JACS JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

pubs.acs.org/JACS

PhnY and PhnZ Comprise a New Oxidative Pathway for Enzymatic Cleavage of a Carbon–Phosphorus Bond

Fern R. McSorley,[†] Peter B. Wyatt,[§] Asuncion Martinez,[‡] Edward F. DeLong,[‡] Bjarne Hove-Jensen,[†] and David L. Zechel*,[†]

[†]Department of Chemistry, Queen's University, 90 Bader Lane, Kingston, Ontario, Canada K7L 3N6

[§]School of Biological and Chemical Sciences, Queen Mary University of London, Mile End Road, London E1 4NS, U.K.

[‡]Division of Biological Engineering, Department of Civil and Environmental Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, United States

Supporting Information

ABSTRACT: The sequential activities of PhnY, an α-ketoglutarate/Fe(II)-dependent dioxygenase, and PhnZ, a Fe(II)-dependent enzyme of the histidine-aspartate motif hydrolase family, cleave the carbon–phosphorus bond of the organophosphonate natural product 2-aminoethylphosphonic acid. PhnY adds a hydroxyl group to the α-carbon, yielding 2-amino-1-hydroxyethylphosphonic acid, which is oxidatively converted by PhnZ to inorganic phosphate and glycine. The PhnZ reaction represents a new enzyme mechanism for metabolic cleavage of a carbon–phosphorus bond.

Among many examples of naturally occurring organophosphonates, 2-aminoethylphosphonic acid (**1**) is the most prevalent in the environment (Scheme 1a).[1,2] Many organisms, particularly phytoplankton, use **1** as a substitute for phosphocholine for the synthesis of phosphonolipids.[3] Although incorporation of **1** into lipids can be a response to phosphate deprivation, the carbon–phosphorus (CP) bond may also afford protection against hydrolytic enzymes, alter membrane bilayer structure, and contribute to intercellular communication.[2] **1** also represents an important source of inorganic phosphate (P_i) for microorganisms living in phosphate-limited environments. Studies have so far uncovered two general enzyme mechanisms for cleaving a CP bond in order to liberate P_i for metabolic use.[1,4] The "electron-sink" mechanism is applied to alkylphosphonates with a β-carbonyl group, where the CP bond is cleaved by direct attack of a nucleophile, either water or an enzyme side chain, on the phosphorus center, with the enzyme using either a Schiff base or interaction of a metal ion with the carbonyl group to assist delocalization of the resulting carbanion. The phosphonoacetaldehyde, phosphonopyruvate, and phosphonoacetate hydrolases typify this electron-sink mechanism.[4] Phosphoenolpyruvate mutase also falls into this category of CP bond cleavage (and is a homologue of phosphonopyruvate hydrolase),[4] although the primary function of this enzyme is to synthesize CP bonds by catalyzing the equilibrium between phosphoenolpyruvate and phosphonopyruvate.[2] The second CP bond cleavage mechanism is found in CP lyase, a multienzyme complex encoded by the *phn* operon (*phnCDEFGHIJKLMNOP*).[5] This mechanism is unusual in that the organophosphonate substrate must be incorporated into ribose as a 5-phospho-α-D-ribosyl-1-alkylphosphonate, whereupon CP bond cleavage yields 5-phospho-α-D-ribosyl-1,2-cyclic phosphate and an alkane (i.e., methylphosphonic acid leads to methane).[6] A milestone study revealed that PhnJ cleaves the CP bond of 5-phospho-α-D-ribosyl-1-alkylphosphonate through a radical reaction that requires a [4Fe-4S] cluster and *S*-adenosylmethionine.[7] Although the precise mechanism of PhnJ has yet to be defined, the formation of the 1,2-cyclic phosphate suggests an essential role for the 2-hydroxyl of ribose in enzyme turnover.

