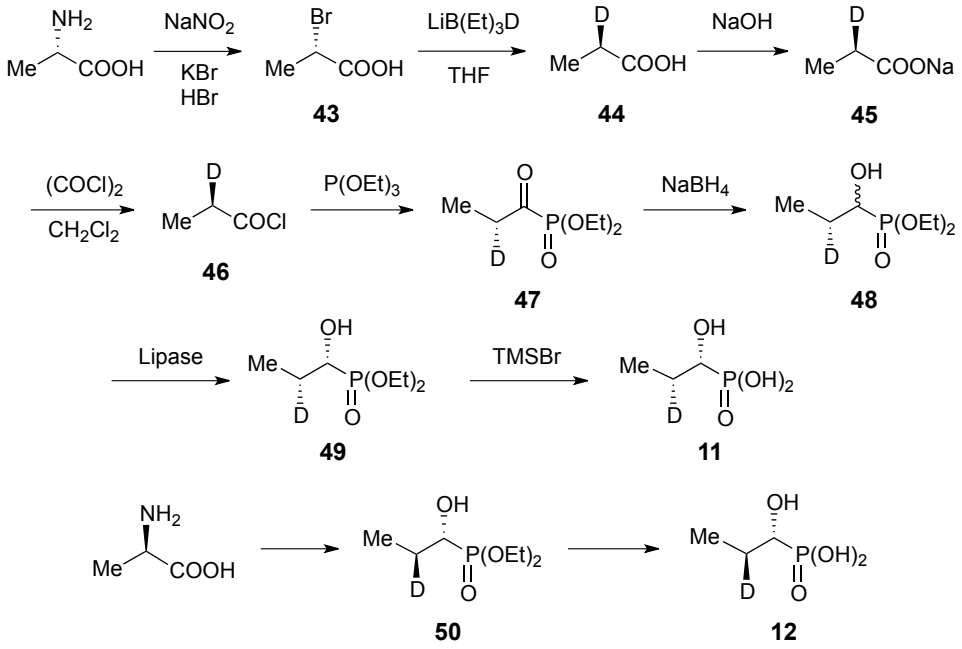
S3. Preparation of Stereospecifically Labeled 1-Hydroxylpropylphosphonic Acids

*S3.1. Preparation of (R)-1-Hydroxypropyl-(R)-[2-$^{2}H_1$]-phosphonic Acid (**11**) and (R)-1-Hydroxypropyl-(S)-[2-$^{2}H_1$]-phosphonic Acid (**12**)*

*(S)-2-Bromopropanoic Acid (**43**) (13).* L-Alanine (4.45 g, 50 mmol) was added to a solution of KBr (20.9 g, 175 mmol) in HBr (48% solution, 50 mL) at room temperature. The reaction mixture was chilled to 0 °C, $NaNO_2$ (8.30 g, 120 mmol) was added in portion, and the reaction was stirred at 0 °C for 2 hr. The reaction mixture was extracted using diethyl ether (200 mL × 4) and the combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo* to afford **43** as a light yellow oil (60%). ^{1}H NMR ($CDCl_3$) δ 4.40 (q, J = 6.8 Hz, 1 H), 1.85 (d, J = 6.8 Hz, 3 H).

*Sodium (R)-[2-$^{2}H_1$] Propionate (**45**) (13).* Compound **43** (7.89 g, 50 mmol) was added to a solution of lithium triethyldeuterioborate (104 mL, 1 M) in THF (200 mL) at 0 °C. The reaction was refluxed for 5 hr and water (30 mL) was added, followed by H_2O_2 (30%)-NaOH (1 M) (1:1, ~ 50 mL), and the mixture was stirred until no more gas was generated. The resulting solution was acidified to pH 2 with conc. H_2SO_4 and distilled under atmospheric pressure (145 °C) to afford

crude **44** as an aqueous solution. The solution was neutralized to pH 8 using NaOH (1 M) and lyophilized to afford **45** as a white solid (2 steps, 75%). ^{1}H NMR ($CDCl_3$) δ 1.96-2.03 (m, 1 H), 0.88 (d, J = 7.6 Hz, 3 H).



*Diethyl (1-Hydroxypropyl)-(R)-[2-$^2H_1$]-phosphonate (***48***).* $(COCl)_2$ (530 μL, 6.18 mmol) was slowly added to a mixture of **45** (500 mg, 5.15 mmol) and CH_2Cl_2 (20 mL) at room temperature. After stirring for 4 hr, $P(OEt)_3$ (1.8 mL, 10.3 mmol) was added to the reaction mixture at 0 °C and the reaction was stirred at room temperature for 1 hr. After chilling the reaction mixture to 0 °C, $NaBH_4$ (380 mg, 10.3 mmol) was added and the mixture was stirred for 1 hr. The reaction was quenched using a dilute H_2SO_4 aqueous solution and extracted with $CHCl_3$ (20 mL × 3). The combined organic layers were concentrated and subjected to silica gel chromatography using hexanes/ethyl acetate (1:1) with 5% Et_3N as the eluent to give **48** as a colorless oil (3 steps, 70%). ^{1}H NMR ($CDCl_3$) δ 4.14-4.18 (m, 4 H), 3.73-3.78 (m, 1 H), 3.13 (br, 1 H, exchangable), 1.64-1.71 and 1.79-1.84 (m, 1 H total), 1.33 (dt, J = 6.8, 1.6 Hz, 6 H), 1.06 (d, J = 7.2 Hz, 3 H); ^{31}P NMR ($CDCl_3$) δ 25.2.

*Diethyl (1R,2R)-1-Hydroxypropyl-[2-$^2H_1$]-phosphonate (***49***).* Following the same procedure described for the synthesis of **35**, **49** was prepared in 40% yield and 99% e.e. using **48** as the starting material. ^{1}H NMR ($CDCl_3$) δ 4.12-4.2- (m, 4 H), 3.76 (m, 1 H), 3.22 (br , 1 H, exchangable), 1.77-1.85 (m, 1 H), 1.33 (t, J = 7.2 Hz, 6 H), 1.06 (d, J = 7.2 Hz, 3 H); ^{31}P NMR ($CDCl_3$) δ 25.2.

*(1R,2R)-1-Hydroxypropyl-[2-$^2H_1$]-phosphonic acid (***11***).* Following the same procedure described for the synthesis of (*S*)-**7**, **11** was prepared from **49** as a white powder in 88% yield. ^{1}H NMR (D_2O) δ 3.40 (m, 1 H), 1.63 (m, 1 H), 0.86 (d, J =

7.8 Hz, 3 H) (see Fig. S2); ^{31}P NMR (D_2O) δ 20.0; ^{13}C NMR (D_2O) δ 71.4 (d, J = 154.4 Hz), 24.4 (t, J = 19.7 Hz), 10.2 (d, J = 13.2 Hz).

*Diethyl (1R,2S)-1-Hydroxypropyl-[2-$^2H_1$]-phosphonate (**50**).* Compound **50** was prepared from D-alanine using the same procedure described above for **49**. ^{1}H NMR ($CDCl_3$) δ 4.12-4.20 (m, 4 H), 3.75 (m, 1 H), 3.20 (br, 1 H, exchangable), 1.63-1.73 (m, 1 H), 1.33 (t, J = 7.2 Hz, 6 H), 1.06 (d, J = 7.2 Hz, 3 H); ^{31}P NMR ($CDCl_3$) δ 25.2.

