

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

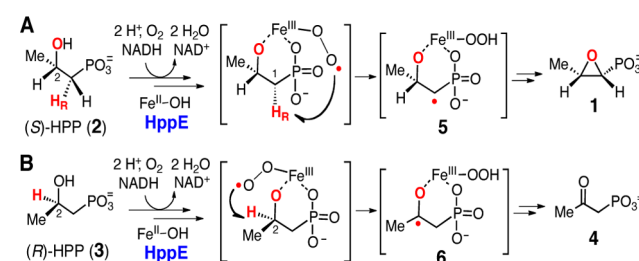
Hui Huang,^{†,‡} Wei-chen Chang,^{†,‡} Pei-Jing Pai,[§] Anthony Romo,[†] Steven O. Mansoorabadi,[†] David H. Russell,[§] and Hung-wen Liu^{*,†}[†]Division of Medicinal Chemistry, College of Pharmacy, and Department of Chemistry and Biochemistry, University of Texas at Austin, Austin, Texas 78712, United States[§]Department of Chemistry, Texas A&M University, College Station, Texas 77843, United States

Supporting Information

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.

Fosfomycin ((1R, 2S)-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-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} ¹⁸O kinetic isotope effects (KIEs) studies of

Scheme 1. Proposed Mechanism for the Oxidation of (S)- and (R)-HPP by HppE^a



^a(A) Conversion of (S)-HPP (**2**) to fosfomycin (**1**). (B) Conversion of (R)-HPP (**3**) to 2-oxopropylphosphonic acid (**4**).

HppE revealed the formation of an Fe^{III}–OOH species in the (partially) rate-limiting step of O₂ 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

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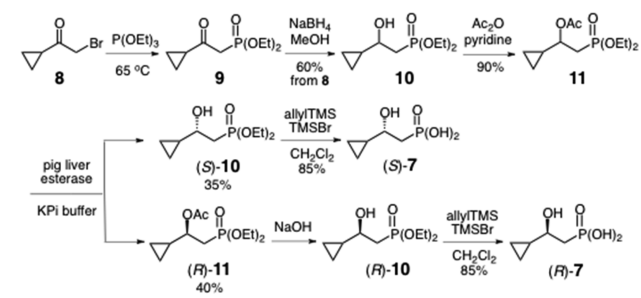
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mechanistic probes of HppE catalysis. The radical-induced ring-opening of cyclopropylcarbinyl or methylenecyclopropylcarbinyl radicals are well-known.^{15,16} Thus, the detection of ring-opened turnover products or radical induced enzyme inactivation 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 8¹⁷ with P(OEt)₃ under

Scheme 2. Synthetic Schemes for the Preparation of (*S*)- and (*R*)-7



Michaelis-Arbuzov reaction conditions followed by reduction of the resulting phosphonate 9 with NaBH₄ 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 toward 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 esterase-catalyzed hydrolysis, both (*R*)- and (*S*)-10 were obtained in >94% enantiomeric excess (ee) as determined by ³¹P 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 ¹H 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 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),

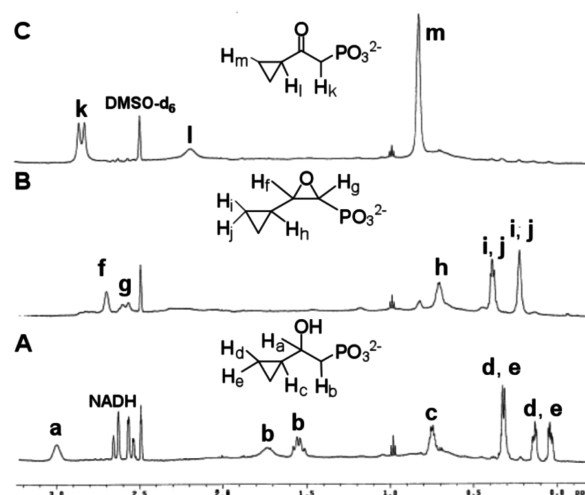
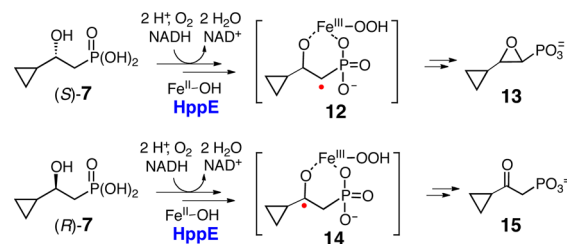


