NIH Public Access
Author Manuscript
J Am Chem Soc. Author manuscript; available in PMC 2013 October 03.

Published in final edited form as:
J Am Chem Soc. 2012 October 3; 134(39): 16171–16174. doi:10.1021/ja3078126.

Evidence for Radical-Mediated Catalysis by HppE – A Study Using Cyclopropyl and Methylenecyclopropyl Substrate Analogues

Hui Huang[1,‡], **Wei-chen Chang**[1,‡], **Pei-Jing Pai**[2], **Anthony Romo**[1], **Steven O. Mansoorabadi**[1], **David H. Russell**[2], and **Hung-wen Liu**[1,*]

[1]Division of Medicinal Chemistry, College of Pharmacy, and Department of Chemistry and Biochemistry, University of Texas at Austin, Austin, Texas 78712, United States.

[2]Department of Chemistry, Texas A&M University, College Station, Texas 77843, United States

Abstract

(*S*)-2-Hydroxypropylphosphonic acid epoxidase (HppE) is an unusual mononuclear iron enzyme that catalyzes the oxidative epoxidation of (*S*)-2-hydroxypropylphosphonic acid ((*S*)-HPP) in the biosynthesis of the antibiotic fosfomycin. HppE also recognizes (*R*)-2-hydroxypropylphosphonic acid ((*R*)-HPP) as a substrate and converts it to 2-oxo-propylphosphonic acid. To probe the mechanisms of these HppE-catalyzed oxidations, cyclopropyl- and methylenecyclopropyl-containing compounds were synthesized and studied as radical clock substrate analogues**.** Enzymatic assays indicated that the (*S*)- and (*R*)-isomers of the cyclopropyl-containing analogues were efficiently converted to epoxide and ketone products by HppE, respectively. In contrast, the ultrafast methylenecyclopropyl-containing probe inactivated HppE, consistent with a rapid radical-triggered ring-opening process that leads to enzyme inactivation. Taken together, these findings provide, for the first time, experimental evidence for the involvement of a C2-centered radical intermediate with a lifetime on the order of nanoseconds in the HppE-catalyzed oxidation of (*R*)-HPP.

Keywords

epoxidase; fosfomycin biosynthesis; catalytic mechanism; cyclopropylcarbinyl radical probes

---

Fosfomycin ((1*R*, 2*S*)-1,2-epoxypropylphosphonic acid, **1**) is a widely used broad-spectrum antibiotic with activity against both Gram-negative and Gram-positive bacterial infections.[1] Fosfomycin blocks bacterial cell wall biosynthesis by inhibiting UDP-*N*-acetyl-glucosamine-3-*O*-enolpyruvyltransferase (MurA) through the covalent modification of an active site cysteine residue.[2] A proposed biosynthetic pathway for fosfomycin was first reported in 1995.[3] One of the key steps in this pathway is the formation of the epoxide ring, which is catalyzed by (*S*)-2-hydroxypropylphosphonic acid epoxidase (HppE), a mononuclear non-heme iron enzyme.[4, 5] Unlike most epoxidases that catalyze direct oxygen atom insertion into an alkene precursor,[6] the oxirane moiety of fosfomycin is generated by forming a C–O bond between C1 and the 2-OH group of the substrate (*S*)-2-

---

*Corresponding Author h.w.liu@mail.utexas.edu.
‡H.H. and W.-c.C. contributed equally.

ASSOCIATED CONTENT Supporting Information Details of synthetic methods for compounds **7**, **15**, **16**, and **24**, and experimental procedures of the enzymatic reactions of HppE with each substrate. This material is available free of charge via the Internet at http://pubs.acs.org.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

hydroxypropylphosphonic acid (**2**, (*S*)-HPP) in a dehydrogenation reaction (Scheme 1A).[3, 7] HppE catalysis employs molecular oxygen as the oxidant and consumes a stoichiometric amount of NADH as the electron donor.[5, 8] ^{18}O kinetic isotope effects (KIEs) studies of HppE revealed the formation of an Fe^{III}-OOH species in the (partially) rate-limiting step of O_2 activation.[9]

Interestingly, HppE can also recognize and efficiently oxidize (*R*)-HPP (**3**), the enantiomer of the natural substrate, to 2-oxopropylphosphonic acid (**4**) (Scheme 1B).[10] The catalytic versatility and substrate flexibility of HppE suggest that both the regiochemistry of the hydrogen atom abstraction steps and the nature of the resulting radical intermediates vary with the stereochemical properties of the substrate. Crystallographic and spectroscopic experiments showed that both enantiomers of HPP (**2** and **3**) act as bidentate ligands to the mononuclear iron, such that only a single hydrogen atom (1-H_R for **2** and 2-H for **3**) is poised for abstraction by a reactive iron-oxygen species (likely Fe^{III}-OO•).[11, 12] These results led to the current working hypothesis for the mechanisms of the HppE-catalyzed reactions shown in Scheme 1.