*(1R,2S)-1-Hydroxypropyl-[2-$^2H_1$]-phosphonic Acid (**12**).* Compound **12** was obtained from **50** by following the same procedure used in the preparation of (*S*)-**7**. ^{1}H NMR (D_2O) δ 3.28 (m, 1 H), 1.31-1.37 (m, 1 H), 0.82 (t, J = 7.2 Hz, 3 H) (see Fig. S2); ^{31}P NMR (D_2O) δ 18.4; ^{13}C NMR (D_2O) δ 72.1 (d, J = 150.0 Hz), 24.8 (t, J = 19.2 Hz), 10.5 (d, J = 12.9 Hz).

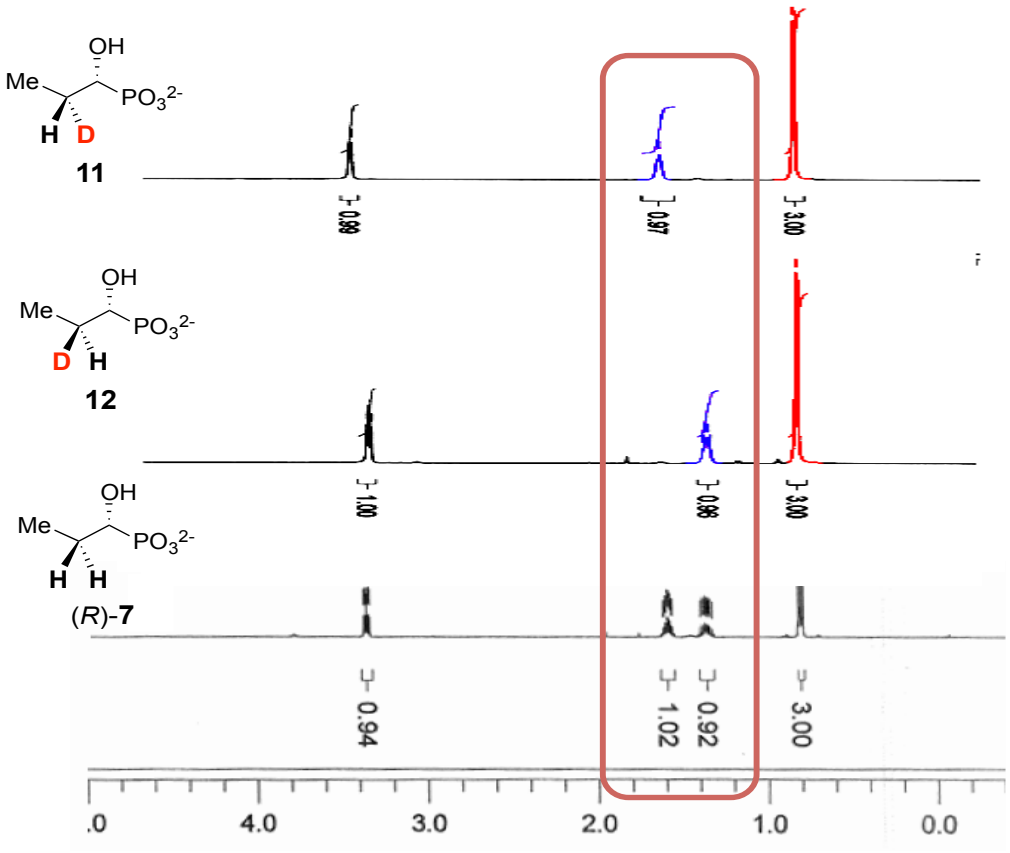


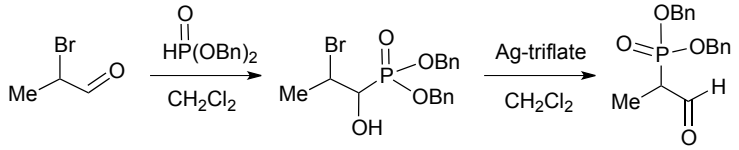
Figure S2: ^{1}H NMR spectra of stereospecifically deuterated substrates 11 and 12, along with (*R*)-7.

S4. Mechanistic Model Studies

S4.1. Model Reaction that Produces Dibenzyl 1-Formyl-ethylphosphonate (20)

*Dibenzyl 2-Bromo-1-hydroxypropylphosphonate (**17**).* To a solution of 2-bromo-propionaldehyde (1.1 g, 8.1 mmol) (*14*) in dry CH_2Cl_2 (20 mL) were added dibenzylphosphite (2.1 g, 8.1 mmol) and a few drops of 1,1,3,3-tetramethyl-

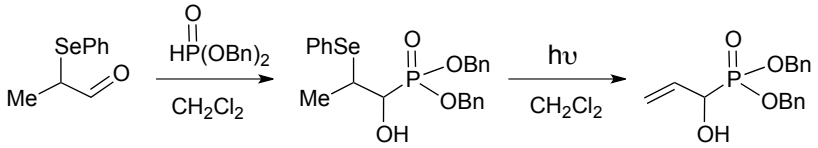
guanidine. The reaction was kept stirring at room temperature for 18 hr. The solvent was then removed under reduced pressure, and the residue was purified by flash chromatography to give **17** as a light yellow oil in 40% yield. ^{1}H NMR ($CDCl_3$) δ 7.35 (s, 10 H), 5.10 (m, 4 H), 4.56 (m, 1 H), 4.37 (dd, *J* = 12.5, 2.4 Hz, 1 H), 1.81 (d, *J* = 6.9 Hz, 3 H); ^{31}P NMR ($CDCl_3$) δ 21.0; ^{13}C NMR ($CDCl_3$) δ 135.7, 135.6, 128.6, 128.5, 128.0, 73.2 (d, *J* = 155.0 Hz), 68.6 (d, *J* = 2.0 Hz), 68.5 (d, *J* = 2.0 Hz), 49.5 (d, *J* = 12.1 Hz), 20.5; High resolution CIMS (NH_3) calcd for $C_{17}H_{21}O_4PBr$ $(M+1)^+$ 399.0361, found 399.0357.



17 **20**

*Dibenzyl 1-Formyl-ethylphosphonate (**20**).* To a solution of compound **17** (0.4 g, 1 mmol) in dry CH_2Cl_2 (20 mL) was added silver trifluormethanesulfonate (0.3 g, 1.2 mmol). After stirring at room temperature for 2 hr, the solvent was removed under reduced pressure. The residue was purified by flash chromatography to afford **20** as a colorless oil in 73% yield. ^{1}H NMR ($CDCl_3$) δ 9.72 (s, 1 H), 7.36 (s, 10 H), 5.03 (4 H, m), 3.07 (m, 1 H), 1.34 (dd, *J* = 17.7, 6.9 Hz, 3 H); ^{31}P NMR ($CDCl_3$) δ 25.3; ^{13}C NMR ($CDCl_3$) δ 196.3, 129.0, 128.7, 128.4, 128.3, 128.3, 128.1, 127.9, 127.7, 68.3, 67.2, 47.9 (d, *J* = 129.0 Hz), 7.84 (d, *J* = 4.9 Hz). High resolution CIMS (NH_3) calcd for $C_{17}H_{20}O_4P$ $(M+1)^+$ 319.1099, found 319.1105.