Figure 1. ¹H NMR end-point assay of HppE with (*R*)- and (*S*)-7. (A) Initial time point recorded 3 min after incubation of substrate with enzyme; (B) 36 min time point showing >90% conversion of (*S*)-7 to 13; (C) 36 min time point showing >90% conversion of (*R*)-7 to 15 (see Figure S4–1, S4–2 for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

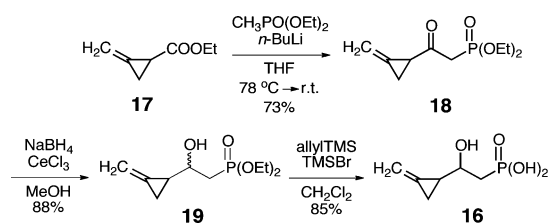
Scheme 3. Conversion of (*S*)-7 to 13 and (*R*)-7 to 15 by HppE



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 ($k = 6 \times 10^9 \text{ s}^{-1}$ at 298 K)¹⁹ is nearly 2 orders of magnitude greater than that of the cyclopropylcarbinyl radical ($k = 8.6 \times 10^7 \text{ s}^{-1}$ 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 methyl-diethylphosphonate to ester 17,²¹ followed by Luche reduction of the resulting ketone (18) to give 19 (Scheme 4).

Scheme 4. Synthetic Scheme for the Preparation of 16



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 (Figure 2).

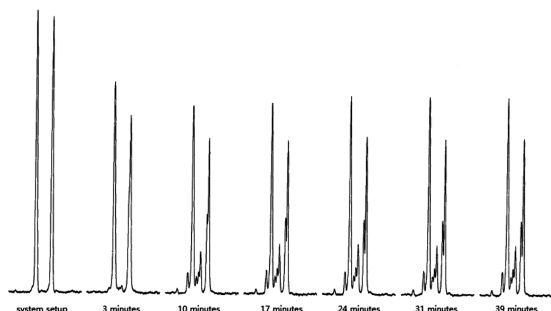


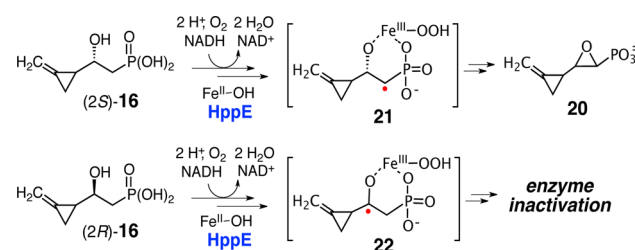
Figure 2. ^1H NMR assay of HppE with **16**. Spectra were recorded at different time points. Only the region (5.1–5.4 ppm) of the olefinic protons is shown. No further consumption of **16** was observed after ~10 min.

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 (*2S*)-epimer of **16** (Scheme 5). The presence of **20** in the reaction system was also confirmed using high resolution mass spectrometry ($[\text{M} - \text{H}]^-$ calcd for $\text{C}_6\text{H}_8\text{O}_4\text{P}^-$, m/z 175.0166; found, m/z 175.0160) (Figure S5–2).

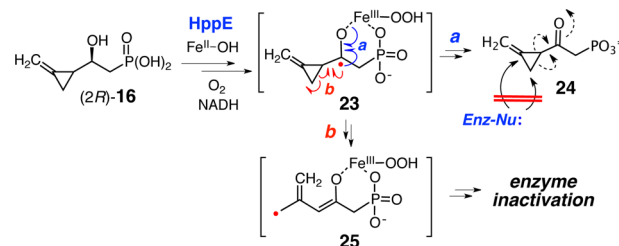
As (*2S*)-**16** is likely to be a substrate of HppE, the inactivation of HppE may thus be attributed to the action of

Scheme 5. Proposed Reactions of (*2S*)- and (*2R*)-**16** with HppE



(*2R*)-**16**. Two mechanistic scenarios can be envisioned for the inactivation of HppE by the *2R*-epimer of **16** (Scheme 6). In

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



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

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 ^1H 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 that 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 (*2S*)-**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 (*2R*)-**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 $6 \times 10^9 \text{ s}^{-1}$ based on

the ring-opening rate constants of the cyclopropylcarbinyl and methylenecyclopropylcarbinyl radicals.

■ 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>.

■ AUTHOR INFORMATION

Corresponding Author

h.w.liu@mail.utexas.edu

Author Contributions

[‡]H.H. and W.-c.C. contributed equally.

Notes

The authors declare no competing financial interest.

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