Biosynthesis

Deutsche Ausgabe: DOI: 10.1002/ange.201510635
Internationale Ausgabe: DOI: 10.1002/anie.201510635

C3′-Deoxygenation of Paromamine Catalyzed by a Radical *S*-Adenosylmethionine Enzyme: Characterization of the Enzyme AprD4 and Its Reductase Partner AprD3

*Hak Joong Kim, Jake LeVieux, Yu-Cheng Yeh, and Hung-wen Liu**

Abstract: *C3′-deoxygenation of aminoglycosides results in their decreased susceptibility to phosphorylation thereby increasing their efficacy as antibiotics. However, the biosynthetic mechanism of C3′-deoxygenation is unknown. To address this issue, aprD4 and aprD3 genes from the apramycin gene cluster in Streptomyces tenebrarius were expressed in E. coli and the resulting gene products were characterized in vitro. AprD4 is shown to be a radical S-adenosylmethionine (SAM) enzyme, catalyzing homolysis of SAM to 5′-deoxyadenosine (5′-dAdo) in the presence of paromamine. [4′-^{2}H]-Paromamine was prepared and used to show that its C4′-H is transferred to 5′-dAdo by AprD4, during which the substrate is dehydrated to a product consistent with 4′-oxolividamine. In contrast, paromamine is reduced to a deoxy product when incubated with AprD4/AprD3/NADPH. These results show that AprD4 is the first radical SAM diol-dehydratase and, along with AprD3, is responsible for 3′-deoxygenation in aminoglycoside biosynthesis.*

The aminoglycosides are an important class of antibiotics in the treatment of Gram-negative bacterial infections.[1–3] Their biological activity is a result of their ability to bind the bacterial 30S ribosome and subsequently inhibit protein synthesis. Many members of this family are characterized by a 2-deoxystreptamine (2-DOS; **1**) core that is decorated with a variety of carbohydrates necessary for activity (Figure 1). In a number of aminoglycosides, such as apramycin (**2**),[4] tobramycin (**3**),[5] nebramycin 5′ (**4**),[6] and lividomycin B (**5**),[7] a 3′-deoxyhexose is found linked through a glycosidic bond to the C4 position of the 2-DOS core. The absence of a C3′ hydroxy group in these species renders them more effective as antimicrobial agents because they are less susceptible to modification by aminoglycoside phosphotransferase (APH(3′)), which is a known mechanism of resistance in some bacteria.[1] Although the biosynthesis of 2-DOS has been investigated in detail,[8–10] much less is known regarding assembly of the sugar appendages[11,12] and in particular the mechanism of C3′-deoxygenation despite its importance to the development and improvement of clinically useful aminoglycoside antibiotics.[13]

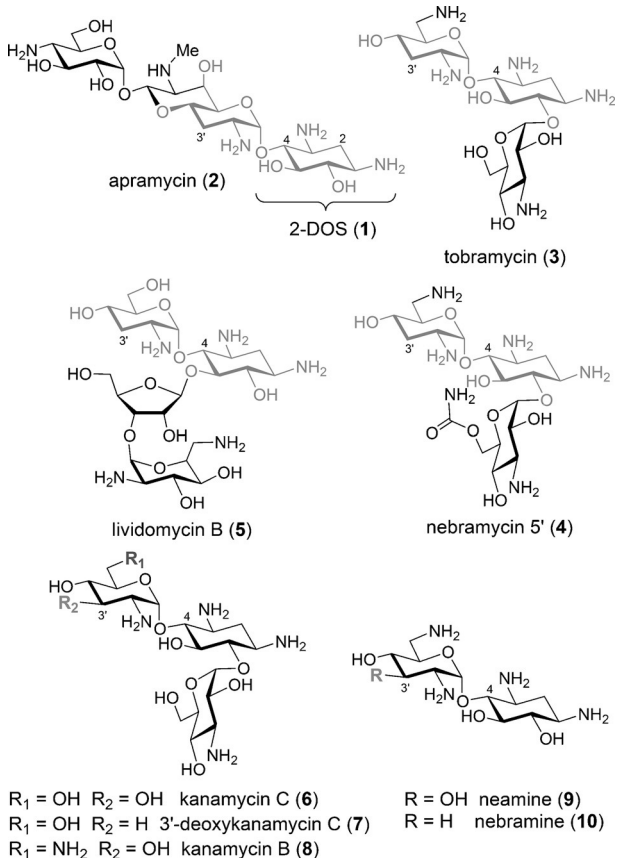


Figure 1. Selected examples of aminoglycosides.