S4.2. Model Reaction that Produces Dibenzyl 1-Hydroxyprop-2-enylphosphonate (22) from Dibenzyl 1-Hydroxy-2-selenophenyl-propylphosphonate (18)



18 **22**

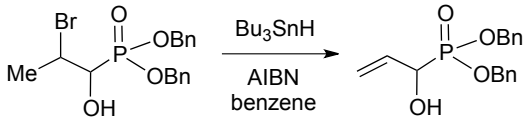
*Dibenzyl 1-Hydroxy-2-selenophenylpropylphosphonate (**18**).* To a solution of 2-selenophenylpropionaldehyde (2 g, 9.3 mmol) (*15*) in dry CH_2Cl_2 (40 mL) were added dibenzylphosphite (2.5 g, 9.3 mmol) and a few drops of 1,1,3,3-tetramethylguanidine. The solution was stirred at room temperature for 18 hr, and then concentrated under reduced pressure. The residue was chromatographed on silica gel using hexanes/ethyl acetate (1:1) as eluent and the partially purified product was re-crystallized (ethyl acetate/hexanes) to give **18** as white needles in 65% yield. ^{1}H NMR ($CDCl_3$) δ 7.56-7.27 (m, 15 H), 5.10 (m, 4 H), 4.08 (dd, *J* = 11.1, 2.4 Hz, 1 H), 3.79 (m, 1 H), 2.64 (br, 1 H, exchangable), 1.57 (d, *J* = 7.2, 3 H); ^{31}P NMR ($CDCl_3$) δ 23.2; ^{13}C NMR ($CDCl_3$) δ 134.5, 129.2, 128.7, 128.5, 128.4, 127.9, 127.8, 70.2 (d, *J* = 156.0

Hz), 68.2, 68.1, 41.5 (d, J = 12.1 Hz), 15.7; High resolution CIMS (NH_3) calcd for $C_{23}H_{25}O_4PSe$ $(M+1)^+$ 476.0656, found 476.0506.

*Dibenzyl 1-Hydroxy-prop-2-enylphosphonate (**22**).* The solution of compound **18** (0.2 g, 0.4 mmol) in 40 mL of dry benzene was exposed to the UV lamp in a Rayonet™ Photochemical Reactor for 3 hr. The solvent was then removed under reduced pressure, and the residue was purified by flash chromatography (hexanes/ethyl acetate (2:1)) to give **22** as a light yellow oil in 45% yield. ^{1}H NMR ($CDCl_3$) δ 7.36 (s, 10 H), 6.02 (m, 1 H), 5.48 (dd, J = 18.3, 6.3 Hz, 1 H), 5.34 (m, 1 H), 5.10 (m, 4 H), 4.55 (dd, J = 16.5, 5.4 Hz, 1 H); ^{31}P NMR ($CDCl_3$) δ 23.4; ^{13}C NMR ($CDCl_3$) δ 136.1, 132.2, 128.5, 128.4, 127.9, 117.6, 117.4, 69.7 (d, J = 159.0), 68.3, 68.2; High resolution CIMS (NH_3) calcd for $C_{17}H_{20}O_4P$ $(M+1)^+$ 319.1099, found 319.1101.

S4.3. Model Reaction that Produces Dibenzyl 1-Hydroxyprop-2-enylphosphonate (22) from Dibenzyl 1-Formyl-ethylphosphonate (17)

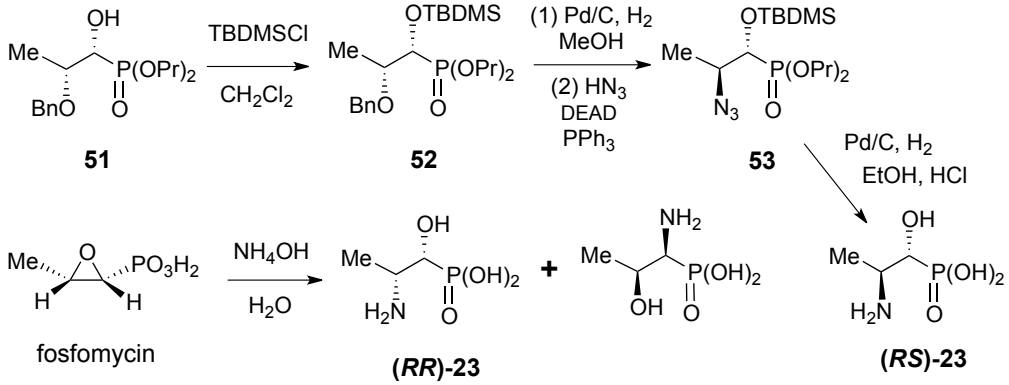
*Dibenzyl 1-Hydroxyprop-2-enylphosphonate (**22**).* To a solution of dibenzyl 2-bromo-1-hydroxypropyl-phosphonate (**17**) (0.2 g, 0.5 mmol) in dry benzene (30 mL) were added tributyltin hydride (0.15 g, 0.5 mmol) and 2,2'-azobisisobutylro-nitrile (0.01 g, 0.06 mmol). The reaction was refluxed for 3 hr. The solvent was then removed under reduced pressure, and the residue was purified by flash chromatography to give **22** as a light colorless oil in 85 % yield. The ^{1}H, ^{31}P and ^{13}C NMR spectra of **22** are the same as the product derived from **18**.



17 **22**

S5. PREPARATION OF 2-AMINO-1-HYDROXYPROPYLPHOSPHONIC ACIDS AND RELATED COMPOUNDS

S5.1. Preparation of (1R,2S)-2-Amino-1-hydroxypropylphosphonic Acid ((RS)-23) and (1R,2R)-2-Amino-1-hydroxypropylphosphonic Acid ((RR)-23)



51 **52** **53** (***RR***)-**23** (***RS***)-**23**

*Dipropyl ((1R,2R)-2-(Benzyloxy)-1-(tert-butyldimethylsilyl)oxy)propylphosphonate (**52**).* Imidazole (960 mg, 14.1 mmol), DMAP (catalytic amount), and TBDMSCl (1.12 g, 7.4 mmol) were added to a solution of **51** (1.06 g, 3.2 mmol) in CH_2Cl_2 (30 mL) (*16*) and the reaction was kept at 40 °C for 48 hr. The reaction mixture was concentrated and subjected to silica gel chromatography using CH_2Cl_2/ethyl acetate (10:1) to afford **52** as a light yellow liquid (60%). The ^{1}H NMR spectrum of **52** is identical to that reported in the literature (*17*).

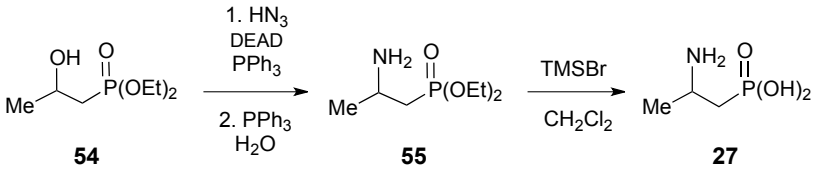
*(1R,2S)-2-Amino-1-hydroxypropylphosphonic acid ((RS)-**23**).* Compound **52** (445 mg, 1.0 mmol) was dissolved in MeOH (20 mL) and hydrogenated using Pd/C (10% Pd, 50 mg) in a Parr apparatus at 60 psi of H_2 and 30 °C for 24 hr. The reaction was filtered through a celite pad and the filtrate was concentrated *in vacuo*. HN_3 (1.1 mL, 1.1 mmol, 1 M) and diethyl azodicarboxylate (DEAD, 175 mg, 1 mmol) were sequentially added to a solution of PPh_3 (261 mg, 1 mml) and the debenzylated product dissolved in toluene (5 mL) at 0 °C. The reaction was warmed to 45 °C and stirred for 2 hr. The reaction was quenched with MeOH and the solvent was evaporated under reduced pressure. The residue was purified by flash silica gel chromatography using CH_2Cl_2/ethyl acetate (8:1) to furnish **53**, which was used in the next step. A solution of **53** (250 mg, 0.66 mmol) was dissolved in EtOH/conc. HCl (10:1, 11 mL) and hydrogenated using Pd/C (10% Pd, 30 mg) in a Parr apparatus at 60 psi of H_2 and 30 °C for 24 hr. The reaction was concentrated and subjected to ion exchange chromatography (Dowex 50 H^+ form) using 1.0 M formic acid as the eluent. Fractions sensitive to ninhydrin were pooled and lyophilized to afford (*RS*)-**23** (3 steps, 40%). ^{1}H NMR (D_2O) δ 3.71 (dd, J = 11.6, 4.4 Hz, 1 H), 3.46-3.54 (m, 1 H), 1.21 (d, J = 7.2 Hz, 3 H); ^{31}P NMR (D_2O) δ 14.8; ^{13}C NMR (D_2O) δ 68.4 (d, J = 153.3 Hz), 49.1 (d, J = 8.3 Hz), 13.2 (d, J = 3.2 Hz).