Although non-heme iron enzymes are thought to initiate substrate oxidation via hydrogen atom abstraction, leading to the formation of substrate-derived radical intermediates,[13] experimental evidence for the involvement of discrete radical intermediates in these enzymatic systems is often lacking.[14] Specifically for the HppE-catalyzed reactions, the intermediacy of the proposed substrate radical species (**5** and **6**) has never been verified experimentally. To address this issue and gain support for the proposed reaction mechanisms, substrate analogues bearing strategically incorporated cyclopropyl or methylenecyclopropyl moieties were prepared and analyzed as mechanistic probes of HppE catalysis. The radical-induced ring opening of cyclopropylcarbinyl or methylenecyclopropylcarbinyl radicals are well known.[15, 16] Thus, the detection of ring-opened turnover products or radical induced enzyme inactivation would provide evidence for the occurrence of radical intermediates during catalysis and their lifetimes. In this paper, we report the syntheses of a set of cyclopropyl- and methylenecyclopropyl-containing substrate analogues ((*S*)-**7**, (*R*)-**7** and **16**), the evaluation of these compounds as radical clock probes for the HppE reaction, and the mechanistic implications of the incubation results.

The syntheses of (*S*)-**7** and (*R*)-**7** are depicted in Scheme 2. Treatment of the bromo compound **8**[17] with $P(OEt)_3$ under Michaelis-Arbuzov reaction conditions followed by reduction of the resulting phosphonate **9** with $NaBH_4$ gave a racemic mixture of alcohol **10**. Resolution of this pair of alcohols was attempted using various lipases. This method attempts to utilize the asymmetric environment in the lipase active site to stereoselectively acetylate one of the enantiomers, rendering the resulting acetate separable from the unreacted alcohol. Unfortunately, all lipase systems tested showed poor stereoselectivity towards **10**.[18] However, it was later found that efficient resolution of the racemic acetate **11** could be achieved through asymmetric hydrolysis with pig liver esterase. Indeed, after three rounds of acetylation and esterasecatalyzed hydrolysis, both (*R*)- and (*S*)-**10** were obtained in >94% enantiomeric excess (*ee*) as determined by ^{31}P NMR analysis using quinine as a chiral shifting reagent (Figure S2.1-1).[18c] Subsequent deprotection of the phosphonate ethyl ester by TMSBr gave the desired substrate analogues (*S*)-**7** and (*R*)-**7**.

The purified (*S*)-**7** and (*R*)-**7** were each incubated with HppE and the reactions were monitored by ^{1}H-NMR spectroscopy (Figure 1). Both compounds were found to be substrates of HppE and each was converted to a single product (Scheme 3). A 1,2-epoxide (**13**) was produced when (*S*)-**7** was employed as the substrate, whereas a keto-product (**15**) was obtained from the incubation with (*R*)-**7**. The chemical nature of these products was

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

further verified by high-resolution mass spectrometry (Figure S5-1), and the structure of **15** was also confirmed by comparison with a synthetic standard. The regio- and stereoselectivity of the turnover of (*S*)-**7** and (*R*)-**7** are consistent with those established for **2** and **3**. Notably, the cyclopropyl group is retained in both products (**13** and **15**).

The conversion of (*S*)-**7** to **13** is not surprising, since the putative C1 radical intermediate (**12**) is not expected to affect the cyclopropyl group at the C3 position. However, the conversion of (*R*)-**7** to **15** as the sole product is in contrast to the anticipation that the C2-centered carbinyl radical (i.e. **14**), once formed, would trigger ring opening of the adjacent cyclopropyl group. At first glance, this observation appears to contradict the proposed radical-mediated oxidation mechanism. However, the retention of the cyclopropyl group in the product may be ascribed to the subsequent one-electron oxidation step (**14**→**15**) being more facile than the competing ring opening reaction.

