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Tryptophan Lyase (NosL): A Cornucopia of 5′-Deoxyadenosyl Radical Mediated Transformations

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Supporting Information

ABSTRACT: Tryptophan lyase (NosL) is a radical *S*-adenosyl-L-methionine (SAM) enzyme that catalyzes the formation of 3-methyl-2-indolic acid from L-tryptophan. In this paper, we demonstrate that the 5′-deoxyadenosyl radical is considerably more versatile in its chemistry than previously anticipated: hydrogen atom abstraction from N_α-cyclopropyltryptophan occurs at Cα rather than the amino group with NosL Y90A and replacing the substrate amine with a ketone or an alkene changes the chemistry from hydrogen atom abstraction to double bond addition. In addition, the 5′-deoxyadenosyl radical can add to the [4Fe−4S] cluster and dithionite can be used to trap radicals at the active site.

Tryptophan Lyase (NosL) is a radical SAM enzyme that catalyzes the complex rearrangement reaction shown in Figure 1.[1] This reaction occurs in the *Streptomyces actuosus*

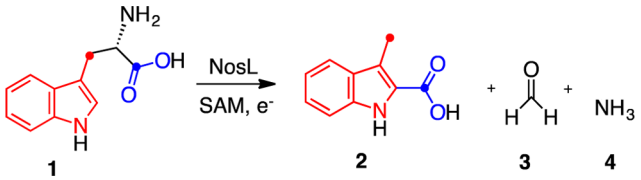

Figure 1. NosL catalyzes the complex conversion of L-tryptophan (**1**) to 3-methyl-2-indolic acid (**2**).

nosiheptide biosynthetic pathway. NosL belongs to the aromatic amino-acid lyase family, which includes ThiH (thiamin biosynthesis), HydG (Fe-Fe hydrogenase maturation) and CofH (F_{420} biosynthesis).[2] The radical SAM superfamily has expanded and is now one of the largest known superfamilies with over 165 000 annotations.[3] Except for Dph2[4] and the glycerol dehydratase activating enzyme,[5] in all reactions with radical SAM enzymes, SAM is reductively cleaved to generate the 5′-deoxyadenosyl radical, which usually then abstracts a hydrogen atom from the substrate directly or via a glycyl radical.[6] To avoid inactivating the enzyme with this highly reactive radical, its reactivity must be tightly controlled at the active site.[7] It is generally assumed that one of the control mechanisms involves formation of the 5′-deoxyadenosyl radical in close proximity to the abstracted hydrogen atom. In this communication we describe studies with enzyme and substrate variants, that reveal unanticipated mobility of the radicals formed at the active site of NosL.

The structure of NosL with bound substrates (Figure 2) suggests that the H atom is abstracted from the amino group of

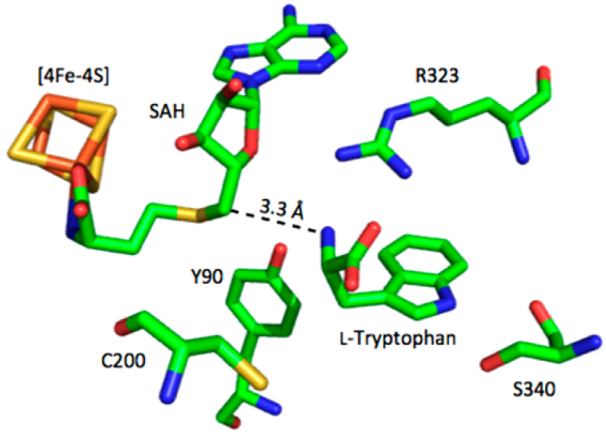

Figure 2. Active site architecture of NosL.[8] Two mechanisms for the rearrangement reaction, that differ in the first bond cleaved (Cα-Cβ or Cα-C carbonyl, Figure S1) have been proposed.[9,10]

1; a result that has been further confirmed with biochemical studies on benzothiophene and benzofuran substrate analogs.[8,11,12] The first hint for relaxed regiocontrol in the initial hydrogen atom abstraction came from studies on the demethylation of N_α-methyl-L-tryptophan (**5**) where the H atom is abstracted from the methyl group instead of the amino group.[12] Cyclopropyltryptophan (**6**) is not a substrate for NosL. Reasoning that this might be due to the size of the cyclopropyl ring, the active site was enlarged by construction of the Y90A variant with the goal of using this analog to determine the relative rate of the β-bond scission of the amino radical.