*(1R,2R)-2-Amino-1-hydroxypropylphosphonic acid ((RR)-**23**) (18).* Fosfomycin (**1**, 1.0 g, 5.5 mmol) was dissolved in 30 mL of H_2O, mixed with 30 mL of a 28% NH_4OH solution, and heated to 60 °C for 72 hr. After cooling to room temperature, solvent was removed by evaporation, the oily residue was dissolved in a minimal amount of H_2O, loaded on to a Dowex 50WX4 (H^+ form) column, and washed with 200 mL of H_2O. The desired compound, (*RR*)-**23**, was eluted from the column with a mixture of 50 mL of H_2O and 50 mL of a 28% NH_4OH solution. The eluate was collected and lyophilized to afford (*RR*)-**23** as a light yellow solid (300 mg, 30%). ^{1}H NMR (D_2O) δ 3.34-3.38 (m, 2 H), 1.15 (d, J = 6.6 Hz, 3 H); ^{31}P NMR (D_2O) δ 14.6; ^{13}C NMR (D_2O) δ 69.0 (d, J = 144.2 Hz), 48.8 (d, J = 1.2 Hz), 15.7 (d, J = 9.3 Hz).

S5.2. Preparation of (2-Aminopropyl)phosphonic Acid ((±)-27)

*Diethyl 2-Amino-propylphosphonate (**55**).* PPh_3 (1.58 g, 6 mmol, in 10 mL of CH_2Cl_2) was added to a solution of DEAD (1.21 g, 6 mmol) in CH_2Cl_2 (5 mL) at -10 °C. A solution of HN_3 (5 mL, 1.8 N in $CHCl_3$) was added dropwise to the

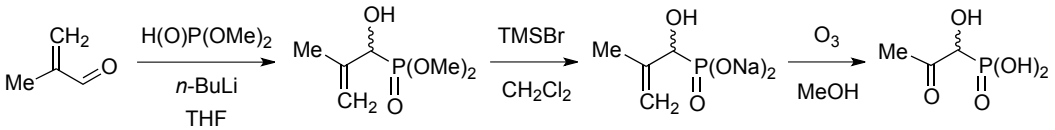
reaction mixture. Compound **54** (*19*) (982 mg, 5 mmol) was then added to the reaction and the resulting mixture was stirred overnight at room temperature. A white precipitate was filtered off and washed with hexanes. The combined filtrates were evaporated and dissolved in benzene (10 mL). PPh_3 (1.31 g, 5 mmol) was then added to this solution. After stirring for 2 hr at room temperature, H_2O was added to the reaction mixture, and the solution was heated to 55 °C and maintained at that temperature for 5 hr. The reaction mixture was cooled to room temperature and extracted with 5% aq. HCl (10 mL). The aqueous layer was neutralized to pH 10 and extracted with ethyl acetate (15 mL × 3). The organic extract was dried over $MgSO_4$ and evaporated to give product **55** in 45% yield. ^{1}H NMR ($CDCl_3$) δ 4.06-4.15 (m, 4 H), 3.35-3.42 (m, 1 H), 1.72-1.92 (m, 2 H), 1.56 (br, 2 H, exchangable), 1.33 (t, *J* = 7.0 Hz, 6 H), 1.18 (dd, *J* = 6.4, 2.0 Hz, 3 H); ^{31}P NMR ($CDCl_3$) δ 30.2; ^{13}C NMR ($CDCl_3$) δ 61.0, 42.2, 35.7 (d, *J* = 137.2 Hz), 25.1 (d, *J* = 16.0 Hz), 16.0 (d, *J* = 3.5 Hz).



54 **55** **27**

*2-Amino-propylphosphonic Acid ((±)-***27***).* TMSBr (1.17 g, 7.67 mmol) was added to a solution of **55** (300 mg, 1.53 mmol) in CH_2Cl_2 (20 mL) at room temperature, and the solution was stirred overnight. Solvent was removed under reduced pressure. The residue was dissolved in $CHCl_3$ (10 mL) and extracted with 10 mL of 0.5 M NH_4OAc solution. The aqueous layer was collected and lyophilized to afford (±)-**27** as a white solid (140 mg, 65%). ^{1}H NMR (D_2O) δ 3.34-3.39 (m, 1 H), 1.48-1.62 (m, 2 H), 1.19 (dd, *J* = 6.6, 1.2 Hz, 3 H); ^{31}P NMR (D_2O) δ 16.9; ^{13}C NMR (D_2O) δ 45.2 (d, *J* = 3.5 Hz), 33.5 (d, *J* = 125.0 Hz), 19.8 (d, *J* = 12.0 Hz); high resolution CIMS calcd for $C_3H_{11}NO_3P$ $(M+1)^+$ 140.0477, found 140.0474.

S5.3. Preparation of (1-Hydroxy-2-oxopropyl)phosphonic Acid Standard (*26*).



56 **57** **58** **26**

*Dimethyl (1-Hydroxy-2-methylallyl)-phosphonate (***57***).* *n*-BuLi (4 mL, 10 mmol, 2.5 M in hexanes) was added to a solution of dimethylphosphite (1.1 g, 10 mmol) in THF (35 mL) at -78 °C and stirred for 10 min. Methacrolein (**56**) (700 mg, 10 mmol) in 15 mL of THF was slowly added to the mixture at -78 °C and the reaction was kept at the same temperature for 30 min. The reaction mixture was quenched by adding 2.0 M AcOH and partitioned between ethyl acetate (30 mL × 3) and water (20 mL). The combined organic layers were washed with brine (40 mL), dried over $MgSO_4$, and

purified by flash chromatography using ethyl acetate/$CHCl_3$ (9:1) to afford product in 80% yield. The ^1H NMR spectrum of **57** is consistent with that reported in the literature (*20*).

*1-Hydroxy-2-oxopropylphosphonic Acid (***26***).* Allyl trimethylsilane (902 mg, 8 mmol) and TMSBr (766 mg, 5 mmol) were added to a solution of **57** (182 mg, 1 mmol) in CH_2Cl_2 (4 mL) at room temperature, and the solution was stirred overnight. Solvent was removed under reduced pressure. The residue was dissolved in $CHCl_3$ (10 mL) and extracted with 15 mL of 0.2 M NaOH. The aqueous layer was collected and lyophilized and directly used in the next step. The crude product **58** (200 mg, 1 mmol) from above was dissolved in 10 mL of MeOH. After cooling to -78 °C, ozone containing air was introduced to the reaction mixture until a faint blue color persisted. The reaction was quenched by adding PPh_3 (20 mg, dissolved in 1 mL of $CHCl_3$) and partitioned between 20 mL of H_2O and 20 mL of $CHCl_3$. The aqueous layer was collected and lyophilized to afford **26** as a very faint yellow solid (100 mg, 50%). The ^1H and ^{31}P NMR spectra of **26** are identical to those reported in the literature (*19*).

