JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
pubs.acs.org/JACS

Reaction of HppE with Substrate Analogues: Evidence for Carbon−Phosphorus Bond Cleavage by a Carbocation Rearrangement

Wei-chen Chang, Steven O. Mansoorabadi, 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

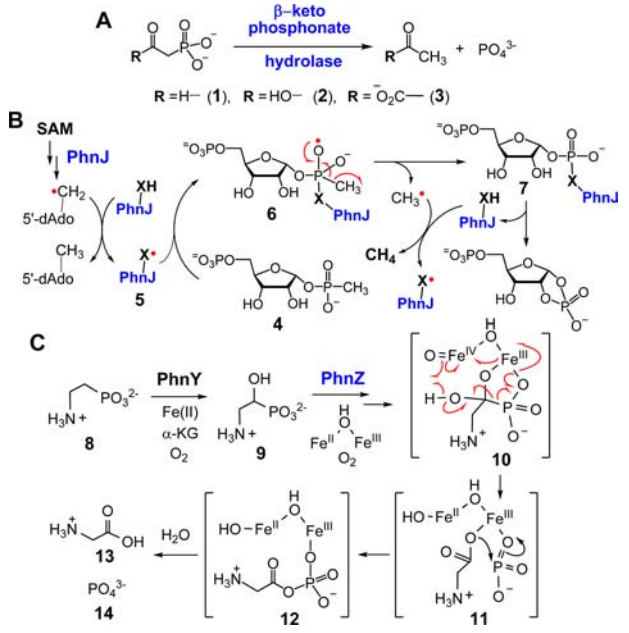
Supporting Information

ABSTRACT: (*S*)-2-Hydroxypropylphosphonic acid ((*S*)-2-HPP) epoxidase (HppE) is an unusual mononuclear non-heme iron enzyme that catalyzes the oxidative epoxidation of (*S*)-2-HPP in the biosynthesis of the antibiotic fosfomycin. Recently, HppE has been shown to accept (*R*)-1-hydroxypropylphosphonic acid as a substrate and convert it to an aldehyde product in a reaction involving a biologically unprecedented 1,2-phosphono migration. In this study, a series of substrate analogues were designed, synthesized, and used as mechanistic probes to study this novel enzymatic transformation. The resulting data, together with insights obtained from density functional theory calculations, are consistent with a mechanism of HppE-catalyzed phosphono group migration that involves the formation of a carbocation intermediate. As such, this reaction represents a new paradigm for biological C−P bond cleavage.

Phosphorus is an essential element for life.[1] In biological systems, P is typically present in the form of inorganic phosphate or derivatives, such as organophosphate esters and anhydrides.[1] In recent years it has become increasingly apparent that more highly reduced P compounds also play prominent roles in biology.[1,2] Many of these compounds, e.g., phosphonic and phosphinic acids, contain stable C−P bonds in place of the labile O−P bonds of the corresponding phosphate esters.[2] Several such "C−P compounds" are bioactive natural products of agricultural (e.g., phosphinothricin tripeptide, phosphonothrixin) and medical (e.g., fosfomycin, fosmidomycin) importance.[1,2] Still other C−P compounds are metabolized as a source of inorganic phosphate by microorganisms living in phosphate-poor environments.[1]

Several distinct mechanisms of biological C−P bond cleavage have been identified (Scheme 1).[1,3−5] For example, C−P bond cleavage in *β*-keto-phosphonates, such as phosphonoacetaldehyde (**1**), phosphonoacetate (**2**), and phosphonopyruvate (**3**), catalyzed by the corresponding hydrolases, proceeds via nucleophilic attack at the P center by a water molecule or an active-site nucleophile. The negative charge developed during turnover is stabilized by metal ions or a Schiff base.[2,4,5] Another example is the bacterial C−P lyase pathway, which utilizes a noncanonical radical *S*-adenosyl-L-methionine (SAM) enzyme, PhnJ, to cleave the C−P bond of 5-phospho-*α*-D-ribosyl-1-alkylphosphonate (**4**).[3] The detailed reaction mechanism of PhnJ is not fully understood, although it has been proposed to