Considering that the ring opening rate constant of (methylenecyclopropyl)carbinyl radical ($k = 6 \times 10^9\ s^{-1}$ at 298K)[19] is nearly two-order of magnitude greater than that of the cyclopropylcarbinyl radical ($k = 8.6 \times 10^7\ s^{-1}$ at 298 K),[20] a substrate analogue carrying a methylenecyclopropyl group (**16**) was designed and synthesized as a more sensitive probe to detect the transient formation of radical intermediate by HppE. The synthetic route to **16** started with nucleophilic addition of methyldiethylphosphonate to ester **17**,[21] followed by Luche reduction of the resulting ketone (**18**) to give **19** (Scheme 4). Since attempts to resolve this racemic alcohol using various lipases and esterases failed, **19** was converted to **16** as a racemic mixture. While enantiomerically pure substrates are preferred, experiments performed with the racemic sample can still provide evidence to discern the possible formation of radical intermediates in the HppE-catalyzed reactions.

When compound **16** was incubated with HppE, formation of a new product was detected by NMR spectroscopy. However, the conversion was incomplete with no further consumption of **16** observed after ~10 min. No fosfomycin formation could be detected when the natural substrate, (*S*)-HPP (**2**), was incubated with HppE that had been pretreated with **16**, suggesting that this compound (**16**) also inactivates the enzyme. The fact that **16** can act as both a substrate and an inactivator for HppE may simply be a consequence of the racemic nature of **16** used for these assays. Namely, the outcome of the incubation is governed by the C2 stereochemistry of **16**, with one epimer serves as the substrate and the other acts as an inactivator.

The structure of the turnover product of **16** is a molecule featuring both an epoxide ring and an intact methylenecyclopropyl group. This is supported by the appearance of new peaks at 2.7–2.9 and 5.3 ppm, which are characteristic resonances for the oxiranyl protons and the olefinic protons of a terminal alkene, respectively. By analogy to the reaction with (*S*)-**7**, this product may be assigned to the corresponding 1,2-epoxyl compound **20** derived from the (2*S*)-epimer of **16** (Scheme 5). The presence of **20** in the reaction system was also confirmed using high resolution mass spectrometry ($[M-H]^-$ calcd for $C_6H_8O_4P^-$: *m/z* 175.0166, found: *m/z* 175.0160) (Figure S5-2).

As (2*S*)-**16** is likely to be a substrate of HppE, the inactivation of HppE may thus be attributed to the action of (2*R*)-**16**. Two mechanistic scenarios can be envisioned for the inactivation of HppE by the 2*R*-epimer of **16** (Scheme 6). In route a, **16** is first converted to the corresponding 2-oxo phosphonate **24** in a manner analogous to the oxidation of (*R*)-**3** and (*R*)-**7** by HppE. Since the cyclopropyl group of **24** is primed by the newly formed electron-withdrawing 2-keto functionality, it may be susceptible to the attack by an active site nucleophile in a Michael-addition reaction.[22] An alternative explanation for the observed inactivation (route b) lies in the ultrafast radical-triggered ring opening of the

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

methylenecyclopropyl group, whereby the C2 radical intermediate (**25**) rearranges to an allylic radical that inactivates the enzyme.

In order to distinguish between these two possibilities, the 2-keto product (**24**) was chemically synthesized and tested as an inhibitor of HppE activity. (*S*)-HPP (**2**) was added to the HppE reaction system that had been preincubated with an excess amount of **24** (20-fold excess than enzyme), and the enzyme activity was determined following fosfomycin formation by ^{1}H-NMR (Figure S4-3). No difference in the rate or extent of fosfomycin formation was detected in the presence or absence of **24**, clearly indicating that this putative turnover product is not an inactivator of HppE. Therefore, a mechanism initiated by a radical-triggered ring opening is a more likely mode of inactivation of HppE by (*R*)-**17**. Consistent with a radical-induced enzyme modification, HppE activity could not be restored after extensive dialysis of the inactivated enzyme. Moreover, given the differences in ring opening rates of the cyclopropylcarbinyl and methylenecyclopropylcarbinyl radicals and the fact inactivation was not observed with (*R*)-**7**, the lifetime of radical **22** can be estimated to be on the order of nanoseconds, placing it within range of a true intermediate.

