

Mechanistic Consequences of Chiral Radical Clock Probes: Analysis of the Mononuclear Non-heme Iron Enzyme HppE with 2-Hydroxy-3-methylenecyclopropyl Radical Clock Substrates

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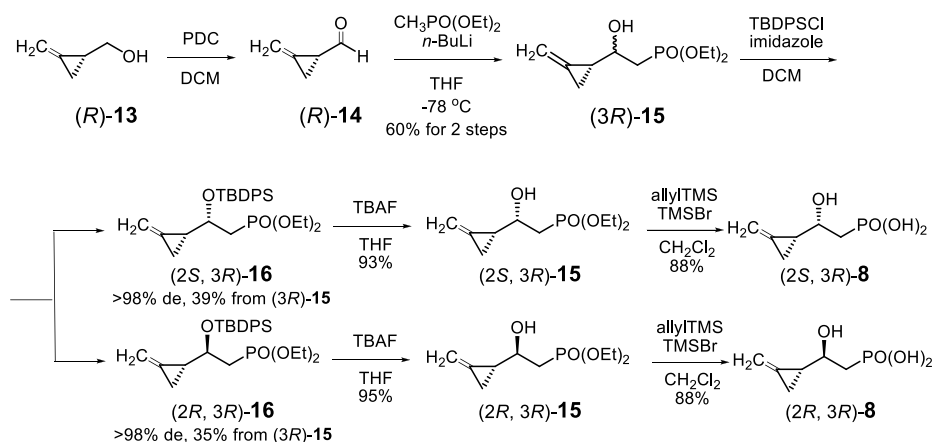
1. General Information

Apo-HppE was expressed and purified as previously described.¹ The enzyme was reconstituted with one equivalent of Fe(II)(NH₄)₂(SO₄)₂·6H₂O before use in assays. Compounds (*R*)-**14** and (*S*)-**14** were prepared from (*R*)-**13** and (*S*)-**13**, respectively, as previously described in literatures.^{2,3} Tetrahydrofuran (THF) was distilled under N₂ atmosphere from Na/benzophenone prior to use. Dichloromethane (DCM, CH₂Cl₂) was distilled under N₂ atmosphere using CaH₂ as a drying agent prior to use. Other dry solvents were purchased from commercial suppliers and used as received. All reagents were used directly as commercially obtained unless otherwise noted. NMR spectra of synthetic samples were recorded on Varian NMR spectrometers operating at 300, 400, 500, or 600 MHz at the NMR facility of the Department of Chemistry, the University of Texas at Austin. Chemical shifts (δ in ppm) are reported relative to that of solvent (CDCl₃ or D₂O) with coupling constants given

in Hertz (Hz). High-resolution mass spectral (HRMS) analyses of all synthesized compounds were carried out at the Mass Spectrometry Facility of the Department of Chemistry, the University of Texas at Austin. MALDI-TOF MS (Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) analyses of trypsin digested HppE and its inactivated form were conducted on the 4800 MALDI TOF/TOF™ Analyzer at the Department of Chemistry, Texas A&M University. Amino acid analysis was performed by the Molecular Structure Facility at the University of California, Davis. Radioactivities of the tritium (³H or T) containing samples were recorded on a Beckman Coulter LS6500 Multi-Purpose Scintillation counter.

2. Chemical Syntheses

2.1. Chemical syntheses of compounds (2*S*, 3*R*)-8 and (2*R*, 3*R*)-8.



Scheme S1. Synthetic scheme for the preparation of (2*S*, 3*R*)-8 and (2*R*, 3*R*)-8.

Diethyl (2-Hydroxy-2-((*R*)-2-methylenecyclopropyl)ethyl)phosphonate ((3*R*)-15). To a solution of diethyl methylphosphonate (456 mg, 3.0 mmol) in THF (15 mL) was added dropwise *n*-BuLi (1.0 mL, 2.5 M solution in hexanes, 2.5 mmol) at -78°C . After stirring for 30 min, the lithium reagent was transferred dropwise to a solution of (*R*)-14 (164 mg, 2.0 mmol) in 15 mL THF at -78°C . The resulting mixture was stirred at -78°C for 45 min and quenched with 8 mL of saturated NH_4Cl . The reaction was slowly warmed to room temperature, and the organic solvent was removed by reduced pressure distillation. The resulting aqueous layer was extracted with ethyl acetate (30 mL \times 3). The combined organic extracts were washed with brine (50 mL \times 2) and dried over Na_2SO_4 . The solvent was removed, and the resulting brown oil was an inseparable mixture of (3*R*)-15 and diethyl methylphosphonate. This material was used for the next reaction without further purification.

Diethyl ((*R*)-2-((*tert*-Butyldiphenylsilyl)oxy)-2-((*R*)-2-methylenecyclopropyl)ethyl) phosphonate ((2*R*,3*R*)-16) and Diethyl ((*S*)-2-((*tert*-Butyldiphenylsilyl)oxy)-2-((*R*)-2-methylenecyclopropyl)ethyl)-phosphonate ((2*S*,3*R*)-16). To a solution of imidazole (170 mg, 2.5 mmol) and compound (3*R*)-15 (440 mg) obtained from the previous reaction in CH_2Cl_2 (DCM, 35 mL) was added dropwise a solution of *tert*-butyl(chloro)diphenylsilane (TBDPSCl, 2.25 mmol, in 15 mL of CH_2Cl_2) at room temperature. The resulting mixture was stirred at 40°C for 16 hr. The mixture was filtered through Celite, and the filter cake was washed with CH_2Cl_2 (30 mL \times 2). The filtrate was concentrated *in vacuo*, and the residue was purified by repeat flash column chromatography (hexanes : ethyl acetate = 5 : 1 to 2 : 1) to give two separate compounds. The less

polar (hexanes : ethyl acetate = 1 : 1, R_f = 0.33) product is (2*S*, 3*R*)-**16** (382 mg, 39% yield from (*R*)-**15**). ^1H NMR (CDCl_3) δ 0.53-0.57 (m, 1H), 0.83 (tt, J = 2.0, 9.2 Hz, 1H); 1.03 (s, 9H); 1.26 (dt, J = 0.8, 7.2 Hz, 6H); 1.77-1.80 (m, 1H), 2.06-2.18 (m, 2H); 3.78 (ddd, J = 5.6, 13.6, 14.4 Hz, 1H), 3.98-4.06 (m, 4H), 5.25 (s, 1H), 5.35 (s, 1H); 7.34-7.43 (m, 6H); 7.72-7.78 (m, 4H); ^{31}P NMR (CDCl_3) δ 28.0; ^{13}C NMR (CDCl_3) δ 9.3, 16.8 (d, J = 3.6 Hz), 16.9 (d, J = 3.6 Hz), 19.9, 23.2 (d, J = 9.9 Hz), 27.2, 34.8 (d, J = 137.6 Hz), 61.8 (d, J = 6.7 Hz), 71.4 (d, J = 2.3 Hz), 104.6, 127.9 (d, J = 20 Hz), 130.1 (d, J = 17 Hz), 133.5, 134.4 (d, J = 41.2 Hz), 136.4; HRMS-ESI calcd for $\text{C}_{52}\text{H}_{74}\text{O}_8\text{P}_2\text{Si}_2\text{Na}$ ($2\text{M}+\text{Na}$) $^+$ 967.4295, found 967.4288.

The more polar (hexanes : ethyl acetate = 1 : 1, R_f = 0.28) product is (2*R*, 3*R*)-**16** (342 mg, 35% yield from (*R*)-**15**). ^1H NMR (CDCl_3) δ 1.03 (s, 9H), 1.08-1.16 (m, 2H), 1.20 (t, J = 8.0 Hz, 6H), 1.79-1.86 (m, 1H), 1.92-2.13 (m, 2H), 3.86-4.03 (m, 5H), 5.16 (t, J = 0.9 Hz, 1H), 5.28 (s, 1H), 7.34-7.39 (m, 6H), 7.69-7.72 (m, 4H); ^{31}P NMR (CDCl_3) δ 27.7; ^{13}C NMR (CDCl_3) δ 7.9, 16.8 (d, J = 4.5 Hz), 19.8, 22.6 (d, J = 5.2 Hz), 27.3, 34.7 (d, J = 135.4 Hz), 61.7 (d, J = 5.2 Hz), 70.5, 104.6, 128.0 (d, J = 7.5 Hz), 130.1 (d, J = 10 Hz), 133.6, 134.2 (d, J = 17 Hz), 136.4 (d, J = 12.6 Hz).

Diethyl ((*S*)-2-hydroxy-2-((*R*)-2-methylenecyclopropyl)ethyl)phosphonate ((2*S*, 3*R*)-15**).** To a stirring solution of compound (2*S*, 3*R*)-**16** (542 mg, 1.15 mmol) in THF (20 mL) was added tetra-*n*-butylammonium fluoride (TBAF, 3 mL, 1 M solution in THF, 3 mmol). After stirring for 10 hr, the organic solvent was removed, and the residue was purified by silica gel flash column chromatography (MeOH : ethyl acetate = 1 : 5) to afford compound (2*S*, 3*R*)-**15** in 93% yield. ^1H NMR (CDCl_3) δ 1.06-1.11 (m, 1H), 1.30 (t, J = 6.3 Hz, 3H), 1.30 (t, J = 6.3 Hz, 3H), 1.28-1.35 (m, 1H) overlapping with previous peak, 1.65-1.70 (m, 1H), 1.98-2.12 (m, 2H), 3.46-3.54 (m, 1H), 3.59 (br s, 1H), 4.04-4.14 (m, 4H), 5.39-5.42 (m, 2H); ^{31}P NMR (CDCl_3) δ 29.7; ^{13}C NMR (CDCl_3) δ 9.0, 16.8 (d, J = 5.2 Hz), 22.8 (d, J = 19.4 Hz), 33.8 (d, J = 136.9 Hz), 62.3 (d, J = 6.3 Hz), 62.4 (d, J = 6.3 Hz), 69.7 (d, J = 5.3 Hz), 104.8, 132.5; HRMS-ESI calcd for $\text{C}_{10}\text{H}_{19}\text{O}_4\text{PNa}$ ($\text{M}+\text{Na}$) $^+$ 257.0919, found 257.0910.

Diethyl ((*R*)-2-hydroxy-2-((*R*)-2-methylenecyclopropyl)ethyl)phosphonate ((2*R*, 3*R*)-15**).** Compound (2*R*, 3*R*)-**15** was obtained in 95% yield, following a similar procedure for the synthesis of (2*S*, 3*R*)-**15**, using (2*R*, 3*R*)-**16** as the starting material. ^1H NMR (CDCl_3) δ 0.97-1.01 (m, 1H), 1.31 (t, J = 7.2, 6H), 1.23-1.32 (m, 1H) overlapping with previous peak, 1.68-1.74 (m, 1H) 1.94-2.13 (m, 2H), 3.37 (br s, 1H), 3.63-3.71 (m, 1H), 4.05-4.15 (m, 4H), 5.42 (s, 1H), 5.35 (s, 1H); ^{31}P NMR (CDCl_3) δ 29.8; ^{13}C NMR (CDCl_3) δ 7.4, 16.8 (d, J = 2.2

Hz), 16.9 (d, $J = 2.2$ Hz), 22.3 (d, $J = 19.7$ Hz), 33.2 (d, $J = 137.6$ Hz), 62.3, 69.3 (d, $J = 4.4$ Hz), 104.9, 133.2 (d, $J = 1.5$ Hz).

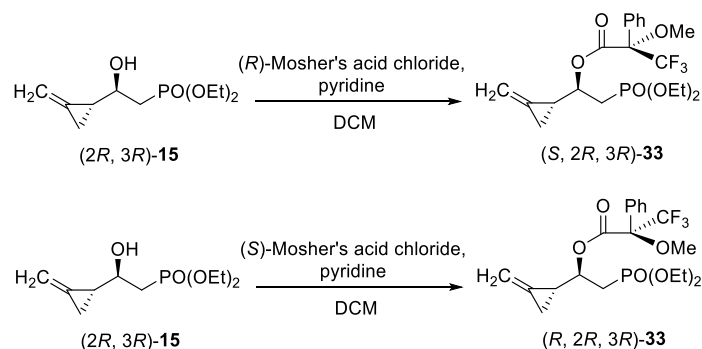
((S)-2-Hydroxy-2-((R)-2-methylenecyclopropyl)ethyl)phosphonic acid ((2S, 3R)-8). A solution of compound (2S, 3R)-**15** (159 mg, 0.68 mmol), bromotrimethylsilane (TMSBr, 420 mg, 2.75 mmol) and allyltrimethylsilane (allylTMS, 542 mg, 4.76 mmol) in CH₂Cl₂ (20 mL) was stirred at room temperature for 12 hr. The solvent was removed by reduced pressure distillation, and the residue was vigorously stirred with 5 mL of water for 10 min. The aqueous solution was neutralized with NH₄HCO₃, washed with chloroform (3 mL \times 3), and lyophilized to give (2S, 3R)-**8** as a white powder (106 mg, 88%). ¹H NMR (D₂O) δ 0.93 (broad s, 1H), 1.19 (t, $J = 8.9$ Hz, 1H), 1.58 (d, $J = 4.2$ Hz, 1H), 1.75-1.82 (m, 2H), 3.33-3.37 (m, 1H), 5.31 (d, $J = 1.2$ Hz, 1H), 5.41 (dd, $J = 0.85, 1.47$ Hz, 1H); ; ³¹P NMR (242 MHz, D₂O) δ 21.3; ¹³C NMR (D₂O) δ 7.5, 22.1 (d, $J = 13.2$ Hz), 33.4 (d, $J = 129$ Hz), 70.5 (d, $J = 3.6$ Hz), 104.2, 132.9; HRMS-ESI calcd for C₆H₁₀O₄P (M-H)⁻ 177.0317, found 177.0322.

((R)-2-Hydroxy-2-((R)-2-methylenecyclopropyl)ethyl)phosphonic acid ((2R, 3R)-8). Compound (2R, 3R)-**8** was obtained in 88% yield by following the same procedure for the synthesis of (2S, 3R)-**8** using (2R, 3R)-**15** as starting material. ¹H NMR (D₂O) δ 0.86-0.89 (m, 1H), 1.16 (tt, $J = 2.2, 9.3$ Hz, 1H), 1.57 (tq, $J = 1.8, 7.2$ Hz, 1H), 1.65 (dt, $J = 9.1, 15.1$ Hz, 1H), 1.81 (ddd, $J = 4.1, 14.9, 17.4$ Hz, 1H), 3.37 (dq, $J = 3.9, 9.8$ Hz, 1H), 5.29 (s, 1H), 5.39 (s, 1H); ³¹P NMR (D₂O) δ 20.5; ¹³C NMR (D₂O) δ 6.9, 21.6 (d, $J = 15.6$ Hz), 35.0 (d, $J = 127.5$ Hz); 70.9 (d, $J = 2.5$ Hz); 103.7, 134.0.

2.2. Chemical syntheses of compounds (2S, 3S)-8 and (2R, 3S)-8.

((R)-2-Hydroxy-2-((S)-2-methylenecyclopropyl)ethyl)phosphonic acid ((2S, 3S)-8) and ((S)-2-hydroxy-2-((S)-2-methylenecyclopropyl)ethyl)phosphonic acid ((2R, 3S)-8). Compounds (2S, 3S)-**8** and (2R, 3S)-**8** were synthesized following the same procedures described for the syntheses of (2R, 3R)-**8** and (2S, 3R)-**8** using (S)-**13** as the starting material. The ¹H and ¹³C-NMR spectra of (2S, 3S)-**8** and (2R, 3S)-**8** are identical to those of (2R, 3R)-**8** and (2S, 3R)-**8**, respectively.