Treatment of **1** with the Y90A variant of NosL generated 3-methylindole and **2** (analogous to the native enzyme (Figures S1 and S2)). However, HPLC analysis of the NosL Y90A-catalyzed reaction with **6** revealed the formation of indole-3-pyruvic acid (**11**, Figure 3). LC-MS analysis of the reaction mixture after treatment with 4-fluoro-7-nitrobenzofurazan (NBD-F, an amine derivatizing reagent) demonstrated the formation of the cyclopropylamine−NBD adduct with no evidence for the anticipated cyclopropyl ring opening (Figure S3).[13] In addition, deuterium incorporation into 5′-deoxyadenosine was not observed when the reaction was carried out in 95% D_2O buffer. These results suggested that the amino

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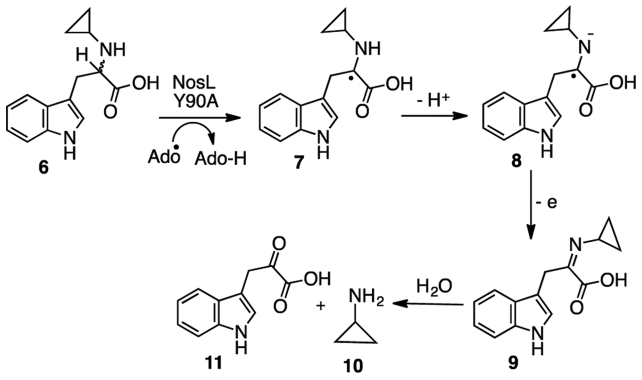

Figure 3. Mechanistic proposal for the NosL Y90A-catalyzed reaction of N_α-cyclopropyltryptophan (**6**).

group is not the site of the initial H atom abstraction. Subsequently, the observation of deuterium transfer from C_α-deuterated-N_α-cyclopropyltryptophan to 5′-deoxyadenosine identified C_α as the hydrogen atom source (Figure S4).

A mechanistic proposal, consistent with these observations, is shown in Figure 3. H atom abstraction from the C_α position of **6** gives **7**. Amine deprotonation followed by electron transfer back to the cluster gives **9**, which then undergoes hydrolysis to give the observed products **10** and **11**. This experiment completes the series shown in Figure 4 in which the active site 5′-deoxyadenosyl radical of NosL abstracts a hydrogen atom not only from the amine of the substrate (**1**) but also from the methyl group of **5** and C*α* of **6**.

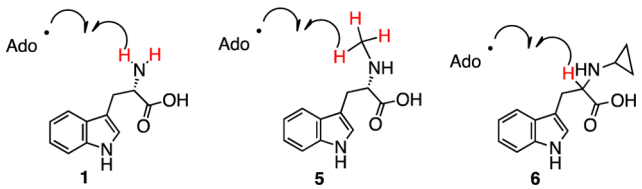

Figure 4. The 5′-deoxyadenosyl radical of NosL shows relaxed regiospecificity with substrate analogs **5** and **6**.

The MqnE-catalyzed formation of aminofutalosine in menaquinone biosynthesis is a recently discovered exception to the general rule that H atom abstraction is the first step in all reactions catalyzed by radical SAM enzymes (Figure 5).[14] In

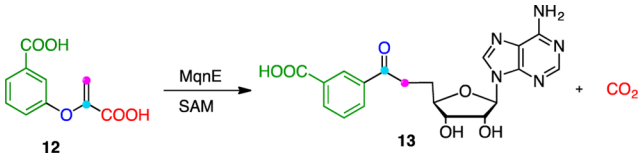

Figure 5. MqnE-catalyzed addition of the 5′-deoxyadenosyl radical to **12** leading to the formation of aminofutalosine (**13**).

this system, the substrate radical is formed by addition of the 5′-deoxyadenosyl radical to the double bond of **12** to give a radical intermediate that then undergoes rearrangement to give **13**. The regiochemical tolerance of the 5′-deoxyadenosyl radical at the active site of NosL suggested that NosL might catalyze a radical addition to the methylene analog of tryptophan (**14**, Figure 6).