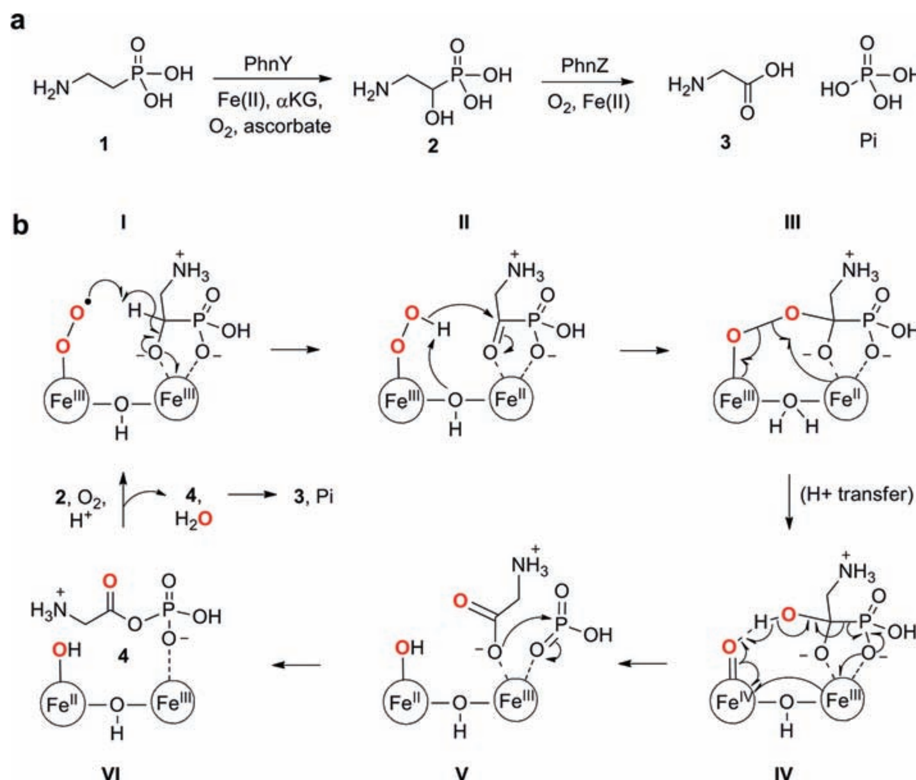
The global distribution of organophosphonates in what are typically phosphate-poor environments leads one to consider that other CP bond-cleaving mechanisms may have evolved to catabolize alternative forms of phosphorus.[8] This was the inspiration behind a recent study that screened marine-derived metagenomic DNA for genes that restored the ability of a Δ*phn* mutant of *Escherichia coli* to grow in the presence of **1** as the sole source of phosphate.[9] In addition to finding genes encoding the phosphonoacetaldehyde hydrolase noted above, a new pair of genes was retrieved, *phnY* and *phnZ*, that complemented the Δ*phn* mutation. PhnY belongs to a large and catalytically diverse class of non-heme Fe(II)/α-ketoglutarate-dependent dioxygenases (family code: pfam05721), which include phytanoyl-CoA dioxygenases and halogenases. PhnY also shows high sequence similarity to *Penicillium decumbens* EpoA and *Pseudomonas stutzeri* WM88 HtxA, which epoxidize *cis*-propenylphosphonic acid and oxidize hypophosphite to phosphite, respectively.[10,11] PhnZ is predicted to be a metal ion-dependent phosphohydrolase of the HD superfamily and belongs to a large subclass of uncharacterized enzymes that are frequently associated with the CP lyase encoding *phn* operon in several species of bacteria (family codes: pfam01966 and TIGR03276).[12] The intriguing functions predicted for PhnY and PhnZ suggest a new strategy for cleavage of a CP bond,[9] which warranted further investigation.