The gene clusters responsible for the biosynthesis of apramycin (**2**), tobramycin (**3**), and lividomycin B (**5**) in *Streptomyces* and *Streptoalloteichius* species have been described,[14–17] and significant progress has been made in elucidating the reactions involved in the tobramycin pathway.[18,19] Although no gene in the tobramycin cluster has been implicated in catalyzing C3′-deoxygenation,[14,15] investigations, including gene-knockout studies[20] into the apramycin and lividomycin B pathways, have identified the AprD4/AprD3 and LivW/LivY enzyme pairs as likely candidates for the activity. For example, incorporation of the *aprD4* and *aprD3* genes into the kanamycin-producing (see **6**) strain *S. kanamyceticus* led to the isolation of 3′-deoxykanamycins (such as **7**),[19] which are not normally produced. Likewise, previous studies have also shown the conversion of neamine (**9**) to nebramine (**10**) in cell-free extracts of *S. venezuelae*

[*] H. J. Kim, J. LeVieux, Y.-C. Yeh, Prof. Dr. H.-w. Liu
Division of Medicinal Chemistry, College of Pharmacy
and Department of Chemistry, University of Texas at Austin
Austin, TX 78712 (USA)
E-mail: h.w.liu@mail.utexas.edu

Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under http://dx.doi.org/10.1002/anie.201510635.

expressing proteins AprD4 and AprD3.[19] However, no conversion of kanamycin B (**8**) to tobramycin (**3**) was observed under the same conditions, leading to the suggestion that the substrate for AprD4/AprD3 may be a pseudo-disaccharide (such as **9**) rather than a pseudo-trisaccharide (such as **8**).[19] Since apramycin (**2**) and tobramycin (**3**) are produced together in the same bacterial strains, it is possible that AprD4/AprD3 serves as the C3′-deoxygenation machinery for both pathways.

Sequence analysis has led to the assignment of AprD3 as an NAD-dependent dehydrogenase (NAD = nicotinamide adenine dinucleotide, oxidized form) and AprD4 as a member of the radical *S*-adenosyl-L-methionine (SAM) family of enzymes.[14,15,18] The radical SAM enzymes are characterized by the presence of a [4Fe-4S] cluster that serves to reductively homolyze SAM and thereby generate methionine (**17**) and a 5′-deoxyadenosyl radical 5′-dAdo·(**18**; see Scheme 2) that initiates a radical-mediated transformation of substrate to product.[21–23] The catalytic [4Fe-4S] cluster is typically coordinated by a highly conserved CxxxCxxC motif;[24] however, in the case of AprD4, a noncanonical CxxxCxxxC motif is found instead. Although a small but growing number of radical SAM enzymes, such as PhnJ,[25] HmdB,[26] QueE,[27] Phd2,[28] and ThiC,[29] do indeed exhibit atypical [4Fe-4S] binding motifs, the noncanonical motif of AprD4 still leads to questions, first as to whether AprD4 operates as a radical SAM enzyme, and second, the mechanism by which C3′-deoxygenation is effected during the biosynthesis of aminoglycosides. Herein, we report the purification and biochemical characterization of AprD4 and AprD3 from *S. tenebrarius* and demonstrate their ability to catalyze 3′-deoxygenation of paromamine (**11**) to lividamine (**13**) in vitro (see Scheme 1 for molecular structures).