S6. NMR Studies of the Reaction of HppE with 1-Hydroxypropylphosphonic Acids, 2-Amino-1-hydroxypropylphosphonic Acids and Related Compounds

Reaction mixtures containing 0.25 mM HppE, 0.25 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 7.5 mM FMN, 25 mM substrate ((*R*)-**7**, (*S*)-**7**, **11**, **12**, (*RR*)-**23**, (*RS*)-**23**, or **27**), and 25 mM NADH in 700 μL of 20 mM Tris buffer (pH 7.5, prepared with H_2O) were prepared and subjected to ^1H NMR analysis (600 MHz spectrometer). The NMR spectra were recorded using selective pre-saturation of the water signal with a 2 s pre-saturation interval. The lock signal is dimethyl-d_6 sulfoxide (DMSO-d_6, 30 μL). Chemical shifts are standardized to the DMSO-d_6 signal at δ 2.49. The enzymatic reactions were initiated by adding reconstituted HppE to the reaction mixture. The ^1H NMR spectra were recorded after aeration of the reaction mixture by bubbling of air through the solution with gentle pipetting. Representative ^1H NMR time-courses for the reaction of HppE with (*R*)-**7** and (*S*)-**7** are given in Fig. 2 (see main text). The spectrum of the reaction product with (*R*)-**7** consists of a doublet of quartets at δ 2.85 (1H, dq, J = 26.0, 6.5 Hz), a doublet of doublets at δ 1.00 (3H, dd, J = 12.5, 6.5 Hz), and a peak at δ 9.5 (1H), consistent with the structure of the aldehyde product **9**. Moreover, the magnitude of the ^{31}P coupling constants with the C2-H (26.0 Hz) and C3-H (12.5 Hz) protons confirms that the phosphonate moiety of the product resides at C2. These results were further confirmed by MS analysis, which shows that the reaction product (m/z 137.00) is 2 a.u. smaller than the substrate (*R*)-**7** (m/z 139.02).

The ^1H NMR spectrum of product **9** derived from **11** (Fig. S3) shows that the C3 methyl signal at δ 1.00 ppm is split into a doublet of doublets ($J_{H\text{-}P}$ = 14.4 Hz and $J_{H\text{-}H}$ = 6.6 Hz). This implies retention of a hydrogen atom in the product. In

contrast, the corresponding signal of the product **13** derived from **12** (Fig. S3) appears as a doublet ($J_{H\text{-}P}$ = 14.4 Hz), indicating retention of deuterium in this product. These results are consistent with the abstraction of the *pro-R* hydrogen atom from C2 of (*R*)-1-HPP ((*R*)-**7**) during turnover.

Formation of product **26** from (*RR*)-**23** and (*RS*)-**23** was observed under the incubation conditions described above. However, the C1-H signal of **26** is buried under peaks from NADH/FMN. Thus, the reaction was repeated using $Ru(NH_3)_6Cl_2$ (2.5 equiv.) instead of NADH/FMN as the reducing agent. While the NMR peaks are broadened might due to the presence of Ru(III), which is paramagnetic (see Fig. S4), the C1-H and C3 Me signals of **26** are clearly visible. The identity of the product generated during the reactions of (*RR*)-**23** and (*RS*)-**23** with HppE was confirmed by spiking the reaction mixture with an authentic standard of **26** (Fig. S4B). To demonstrate that the formation of **26** is enzyme-dependent, a control experiment was conducted under similar conditions by omitting HppE and the result shows no obvious new product could be detected. To verify the formation of **26** using either (*RR*)-**23** or (*RS*)-**23** with HppE is not due to the binding of amino group at C2, similar experiment was carried out using **27** as substrate. When **27** was incubated with HppE, neither substrate consumption nor product formation was observed. In contrast to the reactions with (*R*)- and (*S*)-**7**, which showed >60% conversion to products during the time-scale of the NMR assay, only ~15% of (*RR*)- and (*RS*)-**23** were converted to **26** by HppE (as determined by NMR peak integrations).

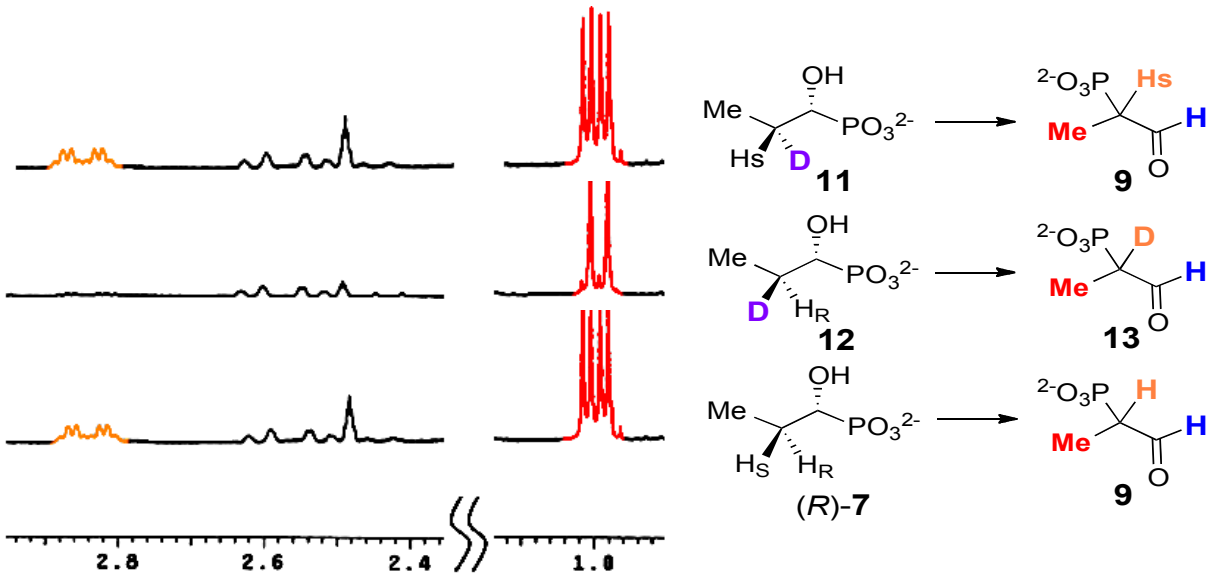


Figure S3: ^{1}H NMR spectra of the products of HppE-catalyzed reactions with stereospecifically deuterated substrates 11 and 12, demonstrating loss of the *pro*-R hydrogen atom.