Scheme 1. Representative Enzyme-Catalyzed C−P Bond Cleavage Reactions



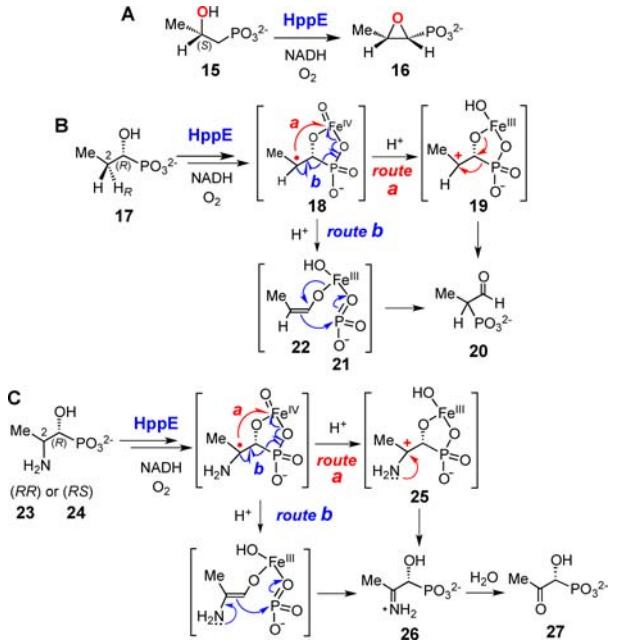
proceed via attack at the P center by a thiyl radical (**5**), leading to homolytic cleavage of the C−P bond through a radical-mediated process (**6**→**7**).[3] Recently, the genes responsible for an alternative organophosphonate catabolic pathway were identified in marine-derived metagenomic DNA. In this pathway, 2-aminoethylphosphonate (**8**) is converted to glycine (**13**) and inorganic phosphate (**14**) by the combined action of PhnY and PhnZ.[6] Specifically, PhnZ, a member of the histidine-aspartate hydrolase superfamily, is proposed to catalyze the oxidative cleavage of the C−P bond of 2-amino-1-hydroxyethylphosphonate (**9**) through a mechanism reminiscent of that used by the diiron enzyme *myo*-inositol oxygenase.[6−8] In this mechanism, the high-valent Fe^{III}-Fe^{IV}-oxo intermediate **10** induces fragmentation of the C−P bond of the substrate, leading to a metaphosphate intermediate (**11**) that recombines with the carboxylate anion of glycine to give **12**. Subsequent hydrolysis yields **13** and **14**.

More recently, an oxidative 1,2-phosphono group migration was observed in the reaction of the non-heme iron enzyme (*S*)-2-

Received: April 6, 2013
Published: May 14, 2013

dx.doi.org/10.1021/ja403441x | J. Am. Chem. Soc. 2013, 135, 8153−8156

Scheme 2. (A) Reaction Catalyzed by HppE, and Mechanisms Proposed to Account for the Reactions of HppE with (B) (*R*)-1-HPP (17) and (C) (1*R*,2*R*)- or (1*R*,2*S*)-2-Amino-1-HPP (23 or 24)[a]

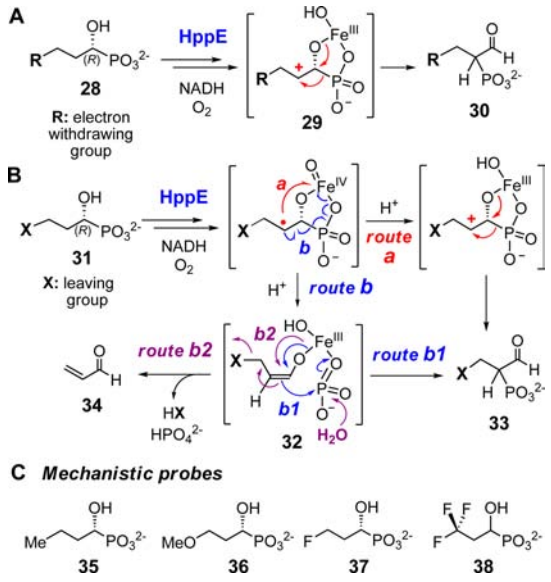


[a]Route a, carbocation rearrangement. Route b, radical-induced fragmentation/recombination.