Both the *aprD3* and *aprD4* genes were amplified by polymerase chain reaction from *S. tenebrarius* genomic DNA, cloned into a pET28 vector, and heterologously overexpressed in *Escherichia coli* (see Figures S1, S2 in the Supporting Information). The His_6-tagged AprD4 was isolated from cell lysates using a nickel–nitrilotriacetate (Ni–NTA) affinity column and was then subjected to anaerobic incubation with $[Fe(NH_4)_2(SO_4)_2]$ and Na_2S in a Coy anaerobic chamber with an atmosphere of more than 97% N_2 and 3% H_2 to reconstitute the [4Fe-4S] cluster. Iron[30] and sulfur analysis[31] of the reconstituted AprD4 revealed the presence of 9.2 ± 0.6 equivalents of iron and 8.2 ± 0.7 equivalents of sulfide per AprD4 monomer. These results indicated the presence of two [4Fe-4S] clusters bound to each AprD4 monomer. Likewise, the UV/Vis absorption spectrum of the reconstituted AprD4 exhibited a shoulder around $\lambda = 420$ nm, which is typical of $[4Fe\text{-}4S]^{2+}$-containing proteins.[32,33] Furthermore, addition of sodium dithionite (2 mM) to the reconstituted enzyme led to a decrease in the absorbance at $\lambda = 420$ nm, implying reduction of the clusters (Figure S2).

Co-incubation of reconstituted AprD4 (0.01 mM) with 1 mM paromamine (**11**) and 2 mM SAM in the presence of 10 mM dithiothreitol (DTT) and 2 mM dithionite (50 mM NH_4HCO_3 buffer, pH 7.8) at room temperature led to the formation of 5′-dAdo (**19**), as detected by monitoring the change in the absorption at $\lambda = 260$ nm (see Figure 2A, trace b). Although exclusion of dithionite from the reaction did not lead to detectable levels of 5′-dAdo formation, the replacement of dithionite with either methyl viologen and NADPH (nicotinamide adenine dinucleotide phosphate, reduced form) or the *E. coli* flavodoxin/flavodoxin reductase/NADPH system were effective (see Figure S4). In contrast, benzyl viologen and NADPH was not suitable for enzyme activity. Likewise, when the incubation was performed without prior reconstitution of the enzyme, no 5′-dAdo was detected (see Figure 2A, trace e). Finally, although uncoupled formation of 5′-dAdo was discernible in the absence of paromamine, the extent of SAM turnover to 5′-dAdo was significantly lower than that of the full reaction (see Figure 2A, traces d and b). These results strongly imply that AprD4 is able to catalyze radical homolysis of SAM consistent with its assignment as a radical SAM enzyme.[21–23]

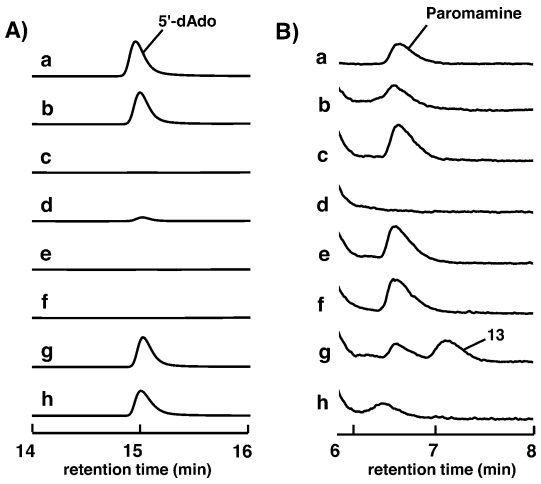


Figure 2. HPLC traces of AprD4 reaction mixtures, showing A) the production of 5′-dAdo (**19**; monitoring at $\lambda = 260$ nm), and B) the consumption of paromamine (**11**), monitored using a Corona CAD (see the text and Supporting Information for details). Trace (a) corresponds to 5′-dAdo (**19**) and paromamine (**11**) standards in parts (A) and (B), respectively. The remaining traces (b–h) in both (A) and (B) are from reaction mixtures containing AprD4 (10 μM), paromamine (1 mM), SAM (2 mM), DTT (10 mM), dithionite (2 mM), in NH_4HCO_3 buffer (50 mM; pH 7.8) buffer with the following modifications: b) no changes, c) no AprD4, d) no paromamine, e) non-reconstituted AprD4, f) no SAM, g) with AprD3 (10 μM) and NADPH (1 mM), and (h) with AprD3 (10 μM) and no NADPH.