Synthetic *phnY* and *phnZ* genes were expressed separately in *E. coli* and the encoded enzymes purified in a single step by affinity chromatography (Supporting Information (SI) Figure

Received: March 1, 2012
Published: May 8, 2012

 © 2012 American Chemical Society

Scheme 1. Proposed Reactions and Mechanisms for PhnY and PhnZ[a]



[a](a) Hydroxylation of **1** by the Fe(II)/α-ketoglutarate-dependent dioxygenase PhnY to give **2** followed by CP bond cleavage by PhnZ to yield glycine **3** and inorganic phosphate (P_i). (b) Hypothetical mechanism for PhnZ by analogy to the calculated mechanism for MIOX.[23]

1). We first examined the activity of PhnY, as the potent oxidizing activity of this class of enzyme seemed ideal for modifying **1** to render the CP bond susceptible to cleavage. The reaction of PhnY with **1** was monitored by ^{31}P NMR spectroscopy. No change in the signal for **1** (δ 17.3 ppm) was observed with PhnY alone or in the presence of α-ketoglutarate (Figure 1a). However, a mixture of Fe(II), α-ketoglutarate, ascorbate, and PhnY enabled conversion of **1** to a new compound with a signal at δ 14.3 ppm (Figure 1b). Omitting ascorbate markedly reduced conversion of **1** by PhnY (SI Figure 2a), reflecting the well-known ability of reducing agents to enhance the activity of α-ketoglutarate/Fe(II)-dependent dioxygenases by maintaining the iron cofactor in the catalytically active ferrous form.[13] Given the precedent of α-hydroxylation of taurine by *E. coli* TauD[14] and 2-hydroxyethylphosphonic acid by *Streptomyces luridis* DhpA,[15] we anticipated that hydroxylation at the α-carbon of **1** by PhnY would yield the known natural product 2-amino-1-hydroxyethylphosphonic acid **2** (Scheme 1a).[9] This was confirmed by adding synthetic (±)-**2** and observing an increase in the signal at δ 14.3 ppm (Figure 1c). In contrast, methylphosphonic and ethylphosphonic acids were not substrates for PhnY (data not shown), which agrees with the previous observation that these compounds do not sustain the growth of *E. coli* Δ*phn* transformed with *phnY* and *phnZ* genes.[9]

We next examined the role of PhnZ in the conversion of **1**. When PhnZ was included with the PhnY reaction, ^{31}P NMR spectroscopy revealed complete conversion of **1** to P_i (δ 2.9 ppm, Figure 1d). Incubation of PhnZ alone with **1** did not afford conversion to a new product. Denaturation of PhnY (95 °C, 10 min) after forming **2** followed by addition of PhnZ also resulted in conversion to P_i (SI Figure 2b). However, reaction of PhnZ directly with (±)-**2** resulted in only 50% conversion (Figure 1e), suggesting that PhnZ (and likewise PhnY) is stereospecific. This indicates that **2** is the substrate for PhnZ, and that PhnZ catalyzes the cleavage of the CP bond of **2** to yield P_i. PhnZ activity is abolished by the addition of EDTA (data not shown), indicating the essential role of a metal ion. After the EDTA was removed by dialysis, PhnZ was incubated with **2** generated by PhnY in the presence of various metal ions (Fe^{2+}, Fe^{3+}, Co^{2+}, Mn^{2+}, Ni^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, or Mg^{2+}, 0.1 mM each). ^{31}P NMR spectroscopy revealed that only Fe(II) enabled PhnZ to completely convert **2** to P_i (SI Figure 3). Partial activity was observed with the other metal ions, presumably due to the presence of residual Fe(II) from the PhnY reaction, with the exception of Mg^{2+} where no conversion was observed, which suggests that this metal ion is inhibitory. Repeating this analysis with synthetic (±)-**2** confirmed the requirement for Fe(II) for activity (SI Figure 4). ICP-MS analysis of the metal ion content of PhnZ confirmed the binding of 1.2 ± 0.1 mol of iron per mole of enzyme, as well as 0.5 ± 0.1 mol of calcium per mole of enzyme. (See SI for more details.)