In this work, cyclopropyl- and methylenecyclopropyl-based radical probes were employed to study the mechanism of HppE-catalysis. Specifically, the cyclopropyl-containing analogues (*S*)-**7** and (*R*)-**7** were found to be processed by HppE to epoxide **13** and ketone **15** products, respectively. Despite the presence of a cyclopropyl radical reporting group in these structures, the reactions of (*S*)-**7** and (*R*)-**7** are free from apparent radical-triggered ring opening events. In contrast, incubation with the ultrafast radical probe **16** led to the formation of **24** and irreversible enzyme inactivation. While product **20** is thought to be generated from (2*S*)-**16** via C1-centered radical intermediate **21**, the observed inactivation is likely a result of radical induced ring opening of the C2-centered radical intermediate (**22**) derived from (2*R*)-**16**. These observations are significant because they provide the first experimental evidence supporting the involvement of substrate radical intermediate(s) in an HppE-catalyzed reactions, and also allow estimation of the rate of the subsequent electron transfer step to be between 8.6×10^7 and 6×10^9 s^{-1} based on the ring-opening rate constants of the cyclopropylcarbinyl and methylenecyclopropylcarbinyl radicals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We wish to thank Steve Sorey for his help on acquiring the NMR spectra.

Funding Sources This work is supported in part by grants from the National Institutes of Health (GM040541 to H.-w.L) and the Robert A. Welch Foundation (F-1511 to H.-w.L. and A-1176 to D.H.R.).

REFERENCES

(1). Raz R. Clin. Microbiol. Infect. 2012; 18:4–7. [PubMed: 21914036]

(2)(a). Marquardt JL, Brown ED, Lane WS, Haley TM, Ichikawa Y, Wong CH, Walsh CT. Biochemistry. 1994; 47:10646–10651. [PubMed: 8075065] (b) Eschenburg S, Priestman M, Schönbrunn E. J. Biol. Chem. 2004; 280:3757–3763. [PubMed: 15531591]

(3). Hidaka T, Goda M, Kuzuyama T, Takei N, Hidaka M, Seto H. Mol. Gen. Genet. 1995; 249:274–280. [PubMed: 7500951]

(4). Liu P, Murakami K, Seki T, He X, Yeung SM, Kuzuyama T, Seto H, Liu H.-w. J. Am. Chem. Soc. 2001; 123:4619–4620. [PubMed: 11457256]

(5). Liu P, Liu A, Yan F, Wolfe MD, Lipscomb JD, Liu H.-w. Biochemistry. 2003; 42:11577–11586. [PubMed: 14529267]

(6). Thibodeaux CJ, Chang W, Liu H.-w. Chem. Rev. 2012; 112:1681–1709. [PubMed: 22017381]

(7)(a). Hammerschmidt F, Bovermann G, Bayer K. Liebigs Ann. Chem. 1990:1055–1061.(b) Hammerschmidt F. J. Chem. Soc. Perkin Trans. 1. 1991:1993–1996.

(8)(a). Yan F, Munos JW, Liu P, Liu H.-w. Biochemistry. 2006; 45:11473–11481. [PubMed: 16981707] (b) Munos JW, Moon S-J, Mansoorabadi SO, Chang W, Hong L, Yan F, Liu A, Liu H.-w. Biochemistry. 2008; 47:8726–8735. [PubMed: 18656958]

(9). Mirica LM, McCusker KP, Munos JW, Liu H.-w. Klinman JP. J. Am. Chem. Soc. 2008; 130:8122–8123. [PubMed: 18540575]

(10). Zhao Z, Liu P, Murakami K, Kuzuyama T, Seto H, Liu H.-w. Angew. Chem. Int. Ed. Engl. 2002; 41:4529–4532. [PubMed: 12458528]

(11)(a). Woschek A, Wuggenig F, Peti W, Hammerschmidt F. Chembiochem. 2002; 3:829–835. [PubMed: 12210983] (b) Higgins LJ, Yan F, Liu P, Liu H.-w. Drennan CL. Nature. 2005; 437:838–844. [PubMed: 16015285]

(12)(a). Yan F, Moon S-J, Liu P, Zhao Z, Lipscomb JD, Liu A, Liu H.-w. Biochemistry. 2007; 46:12628–12638. [PubMed: 17927218] Yun D, Dey M, Higgins LJ, Yan F, Liu H.-w. Drennan CL. J. Am. Chem. Soc. 2011; 133:11262–11269. [PubMed: 21682308]