The AprD4 reaction was also monitored by HPLC using a Corona charged aerosol detector (CAD),[34] because the pseudo-disaccharide substrate paromamine (**11**) and its products lack a UV-absorbing chromophore. However, despite the detected conversion of SAM to 5′-dAdo, neither significant consumption of paromamine nor new product formation was noted, unless AprD3 (10 μM) and NADPH (1 mM) were also included in the reaction mixture (see Figure 2B, traces b and g). In trace g, a new peak with a retention time of 7.2 min was detected, in addition to the consumption of paromamine. Electrospray ionization mass spectra (ESIMS) of the new product revealed a signal at *m/z* 330.1638, consistent with the sodium adduct of lividamine (**13**; calc. 330.1636 $[M + Na]^+$; Figure 3E, Figure S3). These

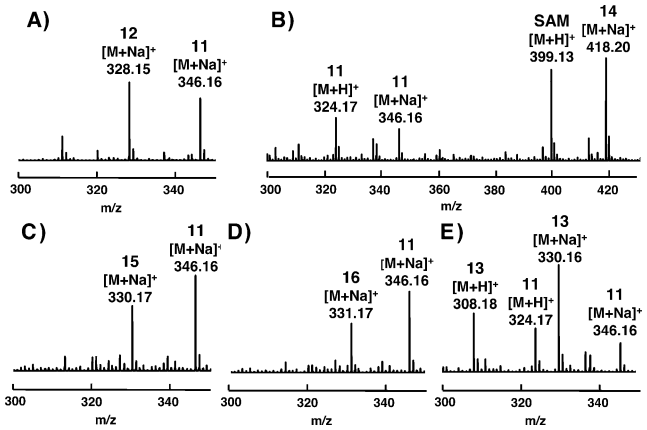
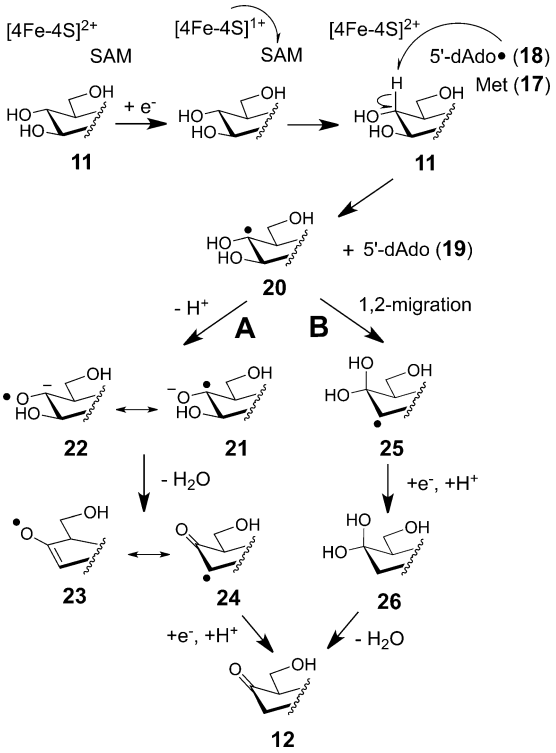


Figure 3. Electrospray ionization (ESI) mass spectrum of A) the AprD4 reaction with paromamine (**11**; in the absence of AprD3). Spectra (B–E) show the ESI mass spectra of the same reaction run in the presence of B) phenylhydrazine, C) $NaBH_4$, or D) $NaBD_4$ (as described in the text). E) ESI mass spectrum of the AprD4/AprD3 reaction with co-incubation with paromamine and NADPH.

findings led to the hypothesis that AprD4 operates as a radical-mediated dehydratase with AprD3 serving as its reductase partner to effect the overall reduction of paromamine to lividamine (**11**→**12**→**13**; see Scheme 1).