2.3. Chemical syntheses of Mosher's esters of (2R,3R)-15: (S, 2R, 3R)-33 and (R, 2R, 3R)-33.

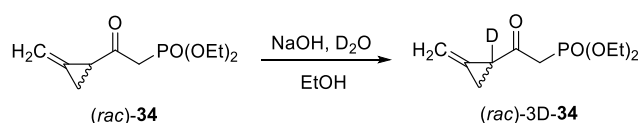


Scheme S2. Synthetic scheme for the preparation of (*S*, 2*R*, 3*R*)-**33** and (*R*, 2*R*, 3*R*)-**33**.

To a stirring solution of (*2R*, *3R*)-**15** (8 mg, 0.034 mmol) and dry pyridine (16 mg, 0.20 mmol, 6 eq) in dry dichloromethane (10 mL) at room temperature was added (*R*)-(-)- α -methoxy- α -(trifluoromethyl)phenylacetyl chloride (1.5 mL, 0.05 M solution in dichloromethane, 2.2 eq). The reaction mixture was stirred for 10 hr followed by the addition of water (1 mL) and then stirred for another one hour. The dichloromethane layer was collected, while the aqueous layer was extracted with Et₂O (5 mL \times 2). The combined organic layers were washed with brine (10 mL) and dried over Na₂SO₄. The solvent was removed and the resulting oil was purified by silica gel flash column chromatography (ethyl acetate : hexanes = 1 : 3) to afford compound (*S*, 2*R*, 3*R*)-**33**. ¹H NMR (CDCl₃) δ 1.17-1.33 (m, 7H), 1.44 (tt, *J* = 2.7, 9.4 Hz, 1H), 1.86-1.94 (m, 1H), 2.16-2.37 (m, 2H), 3.56 (s, 3H), 4.04-4.13 (m, 4H), 4.91-5.05 (m, 1H), 5.36-5.38 (m, 1H), 5.43-5.47 (m, 1H), 7.38-7.42 (m, 3H), 7.55-7.57 (m, 2H); ³¹P NMR (CDCl₃) δ 26.4; ¹⁹F NMR (CDCl₃) δ -71.8.

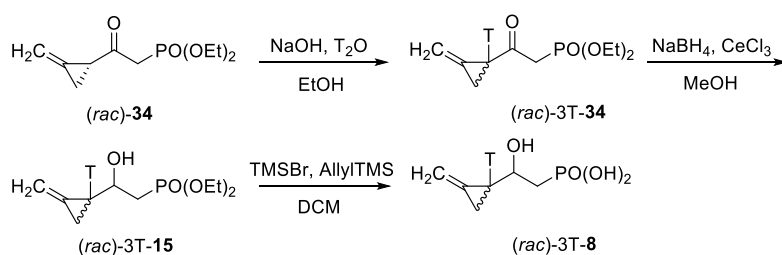
Compound (*R*, 2*R*, 3*R*)-**33** was synthesized following the same procedures described for the syntheses of (*S*, 2*R*, 3*R*)-**33** using (*S*)-(-)- α -methoxy- α -(trifluoromethyl)phenylacetyl chloride as the reagent. ¹H NMR (CDCl₃) δ 1.10-1.12 (m, 1H), 1.24-1.33 (m, 6H), 1.37 (tt, *J* = 2.0, 12.0 Hz, 1H), 1.84-1.90 (m, 1H), 2.19-2.37 (m, 2H), 3.57 (d, *J* = 1.2 Hz, 3H), 4.06-4.15 (m, 4H), 4.91-4.99 (m, 1H), 5.18-5.20 (m, 1H), 5.33-5.35 (m, 1H), 7.38-7.42 (m, 3H), 7.55-7.57 (m, 2H); ³¹P NMR (CDCl₃) δ 25.2; ¹⁹F NMR (CDCl₃) δ -71.4.

2.4. Chemical synthesis of deuterium (D) labeled compound (*rac*)-[3-²H]-**34** and tritium (T) labeled compound (*rac*)-[3-³H]-**8**



Scheme S3. Synthetic scheme for the preparation of deuterium (D) labeled compound (*rac*)-[3-²H]-**34**.

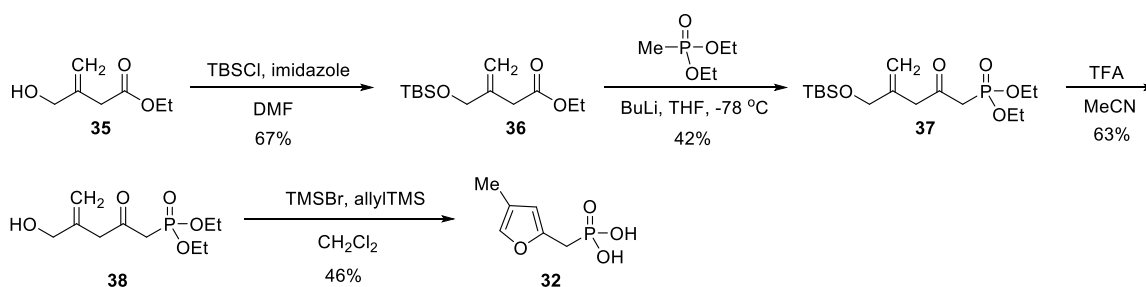
The synthesis of deuterium labeled compound (*rac*)-[3-²H]-**34** was carried out as a model study for the preparation of (*rac*)-[3-³H]-**8**. A solution of (*rac*)-**34**⁴ (16 mg, 0.069 mmol), D₂O (5 mg, 0.25 mmol), and NaOH (10 mg, 0.25 mmol) in EtOH (15 mL) was stirred at room temperature for 72 hr. The organic solvent was removed by reduced pressure distillation, and the resulting mixture was purified by silica gel flash column chromatography (hexanes : ethyl acetate = 1 : 1) to give compound (*rac*)-[3-²H]-**34**. A comparison between the ¹H NMRs of (*rac*)-**34**⁴ and (*rac*)-[3-²H]-**34** clearly revealed that the C3 proton was selectively exchanged under the reaction conditions with ~70% deuterium incorporation (see spectra shown in p. S20). It should be pointed out that solvent deuterium is also expected to be incorporated at C1 position of (*rac*)-**34**. However, due to the greater acidity of the C1 protons, the deuterium incorporated at C1 was exchanged out during the workup process. Thus, deuteration was observed only at C3 but not at C1 at the end of the exchange experiment.



Scheme S4. Synthetic scheme for the preparation of tritium (³H, T) labeled compound (*rac*)-[3-³H]-**8**.

Compound (*rac*)-[3-³H]-**34** was synthesized following the same procedures described for the synthesis of (*rac*)-[3-²H]-**34** using T₂O instead of D₂O as solvent. Then, by applying the same procedure previously described for the synthesis of (*rac*)-**8** from (*rac*)-**34**,⁴ compound (*rac*)-[3-³H]-**8** was obtained from (*rac*)-[3-³H]-**34** with a specific activity of 24.2 μCi/mmol.

2.5. Chemical synthesis of compound **32**



Scheme S5. Synthetic scheme for the preparation of **32**

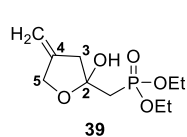
Ethyl 3-(((*tert*-butyldimethylsilyl)oxy)methyl)but-3-enoate (36). A mixture of **35**⁵ (576 mg, 4.0 mmol), *tert*-butyldimethylsilyl chloride (TBSCl, 725 mg, 4.8 mmol) and imidazole (655 mg, 9.6 mmol) in dimethylformamide (DMF, 20 mL) was stirred at room temperature overnight. The reaction mixture was then partitioned between Et₂O (100 mL × 3) and water (50 mL). The ethereal layer was washed with brine (100 mL), dried over Na₂SO₄, filtered, and concentrated to give yellowish crude oil. The crude oil was purified by flash chromatography with silica gel (EtOAc/Hexanes = 5/95) to yield **36** as colorless oil (684 mg, 67%). ¹H NMR (CDCl₃, 400 MHz) δ 5.18 (brd, *J* = 1.6 Hz, 1H, 4-H), 4.98 (brd, *J* = 1.6 Hz, 1H, 4-H), 4.15 (brs, 2H, TBSO - CH₂), 4.12 (q, *J* = 7.2 Hz, 2H, CH₂ of OEt), 3.04 (s, 2H, 2-H), 1.24 (t, *J* = 7.2 Hz, CH₃ of OEt), 0.89 (s, 9H, *t*-Bu of TBS), 0.05 (s, 6H, 2 Me of TBS). ¹³C NMR (CDCl₃, 100 MHz) δ 171.3 (C1), 141.6 (C3), 113.1 (C4), 65.5 (TBS-OCH₂), 60.6 (CH₂ of OEt), 38.6 (C2), 25.9 (OSiCCH₃), 18.3 (OSiCCH₃), 14.2 (CH₃ of OEt), -5.5 (OSiCH₃). HRMS (ESI): calcd for C₁₃H₂₆O₃Si (M+Na⁺) 281.15430, found 281.15410.

Diethyl (4-(((*tert*-butyldimethylsilyl)oxy)methyl)-2-oxopent-4-en-1-yl)phosphonate (37). To a solution of diethyl methylphosphonate (0.41 mL, 2.8 mmol) in THF (5 mL) cooled at -78 °C was added BuLi (2.5 M in hexane, 1.2 mL, 3 mmol) dropwise over 5 min. After stirred at -78 °C for 30 min, the resulting mixture was added dropwise into a solution of **36** (600 mg, 2.32 mmol) in THF (25 mL) cooled at -78 °C over 10 min. The reaction mixture was stirred at the same temperature for another 2 hr before quenched with NH₄Cl_(aq). It was then partitioned between Et₂O (50 mL × 5) and water (50 mL). The combined ethereal layers were washed with brine (100 mL), dried over Na₂SO₄, filtered, and concentrated to give pale yellow oil, which was further chromatographed with silica gel (EtOAc/Hexanes = 1/1 ~ 7/3) to yield **37** as pale-yellow oil (356 mg, 42%). It should be noted that 297 mg (50%) of unreacted **36** was also recovered. ¹H NMR (CDCl₃, 400 MHz) δ 5.23 (brd, *J* = 1.2 Hz, 1H, 5-H), 4.98 (brd, *J* = 1.2 Hz, 1H, 5-H), 4.14 (qd, 2H, *J* = 7.2, 2.0 Hz, CH₂ of OEt), 4.11 (qd, 2H, *J* = 7.2, 1.2 Hz, CH₂ of OEt), 4.06 (brs, 2H, TBS-OCH₂), 3.31 (s, 2H, 3-H), 3.11 (d, *J* = 22.4 Hz, 1-H), 1.32

(td, $J = 7.2, 0.4$ Hz, 6H, two CH₃ of OEt), 0.87 (s, 9H, *t*-Bu of TBS), 0.03 (s, 6H, two Me of TBS). ¹³C NMR (CDCl₃, 100 MHz) δ 199.8 (d, $J = 6.1$ Hz, C2), 141.6 (C4), 114.4 (C5), 65.7 (TBS-OCH₂), 62.5 (d, $J = 6.4$ Hz, CH₂ of OEt), 48.3 (C3), 41.3 (d, $J = 127.3$ Hz, C1), 25.9 (OSiC(CH₃)), 18.3 (OSiC(CH₃)), 16.3 (d, $J = 6.1$ Hz, CH₃ of OEt), 5.5 (OSi(CH₃)). ³¹P NMR (CDCl₃, 162 MHz) δ 19.8. HRMS (ESI): calcd for C₁₆H₃₃O₅PSi (M+Na⁺) 387.17270, found 387.17300.

Diethyl (4-(hydroxymethyl)-2-oxopent-4-en-1-yl)phosphonate (38). To a stirred solution of **37** (37 mg, 0.1 mmol) in acetonitrile (MeCN, 0.4 mL) was added trifluoroacetic acid (TFA, 1% in water, 0.1 mL). After stirred at room temperature for 30 min, the reaction was quenched with NaHCO_{3(aq)}. It was then partitioned between CH₂Cl₂ (5 mL \times 5) and water (5 mL). Combined organic layers were dried over Na₂SO₄, filtered, and concentrated to give crude oil, which was further purified by flash chromatography with silica gel (MeOH/EtOAc = 5/95) to yield **38** as colorless oil (15.9 mg, 63%). The cyclized hemiacetal **39** (7.8 mg, 31%) and trace amount of furan **40** (1.7 mg, 6%), which are difficult to separate, were also isolated as a mixture. It was found that compound **38** spontaneously cyclized to give **39** and then dehydrated to give **40**. Hence, more **40** was generated and the amount of **38** kept decreasing during the course of chromatographic purification (could be monitored by TLC, **40** is less polar). The isolated **38** was therefore used for the next step as soon as possible. ¹H NMR (CDCl₃, 400 MHz) δ 5.21 (brd, $J = 1.2$ Hz, 1H, 5-H), 4.99 (brd, $J = 1.2$ Hz, 1H, 5-H), 4.22-4.20 (m, 6H, two CH₂ of OEt and CH₂OH), 3.41 (s, 2H, 3-H), 3.13 (d, $J = 22.8$ Hz, 2H, 1-H), 1.31 (t, $J = 7.2$ Hz, 6H, two CH₃ of OEt). ¹³C NMR (CDCl₃, 100 MHz) δ 200.4 (d, $J = 5.9$ Hz, C2), 141.9 (C4), 115.9 (C5), 65.7 (CH₂-OH), 62.8 (d, $J = 6.5$ Hz, CH₂ of OEt), 48.7 (C3), 41.8 (d, $J = 126.1$ Hz, C1), 16.3 (d, $J = 6.1$ Hz, CH₃ of OEt). ³¹P NMR (CDCl₃, 162 MHz) δ 19.8. HRMS (ESI): calcd for C₁₀H₁₉O₅P (M+Na⁺) 273.08620, found 273.08590.