hydroxypropylphosphonate (HPP, **15**) epoxidase (HppE), which catalyzes the conversion of **15** to fosfomycin (**16**) (Scheme 2A).[9] When incubated with the alternative substrate (*R*)-1-HPP (**17**), HppE catalyzes the migration of the phosphono group of **17** from C1 to C2 to give aldehyde **20** as the product.[10] From results obtained by X-ray crystallography, model chemistry, and reactions with mechanistic probes (e.g., **23** and **24**), it was proposed that cleavage of the C−P bond of **17** is induced by the formation of a C2-centered carbocation intermediate (**19**, Scheme 2B, route a).[10] If correct, this would represent a new paradigm for the enzymatic cleavage of a C−P bond and would indicate that such reactions can occur via cation- (Scheme 2B, route a) as well as anion- (Scheme 1A) and radical-mediated (Scheme 1B,C) mechanisms. However, by analogy with the proposed mechanism of PhnZ,[6] the HppE-catalyzed C−P bond migration observed with **17** can also be explained by a radical-induced fragmentation to generate metaphosphate (**21**) and an enolate intermediate (**22**), followed by recombination to give **20** (Scheme 2B, route b). Similar mechanisms can also account for the formation of **27** from the corresponding 2-amino analogues **23** and **24** (Scheme 2C). To distinguish between these two fundamentally different mechanisms, additional analogues of **17** bearing electron-withdrawing/leaving groups at C3 were designed, synthesized, and analyzed as mechanistic probes of the HppE-catalyzed 1,2-phosphono migration reaction.

The presence of one or more electron-withdrawing groups on the C adjacent to the carbocation center will dramatically affect the stability of the cation.[11,12] For example, in studies of prenyltransferase, which catalyzes a reaction generally accepted to proceed a carbocation intermediate, the reaction rate was decreased ~3×10^7-fold when one of the methyl groups of the substrate was replaced with a trifluoromethyl group.[13] Thus, one would expect a dramatic rate reduction of HppE-catalyzed

Scheme 3. Possible Outcomes of Reaction of HppE with Substrate Analogues Bearing (A) Electron-Withdrawing and (B) Leaving Groups at C3, and (C) Mechanistic Probes Used in This Study



phosphono migration (**29**→**30**, Scheme 3A) that reflects the electron-withdrawing property of the C3 substituent of the substrate analogues (R in **28**), if the reaction proceeds via the carbocation rearrangement mechanism (Scheme 2B, route a). In contrast, if the radical-induced fragmentation/recombination mechanism is operative (Scheme 2B, route b), analogues containing a leaving group at C3 (e.g., **31**) will partition between formation of the migration product **33** (Scheme 3B, route b1) and elimination of the leaving group from the enolate intermediate **32**, with concomitant formation of acrylaldehyde **34** and inorganic phosphate, respectively (Scheme 3B, route b2).

With these scenarios in mind, we prepared probes bearing methoxy (**36**), monofluoro (**37**), or trifluoro (**38**) groups (Scheme 3C).[14] An analogue with a terminal methyl group (**35**) was also synthesized.[14] Compound **38** was synthesized as a racemic mixture due to poor reactivity with porcine esterase and the various lipases used to resolve the C1-OH chirality. The remaining analogues were obtained in enantiomerically pure form, as determined by measuring the enantiomeric excess by ^{31}P NMR using quinine as a chiral shift reagent[15] (SI).

Following the previously reported procedure,[10] the reaction of HppE with **35** was monitored using ^{1}H NMR spectroscopy over a 30 min period, and the extent of substrate consumption/product formation at various time points was estimated by peak integration and comparison with an internal standard (DMSO-d_6, δ 2.49). A new signal with a chemical shift consistent with an aldehyde proton (δ 9.32) was observed. Its appearance is accompanied by a shift of the methyl resonance from δ 0.75 (of **35**) to 0.68 (of **39**), indicating that HppE can accept **35** as a substrate and convert it nearly quantitatively to the corresponding migration product **39** (Figure 1a and Scheme 4).