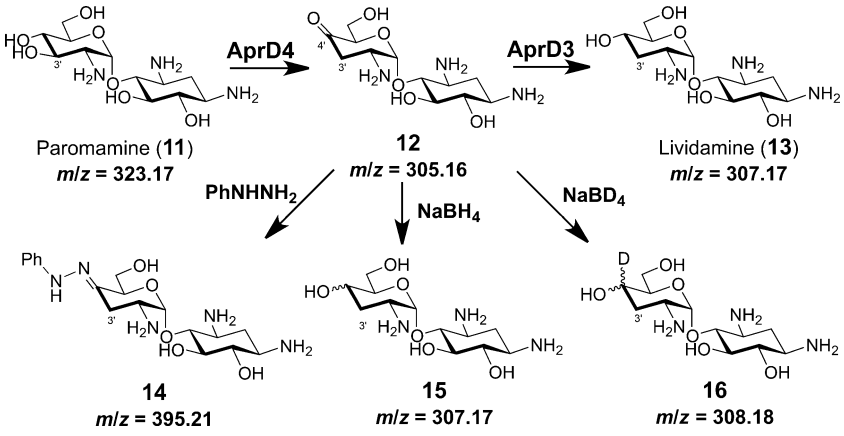
As a first test of this hypothesis, reaction mixtures excluding AprD3 were analyzed by ESIMS to check for low concentrations of the putative dehydration product of paromamine that went undetected by HPLC (Corona CAD). As shown in Figure 3A, a signal was detected at *m*/*z* 328.15, consistent with the sodium adduct of such an intermediary species (**12**). To further support assignment of this MS signal to the putative ketone intermediate, the AprD4 reaction in the absence of AprD3 was run in the presence of phenylhydrazine (10 mM) for 4 h prior to analysis by ESIMS. In this case, a new signal at *m*/*z* 418.20 was detected whereas the signal at *m*/*z* 328.15 was significantly decreased, suggesting formation of a phenylhydrazone derivative (**14**; Figure 3B; Scheme 1). Moreover, treatment of the AprD4-only reaction with $NaBH_4$ or $NaBD_4$ resulted in a shift of the signal at *m*/*z* 328.15 to 330.17 and 331.17, respectively (Figures 3C,D; Scheme 1). These results support the hypothesis that AprD4 catalyzes the first of two half-reactions, resulting in the dehydration of paromamine to form a keto intermediate such as **12**.

Based on these results, at least two mechanisms can be envisioned for the AprD4-catalyzed dehydration of paromamine (Scheme 2). Consistent with radical SAM chemistry,



Scheme 2. Proposed mechanisms for the AprD4-catalyzed dehydration of paromamine (**11**).

both mechanisms begin with initial hydrogen-atom abstraction from the C4′ position of paromamine (**11**→**20**) by the 5′-dAdo• radical (**18**) generated by the reductive homolysis of SAM. The resulting substrate radical (**20**) may then be deprotonated to form a ketyl radical **21**/**22** that subsequently undergoes β-elimination of the 3′-OH group to yield the enol radical **23** (Scheme 2, route A). This mechanism is similar to proposed mechanisms for (*R*)-2-hydroxyacyl-CoA dehydratase[35] and ribonucleotide reductase.[36] One-electron reduction of the resulting enol radical intermediate would then complete the dehydration reaction (**23**/**24**→**12**). Alternatively, the initially formed substrate radical **20** may instead undergo a radical-induced 1,2-hydroxy shift to form the *gem*-diol radical **25** (Scheme 2, route B), in direct analogy to the mechanisms of the B_{12}-dependent diol-dehydratases and ethanolamine ammonia



Scheme 1. The reduction of paromamine (**11**) to lividamine (**13**) catalyzed by AprD4 and AprD3. Derivatization reactions carried out in the absence of AprD3 are also shown.

lyase.[37–39] Following reduction, the *gem*-diol intermediate **26** would then be converted into **12** upon elimination of water.