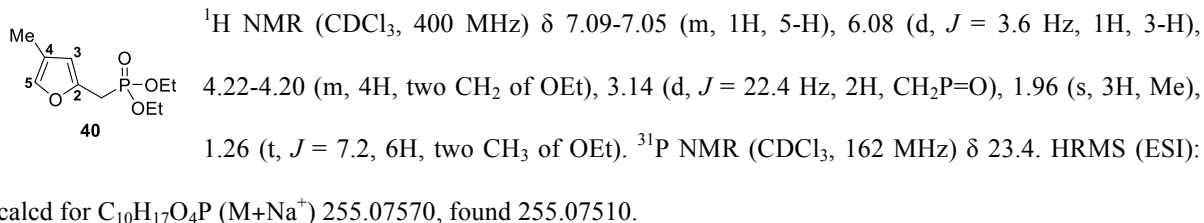
Cyclized side-product: Diethyl ((2-hydroxy-4-methylenetetrahydrofuran-2-yl)methyl)phosphonate (39)



¹H NMR (CDCl₃, 400 MHz) δ 5.21 (brd, $J = 1.2$ Hz, 1H, CH₂=C), 4.99 (brs, 1H, CH₂=C), 4.53 (brd, $J = 12.8$ Hz, 1H, 5-H), 4.53 (brd, $J = 12.8$ Hz, 1H, 5-H), 4.22-4.20 (m, 4H, two CH₂ of OEt), 2.72 (brd, $J = 16.0$ Hz, 1H, 3-H), 2.45 (brd, $J = 16.0$ Hz, 1H, 3-H), 2.35 (dd, $J = 18.0, 15.2$ Hz, 1H, CH₂P=O), 2.35 (dd, $J = 18.4, 15.2$ Hz, 1H, CH₂P=O), 1.31 (t, $J = 7.2$ Hz, 6H, two CH₃ of OEt). ¹³C NMR (CDCl₃, 100 MHz) δ 145.7 (C3), 105.3 (C=C), 103.1 (C2), 70.2 (C5), 62.3 (d, $J = 6.4$ Hz, one CH₂ of OEt), 61.5 (d, $J = 6.6$ Hz, one CH₂ of OEt), 45.4 (C3), 35.7 (d, $J = 134.8$ Hz, CH₂P=O), 16.3 (d, $J =$

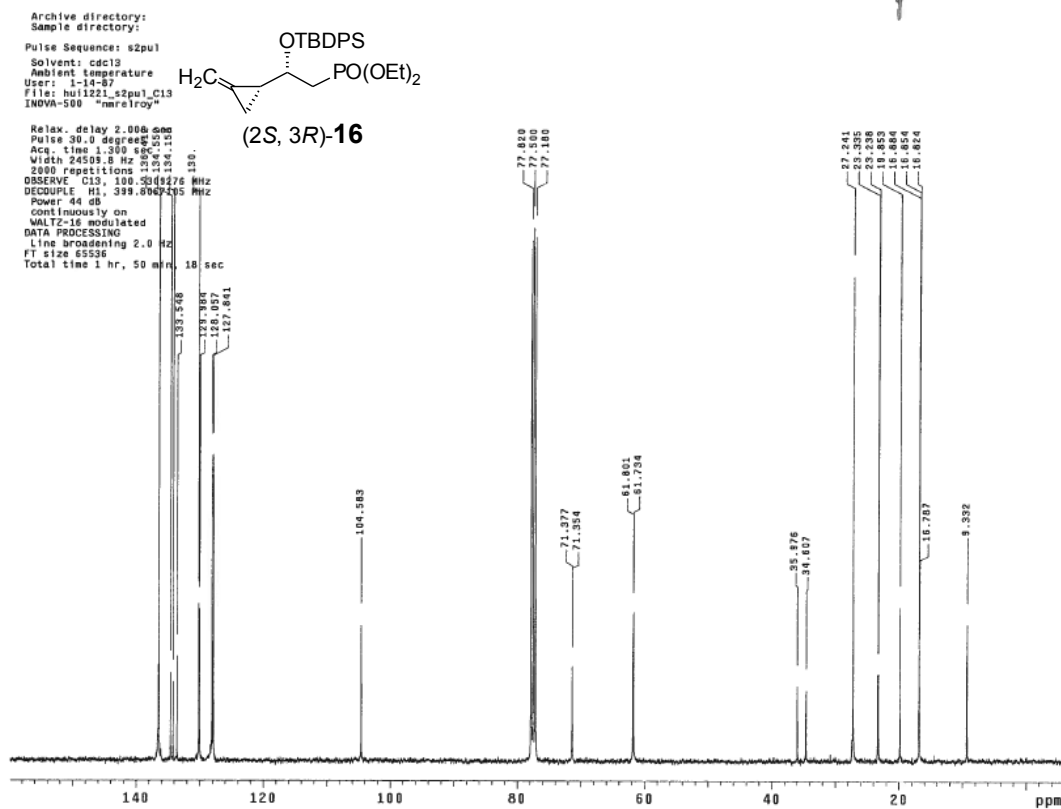
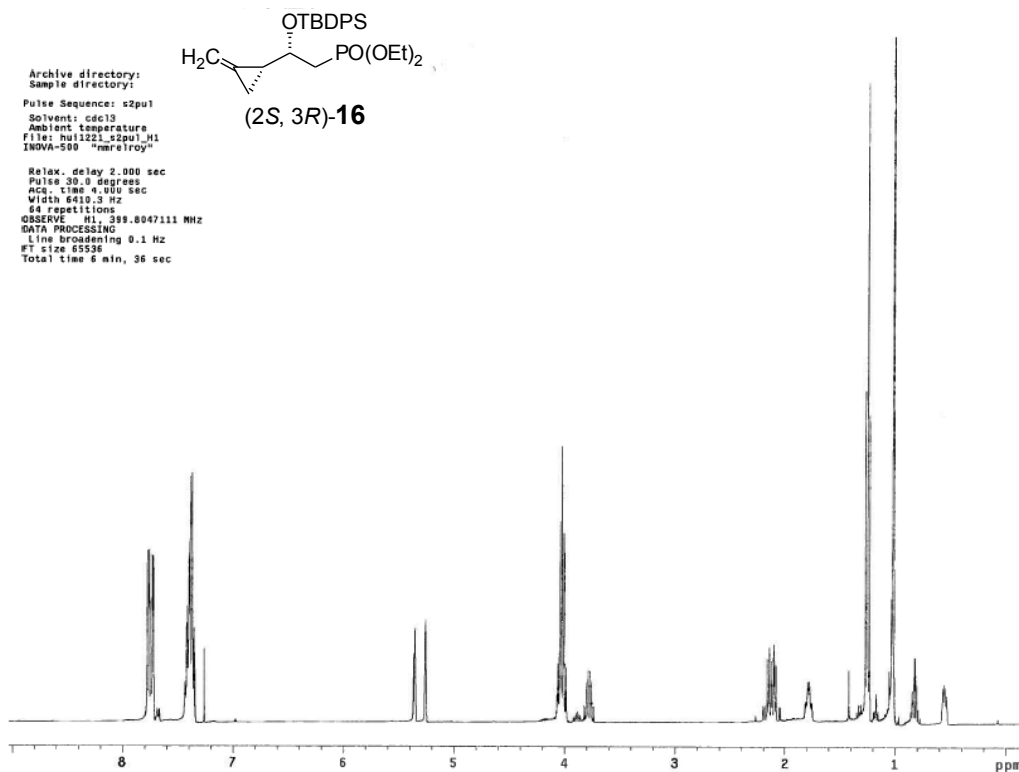
6.1 Hz, CH₃ of OEt). ³¹P NMR (CDCl₃, 162 MHz) δ 27.7. HRMS (ESI): calcd for C₁₀H₁₉O₅P (M+Na⁺) 273.08620, found 273.08590.

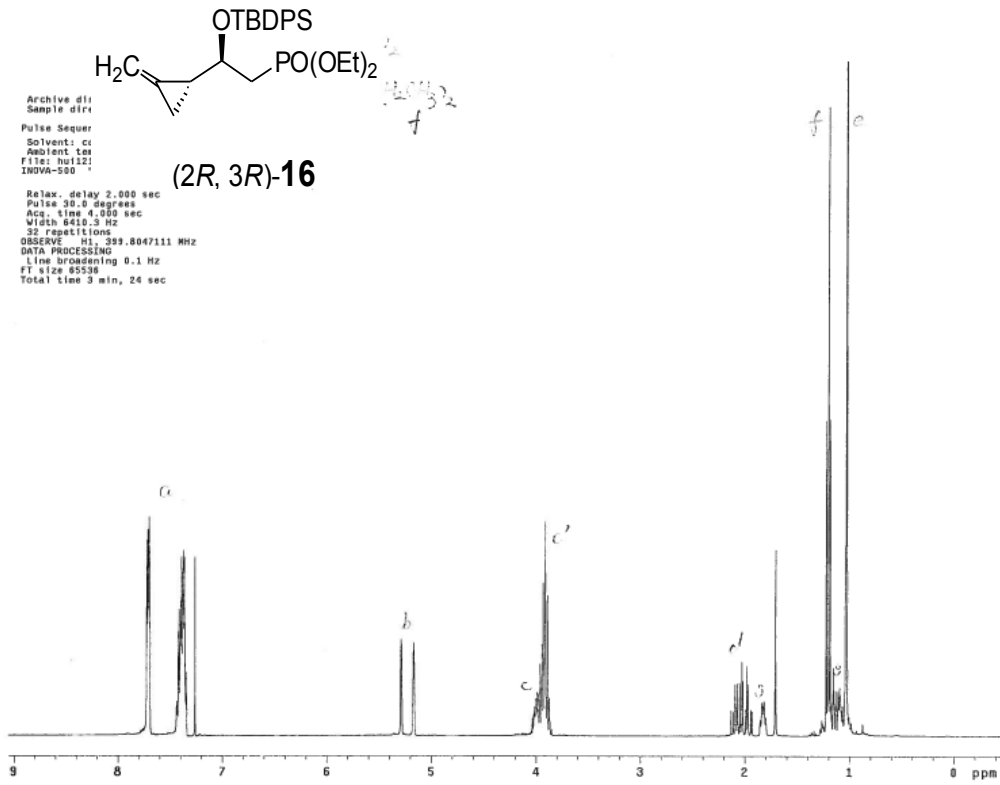
Dehydrated side-product: Diethyl ((4-methylfuran-2-yl)methyl)phosphonate (40)



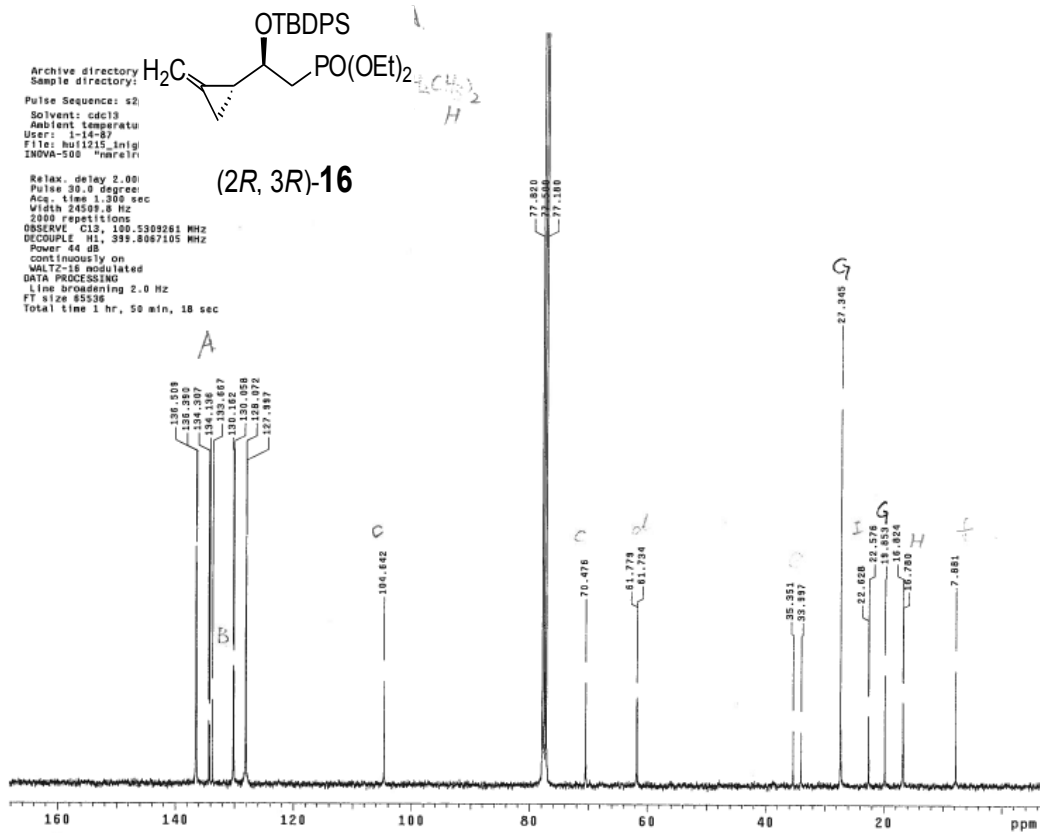
((4-Methylfuran-2-yl)methyl)phosphonic acid (32). A mixture of **38** (28.5 mg, 0.114 mmol), TMSBr (75.2 μL, 0.57 mmol) and allylTMS (145 μL, 0.91 mmol) in CH₂Cl₂ (1 mL) was stirred at room temperature for 30 min. It was then evaporated to remove volatile components. The residue was partitioned between CH₂Cl₂ (2.5 mL) and NH₄HCO₃ (0.2 M, 2.5 mL), and the aqueous layer was lyophilized. Only **32** instead of **30** was obtained as white solid (10.2 mg, 46%). ¹H NMR (D₂O, 400 MHz) δ 7.10-7.07 (m, 1H, 5H), 5.98 (d, *J* = 3.2 Hz, 1H, 3-H), 2.77 (d, *J* = 18.8 Hz, 2H, CH₂P=O), 1.90 (brs, 3H, Me). ³¹P NMR (D₂O, 162 MHz) δ 17.0. HRMS (ESI): calcd for C₆H₉O₄P (M-H⁻) 175.01660, found 175.01660.

3. NMR Spectra of Synthetic Compounds





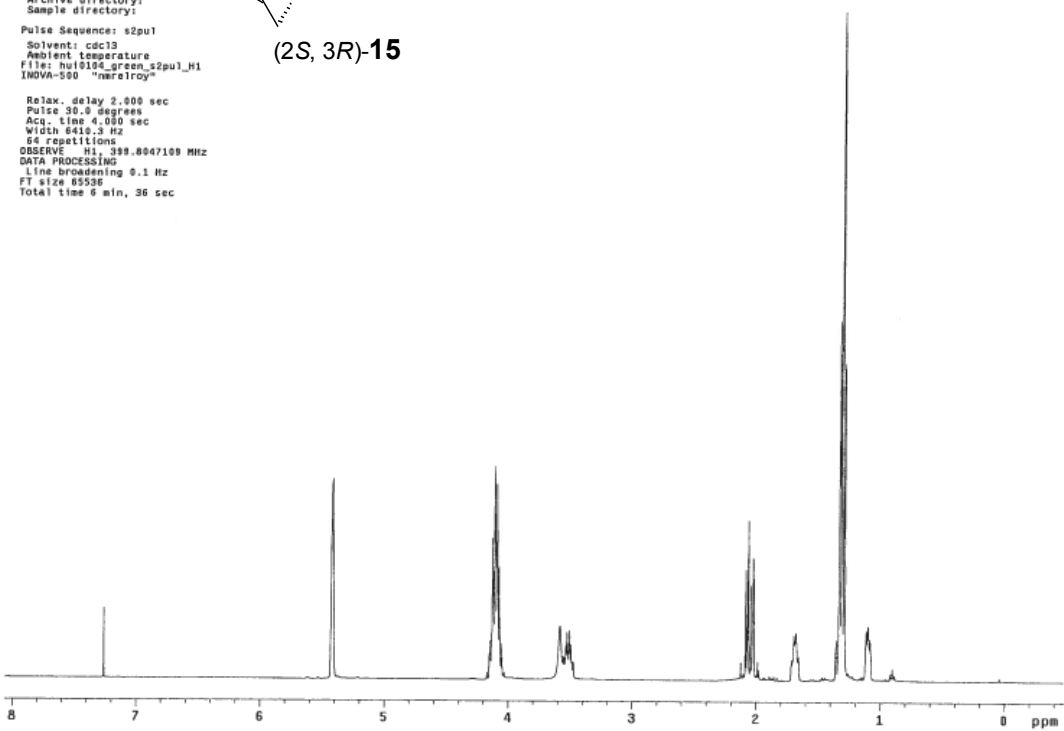
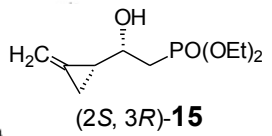
¹H NMR of (2R, 3R)-16