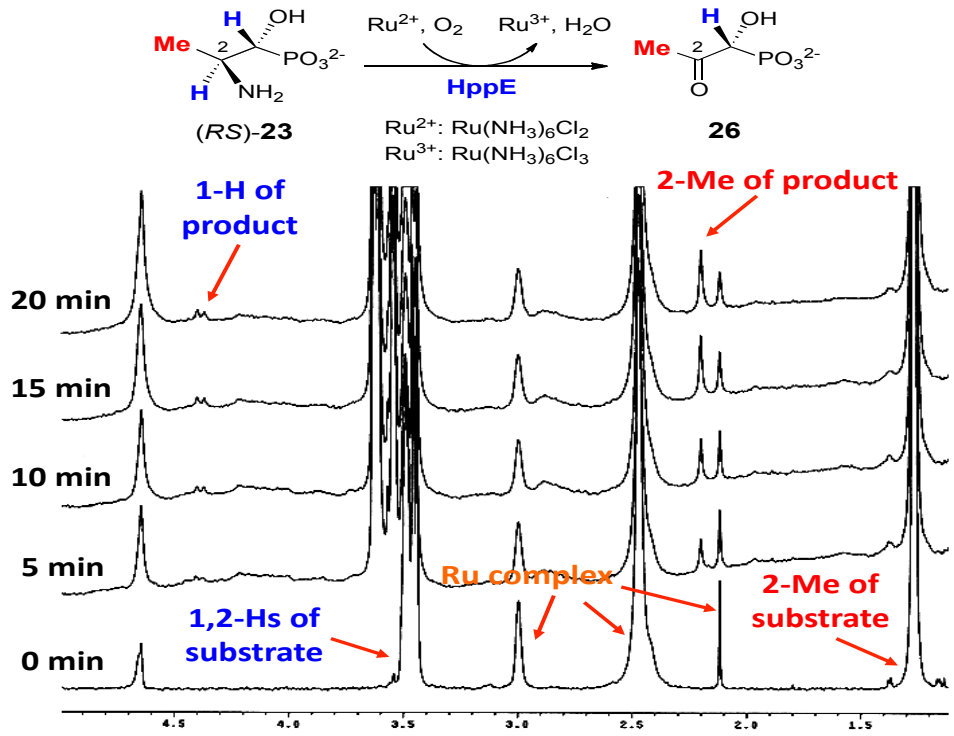


Figure S4: (A) ^{1}H NMR time-course for the HppE-catalyzed conversion of (*RS*)-23 to 26 using $Ru(NH_3)_6Cl_2$ as reducing agent.

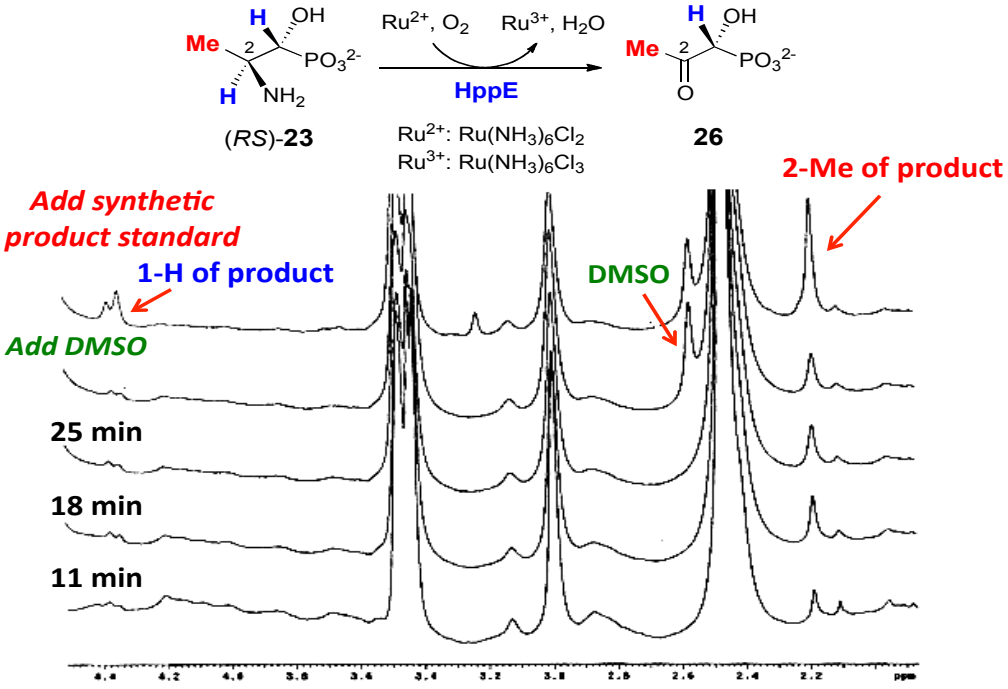


Figure S4: (B) ^{1}H NMR time-course for the HppE-catalyzed conversion of (*RS*)-23 to 26 using $Ru(NH_3)_6Cl_2$ as reducing agent. Additional DMSO-d_6 was introduced as an internal standard prior to the addition of synthetic **26**.

S7. Crystallization of Enzyme-Substrate Complexes

S7.1. Crystallization of Fe^{II}-HppE/(R)-7, Fe^{II}-HppE/(S)-7, and Fe^{II}-HppE/(RR)-23

Apo-HppE was expressed, purified, and assayed as described previously (*1, 21, 22*). The apo-protein was concentrated to 30 mg/mL in 0.02 M Tris•HCl, pH 8.0 and crystallized in an anaerobic glovebox (Coy Scientific, Ar/H_2 gas) using the hanging-drop vapor diffusion method at room temperature. A 2.0 mL solution of 30 mg/mL apo-HppE in 0.02 M Tris-HCl, pH 8.5, was mixed with 2.0 mL of precipitant solution (0.1 M Tris•HCl, pH 8.5, 2.0 M ammonium sulfate) and 0.6 mL of 0.1 M $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$. The Fe^{II}-HppE crystals, which grew in about 24 hrs, were rectangular-shaped. For soaking experiments, these Fe^{II}-HppE crystals were transferred to a new hanging drop, containing 4.4 μL of buffer solution (0.1 M HEPES, pH 7.5, 2.5 M ammonium sulfate, 0.4 M sodium chloride) and 0.6 μL of 0.1 M substrate analogue. The resulting drop was placed over a reservoir of soaking solution for about 6-12 hrs. The crystals were finally transferred to a cryoprotectant solution (0.1 M HEPES, pH 8.0, 2.0 M ammonium sulfate, 30% (w/v) xylitol), soaked for 1-2 min, and flash-frozen in liquid nitrogen.

S7.2. Structure Determinations

The data sets for Fe^{II}-HppE/(*R*)-**7** and Fe^{II}-HppE/(*S*)-**7** were collected at the Advanced Light Source (beamline 8.2.2) at 100 K. The data were integrated in HKL2000 and scaled in SCALEPACK (*23*) (Table S2). Crystals of Fe^{II}-HppE/(*R*)-**7** ($P4_22_12$; a,b = 111.49 Å, c = 151.48 Å) and Fe^{II}-HppE/(*S*)-**7** ($P4_22_12$; a,b = 111.46 Å, c = 151.42 Å) were isomorphous with previous crystals of Fe^{II}-HppE/(*S*)-2-HPP ($P4_22_12$; a,b = 111.65 Å, c = 152.07 Å), and contained three molecules in the asymmetric unit. The structures were determined using isomorphous replacement followed by rigid body refinement in CNS (*24*) with the previously solved Fe^{II}-HppE/(*S*)-2-HPP structure as an initial model (PDB code 1ZZ8) (*22*), but with all waters, substrates, and metal ions removed. Refinement was performed in CNS without a sigma cutoff for the data, and using non-crystallographic symmetry restraints for all protein atoms in the asymmetric unit in all three structures. Iterative rounds of model building in COOT (*25*) and refinement in CNS included simulated annealing, positional refinement, and *B*-factor refinement. The structures were all analyzed using $2F_o$-F_c composite omit maps, with Ramachandran geometries analyzed using PROCHECK (*26-28*). The results from PROCHECK indicate that for the Fe^{II}-HppE/(*S*)-**7** structure 89.5% of the residues are in the most favored, 9.8% of the residues are in additionally allowed, and 0.6% of the residues are in the generously allowed regions of the Ramachandran plot. The Fe^{II}-HppE/(*R*)-**7** structure has 89.0% of the residues in the most favored and 11.0% of the residues in the additionally allowed regions of the Ramachandran plot.