When the experiments were repeated with **36**, the distinctive aldehyde proton peak of the migration product **40** (δ 9.42) was detected, although the extent of conversion was only 16% during the time monitored (Figure 1b). Neither methoxy group elimination nor acrylaldehyde formation was noted. When **37** was incubated with HppE under the same conditions, only a trace amount of the migration product **41** was detected by ^{1}H NMR

dx.doi.org/10.1021/ja403441x | J. Am. Chem. Soc. 2013, 135, 8153−8156

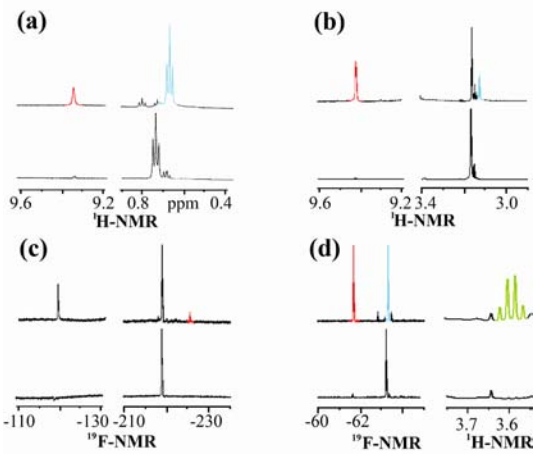
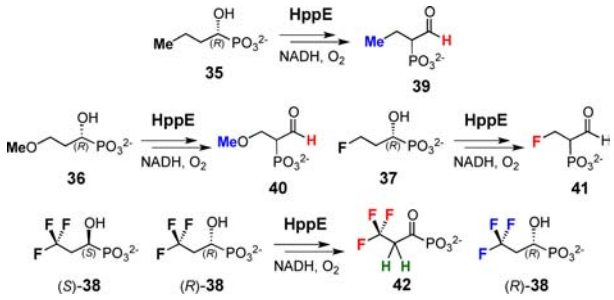


Figure 1. Selected ^{1}H and ^{19}F NMR assays of HppE with substrate analogues (a) **35**, (b) **36**, (c) **37**, and (d) **38**. The bottom trace of each panel is the spectrum taken 3 min after mixing HppE with the substrate analogue, and the top trace is recorded 27 min after initiation of the reaction. The NMR signals and the corresponding protons in the structures shown in Scheme 4 are color-coded.

Scheme 4. Reactions of HppE with 35−38



(Scheme 4 and Figure S1). The conversion was <5% based on integration of the ^{19}F NMR peaks (Figure 1c). Interestingly, in addition to the fluoro resonances of the substrate and product (δ −218 and −225, respectively), a fluoride anion signal at δ −121 was detected. However, this F^- peak remains visible in the absence of enzyme, and no inorganic phosphate formation was discernible using ^{31}P NMR. Thus, the appearance of F^- during incubation is unlikely a catalytically relevant event.

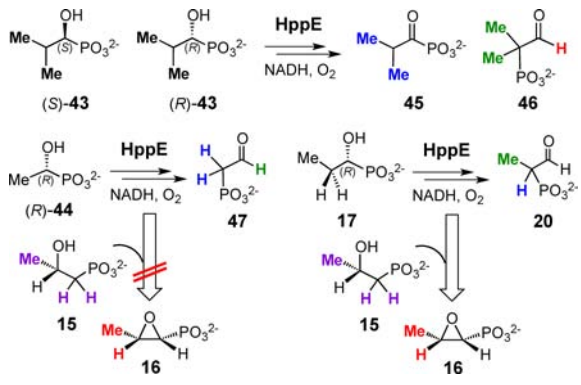
When the substrate analogue **38** was incubated with HppE, ~50% of it was converted to acylphosphonate **42** (estimated by integration of the ^{19}F NMR resonances at δ −63.2 of **38** and δ −61.6 of **42**, Figure 1d). A quintet (J_{H-F} = 10.8 Hz) at δ 3.60 in the ^{1}H NMR spectrum (Figure 1d) is characteristic for the C2 methylene protons of **42**. No obvious formation of the migration product was discernible. The fact that only half of **38** was consumed during the course of the reaction is not surprising since **38** used in the incubation is a racemic mixture. It appears that only the *S*-epimer is accepted as a substrate by HppE and is converted to the expected acylphosphonate product **42**, whereas the *R*-epimer, which should give rise to the migration product, does not react (Scheme 4).[10,16] Given the inverse correlation between the extent of conversion of the analogues to the corresponding migration products and the electron-withdrawing ability of the substituents, these results are more consistent with a mechanism involving the formation of a carbocation intermediate along the reaction coordinate.