When **14** was treated with NosL, three new products were observed by LC-MS analysis (Figure S6). These products had

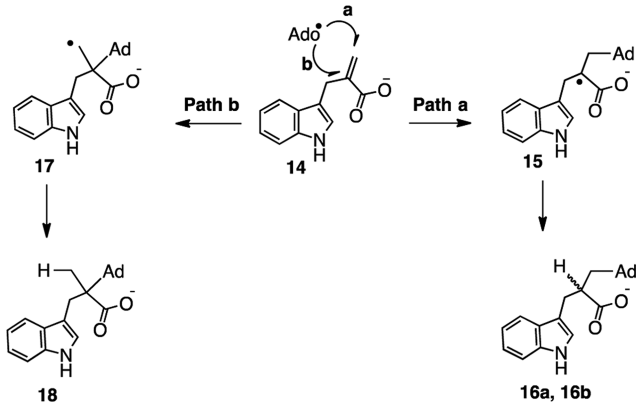

Figure 6. Addition of the 5′-deoxyadenosyl radical to the double bond of indole methyl acrylate **14**.

identical *m*/*z* values that were consistent with the expected mass of adducts between the substrate **14** and the 5′-deoxyadenosyl radical, suggesting that the latter radical added to either carbon of the double bond of **14**. This addition will be facially specific and controlled by the active site structure. Quenching of **15** uses two different hydrogen atom donors and gives product as a mixture of two epimers (**16a**,**16b**, path a, Figure 6). Quenching of radical **17** will give **18** as a single isomer (path b, Figure 6). When the reaction was performed in 95% D_2O buffer, deuterium incorporation was only observed in product **18** (Figure S7). This may result from quenching of radical intermediate **17** by a solvent exchangeable Y90 residue in the active site. 5′-Deoxyadenosine was not detected suggesting efficient radical capture by **14**. To reduce the unfavorable interaction between the methylene group in **14** and Arg323, we next investigated the reaction of **14** with NosL R323K. This variant showed ∼10-fold enhancement in the formation of all three adducts (Figure S6). It was not possible to characterize the reaction products by NMR because of their instability during purification.

In search of a tryptophan analog that would give more stable products, indole-3-pyruvic acid (**11**) was next tested using NosL R323K. HPLC analysis of the resulting reaction mixture indicated the absence of 5′-deoxyadenosine and the formation of a new compound eluting at 24 min (Figure S8). LC-MS analysis of this product gave a mass that was consistent with **22**, generated by 5′-deoxyadenosyl radical addition to the carbonyl followed by radical induced decarboxylation of **20** and electron transfer back to the cluster (Figure 7). This product was stable and was purified by HPLC and characterized by NMR (Figure S9).

The rational design of substrate analogs capable of shifting the reactivity of the 5′-deoxyadenosyl radical from hydrogen atom abstraction to double bond addition may be generally useful for the design of "bisubstrate inhibitors" of radical SAM enzymes. Such inhibitors would be of use for structural studies and as lead compounds for antibiotic development.

The Y90F and Y90A NosL variants, in the absence of tryptophan or its analogs, catalyzed the conversion of SAM to a new product, as indicated by HPLC analysis. UV−vis analysis indicated the presence of the adenosine chromophore. LC-MS analysis gave an $[M - H]^-$ = 282 Da consistent with structure **23** (Figure S10). This was confirmed by derivatization with iodoacetamide (**24**), which resulted in a new species with $[M - H]^-$ = 339 Da consistent with **25** (Figure 8). This product was purified by HPLC and its structure was confirmed by NMR

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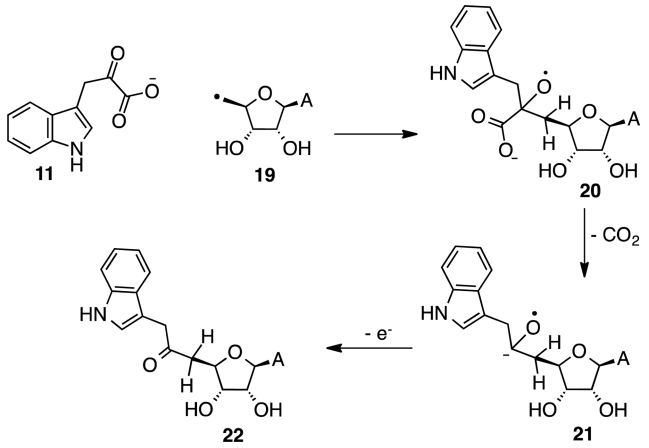

Figure 7. NosL R323K-catalyzed addition of the 5′-deoxyadenosyl radical to indole-3-pyruvic acid (**11**).