Parameter and topology files for substrate analogues were obtained using Chem3D Pro 12.0 (*29*) and XPLO2D (*30*), while those for the iron coordination sphere were generated using weak restraints to allow movement of atoms. (*R*)- and (*S*)-1-HPP (**7**) substrates were positioned in the structures based on omit electron density maps (Fig. S6), using high-sigma electron density peaks to indicate the location of the substrate phosphorus atom (Fig. S5). Both molecules bind in a bidentate mode to the iron atom at the active site, and refinement of these substrates shows no positive or negative difference electron density, indicating a single conformation of each molecule. All protein residues are modeled except for the first four residues in structures of Fe^{II}-HppE/(*R*)-**7,** and the first five residues in chains A and B and the first three in chain C in the structures of Fe^{II}-HppE/(*S*)-**7**. Structural figures were generated using PyMOL (*31*). Structure refinement statistics are summarized in Table S2.

S7.3. Data Collection and Analysis

Data sets for Fe^{II}-HppE/(*S*)-**7** and Fe^{II}-HppE/(*R*)-**7** were collected at 100K, using 1.00 Å wavelength at Advanced Light Source. The data were subsequently integrated and scaled in DENZO and SCALEPACK (*32*), respectively (Table S2).

Table S2. Data Collection and Refinement Statistics (Molecular Replacement)

	Fe^{II}-HppE/(*S*)-**7**	Fe^{II}-HppE/(*R*)-**7**
Data collection		
Space group	$P4_22_12$	$P4_22_12$
Cell dimensions		
a, *b*, *c* (Å)	111.46, 111.46, 151.42	111.49, 111.49, 151.48
α, β, γ (°)	90.00, 90.00, 90.00	90.00, 90.00, 90.00
Resolution (Å)	50-2.80 (2.85-2.80)*	50-2.71 (2.76-2.71)*
R_{svm} or R_{merge}	9.1 (74.1)	9.5 (66.3)
I / σI	23.1 (2.7)	20.2 (2.2)
Completeness (%)	99.3 (99.6)	96.9 (99.1)
Redundancy	8.6 (8.7)	6.9 (6.9)
Refinement		
Resolution (Å)	50-2.80	50.0-2.72
No. reflections	23510	24490
R_{work} / R_{free}	25.1 (26.3)	24.2 (27.5)
No. atoms		
Protein	4405	4404
Iron	3	3
(*S*)-**7**	24	
(*R*)-**7**		24
Water	31	41
B-factors		
Protein	59.1	55.3
Iron	58.1	52.3
(*S*)-**7**	65.1	

(*R*)-**7**		68.8
Water	42.8	44.5
R.m.s. deviations		
Bond lengths (Å)	0.009	0.008
Bond angles (°)	1.6	1.5

*One crystal used for each structure. *Values in parentheses are for highest-resolution shell.

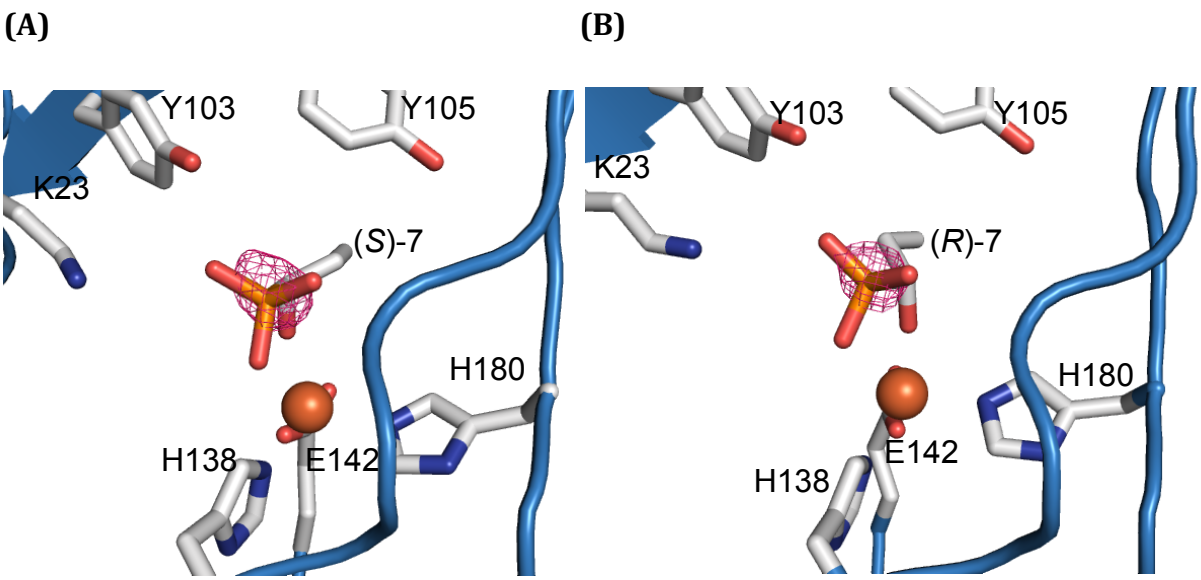


Figure S5. Identification of Phosphorus Positions for HppE Substrates in X-ray Structures Using High Sigma Electron Density Peaks (**A**) (*S*)**-7** F*o*-F*c* omit density (in magenta) around phosphorus at 8.5 σ. (**B**) (*R*)-**7** F*o*-F*c* omit density (in magenta) around phosphorus at 9.0 σ.

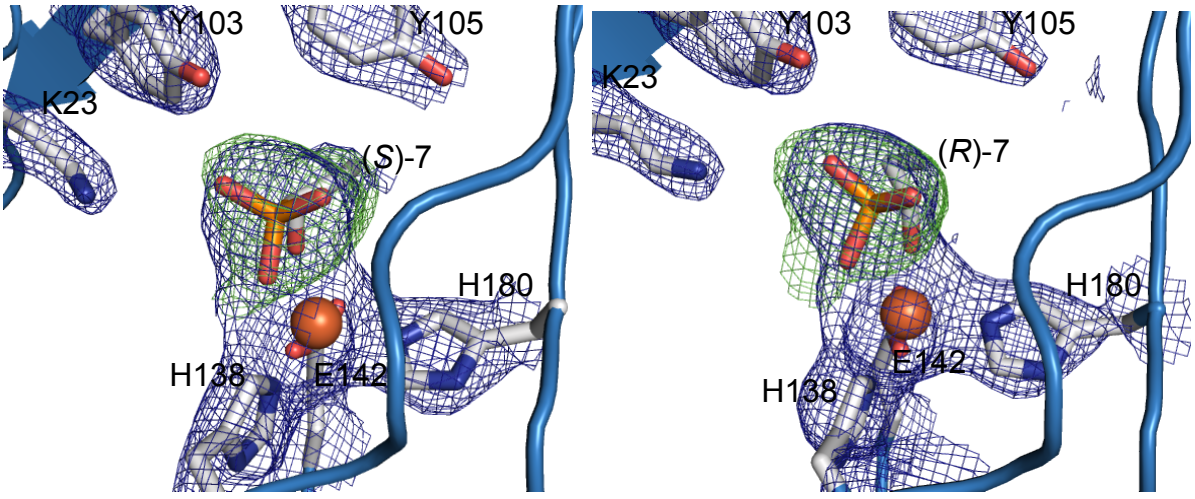


Figure S6. Additional Views of Electron Density for (*S*)-7 and **(*R*)-7 HppE Substrates.** 2F*o*-F*c* electron density map at 1 σ (blue) with F*o*-F*c* omit map for substrate at 4.5 σ (in green) for (**A**) (*S*)-**7**, (**B**) (*R*)-**7**.