To further investigate the mechanism of AprD4-catalyzed dehydration and establish the site of hydrogen-atom abstraction from **11**, the [4′-^{2}H] isotopologue of paromamine was prepared (see the Supporting Information for details). When AprD4 (10 μM) was incubated with [4′-^{2}H]-paromamine (1 mM), SAM (2 mM), and dithionite (2 mM) in NH_4HCO_3 (50 mM; pH 7.8) for 8 h at room temperature, formation of 5′-dAdo was again detected and this time with single-deuterium incorporation beyond natural abundance as determined by ESIMS (Figure S5). A complimentary assay with unlabeled paromamine (**11**) and AprD4 using the dideuterated [5′-$^2H_2$]-SAM isotopologue[40] in the incubation revealed hydrogen transfer from **11** to [5′-$^2H_2$]-5′-dAdo• to yield [5′-$^2H_2$]-5′-dAdo as the product (Figure S6). These results further substantiate the proposed early steps of AprD4 catalysis shown Scheme 2, whereby AprD4 acts as a radical SAM enzyme that initiates a radical-mediated dehydration of paromamine through hydrogen-atom abstraction from the C4′ position.

In summary, the results reported herein provide preliminary evidence for the assignment of functions of enzymes AprD4 and AprD3 in the biosynthesis of C3′-deoxyaminoglycosides. AprD4 appears to be the first example of a radical SAM diol-dehydratase and catalyzes the radical-mediated dehydration of paromamine (**11**). This transformation is reminiscent of the radical-mediated dehydration reactions catalyzed by the B_{12}-dependent diol-dehydratases[38,39] as well as the B_{12}-independent glycerol dehydratase,[41] which employs a radical SAM activating enzyme to facilitate turnover.[41a,42] However, the reactive 5′-dAdo• radical (**18**) is consumed in vitro to give 5′-dAdo (**19**) during the AprD4 catalytic cycle, whereas the radical initiator is typically regenerated in the case of other radical-mediated dehydratases.[38,39]

AprD3 is shown to be a dehydrogenase acting as the reductase counterpart to AprD4 in order to facilitate the net C3′-deoxygenation of the pseudo-disaccharide substrates in the biosynthesis of apramycin (**2**) and tobramycin (**3**). Enzymes LivW and LivY are expected to play analogous roles in the lividomycin (**5**) biosynthetic pathway. The dehydration reaction catalyzed by AprD4 bears a close resemblance to the deamination catalyzed of DesII, which is also a radical SAM enzyme catalyzing the conversion of TDP-4,6-dideoxy-4-amino-D-glucose to TDP-4,6-dideoxy-3-keto-D-glucose (TDP = thymidine diphosphate).[40,43–49] However, the latter is unable to catalyze dehydration upon direct substitution of the eliminated amine with a hydroxy group and instead acts as a dehydrogenase.[45] This feature makes the comparative study of the AprD4 and DesII enzymes of particular interest for understanding how enzymes are able to control the fate of their radical intermediates during turnover.

Acknowledgements

This work was supported by grants from the National Institutes of Health (GM035906) and the Welch Foundation (F-1511). We would like to thank Dr. Mark Ruszczycky for his help with manuscript preparation and critical comments. We would also thank Dr. Reid McCarty for his help with the early experiments.

