Published in final edited form as:

Biochemistry. 2020 June 02; 59(21): 1961–1965. doi:10.1021/acs.biochem.0c00289.

Alternative reactivity of leucine 5-hydroxylase using an olefin-containing substrate to construct substituted piperidine ring

Lide Cha^a, Sergey Milikisiyants^a, Madison Davidson^a, Shan Xue^b, Tatyana Smirnova^a, Alex Smirnov^{*,a}, Yisong Guo^{*,b}, Wei-chen Chang^{*,a}

^aDepartment of Chemistry, North Carolina State University, Raleigh, NC 27695

^bDepartment of Chemistry, Carnegie Mellon University, Pittsburgh, PA 15213

Abstract

Applying enzymatic reactions to produce useful molecules is a central focus of chemical biology. Iron and 2-oxoglutarate (Fe/2OG) enzymes are found in all kingdoms of life and catalyze a broad array of oxidative transformations. Herein, we demonstrate that the activity of an Fe/2OG enzyme can be redirected when changing the targeted carbon hybridization from sp³ to sp². During leucine 5-hydroxylase catalysis, installation of an olefin group onto the substrate redirects the Fe(IV)-oxo species reactivity from hydroxylation to asymmetric epoxidation. The resulting epoxide subsequently undergoes intramolecular cyclization to form the substituted piperidine, 2*S*,5*S*-hydroxypiperic acid.

Piperidine alkaloids possess a wide range of biological activities and are well represented among pharmaceutically important compounds such as febrifugine, palinavir, and prosopinine.^{1–4} Chiral piperidines, such as 5-hydroxypiperic acid (**1**), serve as key precursors in the preparation of piperidine-containing molecules.⁵ Thus, the chiral hydroxypiperic acids represent an important synthetic target. Currently, the majority of approaches to prepare hydroxypiperic acids require chiral building blocks, enzymatic resolution, or asymmetric syntheses.^{6–10} An alternative approach based on enzymes to install the stereocenter via hydroxylation would be advantageous because the biocatalysts have exceptional functional group tolerance, evolvability, and sustainability. For example, proline 4-hydroxylase has been investigated in the preparation of hydroxypiperic acids.^{11, 12} A major drawback of such an enzymatic approach is, however, the specific reactivity of the enzymes. Therefore, it is of interest to identify the key determinants by which the reactivity of an enzyme can be reprogrammed so it could be repurposed to catalyze synthetically useful reactions.

Non-heme iron and 2-oxoglutarate (Fe/2OG) enzymes catalyze diverse, but well-controlled reactions.^{13–19} In some cases, a divergence of reactivity has been reported when substrate

*Corresponding Author: aismirno@ncsu.edu, ysguo@andrew.cmu.edu, wchang6@ncsu.edu.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Supporting Information. The Supporting Information is available free of charge on the ACS Publications website. Experimental methods, Figures S1–26, Tables S1–S2, and references (PDF).

GenBank Accession ID for protein used in this study: [ACC80786](#)

analogues and/or enzyme variants are employed.^{20–22} In the last decade, tremendous efforts have been devoted to understanding the chemistry of Fe/2OG enzyme catalysis.^{13–16} By tuning the regioselectivity of hydroxylation, Fe/2OG hydroxylases have been applied to the preparation of a number of important molecules.^{23–25} Given the importance of functionalized piperidines and aiming at developing a potentially generalizable method for controlling the Fe/2OG enzyme reactivities, we considered the plausibility of preparing hydroxypipicolinic acids using (*S*)-2-amino-5-hexenoic acid (**2**) as an alternative substrate for leucine 5-hydroxylase (LdoA). LdoA, found in *Nostoc punctiforme* (ACC80786), catalyzes C5-hydroxylation of L-leucine and L-norleucine to produce (2*S*,4*S*)-5-hydroxyleucine and (2*S*)-5-hydroxynorleucine.²⁶ In addition, when a targeted carbon is replaced by a sulfur atom (e.g. by using L-methionine or ethionine as substrates), LdoA can catalyze the sulfoxide formation.²⁶ Herein, we demonstrate that by converting the targeted carbon hybridization from sp³ to sp² through introduction of an olefin moiety (**2**), the LdoA reactivity is redirected to non-native epoxidation. It may then be possible to convert the resulting epoxide (**3**) to 5-hydroxypipicolinic acid (**1**) via intramolecular cyclization (Scheme 1).