(13)(a). Kappock TJ, Caradonna JP. Chem. Rev. 1996; 96:2659–2756. [PubMed: 11848840] (b) Hegg EL, Que L. Eur. J. Biochem. 1997; 250:625–629. [PubMed: 9461283] (c) Rocklin AM, Tierney DL, Kofman V, Brunhuber NMW, Hoffman BM, Christoffersen RE, Reich NO, Lipscomb JD, Que L. Proc. Natl. Acad. Sci. U.S.A. 1999; 96:7905–7909. [PubMed: 10393920] (d) Fitzpatrick PF. Annu. Rev. Biochem. 1999; 68:355–381. [PubMed: 10872454] (e) Gibson DT, Parales RE. Curr. Opin. Biotechnol. 2000; 11:236–243. [PubMed: 10851146] (f) Prescott AG, Lloyd MD. Nat. Prod. Rep. 2000; 17:367–383. [PubMed: 11014338] (g) Costas M, Mehn MP, Jensen MP, Que L Jr. Chem. Rev. 2004; 104:939–986. [PubMed: 14871146] (h) Kovaleva EG, Lipscomb JD. Nat. Chem. Biol. 2008; 4:186–193. [PubMed: 18277980] (i) Bollinger JM Jr. Krebs C. Curr. Opin. Chem Biol. 2007; 11:151–158. [PubMed: 17374503] (j) van der Donk WA, Krebs C, Bollinger JM Jr. Current Opinion Struct. Biol. 2010; 20:673–683.

(14). For examples see: Ryle MJ, Liu A, Muthukumaran RB, Ho RYN, Koehntop KD, McCracken J Jr. Que L, Hausinger RP. Biochemistry. 2003; 42:1854–1862. [PubMed: 12590572] ; Howard-Jones AR, Elkins JM, Clifton IJ, Roach PL, Adlington RM, Baldwin JE, Rutledge PJ. Biochemistry. 2007; 46:4755–4762. [PubMed: 17397141]

(15)(a). Griller D, Ingold KU. Acc. Chem. Res. 1980; 13:317–323.(b) Newcomb M. Tetrahedron. 1993; 49:1151–1176.(c) Silverman RB. Acc. Chem. Res. 1995; 28:335–342.(d) Newcomb M, Toy PH. Acc. Chem. Res. 2000; 33:449–455. [PubMed: 10913233] (e) Salaun, J. Topics in Current Chemistry. Vol. Vol. 207. Springer-Verlag; Berlin: 2000. p. 1-67.(f) Ortiz de Montellano PR. Chem. Rev. 2010; 110:932–948. [PubMed: 19769330]

(16)(a). Lenn ND, Shih Y, Stankovuch MT, Liu H.-w. J. Am. Chem. Soc. 1989; 111:3065–3067.(b) Lai, M.-t.; Li, D.; Oh, E.; Liu, H.-w. J. Am. Chem. Soc. 1993; 115:1619–1628.

(17). Laurent M, Ceresiat M, Marchand-Brynaert J. Eur. J. Org. Chem. 2006:3755–3766.

(18)(a). Zhang YH, Yuan CY, Li ZY. Tetrahedron. 2002; 58:2973–2978.(b) Zurawinski R, Nakamura K, Drabowicz J, Kielbasinski P, Mikolajczyk M. Biocatalytic, Tetrahedron Asymm. 2001; 12:3139–3145.(c) yma czyk-Duda E, Skwarczy ski M, Lejczak B, Kafarski P. Tetrahedron Asymm. 1996; 7:1277–1280.

(19). Horner JH, Johnson CC, Lai MT, Lin H.-w. Martinesker AA, Newcomb M, Oh E. Bioorg. Med. Chem. Lett. 1994; 4:2693–2696.

(20). Bowry VW, Lusztyk J, Ingold KU. J. Am. Chem. Soc. 1991; 113:5687–5698.

(21). Lai MT, Liu LD, Liu H.-w. J. Am. Chem. Soc. 1991; 113:7388–7397.