Keywords: aminoglycosides · biosynthesis · enzyme catalysis · radical enzymes · reaction mechanisms

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 3724–3728
Angew. Chem. **2016**, *128*, 3788–3792

[1] S. Magnet, J. Blanchard, *Chem. Rev.* **2005**, *105*, 477–498.
[2] D. Moazed, H. F. Noller, *Nature* **1987**, *327*, 389–396.
[3] B Becker, M. A. Cooper, *ACS Chem. Biol.* **2013**, *8*, 105–115.
[4] S. O'Connor, L. K. T. Lam, N. D. Jones, M. O. Chaney, *J. Org. Chem.* **1976**, *41*, 2087–2092.
[5] W. M. Stark, M. M. Hoehn, N. G. Knox, *Antimicrob. Agents Chemother.* **1967**, *7*, 314–323.
[6] K. F. Koch, F. A. Davis, J. A. Rhoades, *J. Antibiot.* **1973**, *26*, 745–751.
[7] T. Mori, Y. Kyotani, I. Watanabe, T. Oda, *J. Antibiot.* **1972**, *25*, 149–150.
[8] F. Kudo, T. Eguchi, *Methods Enzymol.* **2009**, *459*, 493–519.
[9] N. M. Llewellyn, J. B. Spencer, *Nat. Prod. Rep.* **2006**, *23*, 864–874.
[10] F. Kudo, T. Eguchi, *J. Antibiot.* **2009**, *62*, 471–481.
[11] J. W. Park, J. S. J. Hong, N. Parajuli, W. S. Jung, S. R. Park, S.-K. Lim, J. K. Sohng, Y. J. Yoon, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 8399–8404.
[12] T. Dairi, T. Ohta, E. Hashimoto, M. Hasegawa, *Mol. Gen. Genet.* **1992**, *236*, 39–48.
[13] S. Kondo, K. Hotta, *J. Infect. Chemother.* **1999**, *5*, 1–9.
[14] W. Piepersberg, K. M. Aboshanab, H. Schmidt-Beissner, U. F. Wehmer, in *Aminoglycoside Antibiotics* (Ed.: D. P. Arya), Wiley-VCH, Weinhein, **2007**, pp. 15–118.
[15] T. Hirayama, H. Tamegai, F. Kudo, K. Kojima, K. Kakinuma, T. Eguchi, *J. Antibiot.* **2006**, *59*, 358–361.
[16] T. Oda, T. Mori, H. Ito, T. Kunieda, K. Munakata, *J. Antibiot.* **1971**, *24*, 333–338.
[17] T. Mori, R. Ichiyanagi, H. Kondo, K. Tokunaga, T. Oda, K. Munakata, *J. Antibiot.* **1971**, *24*, 339–346.
[18] S. R. Park, J. W. Park, Y. H. Ban, J. K. Sohng, Y. H. Yoon, *Nat. Prod. Rep.* **2013**, *30*, 11–20.
[19] J. W. Park, S. Y. Park, K. K. Nepal, A. R. Han, Y. H. Ban, Y. J. Yoo, E. J. Kim, E. M. Kim, D. Kim, J. K. Sohng, Y. J. Yoon, *Nat. Chem. Biol.* **2011**, *7*, 843–852.
[20] X. Ni, D. Li, L. Yang, T. Huang, H. Li, H. Xia, *Appl. Microbiol. Biotechnol.* **2011**, *89*, 723–731.
[21] J. B. Broderick, B. T. Duffuns, K. S. Duschene, E. M. Shepard, *Chem. Rev.* **2014**, *114*, 4229–4317.
[22] P. A. Frey, *Acc. Chem. Res.* **2014**, *47*, 540–549.
[23] P. A. Frey, A. D. Hegeman, F. J. Ruzicka, *Crit. Rev. Biochem. Mol. Biol.* **2008**, *43*, 63–88.
[24] H. J. Sofia, G. Chen, B. H. Hetzler, J. F. Reyes-Spindola, N. E. Miller, *Nucleic Acids Res.* **2001**, *29*, 1097–1106.
[25] S. S. Kamat, H. J. Williams, F. M. Raushel, *Nature* **2011**, *480*, 570–573.
[26] S. E. McGlynn, E. S. Boyd, E. M. Shepard, R. K. Lange, R. Gerlach, J. B. Broderick, J. W. Peters, *J. Bacteriol.* **2010**, *192*, 595–598.
[27] D. P. Dowling, N. A. Bruender, A. P. Young, R. M. McCarty, V. Bandarian, C. L. Drennan, *Nat. Chem. Biol.* **2014**, *10*, 106–112.
[28] Y. Zhang, X. Zhu, A. T. Torelli, M. Lee, B. Dzikovski, R. M. Koralewski, E. Wang, J. Freed, C. Krebs, S. E. Ealick, H. Lin, *Nature* **2010**, *465*, 891–896.