¹³C NMR of (2R, 3R)-16

Archive directory:
 Sample directory:
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 Solvent: cdcl3
 Ambient temperature
 File: hu10104_green_s2pu1_H1
 INOVA-500 "nmrelroy"

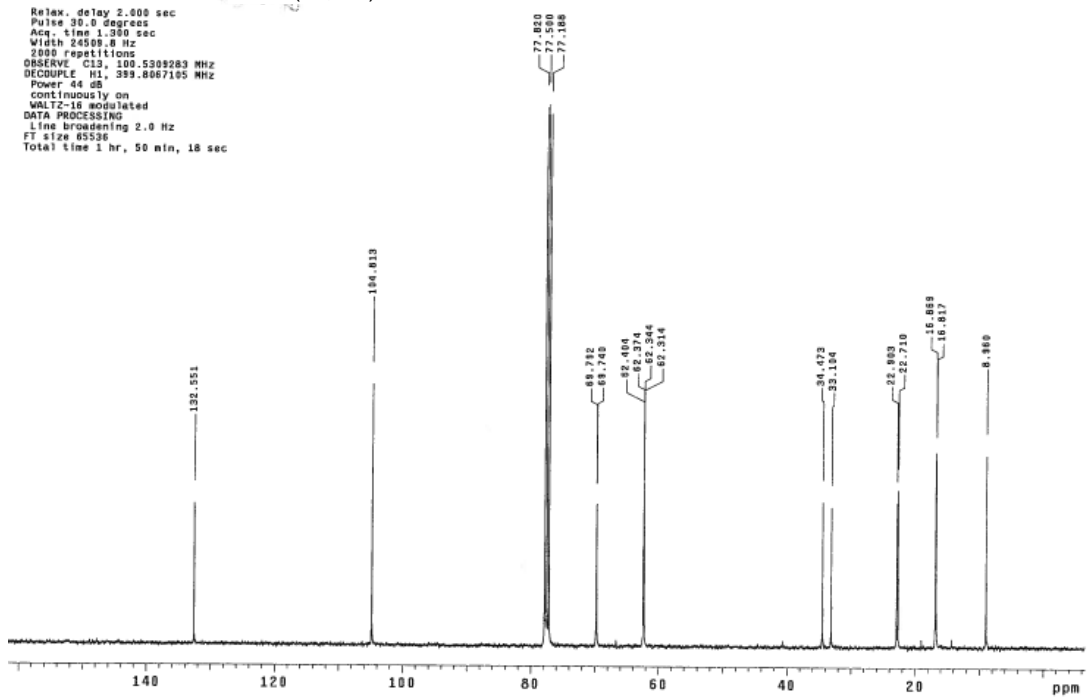
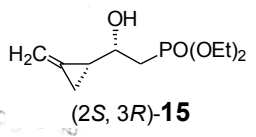
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 Acq. time 4.000 sec
 Width 6410.3 Hz
 64 repetitions
 OBSERVE F1, 399.0047109 MHz
 DATA PROCESSING
 Line broadening 0.1 Hz
 FT size 65536
 Total time 6 min, 36 sec



¹H NMR of (2*S*, 3*R*)-15

Archive directory:
 Sample directory:
 Pulse Sequence: s2pu1
 Solvent: cdcl3
 Ambient temperature
 User: 1-14-87
 File: hu10104_green_s2pu1_C13
 INOVA-500 "nmrelroy"

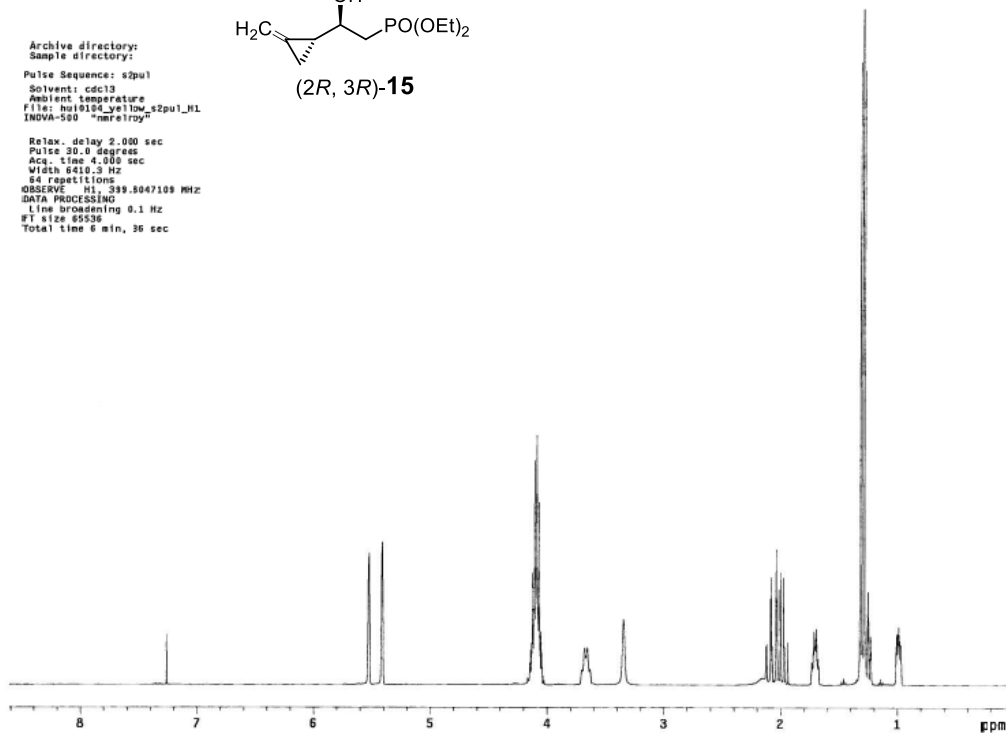
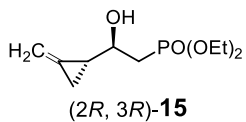
Relax. delay 2.000 sec
 Pulse 30.0 degrees
 Acq. time 1.300 sec
 Width 20569.0 Hz
 2000 repetitions
 OBSERVE C13, 100.5309203 MHz
 DECOUPLE H1, 399.8667105 MHz
 Power 44 dB
 continuously on
 WALTZ-16 modulated
 DATA PROCESSING
 Line broadening 2.0 Hz
 FT size 65536
 Total time 1 hr, 50 min, 18 sec



¹³C NMR of (2*S*, 3*R*)-15

Archive directory:
 Sample directory:
 Pulse Sequence: s2pu1
 Solvent: cdcl3
 Ambient temperature
 File: hu10104_yellow_s2pu1_M1
 INOVA-500 "nmrelroy"

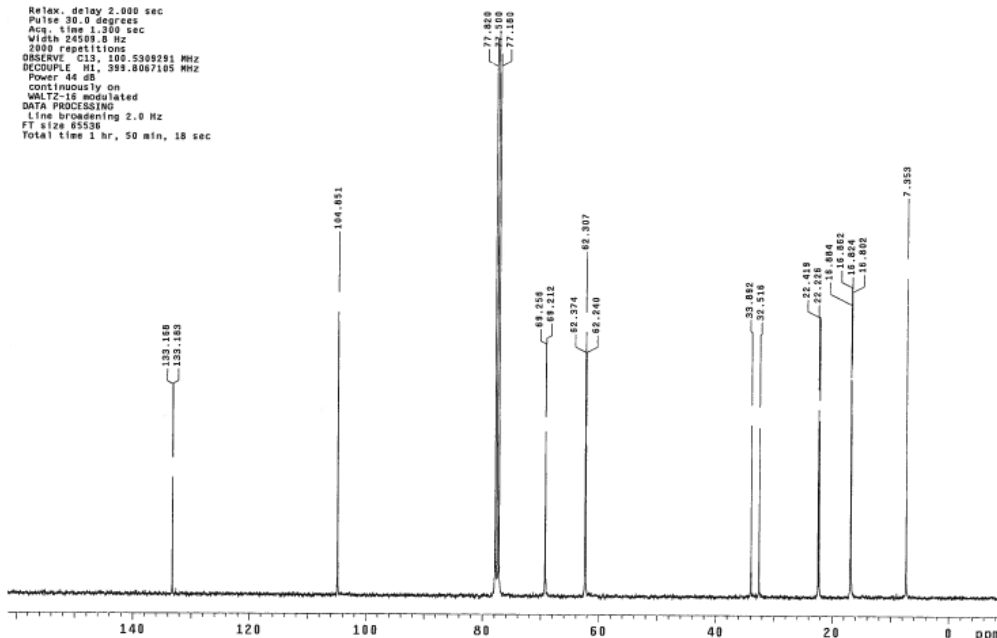
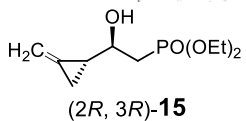
Relax. delay 2.000 sec
 Pulse 30.0 degrees
 Acq. time 4.000 sec
 Width 6410.0 Hz
 64 repetitions
 OBSERVE M1, 399.8047109 MHz
 DATA PROCESSING
 Line broadening 0.1 Hz
 FT size 65536
 Total time 6 min, 36 sec



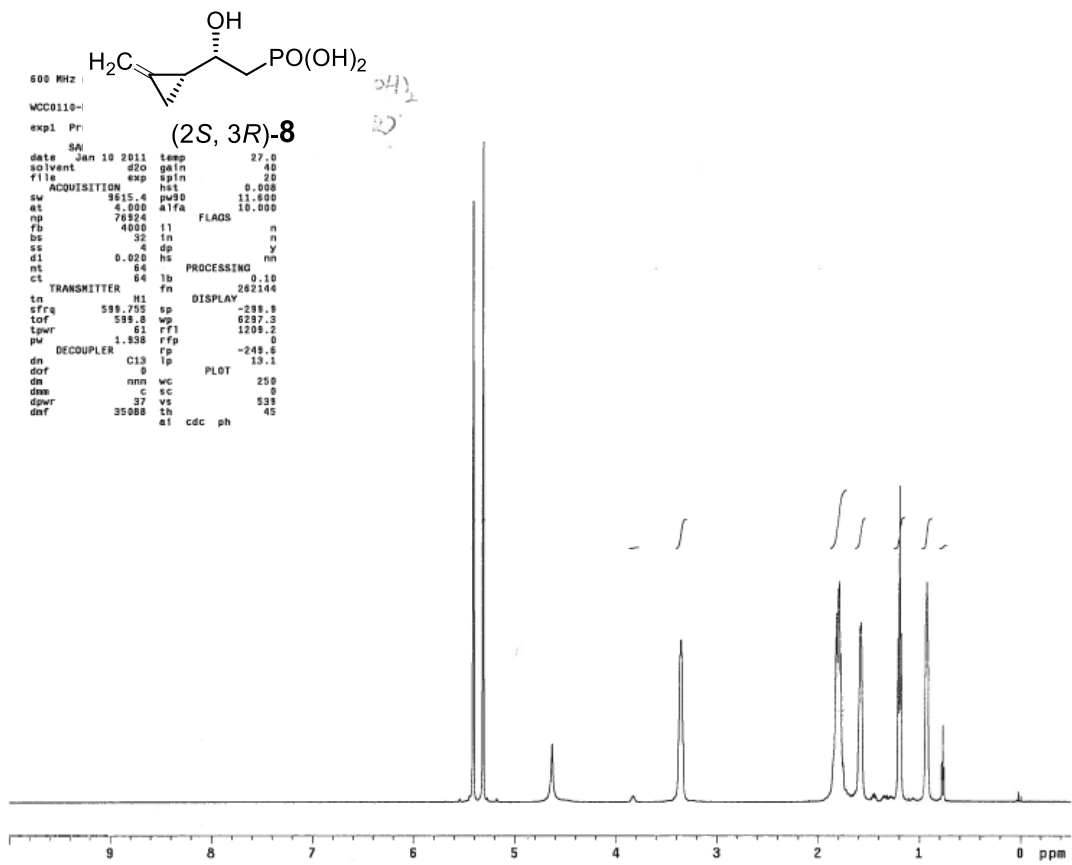
¹H NMR of (2*R*, 3*R*)-15

Archive directory:
 Sample directory:
 Pulse Sequence: s2pu1
 Solvent: cdcl3
 Ambient temperature
 User: i-14-87
 File: hu10104_yellow_s2pu1_M1
 INOVA-500 "nmrelroy"

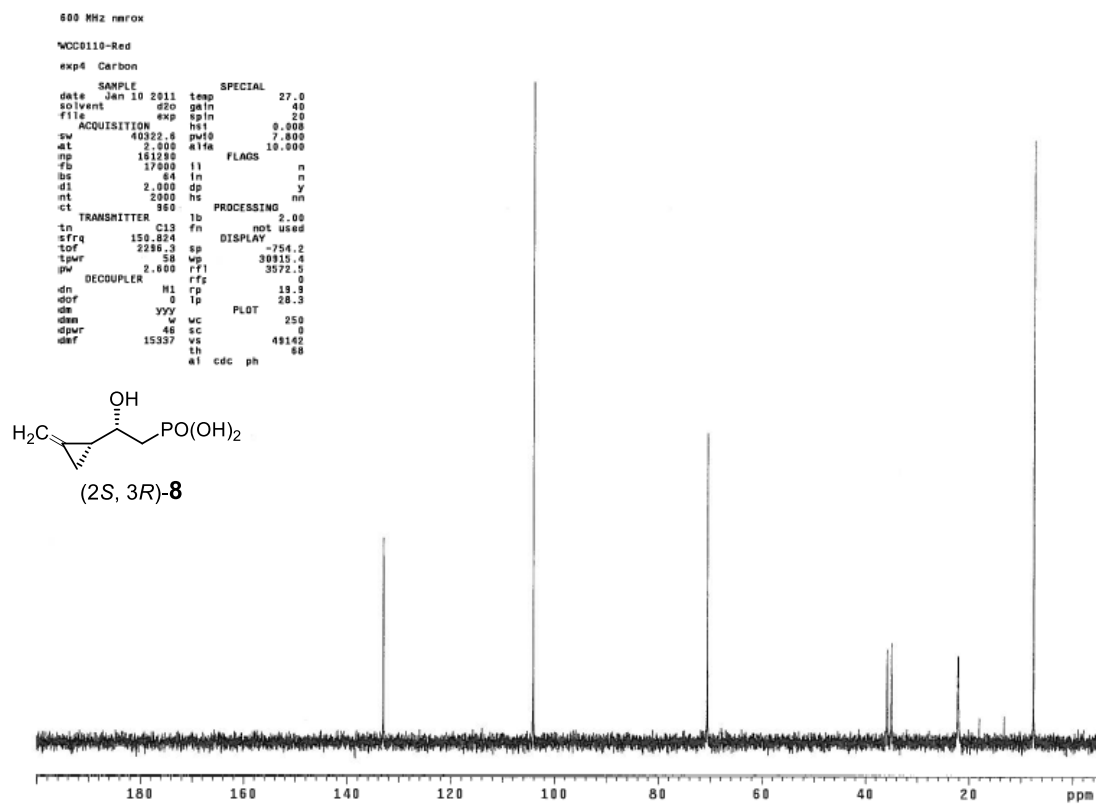
Relax. delay 2.000 sec
 Pulse 30.0 degrees
 Acq. time 1.300 sec
 Width 24500.0 Hz
 2000 repetitions
 OBSERVE C13, 100.5309291 MHz
 DECOUPLE M1, 399.8067105 MHz
 Power 48 dB
 continuously on
 WALTZ-16 modulated
 DATA PROCESSING
 Line broadening 2.0 Hz
 FT size 65536
 Total time 1 hr, 50 min, 18 sec