(22). Agnihotri G, He SM, Hong L, Dakoji S, Withers SG, Liu H.-w. Biochemistry. 2002; 41:1843–1852. [PubMed: 11827529]

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

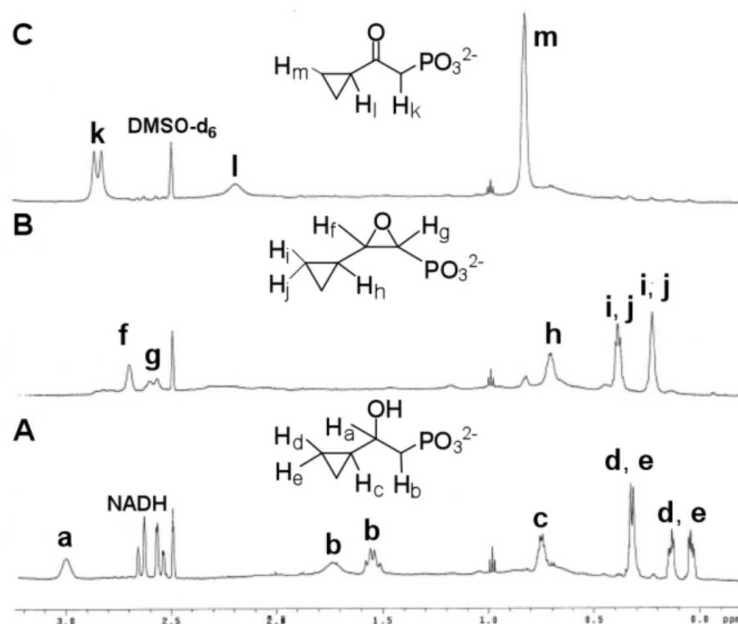


Figure 1.

^{1}H-NMR end-point assay of HppE with (*R*)- and (*S*)-**7**. (A) Initial time point recorded 3 min after incubation of substrate with enzyme. (B) 36 min time point showing >90% conversion of (*S*)-**7** to **13**. (C) 36 min time point showing >90% conversion of (*R*)-**7** to **15**. (See Figure S4-1, S4-2 for details).

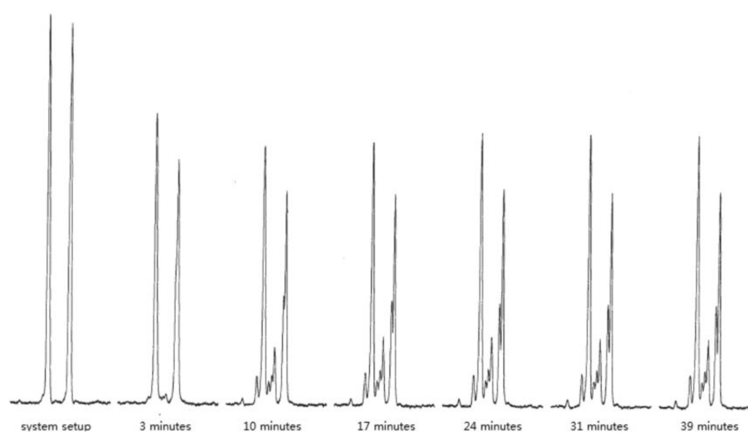


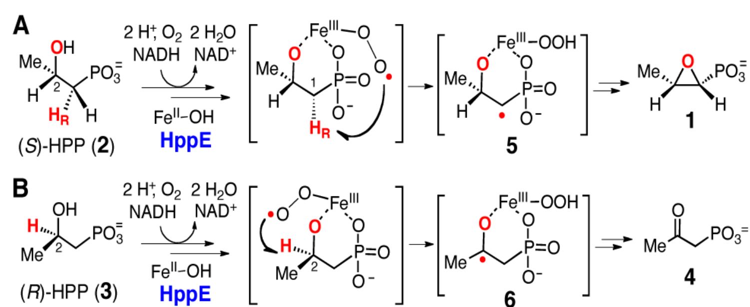
Figure 2.
^{1}H-NMR assay of HppE with **16**. Spectra were recorded at different time points. Only the region (5.1–5.4 ppm) of the olefinic protons is shown. No further consumption of **16** was observed after ~10 min.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript



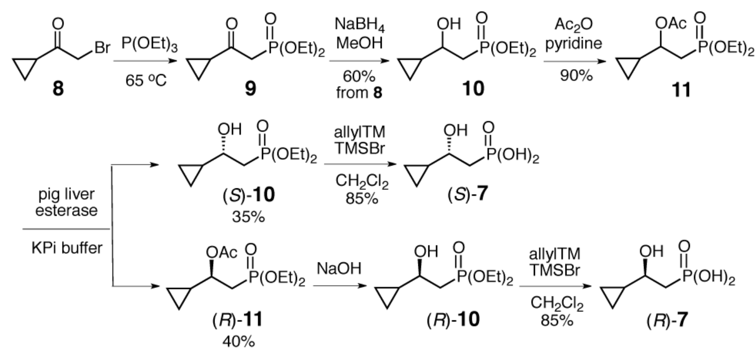
Scheme 1.