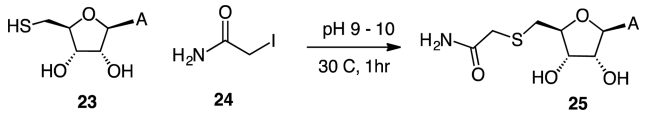

Figure 8. Derivatization of (**23**) to confirm the presence of a thiol in the molecule.

analysis (Figure S11). Low levels of this compound were also observed with native NosL (Figure S10) and with MoaA,[15] BzaF,[16] MqnC,[17] and MqnE[14] in the absence of the substrate (D. Bhandari, S. Joshi, and T. P. Begley, unpublished).

A mechanistic proposal for the formation of 5′-deoxy-5′-thioadenosine (**23**) is shown in Figure 9 (path a). In this

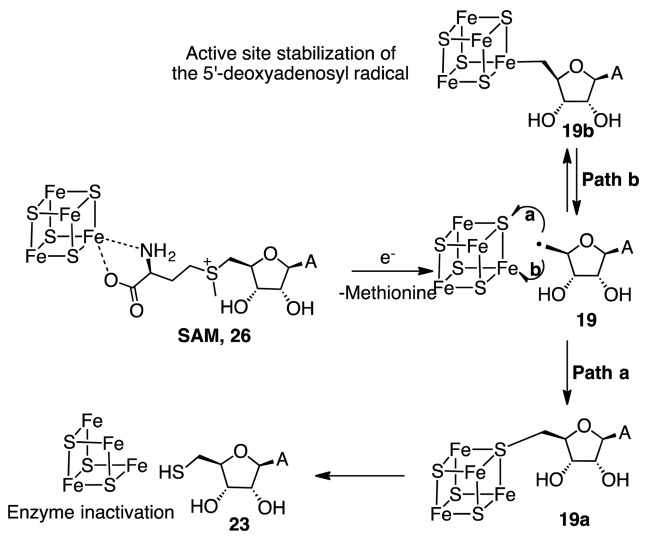

Figure 9. Mechanistic proposal for the formation of 5′-deoxy-5′-thioadenosine (**23**) involving C−S bond formation with the [4Fe−4S] cluster.

proposal, the 5′-deoxyadenosyl radical reacts with one of the sulfurs in the oxidized cluster to form **19a**, which decomposes during workup of the reaction to give **23**. Organic radical attack on the [2Fe−2S] cluster of biotin synthase to form a thiol gives good precedence for this reaction (Figure S13).[18] It has also been reported that the 5′-deoxyadenosyl radical in BioB variants (D155N and N153A) can abstract sulfur from the [4Fe−4S] cluster leading to the formation of **23**.[19] In NosL, formation of **23** is observed in higher amounts in the Y90A and Y90F mutants when compared to WT. This suggests that residues like Y90 in the active site might act as H atom donors to the 5′-deoxyadenosyl radical during the uncoupled reaction.

Recent EPR studies indicate that the 5′-deoxyadenosyl radical in the pyruvate formate lyase activating enzyme forms a covalent adduct with one of the iron atoms in the [4Fe−4S] cluster and that this interaction stabilizes the 5′-deoxyadenosyl radical.[20] This is a surprising result because the C5′ carbon of bound SAM is 5.5 Å from the closest iron[21] and suggests that the 5′-deoxyadenosyl radical has considerable mobility in the active site and should therefore also be able to form a carbon−sulfur (C−S) bond with the cluster. In contrast to the carbon−iron (C−Fe) bond, C−S bond formation leading to **23** would result in enzyme inactivation and may be one of the contributing factors to the low turnover of enzymes in this superfamily (Figure 9).

Enzyme-generated radical intermediates are sequestered from the buffer and generally cannot be trapped by exogenous reagents such as spin traps or hydrogen atom donors. Dithionite is emerging as an exception to this, and sulfinic acid products have been reported for the spore photoproduct lyase, Dph2 and DesII systems,[4,22,23] suggesting that **30** (Figure 10) can diffuse into active sites and trap radical intermediates. This hypothesis was tested for the NosL catalyzed reaction.