Hydroxylation and epoxidation are the two common reactivities associated with reactive iron-oxo species. Hydroxylation proceeds with a hydrogen atom transfer (HAT) to the iron-oxo species (e.g., Fe(IV)-oxo porphyrin cation radical (Cpd I) in cytochrome P-450 enzymes and the Fe(IV)-oxo (ferryl) in Fe/2OG enzymes) to yield a substrate radical and a hydroxyl ligand coordinated to the iron.^{15, 27} Subsequent addition of the hydroxyl group to the substrate radical results in formation of the C–OH bond and reduction of the iron in a process known as hydroxyl group rebound.²⁸ In contrast, epoxidation proceeds by electrophilic addition of the iron-oxo species onto an olefin moiety via oxygen atom transfer (OAT).²⁹

When chloroperoxidases and P-450s were tested with substrates containing both olefin and allylic hydrogen, mixtures of hydroxylation and epoxidation products were observed.^{30–33} It has thus been postulated that the chemo-selectivity, e.g., HAT vs. OAT, of Fe-oxo complexes is affected by a number of factors including solvent, temperature, ligand, as well as the geometric and electronic nature of the supporting ligand and substrate.^{34–38} Likewise, the thermodynamic factors such as ionization energy of the olefin and the strength of the targeted C–H bond also contribute to chemo-selectivity.^{39, 40}

Although native hydroxylation and epoxidation are performed by Fe/2OG enzymes,^{13, 15, 16} the chemo-selectivity of a ferryl species remains to be elucidated when it is challenged with a substrate poised for both HAT and OAT. In order to investigate possible factors governing the chemo-selectivity and then be able to apply these principles in the preparation of hydroxypipicolinic acids, a mechanistic probe (**2**) bearing both an olefin and allylic C–H bonds was tested with LdoA (Scheme 1). It was found that with this substrate the LdoA reaction proceeds entirely via OAT to produce the epoxide. Characterization by EPR and hyperfine sublevel correlation spectroscopy (HYSCORE) along with NMR and MS reveal that converting the targeted carbon (C5) hybridization from sp³ to sp² through introduction of an olefin moiety provides an alternative pathway that redirects the reaction flux from HAT to OAT. The resulting epoxide can then undergo intramolecular cyclization to afford the

hydroxypipicolinic acid synthon. These conclusions were based on the observations detailed below.

First, enzyme activity was established by incubating LdoA with L-norleucine (see SI for details). Formation of the hydroxylated product (m/z 132.1 $\rightarrow$ 148.1, ESI, positive ion mode) in the presence of O₂ and 2OG is consistent with the reported C5-hydroxylation reactivity (Figure 1).²⁶ When using unlabeled **2**, a single product was observed by LC-MS with an m/z increase of +16 (130.1 $\rightarrow$ 146.1). This is consistent with formation of a hydroxylation and/or an epoxidation product, both of which have the same m/z value. However, when 4-D₂-**2** and 5-D₁-**2** were used, the only observable product had m/z value of 148.1 and 147.1, respectively. This is inconsistent with C4- or C5-HAT as either would have resulted in a loss of deuterium at C4 or C5 position in the hydroxylated product (4-D₂-**2**, 132.1 $\rightarrow$ 147.1 or 5-D₁-**2**, 131.1 $\rightarrow$ 146.1, m/z = +15). This result suggests that deuterons at C4 and C5 are retained and the reaction likely proceeds through the OAT leading to epoxide formation.