The dependence on Fe(II) for activity suggested that PhnZ might cleave the CP bond of **2** through an oxidative mechanism. A precedent for such a reaction was reported for the Fe(II)-dependent enzyme 2-hydroxyethylphosphonate dioxygenase (HEPD). Although HEPD normally catalyzes oxidative cleavage of a carbon−carbon bond,[16] elegant studies with substrate analogues revealed promiscuous CP bond cleaving activity toward 1-hydroxyalkylphosphonic acids.[17,18] This suggests that 1-hydroxyalkylphosphonic acids are

 dx.doi.org/10.1021/ja302072f | J. Am. Chem. Soc. 2012, 134, 8364−8367

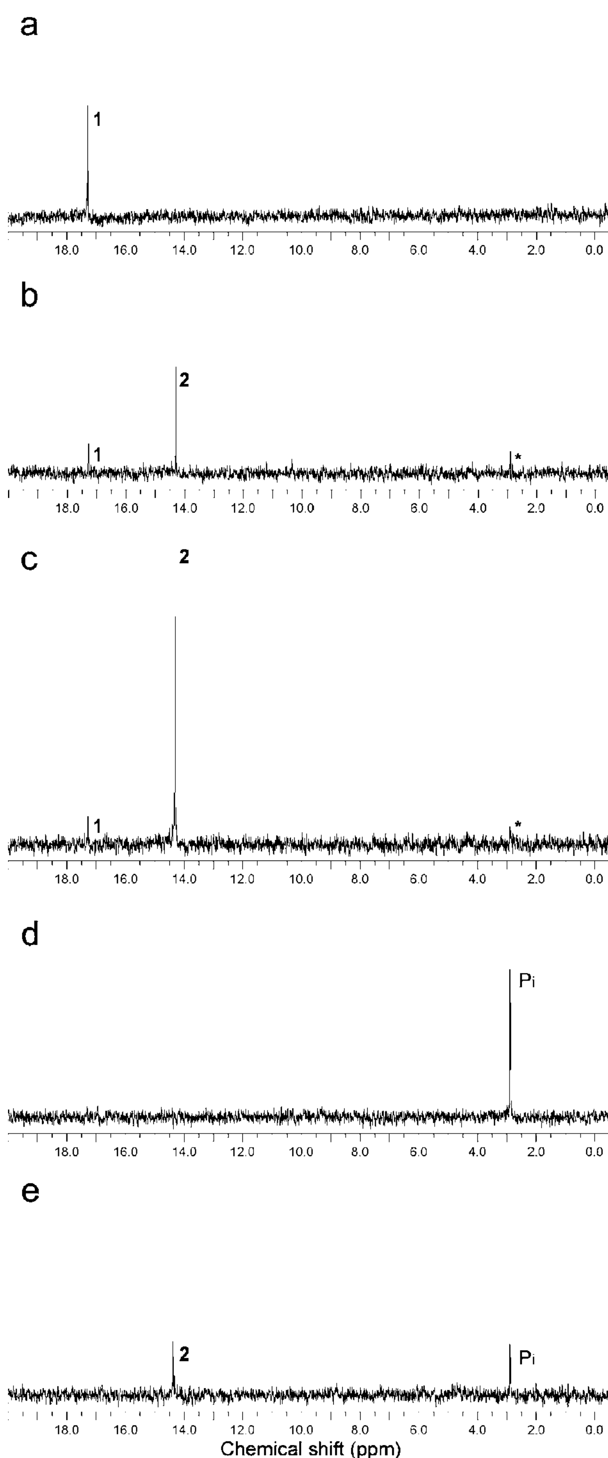


Figure 1. *In vitro* reconstitution of the degradation of 2-aminoethylphosphonate **1** to inorganic phosphate by PhnY and PhnZ monitored by ^{31}P NMR spectroscopy. (a) Spectrum of the reaction of **1** (δ 17.3 ppm) with PhnY and α-ketoglutarate. (b) Reaction of **1** (17.3 ppm) with PhnY in the presence of Fe(II), α-ketoglutarate, and ascorbate, yielding 2-amino-1-hydroxyethylphosphonic acid (**2**) (δ 14.3 ppm). (c) Addition of synthetic ($\pm$)-**2** to the PhnY reaction described in (b). (d) Result of the addition of PhnZ to the PhnY reaction described in (b). Inorganic phosphate appears at δ 2.9 ppm. (e) Result of the reaction of PhnZ with synthetic ($\pm$)-**2** (δ 14.3 ppm) to yield P_i (δ 2.9 ppm). *Residual P_i from purification of PhnY.