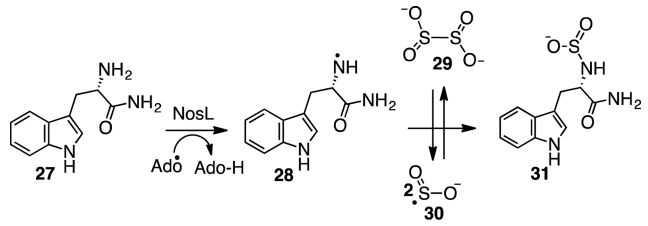

Figure 10. NosL-catalyzed generation of the sulfuramidite (**31**) with L-tryptophanamide (**27**).

LC-MS analysis of the NosL-catalyzed reaction of L-tryptophanamide (**27**) demonstrated the formation of a new product (Figure S16) having a mass consistent with sulfuramidite **31** resulting from the trapping of **28** by **30**.[24] Similar adducts were observed with several other tryptophan analogs (*N*-methyl-L-tryptophanamide, *N*-ethyl-L-tryptophanamide, L-tryptophan methyl ester, L-tryptophan ethyl ester, methyl-D,L-indole-3-lactate, ethyl-D,L-indole-3-lactate). In addition, 5′-deoxyadenosyl-5′-sulfinic acid was observed in the absence of substrate and with enhanced levels in the presence of L-tryptophan ethyl ester (Figures S16 −S19). 5′-Deoxyadenosyl-5′-sulfinic acid was also detected for MoaA, BzaF, MqnC, and MqnE in the absence of the substrate (D. Bhandari, S. Joshi, and T. P. Begley, unpublished). These experiments suggest that dithionite may be a particularly useful active site radical trap for radical SAM enzymes because in many cases dithionite is used to activate the enzyme by reduction of the cluster and is therefore present in the reaction buffer. However, we did not detected any dithionite trapped products with the native substrate **1** and various other substrate analogs reported in this and previous work.[12]

In conclusion, we have demonstrated that N_α-alkylated tryptophan analogs are substrates for NosL and that the alkyl group (R) can alter the regiochemistry of the initial hydrogen atom abstraction from the amino group (R = H) to the alkyl

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group (R = methyl) or to the α-carbon (R = cyclopropyl). We have also demonstrated that replacing the substrate amine with a ketone or an alkene changes the 5′-deoxyadenosyl radical chemistry from hydrogen atom abstraction to double bond addition, and we propose that this strategy may be useful for the design of bisubstrate inhibitors of radical SAM enzymes. We have also demonstrated that the 5′-deoxyadenosyl radical can add to one of the sulfurs in the [4Fe−4S] cluster and that dithionite can be used to trap radicals at the NosL active site with substrate analogs suggesting that this reagent has potential for the trapping of intermediates at the active sites of radical SAM enzymes. Preliminary experiments with MoaA, BzaF, MqnC, and MqnE suggest that many of these findings will have some generality in radical SAM enzymology.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b06139.

Detailed procedures for substrate synthesis, enzymatic reaction conditions, HPLC and LC-MS chromatograms of all enzymatic reaction products, and NMR spectra of synthesized compounds (PDF)

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Notes

While this paper was under review, Professor Zhang also reported the addition of the adenosyl radical to methylene analogue of tryptophan.[25]

The authors declare no competing financial interest.

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■ REFERENCES

(1) Zhang, Q.; Li, Y.; Chen, D.; Yu, Y.; Duan, L.; Shen, B.; Liu, W. *Nat. Chem. Biol.* **2011**, *7* (3), 154−160.

(2) Mehta, A. P.; Abdelwahed, S. H.; Mahanta, N.; Fedoseyenko, D.; Philmus, B.; Cooper, L. E.; Liu, Y.; Jhulki, I.; Ealick, S. E.; Begley, T. P. *J. Biol. Chem.* **2015**, *290* (7), 3980−3986.

(3) Gerlt, J. A.; Bouvier, J. T.; Davidson, D. B.; Imker, H. J.; Sadkhin, B.; Slater, D. R.; Whalen, K. L. *Biochim. Biophys. Acta, Proteins Proteomics* **2015**, *1854* (8), 1019−1037.

(4) Zhang, Y.; Zhu, X.; Torelli, A. T.; Lee, M.; Dzikovski, B.; Koralewski, R. M.; Wang, E.; Freed, J.; Krebs, C.; Ealick, S. E.; Lin, H. *Nature* **2010**, *465* (7300), 891−896.