[29] A. Chatterjee, Y. Li, Y. Zhang, T. L. Grove, M. Lee, C. Krebs, S. J. Booker, T. P. Begley, S. E. Ealick, *Nat. Chem. Biol.* **2008**, *4*, 758 – 765.
[30] W. W. Fish, *Methods Enzymol.* **1988**, *158*, 357 – 364.
[31] H. Beinert, *Anal. Biochem.* **1983**, *131*, 373 – 378.
[32] N. B. Ugulava, B. R. Gibney, J. T. Jarrett, *Biochemistry* **2000**, *39*, 5206 – 5214.
[33] S. Ollagnier, C. Meier, E. Mulliez, J. Gaillard, V. Schuenemann, A. Trautwein, T. Mattioli, M. Lutz, M. Fontecave, *J. Am. Chem. Soc.* **1999**, *121*, 6344 – 6350.
[34] T. Vehovec, A. Obreza, *J. Chromatogr. A* **2010**, *1217*, 1549 – 1556.
[35] W. Buckel, *Angew. Chem. Int. Ed.* **2009**, *48*, 6779 – 6787; *Angew. Chem.* **2009**, *121*, 6911 – 6920.
[36] J. Stubbe, W. A. van der Donk, *Chem. Biol.* **1995**, *2*, 793 – 801.
[37] V. Bandarian, G. H. Reed, in *Chemistry and Biochemistry of B_{12}* (Ed.: R. Banerjee), Wiley, New York, **1999**, pp. 811 – 833.
[38] T. Toraya, *Chem. Rev.* **2003**, *103*, 2095 – 2127.
[39] T. Toraya, *Arch. Biochem. Biophys.* **2014**, *544*, 40 – 57.
[40] Y. Ko, M. W. Ruszczycky, S.-h. Choi, H.-w. Liu, *Angew. Chem. Int. Ed.* **2015**, *54*, 860 – 863; *Angew. Chem.* **2015**, *127*, 874 – 877.
[41] a) J. R. O'Brien, C. Raynaud, C. Croux, L. Girbal, P. Soucaille, W. N. Lanzilotta, *Biochemistry* **2004**, *43*, 4635 – 4645; b) C. Raynaud, P. Sarcabal, I. Meynial-Salles, C. Croux, P. Soucaille, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 5010 – 5015.
[42] J. M. Demick, W. N. Lanzilotta, *Biochemistry* **2011**, *50*, 440 – 442.
[43] G.-M. Lin, S.-H. Choi, M. W. Ruszczycky, H.-w. Liu, *J. Am. Chem. Soc.* **2015**, *137*, 4954 – 4967.
[44] M. W. Ruszczycky, S.-h. Choi, H.-w. Liu, *J. Am. Chem. Soc.* **2010**, *132*, 2359 – 2369.
[45] P. H. Szu, M. W. Ruszczycky, S.-h. Choi, F. Yan, H.-w. Liu, *J. Am. Chem. Soc.* **2009**, *131*, 14030 – 14042.
[46] M. W. Ruszczycky, S.-h. Choi, S. O. Mansoorabadi, H.-w. Liu, *J. Am. Chem. Soc.* **2011**, *133*, 7292 – 7295.
[47] M. W. Ruszczycky, Y. Ogasawara, H.-w. Liu, *Biochim. Biophys. Acta Proteins Proteomics* **2012**, *1824*, 1231 – 1244.
[48] M. W. Ruszczycky, S.-h. Choi, H.-w. Liu, *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 2088 – 2093.
[49] M. W. Ruszczycky, H.-w. Liu, *Isr. J. Chem.* **2015**, *55*, 315 – 324.

Received: November 16, 2015
Published online: February 15, 2016