To further explore the effects of electron-withdrawing substitution with the (*R*)-1-HPP (**17**) analogues on the energetics of the HppE-catalyzed reactions, density functional theory (DFT) calculations were performed (SI). The calculated C2−H bond dissociation energies (BDEs) for the methoxy-containing substrate analogue **36** (93.9 kcal/mol) and the monofluoro analogue **37** (93.5 kcal/mol) are very similar to that of **17** (93.8 kcal/mol),[10] whereas the BDE for the trifluoro analogue **38** is significantly higher (98.4 kcal/mol). The increased strength of the C−H bond of **38** may explain the inability of HppE to accept the *R*-isomer as a substrate, but BDEs alone cannot explain the observed differences in yield with **36** and **37** (16% and <5% versus **17**, respectively). This prompted an examination of the relative ionization energies (IEs) of each of the corresponding C2-centered radicals of **36**−**38**.[17] The IEs of the substrate analogue radicals were found to increase as a function of the electron-withdrawing ability of the C3 substituent, from 4.5 to 15.2 to 26.3 kcal/mol (relative to the IE of the (*R*)-1-HPP radical) for analogues **36**−**38**, respectively. This increase in IE closely follows the observed trend in reactivity of HppE with the substrate analogues, supporting carbocation formation in the 1,2-phosphono migration reaction (Scheme 2B, route a).

The C2 radicals/cations formed in the reactions of HppE with **17** and analogues **36**−**38** are centered at a 2° C. It is well known that the stability of both radicals and cations increases in the order 1° < 2° < 3°.[11,12] Moreover, the stabilization effects are more dramatic for cations than the corresponding radicals.[12] As a result, an analogue of **17** proceeding through a 3° carbocation intermediate is expected to be readily converted by HppE to the subsequent migration product, while an analogue that requires the formation of a 1° carbocation is likely to be a poor substrate. Two compounds, **43** and **44**, were therefore prepared to test this hypothesis and gain further evidence for the proposed carbocation mechanism (Scheme 5).[14]

Compound **43** was synthesized as a racemic mixture. Upon incubation with HppE, **43** was completely consumed to produce acylphosphonate **45** (CH_3, δ 0.86, d, J_{H-H} = 7.2 Hz) and the migration product **46** (CH_3, δ 1.05, d, J_{P-H} = 13.8 Hz; aldehyde-H, δ 9.41) in a 1:1 ratio (as determined by integration of the methyl signals of the products, Figure 2a). Unlike **43**, the *R*- and *S*-isomers of **44** were prepared separately.[14] When (*S*)-**44** was incubated with HppE, quantitative turnover to the acylphosphonate product was observed (Figure S2). However, under the same conditions, <15% of (*R*)-**44** was converted to the migration product **47** (methylene protons, δ 2.72, dd, J_{P-H} = 19.8 Hz, J_{H-H} = 4.2 Hz; aldehyde-H, δ 9.40, t, J_{H-H} = 4.2 Hz, Figure