(5) Demick, J. M.; Lanzilotta, W. N. *Biochemistry* **2011**, *50* (4), 440−442.

(6) Broderick, J. B.; Duffus, B. R.; Duschene, K. S.; Shepard, E. M. *Chem. Rev.* **2014**, *114* (8), 4229−4317.

(7) Horitani, M.; Byer, A. S.; Shisler, K. A.; Chandra, T.; Broderick, J. B.; Hoffman, B. M. *J. Am. Chem. Soc.* **2015**, *137* (22), 7111−7121.

(8) Nicolet, Y.; Zeppieri, L.; Amara, P.; Fontecilla-Camps, J. C. *Angew. Chem., Int. Ed.* **2014**, *53* (44), 11840−11844.

(9) Ji, X.; Li, Y.; Jia, Y.; Ding, W.; Zhang, Q. *Angew. Chem., Int. Ed.* **2016**, *55* (10), 3334−3337.

(10) Sicoli, G.; Mouesca, J.-M.; Zeppieri, L.; Amara, P.; Martin, L.; Barra, A. L.; Fontecilla-Camps, J. C.; Gambarelli, S.; Nicolet, Y. *Science* **2016**, *351* (6279), 1320−1323.

(11) Ji, X.; Li, Y.; Ding, W.; Zhang, Q. *Angew. Chem., Int. Ed.* **2015**, *54* (31), 9021−9024.

(12) Bhandari, D. M.; Xu, H.; Nicolet, Y.; Fontecilla-Camps, J. C.; Begley, T. P. *Biochemistry* **2015**, *54* (31), 4767−4769.

(13) Imai, K.; Watanabe, Y. *Anal. Chim. Acta* **1981**, *130* (2), 377−383.

(14) Mahanta, N.; Fedoseyenko, D.; Dairi, T.; Begley, T. P. *J. Am. Chem. Soc.* **2013**, *135* (41), 15318−15321.

(15) Mehta, A. P.; Hanes, J. W.; Abdelwahed, S. H.; Hilmey, D. G.; Hänzelmann, P.; Begley, T. P. *Biochemistry* **2013**, *52* (7), 1134−1136.

(16) Mehta, A. P.; Abdelwahed, S. H.; Fenwick, M. K.; Hazra, A. B.; Taga, M. E.; Zhang, Y.; Ealick, S. E.; Begley, T. P. *J. Am. Chem. Soc.* **2015**, *137* (33), 10444−10447.

(17) Cooper, L. E.; Fedoseyenko, D.; Abdelwahed, S. H.; Kim, S.-H.; Dairi, T.; Begley, T. P. *Biochemistry* **2013**, *52* (27), 4592−4594.

(18) Ugulava, N. B.; Sacanell, C. J.; Jarrett, J. T. *Biochemistry* **2001**, *40* (28), 8352−8358.

(19) Farrar, C. E.; Jarrett, J. T. *Biochemistry* **2009**, *48* (11), 2448−2458.

(20) Horitani, M.; Shisler, K.; Broderick, W. E.; Hutcheson, R. U.; Duschene, K. S.; Marts, A. R.; Hoffman, B. M.; Broderick, J. B. *Science* **2016**, *352* (6287), 822−825.

(21) Vey, J. L.; Yang, J.; Li, M.; Broderick, W. E.; Broderick, J. B.; Drennan, C. L. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105* (42), 16137−16141.

(22) Chandor-Proust, A.; Berteau, O.; Douki, T.; Gasparutto, D.; Ollagnier-de-Choudens, S.; Fontecave, M.; Atta, M. *J. Biol. Chem.* **2008**, *283* (52), 36361−36368.

(23) Ko, Y.; Ruszczycky, M. W.; Choi, S. H.; Liu, H. w. *Angew. Chem.* **2015**, *127* (3), 874−877.

(24) Rinker, R. G.; Gordon, T. P.; Mason, D. M.; Corcoran, W. H. *J. Phys. Chem.* **1959**, *63* (2), 302−302.

(25) Ji, X.; Li, Y.; Xie, L.; Lu, H.; Ding, W.; Zhang, Q. *Angew. Chem., Int. Ed.* **2016**, *55* (39), 11845−11848.

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