inherently sensitive to oxidative CP bond cleavage, and that a similar oxidative reaction by PhnZ with **2** would yield P_i and glycine **3** (Scheme 1a). Indeed, treatment of a PhnY/PhnZ reaction mixture with phenyl isothiocyanate followed by HPLC analysis revealed that glycine **3** is the alkyl product resulting from CP bond cleavage of **2** (Figure 2).

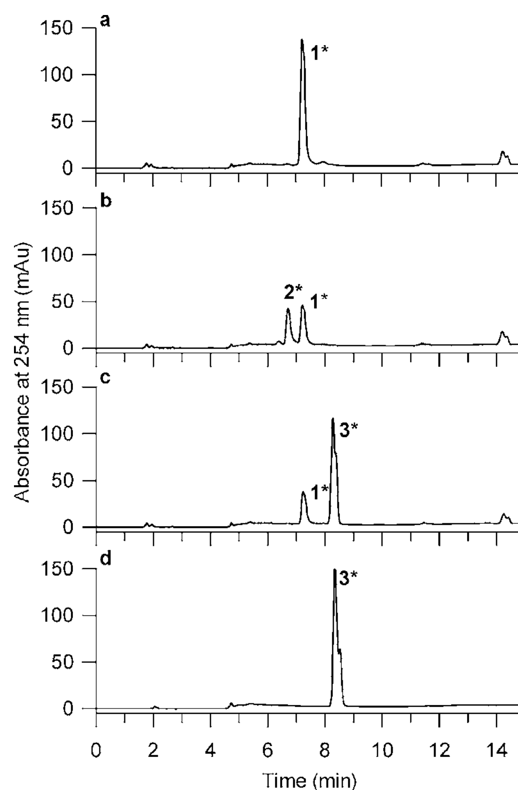


Figure 2. HPLC detection of glycine as a product of the PhnY and PhnZ reaction. All solutions were reacted with phenyl isothiocyanate prior to HPLC analysis (see SI). Phenyl isothiocyanate adducts are denoted with *. (a) Chromatogram of the phenyl isothiocyanate adduct of **1**. (b) Reaction of **1** with PhnY in the presence of Fe(II), α-ketoglutarate, and ascorbate yielding **2**. (c) Addition of PhnZ to the PhnY reaction described in (b). (d) Chromatogram of the phenyl isothiocyanate adduct of glycine **3**.

Although PhnZ and HEPD catalyze similar reactions, the mechanism of PhnZ may actually more closely resemble that proposed for *myo*-inositol oxygenase (MIOX).[19] Unlike HEPD, a mononuclear enzyme that belongs to the cupin superfamily, MIOX has significant structural homology to the HD hydrolase family.[20] Indeed, PhnZ and MIOX share the conserved "HD" motif that distinguishes this family of enzymes, despite sharing little overall sequence homology (10% identity, 19% similarity, see SI). In the case of MIOX, the HD residues act as ligands for a mixed-valence Fe(II)/Fe(III) cofactor to catalyze the oxidative cleavage of a carbon−carbon bond.[21] Thus, analogous to MIOX, PhnZ may utilize the Fe(II) ion to reduce molecular oxygen to form a superoxo-Fe(III) species in proximity of the substrate **2** (**I** in Scheme 1b).[22,23] MIOX employs the second Fe(III) ion to bind the vicinal hydroxyl groups of *myo*-inositol in a bidentate fashion.[20] A similar Lewis acid interaction with the phosphonate and α-hydroxyl oxygens of **2** can be envisioned for PhnZ (Scheme 1b). Although PhnZ co-purifies with approximately one equivalent of iron rather than two, it is possible that the purified enzyme is not fully loaded with metal ion. Also, spontaneous oxidation of excess Fe(II) in the PhnZ

dx.doi.org/10.1021/ja302072f | J. Am. Chem. Soc. 2012, 134, 8364−8367

reactions described above would provide Fe(III) to serve this substrate binding role.