¹³C NMR of (2*R*, 3*R*)-15



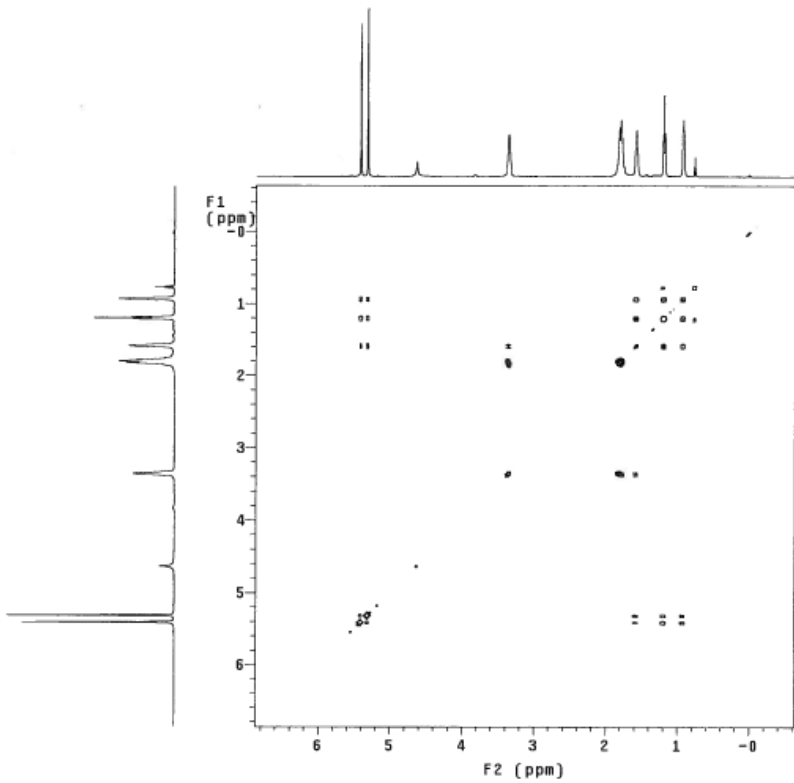
¹H NMR of (2*S*, 3*R*)-**8**



¹³C NMR of (2*S*, 3*R*)-**8**

exp3 Gcosy

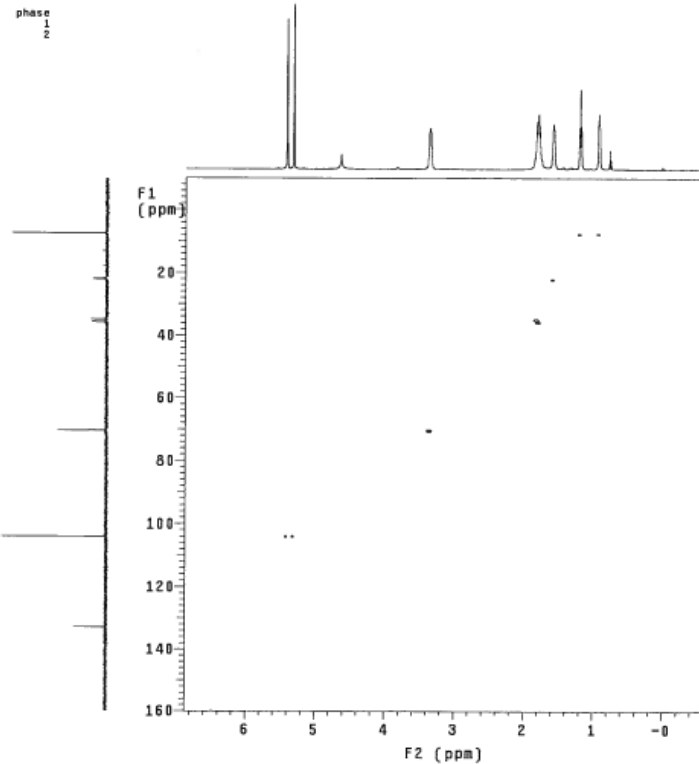
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solvent d2o sspul                y
sample hsglv1 SPECIAL            7042
ACQUISITION
sw 4496.4 temp 27.0
at 0.228 gain 4
np 2048 spin 0
fb 4000 F2 PROCESSING
ss 16 sb -0.114
d1 0 sbs not used
nt 2 fn 4096
2D ACQUISITION F1 PROCESSING
sw1 4496.4 sb1 -0.114
nl 512 sbs1 not used
PRESATURATION proc1 1p
satmode y fn1 4096
satdly 2.000 DISPLAY
satfrq -220.3 sp -374.4
satpwr -13 wp 4494.2
TRANSMITTER sp1 -374.4
tn H1 wp1 4494.2
sfrq 599.753 rf1 376.6
tof -1127.1 rfp 0
tpwr 61 rf11 376.6
pw 11.600 rfp1 0
tpwr_cf 1.064 PLOT 0
GRADIENTS wc 116.0
gzlv11 9389 sc 10.0
gt1 0.001000 wc2 116.0
gstab 0.000500 sc2 0
DECOUPLER vs 33140
dn C13 th cdc av 2
dm nnn at cdc av 2
```



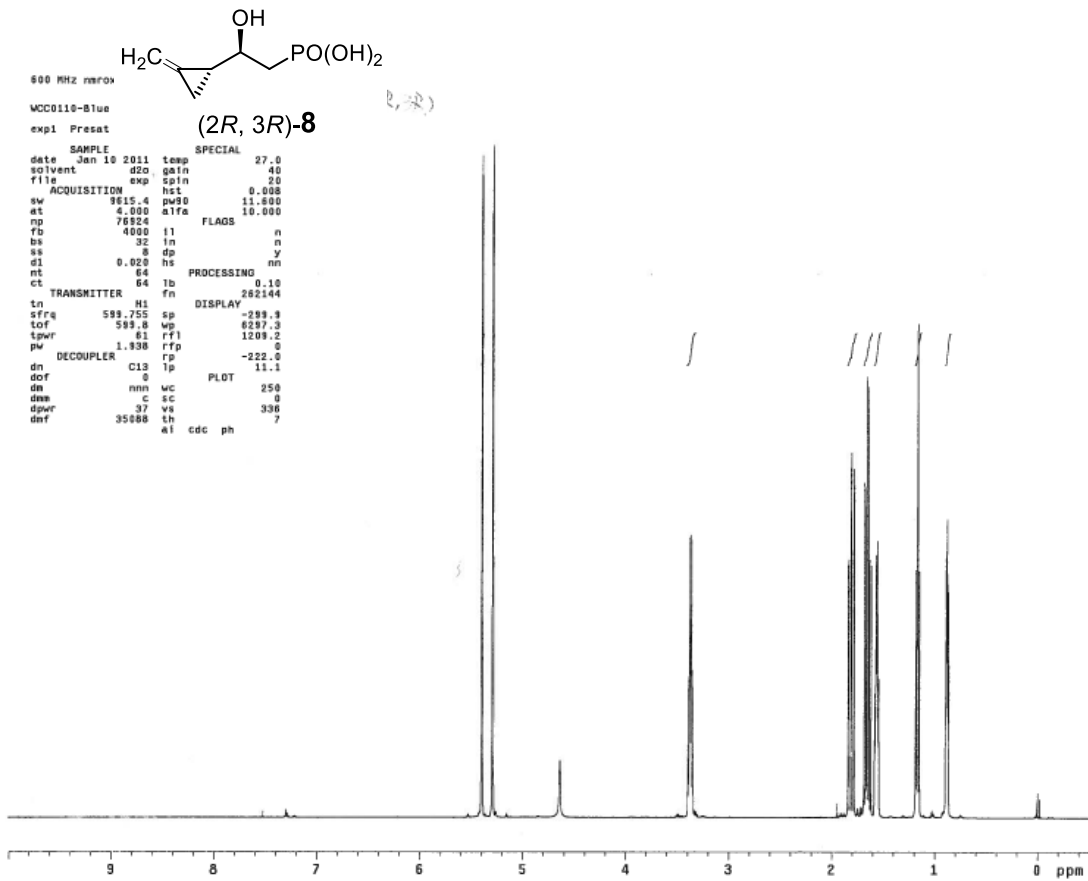
COSY of (2S, 3R)-8

exp5 Ghsqc

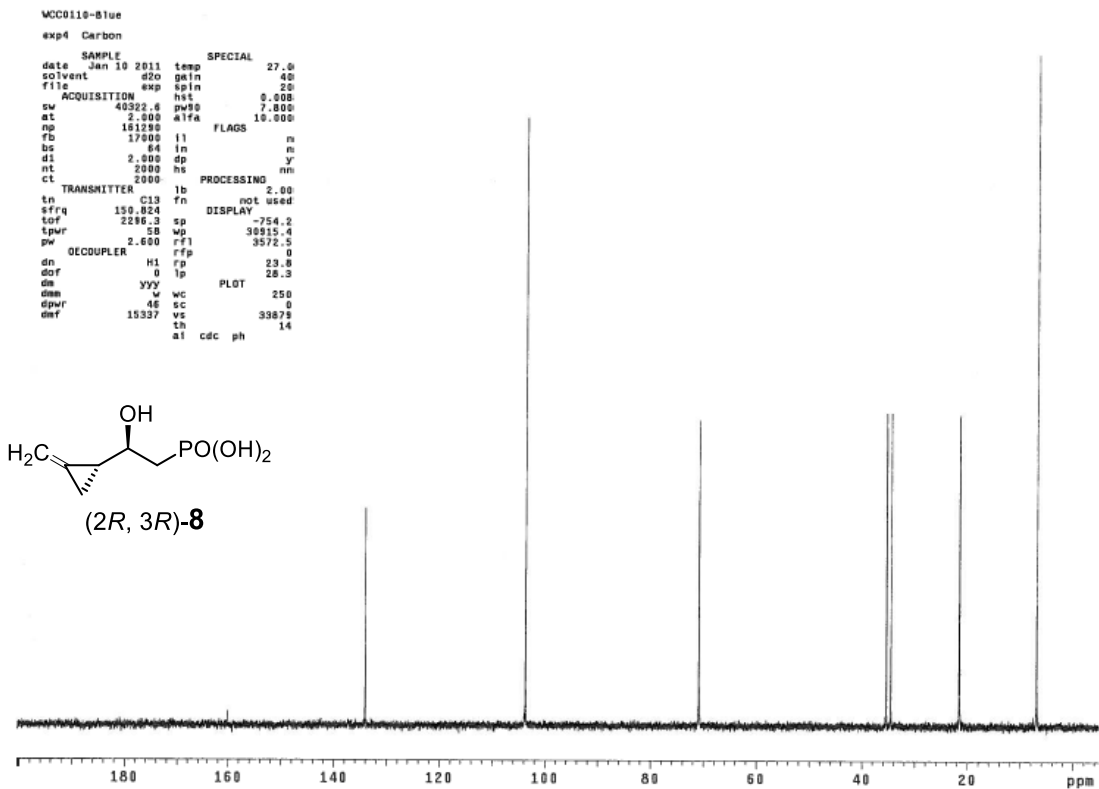
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date Jan 10 2011 hc              array phase
solvent d2o sspul                y arraydim 512
sample hsglv1 SPECIAL            8802 1 phase 1
ACQUISITION
sw 4496.4 temp 27.0
at 0.198 gain 30
np 4096 spin 0
fb 4000 F2 PROCESSING
ss 8 GRADIENTS
d1 0 gzlv11 11737
nt 4 gt1 0.001000
2D ACQUISITION gzlv13 5901
sw1 29641.0 gt3 0.000500
nl 256 gstab 0.000500
phase arrayed F2 PROCESSING
PRESATURATION gf 0.582
satmode yy gfs not used
satdly 2.000 fn 4096
satfrq -220.3 F1 PROCESSING
satpwr -13 gfl 0.009
TRANSMITTER gfs1 not used
tn H1 proc1 1p
sfrq 599.753 rf1 2048
tof -1127.1 fn1 DISPLAY
tpwr 61 sp -374.4
pw 11.600 wp 4494.2
DECOUPLER sp1 -1484.9
dn C13 wp1 25816.0
dof -2981.9 rf1 376.6
dm nny rfp 0
daf 35088 rf11 1509.8
dpxr 37 rfp1 0
dpxlv1 57 PLOT 0
pwx HSQC 8.800 wc 116.0
j1xh 140.0 wc2 116.0
nullfg y sc2 0
mult 2 vs 2671
at cdc ph
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HSQC of (2S, 3R)-8



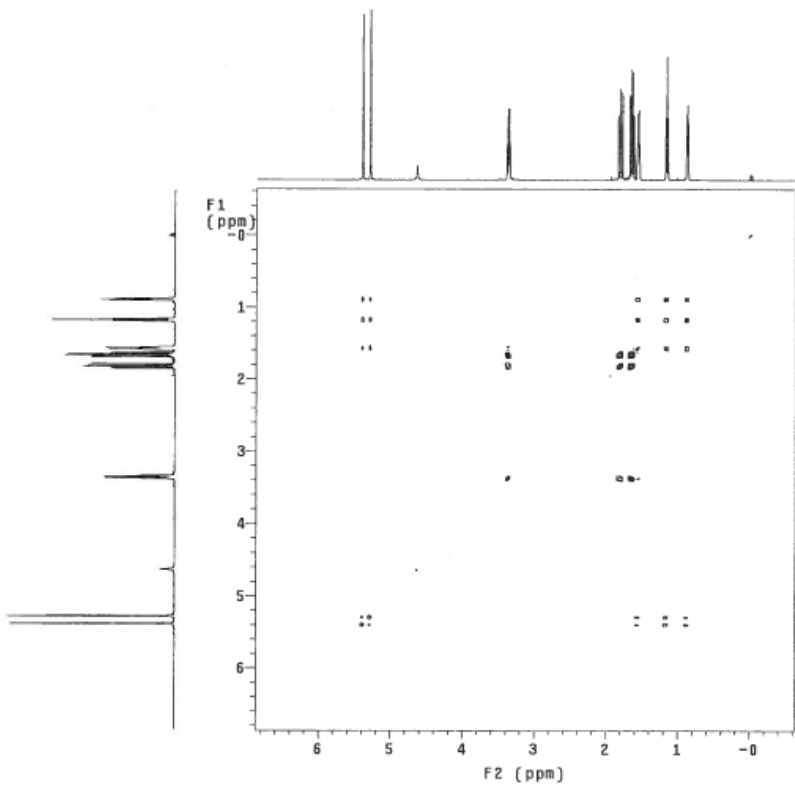
^1H NMR of (2*R*, 3*R*)-**8**



^{13}C NMR of (2*R*, 3*R*)-**8**

exp3 Gcoxy

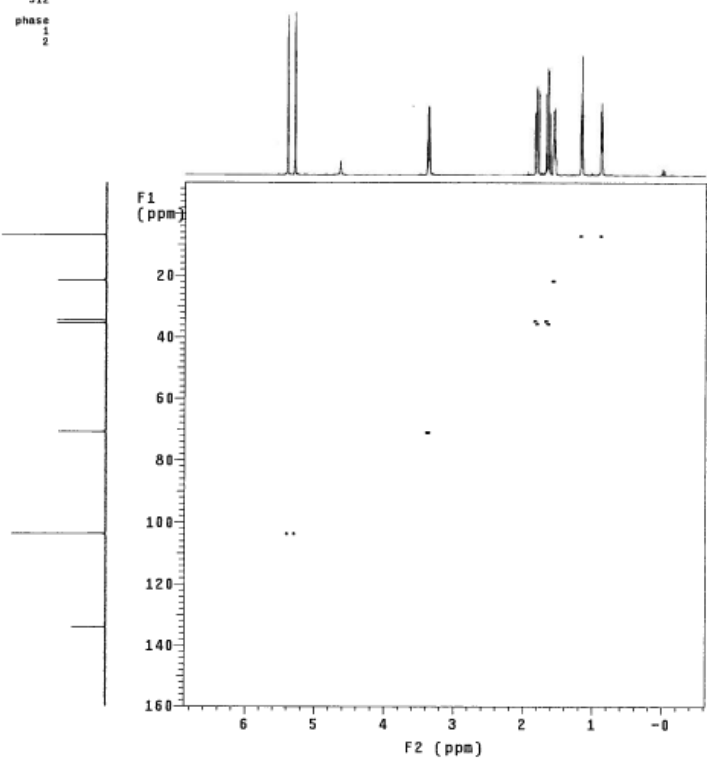
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sample          hsglv1 704Z
ACQUISITION     SPECIAL
sv 4498.4 temp 27.0
at 0.228 gain 4
np 2048 spin 0
fb 4000 F2 PROCESSING
ss 16 sb -0.114
dl 0 sbs not used
nt 2 fn 4096
2D ACQUISITION F1 PROCESSING
sw1 4498.4 sb1 -0.114
nl 512 sbs1 not used
PRESATURATION  proc1 lp
satmode y fn1 4096
satdly 2.000 DISPLAY
satfrq -220.3 sp -374.4
satpwr -13 wp 4494.2
TRANSMITTER    sp1 -374.4
tn H1 wp1 4494.2
sfrq 599.753 rf1 376.6
tof -1127.1 rfp 0
tpwr 61 rfp1 376.6
pw 11.600 rfp1 0
tpwr_cf 1.064 PLOT
GRADIENTS      wc 116.0
gzlv1 8389 sc 10.0
gt1 0.001000 wc2 116.0
gstab 0.000500 sc2 0
DECOUPLER      vs 331.0
dn C13 th 2
dm nnn ai cdc av
```