Scheme 5. Reactions of HppE with 43 and 44



dx.doi.org/10.1021/ja403441x | J. Am. Chem. Soc. 2013, 135, 8153−8156

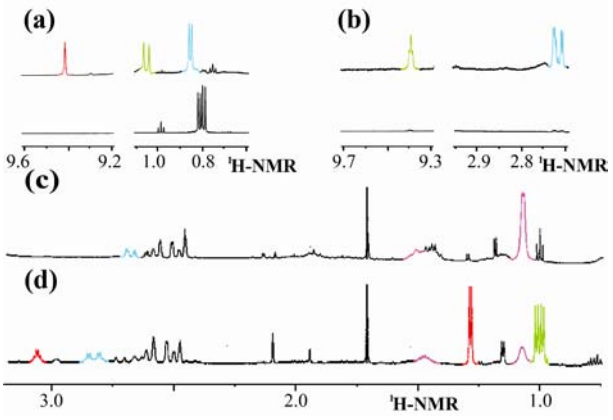


Figure 2. Selected ^{1}H NMR assays of HppE with substrate analogues (a) **43** and (b) (*R*)-**44**, (c) preincubation with (*R*)-**44** followed by addition of **15**, and (d) preincubation with **17** followed by addition of **15**. The bottom trace in panels (a) and (b) is the spectrum taken 3 min after mixing HppE with the substrate analogue, and the top trace is recorded 27 min after initiation of the reaction. The NMR signals and the corresponding protons in the structures shown in Scheme 5 are color-coded.

2b). Further experiments showed that preincubation of HppE with (*R*)-**44** led to enzyme inactivation, as the resulting enzyme failed to convert **15** to **16** (Figure 2c). In control reactions where HppE was preincubated with **17**, the enzyme remained active and capable of catalyzing fosfomycin production from **15** (Figure 2d). These data suggest that HppE is able to abstract a H atom from C2 of (*R*)-**44** to initiate the reaction, but only a fraction of the radicals generated are oxidized to the corresponding cation and converted to the migration product **47**; the remaining radicals react with the enzyme, rendering it inactive. This interpretation is consistent with the results of DFT calculations on **43** and **44**, which show that the C2−H BDE and IE of the 3° analogue **43** are 3.3 and 7.3 kcal/mol lower, respectively, than for **17**, while those for the 1° analogue **44** are 3.7 and 10.5 kcal/mol greater than for **17**.

In summary, these results, coupled with information obtained from previous mechanistic studies,[10,18] are consistent with the following mechanism of HppE-catalyzed 1,2-phosphono migration (Scheme 2B and Figure S3). First, (*R*)-1-HPP (**17**) binds to the ferrous iron of HppE in a bidentate fashion through its hydroxyl and phosphonate moieties and organizes the iron center to coordinate molecular oxygen. O_2 then binds, generating a ferric-superoxo species that abstracts the *pro-R* H atom from C2. Proton-coupled electron transfer to the resulting ferric-hydroperoxo species generates a highly reactive ferryl intermediate (**18**) that oxidizes the C2-centered substrate radical to the corresponding 2° carbocation (**19**). The carbocation thus formed induces a 1,2-shift of the phosphono moiety to generate the aldehyde product (**20**). Finally, product release and reduction of the ferric iron back to the ferrous state completes the catalytic cycle. To our knowledge, this is the first example of enzymatic cleavage of a C−P bond induced by the formation of a carbocation. As such, it represents a new paradigm for biological C−P bond cleavage.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details and computational methods. 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

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work is supported in part by grants from the National Institutes of Health (GM040541) and the Welch Foundation (F-1511). Dedicated to Prof. Koji Nakanishi on the occasion of his 88th birthday. We thank Steve Sorey for help in acquiring the NMR spectra.

■ REFERENCES

(1) (a) Seto, H.; Kuzuyama, T. *Nat. Prod. Rep.* **1999**, *16*, 589. (b) White, A. K.; Metcalf, W. W. *Annu. Rev. Microbiol.* **2007**, *61*, 379.

(2) Metcalf, W. W.; van der Donk, W. A. *Annu. Rev. Biochem.* **2009**, *78*, 65.

(3) Kamat, S. S.; Williams, H. J.; Raushel, F. M. *Nature* **2011**, *480*, 570.

(4) Quinn, J. P.; Kulakova, A. N.; Cooley, N. A.; McGrath, J. W. *Environ. Microbiol.* **2007**, *9*, 2392.