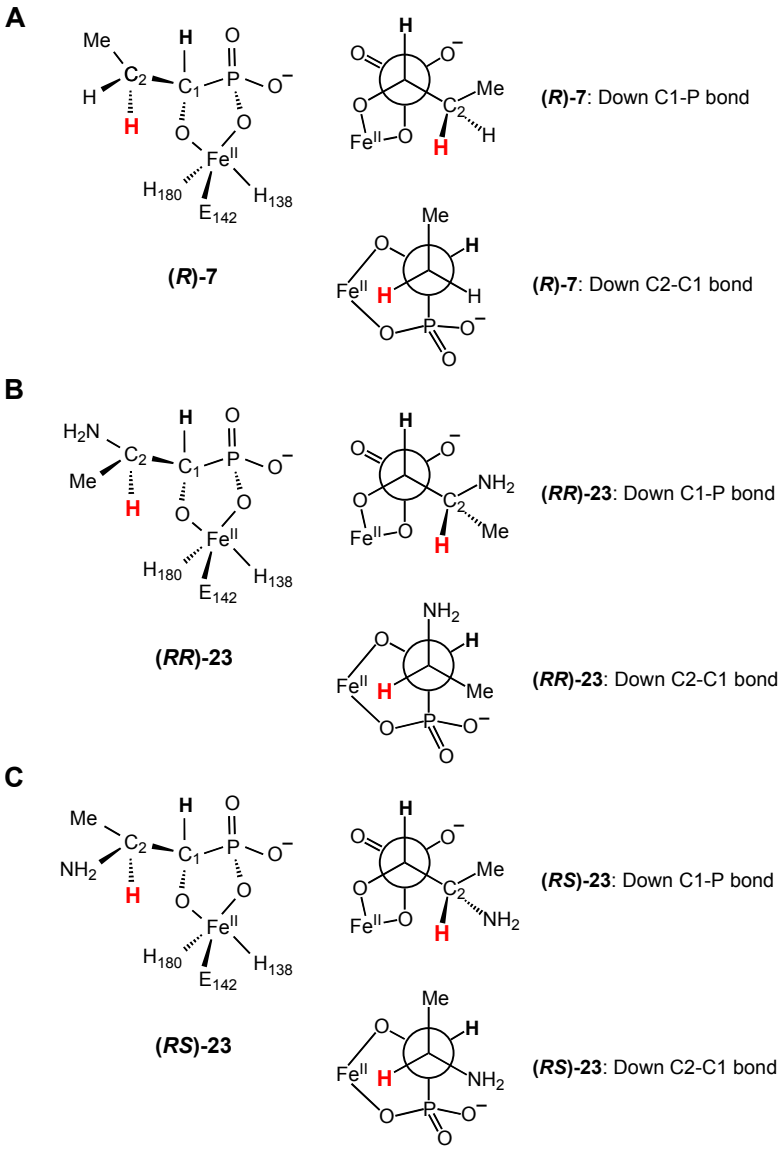


Figure S7. Newman Projections Showing Hydrogen Atom Accessibility with HppE Substrates bound in Staggered Conformations. All crystal structures of HppE with bound substrates determined to date show substrates bound to iron in extended low energy conformations. Here substrates are drawn looking down the C1-P bond (top) and C2-C1 bond (bottom) illustrating bidentate substrate binding in a staggered conformation. Hydrogen atoms expected to be accessible for abstraction are shown in red. (**A**) This low energy staggered conformation of (*R*)-**7** is consistent with the X-ray structure and with the established stereospecificity of the reaction (see text). (**B**) Modeling of (*RR*)-**23** in a low energy staggered conformation is consistent with hydrogen atom abstraction by an Fe-oxygen intermediate of the C2 hydrogen. (**C**) Modeling of (*RS*)-**23** in a low energy staggered conformation shows that the C2 hydrogen would also be available for hydrogen atom abstraction. Also see Fig. S8.

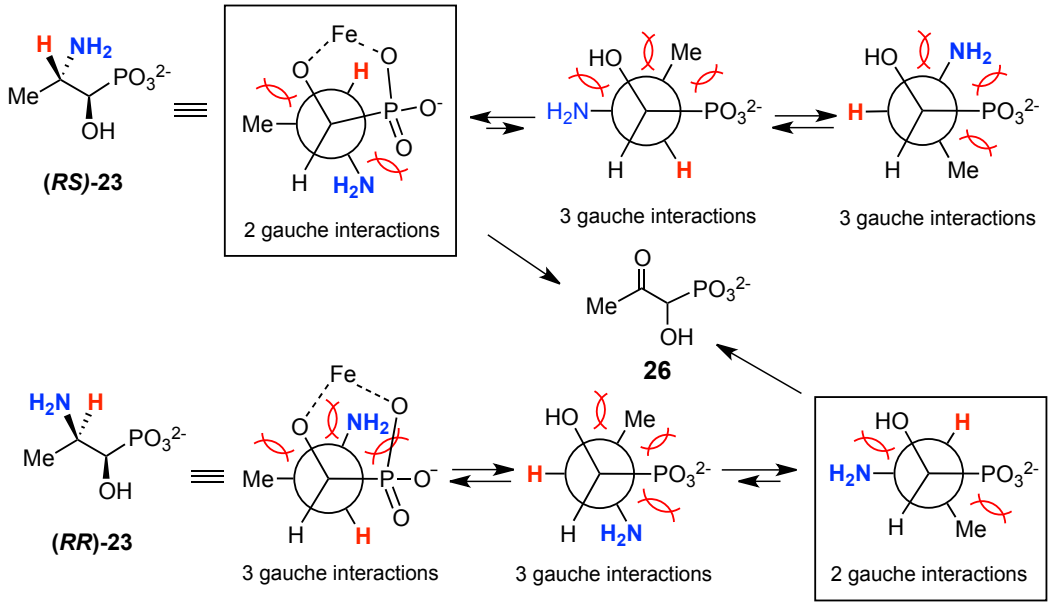


Figure S8. Explanation of the observed stereochemistry for the conversion of (*RR*)- and (*RS*)-23 to 26. The most stable binding conformation (with two gauche interactions) of each substrate positions the C2 hydrogen atom for abstraction by the reactive iron-oxygen intermediate, leading to formation of the same reaction product.

S8. Density Functional Theory Calculations

Bond dissociation enthalpies for the C1-H of (*S*)-2-HPP and the C2-H of (*R*)-1-HPP (in their monoanionic forms) were obtained using Becke-style three-parameter density functional theory (DFT) with the Lee-Yang-Parr correlation functional (B3LYP) and Pople's diffuse polarized triple-ζ 6-311+G(d,p) basis set, as implemented in *Gaussian 98* (*33*). Vibrational frequency calculations were performed on the geometry optimized structures at 25 °C and 1 atm pressure in the gas phase using a scaling factor of 0.9877 to correct the zero-point vibrational energies (*34*).

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