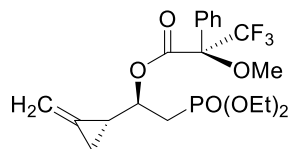
COSY of (2R, 3R)-8

exp6 Hsqc

```
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solvent d2o      sspul y          arraydim 512
sample          PFGFlg
ACQUISITION     SPECIAL      8802 1 phase
sv 4498.4 temp 27.0          2 1
at 0.198 gain 30
np 2048 spin 0
fb 4000 F2 PROCESSING
ss 8 gztv11 11737
dl 4 gt1 0.001000
nt 4 gztv13 3901
2D ACQUISITION gztv13 3901
sw1 2584.0 st3 0.000500
nl 256 gstab 0.000500
phase arrayed  F2 PROCESSING
PRESATURATION  pf 0.092
satmode yy gfs not used
satdly 2.000 fn 4096
satfrq -220.3 F1 PROCESSING
satpwr -13 gf1 0.009
TRANSMITTER    gfs1 not used
tn H1 proc1 lp
sfrq 599.753 fn1 2048
tof -1127.1 sp -374.4
tpwr 61 wp 4494.2
pw 11.600 wp1 -1484.9
DECOUPLER      sp1 25816.0
dn C13 wp1 376.6
dof -2881.8 rf1 0
dm nny rfp 0
dof 35088 rf1 1509.9
dpwr 37 rfp1 0
pwxlv1 57 PLOT
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j1xb sc 10.0
multfg 140.0 wc2 116.0
mult 2 vs 1571
mult th 2
ai cdc ph
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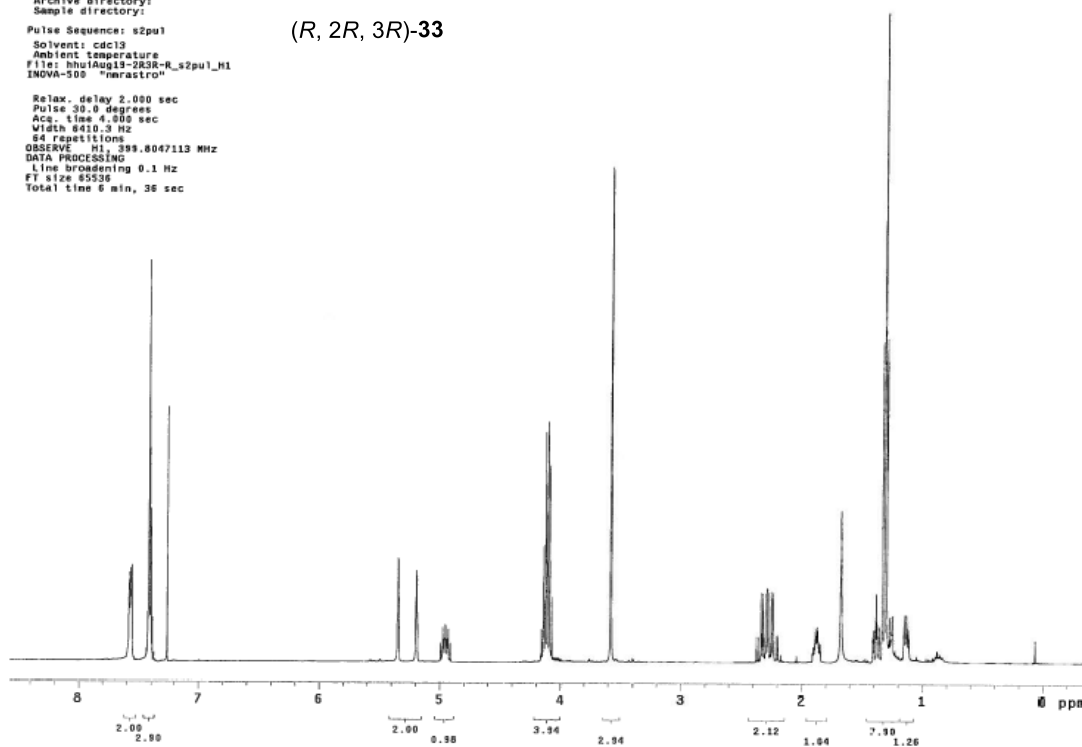


HSQC of (2R, 3R)-8

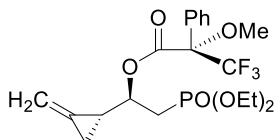


(*R*, 2*R*, 3*R*)-**33**

Archive directory:
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 Pulse Sequence: s2pu1
 Solvent: cdCl3
 Ambient temperature
 File: hh1Aug19-2R3R-R_s2pu1_H1
 INOVA-500 "nraastro"
 Relax. delay 2.000 sec
 Pulse 30.0 degrees
 Acq. time 4.300 sec
 Width 6410.3 Hz
 64 repetitions
 OBSERVE H1, 399.8047113 MHz
 DATA PROCESSING
 Line broadening 0.1 Hz
 FT size 65536
 Total time 6 min, 36 sec

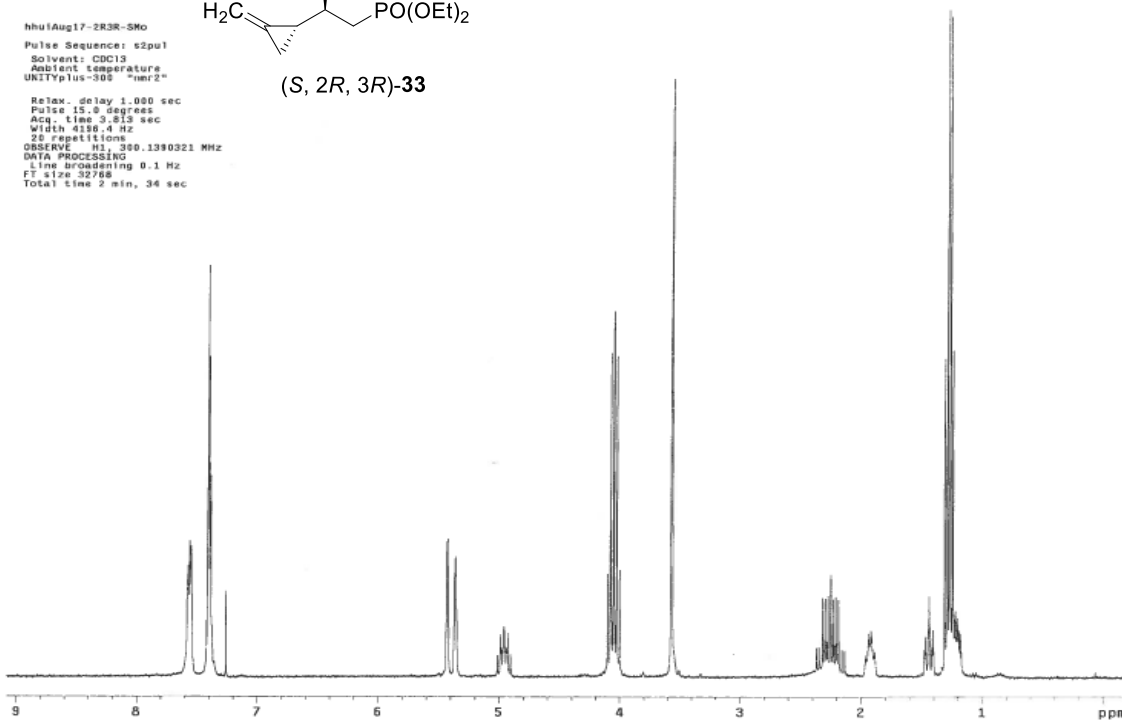


¹H NMR of (*R*, 2*R*, 3*R*)-**33**

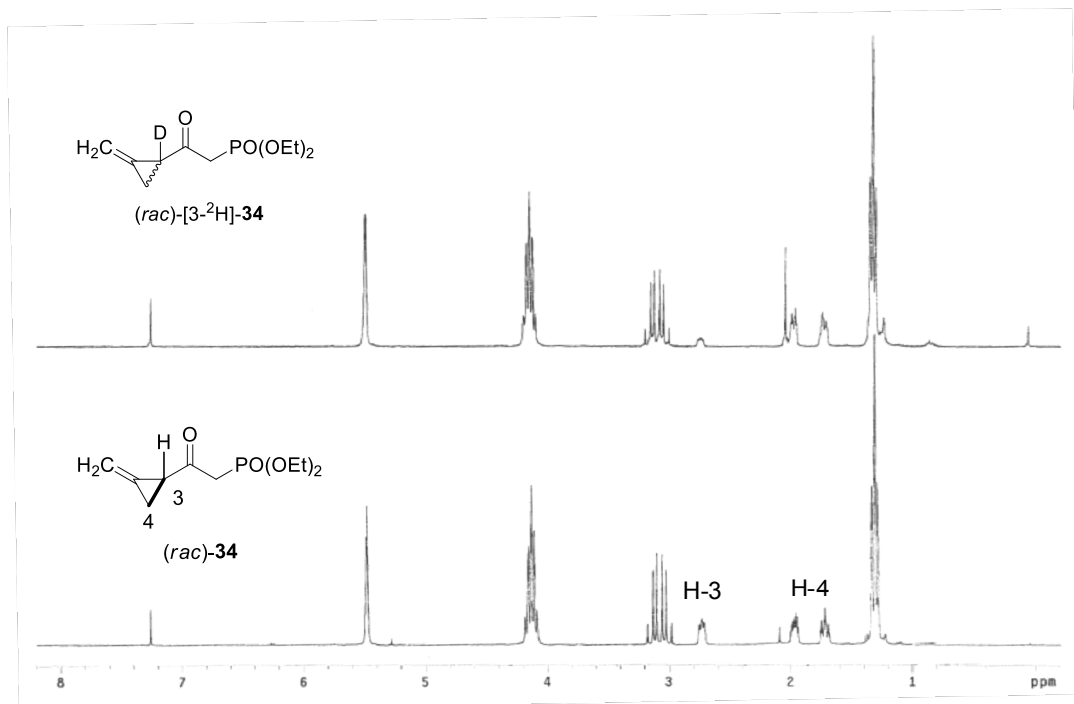


(*S*, 2*R*, 3*R*)-**33**

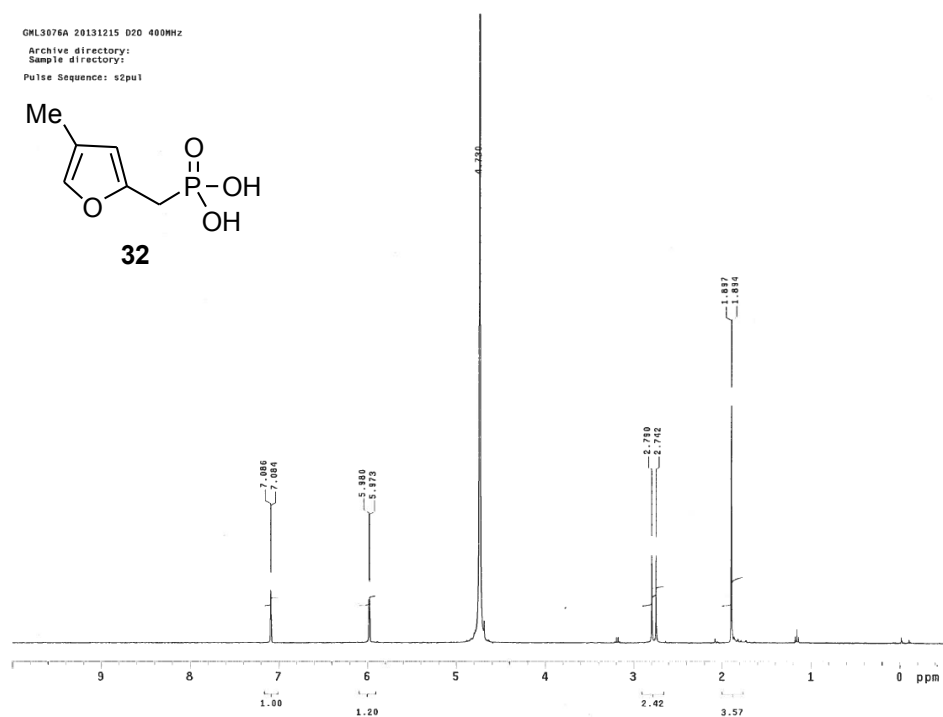
hh1Aug17-2R3R-SM
 Pulse Sequence: s2pu1
 Solvent: CDCl3
 Ambient temperature
 UNITYplus-300 "mr2"
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 Pulse 15.0 degrees
 Acq. time 3.919 sec
 Width 4186.4 Hz
 32 repetitions
 OBSERVE H1, 300.1390321 MHz
 DATA PROCESSING
 Line broadening 0.1 Hz
 FT size 32768
 Total time 2 min, 36 sec



¹H NMR of (*S*, 2*R*, 3*R*)-**33**



^1H NMR of (rac) -**34** (bottom) and (rac) -[3- ^2H]-**34** (top)



^1H NMR of **32**

4. NMR-monitored Enzymatic Assays of (2*S*, 3*S*)-**8**, (2*S*, 3*R*)-**8**, (2*R*, 3*R*)-**8** and (2*R*, 3*S*)-**8**

Reaction mixtures containing 0.25 mM HppE, 0.25 mM Fe(NH₄)₂(SO₄)₂•6H₂O, 7.5 mM FMN, 5 mM of each substrate, and 25 mM NADH in 700 μL of 20 mM Tris buffer (pH 7.5, prepared with H₂O) 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 dimethylsulfoxide-d₆ (DMSO-d₆, 30 μL). Chemical shifts are standardized to the DMSO-d₆ signal at δ 2.49. The enzymatic reactions were initiated by adding reconstituted HppE to the reaction mixture. The authentic racemic ketone standard [(*rac*)-**23/26**] was synthesized from (*rac*)-**34** according to the literature.⁴ 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-course spectra for the reaction of HppE with various substrates are given below.

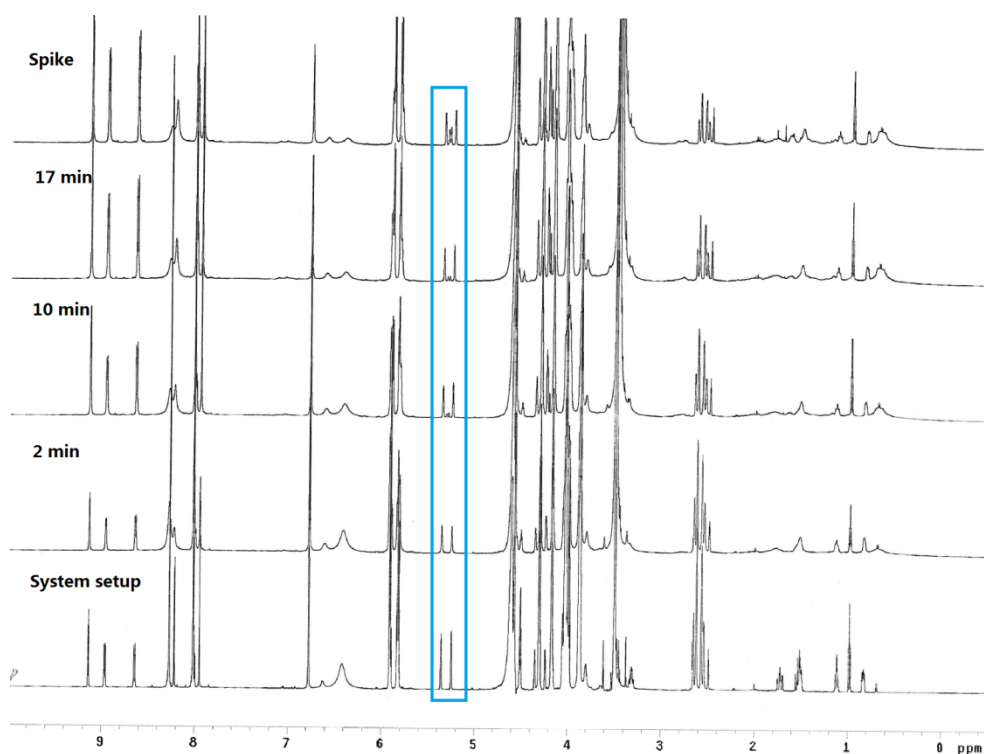


Figure S1a. ^1H NMR time-course of HppE incubation with $(2R, 3R)$ -**8**. A synthetic standard $[(rac)\text{-}23/26]$ was added to the NMR tube at the end of the experiment. The characteristic alkene peaks were circled with blue box and magnified as showed in **Figure S1b**.

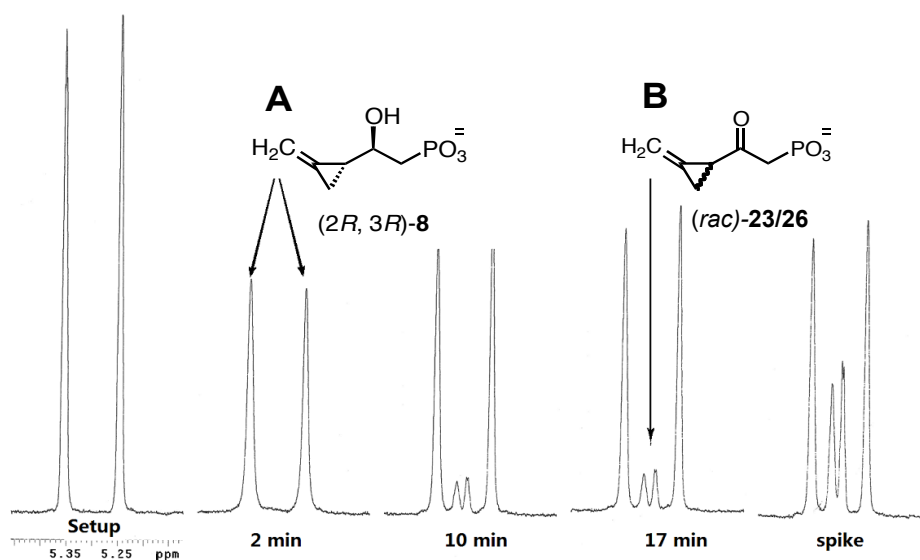


Figure S1b. ^1H NMR time-course of HppE incubation with $(2R, 3R)$ -**8** (the alkene region). A synthetic standard $[(rac)\text{-}23/26]$ was added to the NMR tube at the end of the experiment.

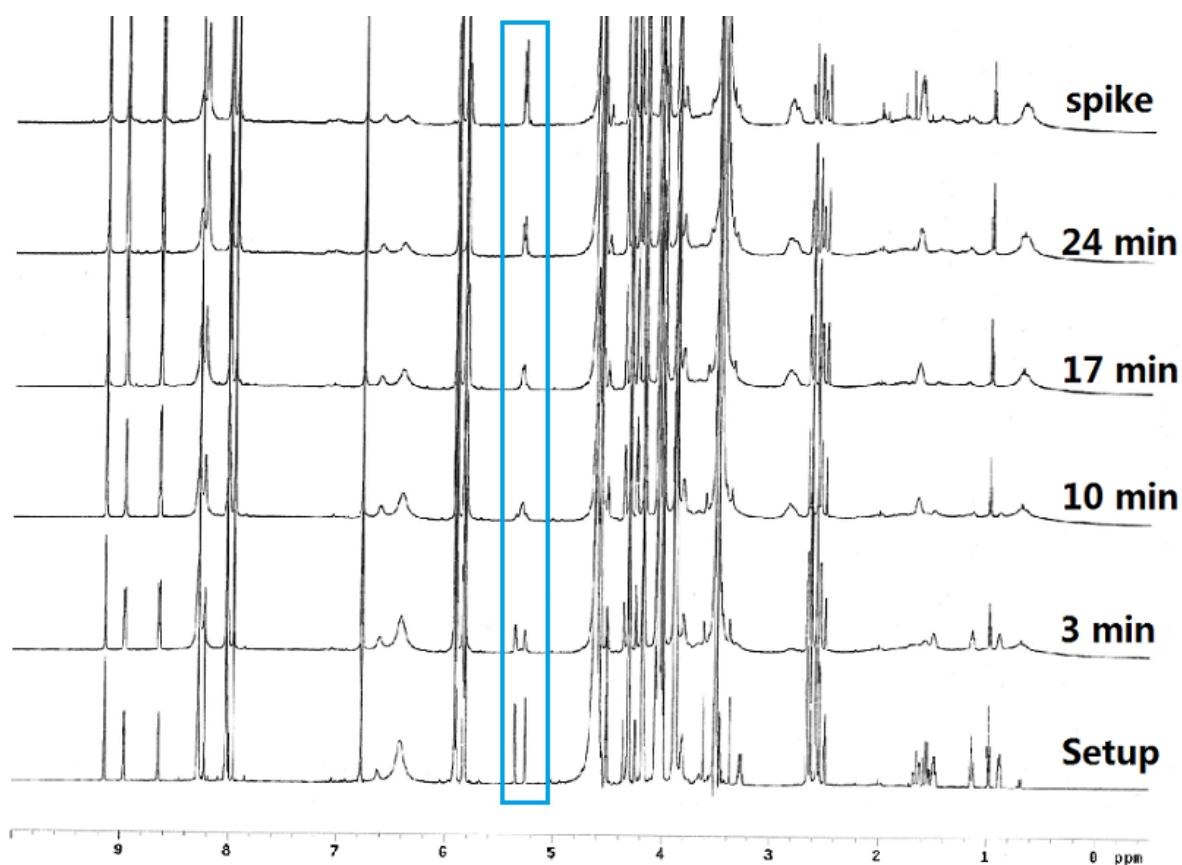


Figure S2a. ^1H NMR time-course of HppE incubation with $(2R, 3S)$ -**8** to **26**. A synthetic standard $[(rac)\text{-}23/26]$ was added to the NMR tube at the end of the experiment. The characteristic alkene peaks were circled with blue box and magnified as showed in **Figure S2b**.