(5) Kim, J.; Dunaway-Mariano, D. *Biochemistry* **1996**, *35*, 4628.

(6) McSorley, F. R.; Wyatt, P. B.; Martinez, A.; Delong, E. F.; Hove-Jensen, B.; Zechel, D. L. *J. Am. Chem. Soc.* **2012**, *134*, 8364.

(7) Xing, G.; Barr, E. W.; Diao, Y.; Hoffart, L. M.; Prabhu, K. S.; Arner, R. J.; Reddy, C. C.; Krebs, C.; Bollinger, J. M., Jr. *Biochemistry* **2006**, *45*, 5402.

(8) Xing, G.; Diao, Y.; Hoffart, L. M.; Barr, E. W.; Prabhu, K. S.; Arner, R. J.; Reddy, C. C.; Krebs, C.; Bollinger, J. M., Jr. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 6130.

(9) (a) Liu, P.; Murakami, K.; Seki, T.; He, X.; Yeung, S. M.; Kuzuyama, T.; Seto, H.; Liu, H.-w. *J. Am. Chem. Soc.* **2001**, *123*, 4619. (b) Zhao, Z.; Liu, P.; Murakami, K.; Kuzuyama, T.; Seto, H.; Liu, H.-w. *Angew. Chem., Int. Ed.* **2002**, *41*, 4529. (c) Liu, P.; Liu, A.; Yan, F.; Wolfe, M. D.; Lipscomb, J. D.; Liu, H.-w. *Biochemistry* **2003**, *42*, 11577. (d) Higgins, L. J.; Yan, F.; Liu, P.; Liu, H.-w.; Drennan, C. L. *Nature* **2005**, *437*, 838.

(10) Chang, W.-c.; Dey, M.; Liu, P.; Mansoorabadi, S. O.; Moon, S.-J.; Zhao, K. Z.; Drennan, C. L.; Liu, H.-w. *Nature* **2013**, *496*, 114.

(11) Blanksby, S. J.; Ellison, G. B. *Acc. Chem. Res.* **2003**, *36*, 255.

(12) Kim, C. K.; Lee, K. A.; Bae, S. Y.; Han, I. S.; Kim, C. K. *Bull. Kor. Chem. Soc.* **2004**, *25*, 311.

(13) (a) Poulter, C. D.; Rilling, H. C. *Biochemistry* **1976**, *15*, 1079. (b) Poulter, C. D.; Satterwhite, D. M. *Biochemistry* **1977**, *16*, 5470.

(14) See SI for experimental details.

(15) Żymańczyk-Duda, E.; Skwarczyński, M.; Lejczak, B.; Kafarski, P. *Tetrahedron: Asymmetry* **1996**, *7*, 1277.

(16) The configuration of the substrate governs its enzymatic fate.[9b] The *S*-epimer of 1-HPP is converted to an acylphosphonate, whereas the phosphonate migration reaction is observed with the *R*-epimer.[10]

(17) The calculated IEs were obtained as the difference in the electronic energies of the radical and cationic species using the optimized structure of the corresponding radical. This was done because the phosphonate group was found to migrate to the 2 position during geometry optimization of the cationic species. The reported values can thus be considered an upper limit for the IE differences.

(18) (a) Yan, F.; Moon, S. J.; Liu, P.; Zhao, Z.; Lipscomb, J. D.; Liu, A.; Liu, H.-w. *Biochemistry* **2007**, *46*, 12628. (b) Mirica, L. M.; McCusker, K. P.; Munos, J. W.; Liu, H.-w.; Klinman, J. P. *J. Am. Chem. Soc.* **2008**, *130*, 8122. (c) Yun, D.; Dey, M.; Higgins, L. J.; Yan, F.; Liu, H.-w.; Drennan, C. L. *J. Am. Chem. Soc.* **2011**, *133*, 11262. (d) Huang, H.; Chang, W.-c.; Pai, P.-J.; Romo, A.; Mansoorabadi, S. O.; Russell, D. H.; Liu, H.-w. *J. Am. Chem. Soc.* **2012**, *134*, 16171.