The calculated reaction manifold for MIOX provides a plausible template for CP bond cleavage by PhnZ.[23] The superoxo-Fe(III) species can be expected to abstract the α-hydrogen of **2** (**I** to **II**, Scheme 1b). Hydroperoxylation or hydroxylation of a substrate radical intermediate utilizing low valence Fe(III) or high valence Fe(IV)-oxo species as electron acceptors, respectively, have been proposed to follow hydrogen abstraction by MIOX.[19] However, calculations suggest that rather than forming a carbon-centered radical, hydrogen abstraction by MIOX is accompanied by single electron oxidation of the substrate to form a carbonyl,[23] which in the case of the PhnZ reaction would correspond to the α-ketophosphonate intermediate **II**. Interestingly, calculations on HEPD predict formation of a similar intermediate during CP bond cleavage of 1-hydroxyalkylphosphonic acids.[24] Subsequently, a metal ion bridging hydroxide is expected to facilitate nucleophilic attack of the Fe(III)-hydroperoxide on the α-carbonyl of **II**, forming a peroxide-hemiketal **III**. Previous studies have suggested that intermediates like **III** may undergo CP bond cleavage through a Criegee-like rearrangement, yielding acyl phosphates.[17,18,25] However, calculations for dinuclear MIOX and mononuclear HEPD favor homolytic cleavage of the peroxide-hemiketal O−O bond,[23,24] leading in the dinuclear case to a Fe(IV)-oxo species and the geminal diolate intermediate **IV**. Abstraction of the hydroxyl hydrogen of **IV** by the ferryl oxygen then initiates CP bond cleavage, leading to glycine and metaphosphate (**IV** to **V**, Scheme 1b). This step also regenerates the Fe(II) ion, which would account for the observation that PhnZ does not require external reducing equivalents to perform multiple turnovers of substrate. This is demonstrated by the ability of purified PhnZ (5 μM) to convert ~50% of a large excess of synthetic (±)-**2** (1 mM) to P_i (Figure 1e). Capture of metaphosphate by glycine would yield glycyl phosphate **4** (**V** to **VI**, Scheme 1b). This step is predicted for the HEPD reaction with 1-hydroxyalkylphosphonic acids[23] and acyl phosphates have been observed as products.[17] However, a ^{31}P NMR spectroscopic signal was not observed for **4** in PhnY/PhnZ reactions, suggesting that this species, if formed, is rapidly hydrolyzed.[26] In sum, the reaction catalyzed by PhnZ is clearly distinct from other dedicated CP bond cleaving enzymes, and thus represents a third class of mechanism for CP bond cleavage. The oxidative cleavage of a CP bond by PhnZ also extends the scope of reactions catalyzed by the HD hydrolase family.

■ ASSOCIATED CONTENT

Supporting Information

Expression of *phnY* and *phnZ* in *E. coli*, enzyme purification, activity assays, ICP-MS analysis, as well as additional figures and schemes. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

dlzechel@chem.queensu.ca

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the Gordon and Betty Moore Foundation (AM, EFD), the Natural Sciences and Engineering Research Council of Canada (NSERC), and Queen's University. D.L.Z. is additionally supported by an Ontario Early Researcher Award. We thank Prof. D. Beauchemin and Dr. A. Asfaw for assistance with ICP-MS analysis, as well as Prof. R. S. Brown for assistance with HPLC analyses. We also thank Profs. N. H. Williams, R. Kluger, and D. A. Pratt for inspiring discussions on the mechanism of PhnZ.