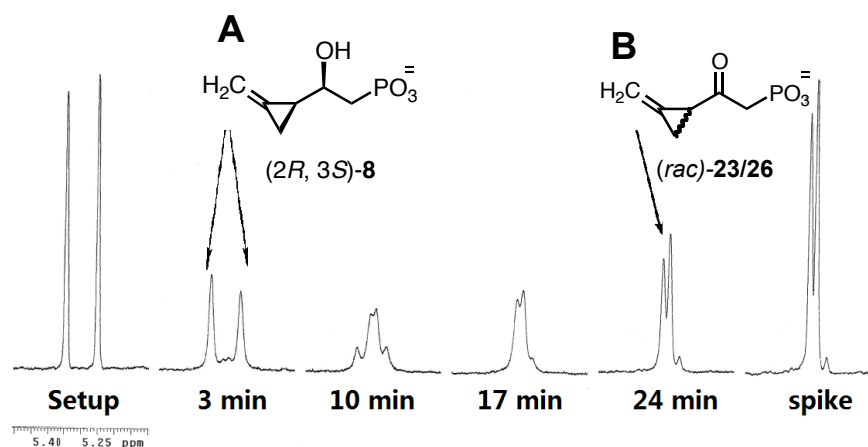


Figure S2b. ^1H NMR time-course of HppE incubation with $(2R, 3S)$ -**8** (the alkene region). A synthetic standard $[(rac)\text{-}23/26]$ was added to the NMR tube at the end of the experiment.

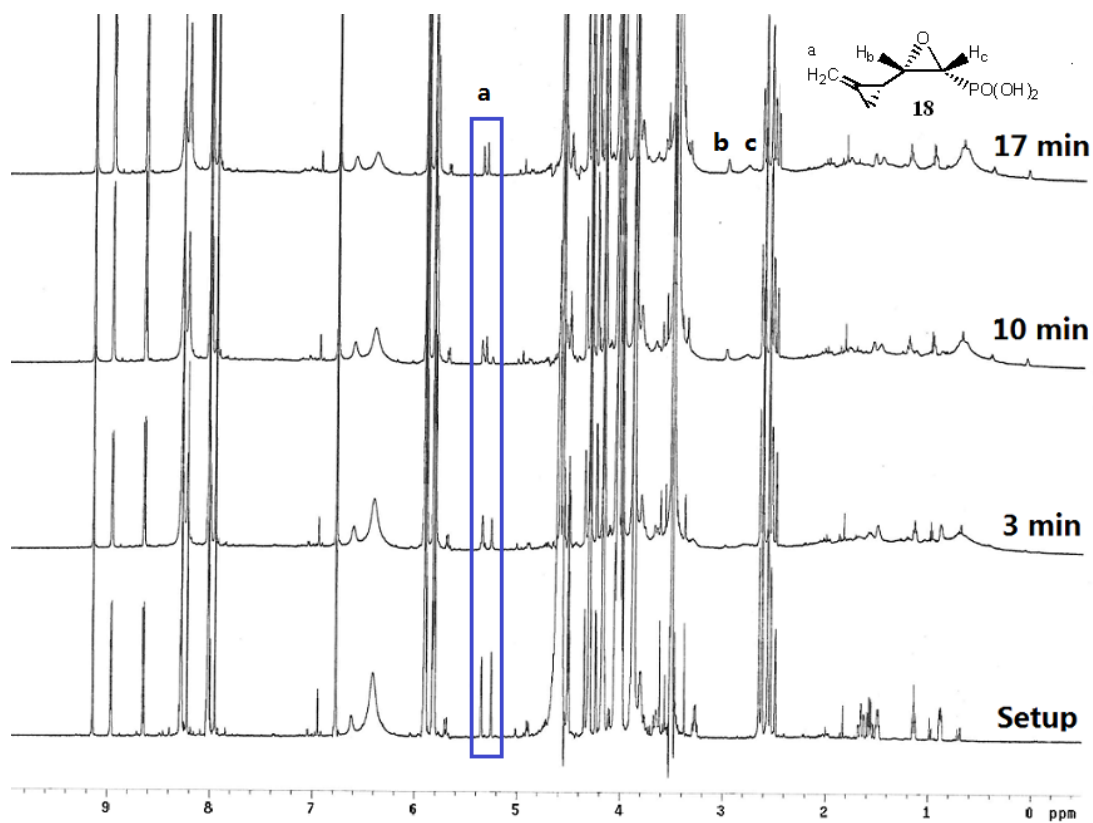


Figure S3a. ^1H NMR time-course of HppE incubation with $(2S, 3R)$ -8 to 18. The characteristic alkene peaks were circled with blue box and magnified as showed in **Figure S3b**.

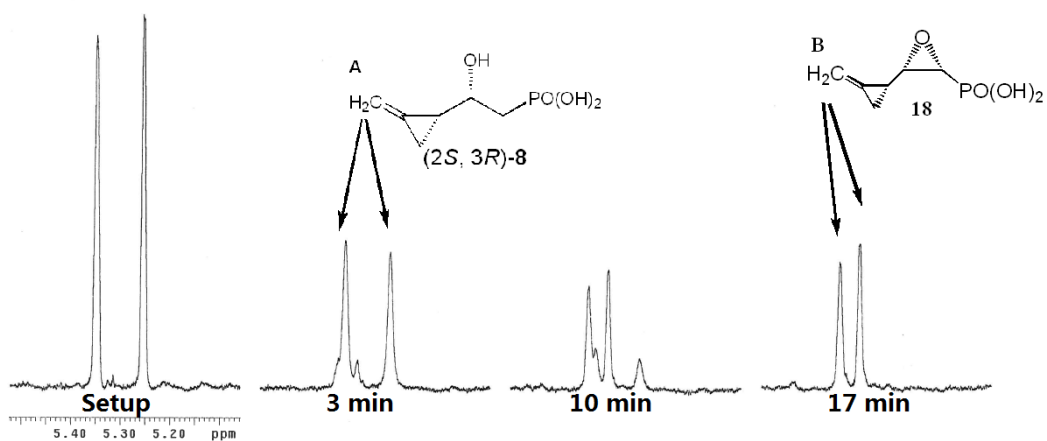


Figure S3b. ^1H NMR time-course of HppE incubation with $(2S, 3R)$ -8 (the alkene region).

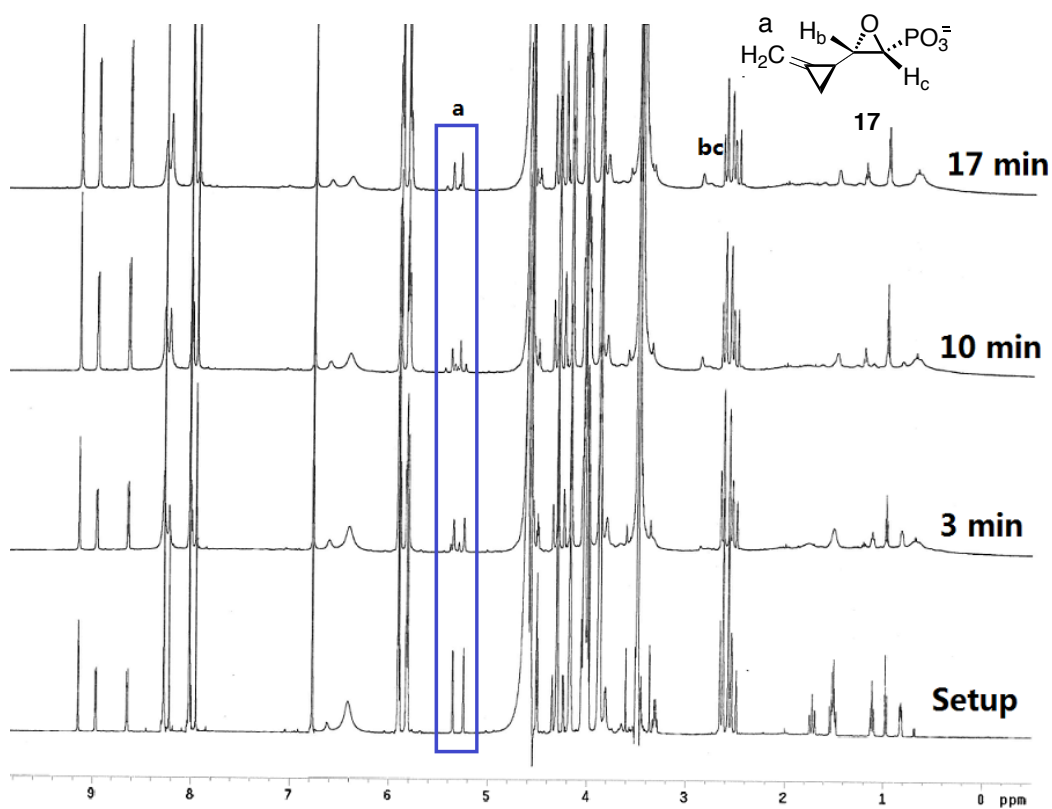


Figure S4a. ^1H NMR time-course of HppE incubation with (2*S*, 3*S*)-8 to 17. The characteristic alkene peaks were circled with blue box and magnified as shown in **Figure S4b**.

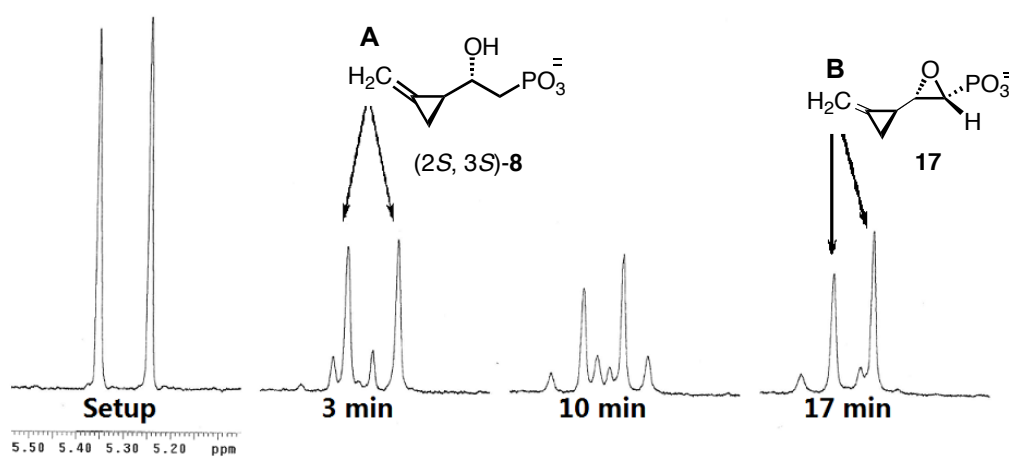


Figure S4b. ^1H NMR time-course of HppE incubation with (2*S*, 3*S*)-8 (the alkene region).

5. HPLC Analysis of Fosfomycin Formation Using HppE Pre-incubated with (2*R*, 3*R*)-8

The inactivation of HppE by (2*R*, 3*R*)-8 was determined by HPLC analysis of fosfomycin formation using enzyme which was pre-incubated with (2*R*, 3*R*)-8 for varied length of time as described below. For the time-dependent enzyme inactivation experiment, a freshly prepared reaction solution of 0.05 mM HppE, 1.5 mM FMN and 5 mM NADH in 50 mM Tris buffer (pH 7.5) was incubated with 0.5 mM (2*R*, 3*R*)-8 for 0, 20, 40, 60, or 90 seconds. To each of the pre-incubated enzyme solution was then added an equal volume of (*S*)-HPP (10 mM) solution making the final concentration ratio of HppE : (2*R*, 3*R*)-8 : (*S*)-HPP = 1 : 10 : 200. These samples were allowed to react for another 20 min before being quenched and analyzed by HPLC using a Dionex[®] column. The column was eluted with water and NH₄OAc solution using the literature reported procedure and the elution was monitored using a Corona detector.¹ The residual enzyme activity was determined based on the amount of fosfomycin produced.

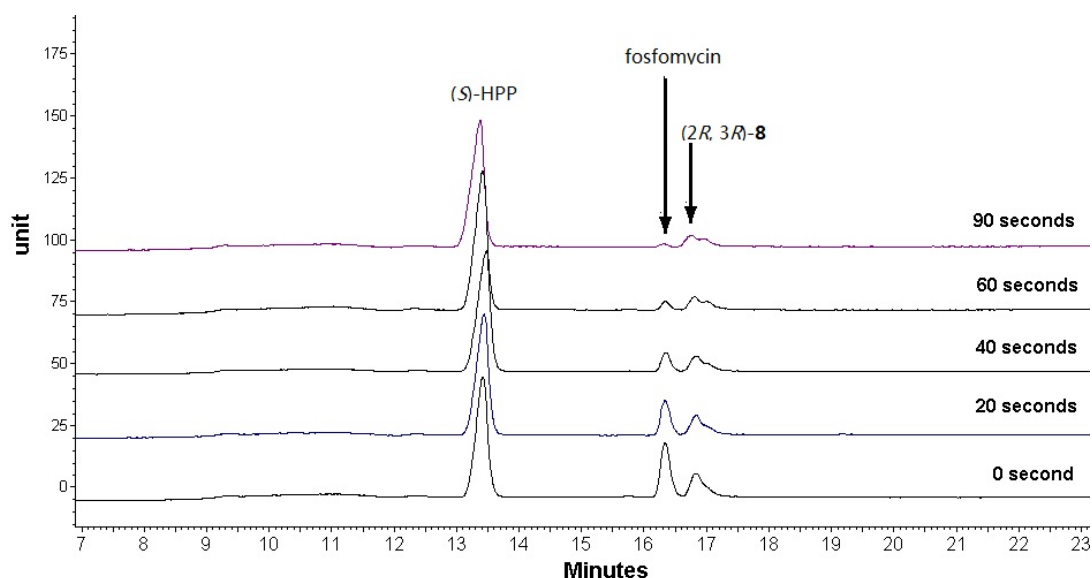


Figure S5. HPLC trace of time dependent HppE inactivation assay using (2*R*, 3*R*)-8

To determine the partition ratio of HppE inactivation by (2*R*, 3*R*)-8, a freshly prepared reaction solution of 0.05 mM HppE, 1.5 mM FMN and 5 mM NADH in 50 mM Tris buffer (pH 7.5) was incubated with 0, 0.015, 0.03, 0.05, 0.07, 0.09, 0.1, or 0.125 mM (2*R*, 3*R*)-8 for 10 min. To each of the pre-incubated enzyme solution was added an equal volume (*S*)-HPP (10 mM) solution. This mixture was allowed to react for another 20 min before being quenched and analyzed by HPLC using a Dionex[®] column eluted with water and NH₄OAc solution. The elution was monitored using a Corona detector. The residual enzyme activity was determined

based on the amount of fosfomycin formation. Plot of residual activity versus the ratio of [(2*R*, 3*R*)-**8**]/[HppE] used in the incubation gives partition ratio (Figure S6). It should be pointed out that mass analysis of the whole protein after inactivation was attempted. However, no apparent MS change was observed. Perhaps, the enzyme-inhibitor complex is unstable under the MS conditions.

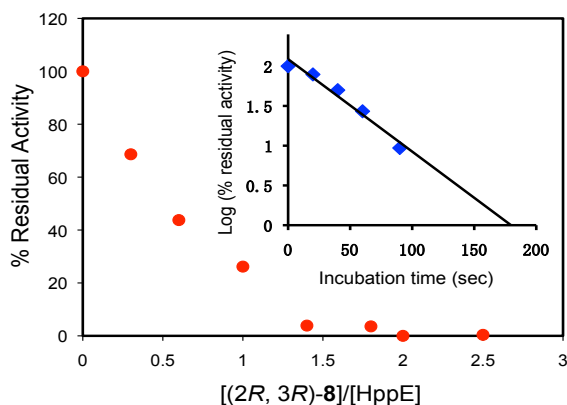


Figure S6. Plot of percentage of residual activity versus ratio of the inhibitor and enzyme concentrations $\{[(2*R*, 3*R*)-\mathbf{8}]/[\text{HppE}]\}$ used in the experiments. The inset is the plot of logarithm of the residual enzyme activity versus the incubation time.

6. Radioactivity Assays Using (*rac*)-[3-³H]-**8**