Proposed mechanism for the oxidation of (*S*)- and (*R*)-HPP by HppE. (A) Conversion of (*S*)-HPP (**2**) to fosfomycin (**1**). (B) Conversion of (*R*)-HPP (**3**) to 2-oxopropylphosphonic acid (**4**).

NIH-PA Author Manuscript

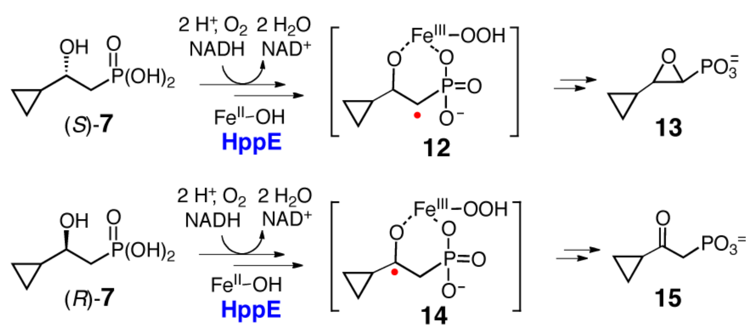
NIH-PA Author Manuscript

NIH-PA Author Manuscript



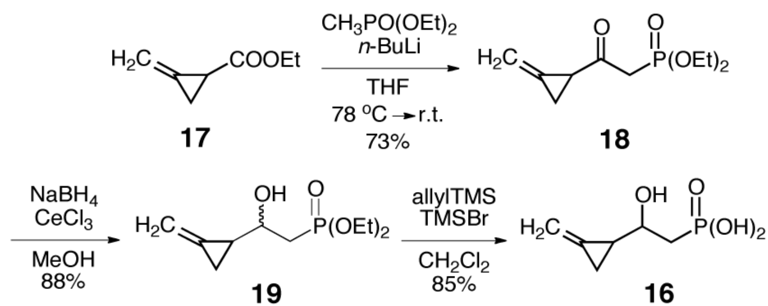
Scheme 2.

Synthetic schemes for the preparation of (*S*)- and (*R*)-**7**.



Scheme 3.
Conversion of (*S*)-**7** to **13** and (*R*)-**7** to **15** by HppE.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript



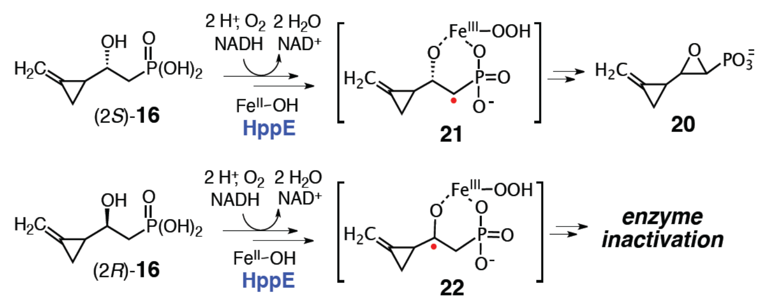
Scheme 4.
Synthetic scheme for the preparation of **16**.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript



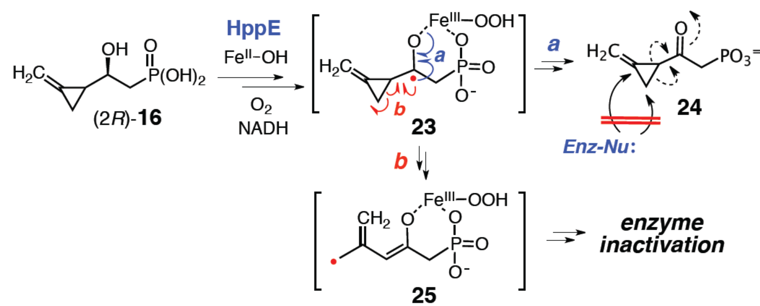
Scheme 5.

Proposed reactions of (2*S*)- and (2*R*)-**16** with HppE.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript



Scheme 6.
Proposed mechanisms for the inactivation of HppE by (2*R*)-**16**.