■ REFERENCES

(1) 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) Van Mooy, B. A.; Fredricks, H. F.; Pedler, B. E.; Dyhrman, S. T.; Karl, D. M.; Koblizek, M.; Lomas, M. W.; Mincer, T. J.; Moore, L. R.; Moutin, T.; Rappe, M. S.; Webb, E. A. *Nature* **2009**, *458*, 69.
(4) Quinn, J. P.; Kulakova, A. N.; Cooley, N. A.; McGrath, J. W. *Environ. Microbiol.* **2007**, *9*, 2392.
(5) Jochimsen, B.; Lolle, S.; McSorley, F. R.; Nabi, M.; Stougaard, J.; Zechel, D. L.; Hove-Jensen, B. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 11393.
(6) Hove-Jensen, B.; McSorley, F.; Zechel, D. *J. Am. Chem. Soc.* **2011**, *133*, 3617.
(7) Kamat, S. S.; Williams, H. J.; Raushel, F. M. *Nature* **2011**, *480*, 570.
(8) Villarreal-Chiu, J. F.; Quinn, J. P.; McGrath, J. W. *Front. Microbiol.* **2012**, *3*, 1.
(9) Martinez, A.; Tyson, G. W.; Delong, E. F. *Environ. Microbiol.* **2010**, *12*, 222.
(10) Watanabe, M.; Sumida, N.; Murakami, S.; Anzai, H.; Thompson, C. J.; Tateno, Y.; Murakami, T. *Appl. Environ. Microbiol.* **1999**, *65*, 1036.
(11) White, A. K.; Metcalf, W. W. *J. Biol. Chem.* **2002**, *277*, 38262.
(12) Aravind, L.; Koonin, E. V. *Trends Biochem. Sci.* **1998**, *23*, 469.
(13) Costas, M.; Mehn, M. P.; Jensen, M. P.; Que, L., Jr. *Chem. Rev.* **2004**, *104*, 939.
(14) Price, J. C.; Barr, E. W.; Glass, T. E.; Krebs, C.; Bollinger, J. M., Jr. *J. Am. Chem. Soc.* **2003**, *125*, 13008.
(15) Circello, B. T.; Eliot, A. C.; Lee, J. H.; van der Donk, W. A.; Metcalf, W. W. *Chem. Biol.* **2010**, *17*, 402.
(16) Cicchillo, R. M.; Zhang, H.; Blodgett, J. A.; Whitteck, J. T.; Li, G.; Nair, S. K.; van der Donk, W. A.; Metcalf, W. W. *Nature* **2009**, *459*, 871.
(17) Whitteck, J. T.; Cicchillo, R. M.; van der Donk, W. A. *J. Am. Chem. Soc.* **2009**, *131*, 16225.
(18) Peck, S. C.; Cooke, H. A.; Cicchillo, R. M.; Malova, P.; Hammerschmidt, F.; Nair, S. K.; van der Donk, W. A. *Biochemistry* **2011**, *50*, 6598.
(19) Bollinger, J. M., Jr.; Diao, Y.; Matthews, , M. L.; Xing, G.; Krebs, C. *Dalton Trans.* **2009**, 905.
(20) Brown, P. M.; Caradoc-Davies, T. T.; Dickson, J. M.; Cooper, G. J.; Loomes, K. M.; Baker, E. N. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 15032.
(21) 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.
(22) 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.
(23) Hirao, H.; Morokuma, K. *J. Am. Chem. Soc.* **2009**, *131*, 17206.
(24) Hirao, H.; Morokuma, K. *J. Am. Chem. Soc.* **2011**, *133*, 14550.
(25) Gordon, N.; Evans, S., Jr. *J. Org. Chem.* **1993**, *58*, 4516.
(26) Billhardt, U.; Stein, P.; Whitesides, G. *Bioorg. Chem.* **1989**, *17*, 1.