A freshly prepared solution of 0.25 mM HppE, 7.5 mM FMN, 10 mM NADH in 50 mM Tris buffer (pH 7.5) was incubated with (*rac*)-[3-³H]-**8** (75 mM, specific activity: 24.2 $\mu\text{Ci}/\text{mmol}$) at room temperature for 40 min. The reaction mixture was then loaded onto an Econo-Pac[®]10DG desalting column and eluted with 50 mM Tris buffer (pH 7.5). The eluant containing HppE was combined and dialyzed in 50 mM Tris buffer (pH 7.5) at 4 °C for 12 hr to ensure all of the unbound small molecules were removed. After dialysis, an aliquot of the protein solution was taken for radioactivity counting, which gave 27.0 $\mu\text{Ci}/\text{mmol}$ (2.42×10^{-3} μCi for 8.95×10^{-5} mmol HppE protein). Since the specific activity of (*rac*)-[3-³H]-**8** used in this experiment was 24.2 $\mu\text{Ci}/\text{mmol}$, this result suggested approximately 1.1 stoichiometry of tritiated compound bound to the inactivated HppE. It should be noted that the concentration of HppE used in this experiment was determined based the results of amino acid analysis.

The protein solution obtained above was then denatured in boiling water. After cooled down to room temperature, this whole sample was diluted with water, transferred onto an Amicon Ultra-15 centrifugal filter device and centrifuged at 4000 rpm for 15 min. Analysis of the resulting protein solution let to a specific activity

of 5.2 $\mu\text{Ci}/\text{mmol}$ ($2.98 \times 10^{-4} \mu\text{Ci}$ for $5.71 \times 10^{-5} \text{mmol}$ protein) which suggested the loss of $\sim 80\%$ of the radioactivity during this denaturing process.

7. MALDI-TOF MS Analyses of Trypsin Digested HppE and Its Inactivated Form

The reaction solutions from HppE control sample and the inactivated HppE sample were diluted 10 times with 50 mM ammonium bicarbonate (pH 7.8), and the protein denaturation were performed at 90 °C for 20 min. The resulting samples were reduced with 10 mM dithiothreitol (DTT) at 60 °C for 45 min and then alkylated with 20 mM iodoacetamide (IAA) at room temperature for 30 min. The excess amounts of IAA were quenched by 10 mM DTT. Trypsin solutions were added to the substrate protein solutions (w/w = 1:50) and followed by incubation at 37 °C overnight. After desalting by C18 ZipTip pipette tips (EMD Millipore Corporation, Billerica, MA), trypsin digested samples were mixed 1:1 (v/v) with the MALDI matrix (7 mg/mL α -cyano-4-hydroxycinnamic acid, 50% (v/v) acetonitrile, 10 mM ammonium dihydrogen phosphate, 0.1% trifluoroacetic acid). One μL of the resulting mixtures were spotted onto a MALDI target plate. MALDI-MS and MS/MS experiments were performed using the 4800 MALDI TOF/TOFTM Analyzer (Applied Biosystems, Framingham, MA). Collision-induced dissociation (CID) spectra were acquired using air as the collision gas (medium pressure setting) at 1 kV of collision energy.

The sequence coverage for trypsin digested HppE control and the inactivated HppE detected by MALDI-TOF MS are 86%, which covers the active site of HppE. No mass shift was observed for the corresponding peptides between the tryptic digested HppE control and the inactivated HppE. This result suggests that there is no covalent modification for the inactivated HppE.

(A)

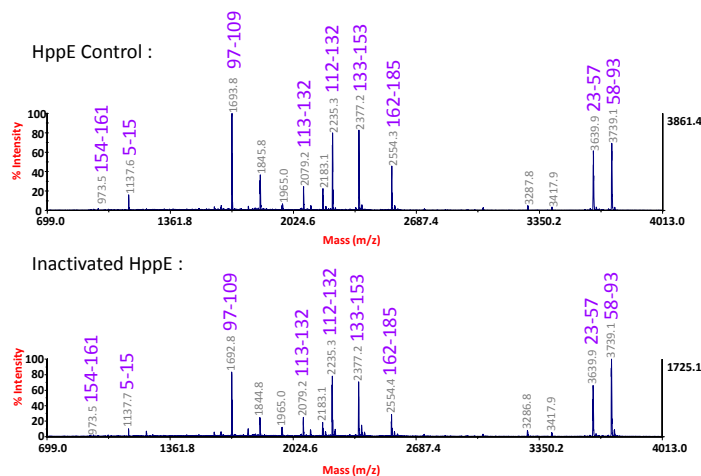


Figure S7. (A) The MALDI-TOF mass spectra of trypsin digested HppE control (top) and its inactivated form (bottom). (B) Summary of sequence coverage from MALDI-TOF MS results. All of the sequences observed in MALDI-TOF MS are labeled in red.

(B)

HppE sequence :

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      10      20      30      40      50      60
SNTKTASTGF AELLKDRREQ VKMDHAALAS LLGETPETVA AWENGGEGEL TLTQLGRIAH
      70      80      90     100     110     120
VLGTSIGALT PPAGNDLDDG VIIQMPDERP ILKGVDRNVD YVYVYVCLVRT KRAPSLVPLV
      130     140     150     160     170     180
VDVLTDPDD AKFNSGHAGN EFLVLEGEI HMKWGDKENP KEALLPTGAS MFVEEHVPHA
      190
FTAAKGTGSA KLIAVNF

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HppE total 197 amino acids.
Sequence coverage : 169/197=86% coverage

8. References

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