In order to establish the reaction outcome, two regiospecifically ¹³C-labelled substrates (5-¹³C-**2** and 6-¹³C-**2**) were prepared (see SI for preparation) and the LdoA reaction was monitored by ¹³C-NMR as shown in Figures 2 and S26. After incubating LdoA with ¹³C-labelled substrate in the presence of O₂ and 2OG, LdoA was removed and the reaction mixture was analyzed. When using 6-¹³C-**2**, two signals with ¹³C chemical shifts of 49.0 and 61.7 ppm were produced. Under the same conditions with the 5-¹³C-**2**, two signals were observed at 63.2 and 64.9 ppm. Requirement of both O₂ and 2OG for the formation of these new peaks is consistent with their assignment to the reaction products. Furthermore, the signals with chemical shifts of 63.2 (C5) and 61.7 (C6) ppm imply a species with both C5 and C6 carbons linked to an oxygen via a single C–O bond. In contrast, due to differences in the electronegativity of oxygen versus nitrogen, the signals at 64.9 (C5) and 49.0 (C6) ppm are consistent with a species retaining a C–O bond at C5 but now containing a C–N bond at C6. The ¹³C NMR resonances of the reaction products were also compared with those of the (2*S*,5*R*)- and (2*S*,5*S*)-5-hydroxypipicolinic acid standards. Neither C5 nor C6 ¹³C NMR resonances of LdoA reaction products were found to correlate with the corresponding signals in the (2*S*,5*R*)-5-hydroxypipicolinic acid. In contrast, the C5 and C6 chemical shifts of the presumptive 5-hydroxypipicolinic acid product were found to correlate very well with C5 and C6 in the (2*S*, 5*S*)-5-hydroxypipicolinic acid (Figure 2). This observation supports the hypothesis that the immediate product of the LdoA reaction with **2** is a 5,6-epoxide that undergoes intramolecular cyclization to yield a 5-hydroxypipicolinic acid.

LdoA catalyzed reaction was also monitored by LC-MS in a time-dependent manner. Specifically, a reaction mixture containing LdoA, Fe(II), 2OG, **2** was prepared anaerobically. After exposure to air for 1 h along with the substrate consumption, two peaks with different retention times (11.1 and 11.4 min) but the same m/z value (146.1) and the isotope distribution were detected (Figure 2). Following a longer incubation time (14 h at RT), in a filtrate from which the protein was removed using a centrifugal filter (10K, VWR®) after the enzymatic reaction (1h), the peak with the retention time of 11.4 min became the major product. This observation suggested that the immediate product is converted to the second species under the experimental conditions (aqueous buffer, pH $\approx$ 7.6). Another possibility is

that upon formation of an epoxide product, it may undergo intramolecular cyclization to generate 5-hydroxypipelicolic acid. In comparison with the standards of (2*S*, 5*S*)- and (2*S*, 5*R*)-5-hydroxypipelicolic acid, only the (2*S*, 5*S*)-5-hydroxypipelicolic acid standard was found to have the same retention time with the second reaction product. Taken together, these results suggest that LdoA catalyzes asymmetric epoxidation of **2** to generate epoxide **3** possessing a *S*-configuration at C5. Subsequently, it undergoes spontaneous cyclization to yield stable (2*S*, 5*S*)-5-hydroxypipelicolic acid.

To provide further insights into the observations discussed above, pulsed EPR measurements were carried out using analogs that contain deuterium at the C4, C5 or C6 positions (4-D₂-**2**, 5-D₁-**2** and 6-D₂-**2**, see SI for preparation) in order to estimate the distances between the iron and the hydrogens susceptible to HAT. Nitric oxide (NO) was used as the O₂ surrogate.

Addition of NO to non-heme iron enzymes yields an active site {FeNO}⁷ complex with an EPR signal that can be used to elucidate the interactions between the substrate and the iron.^{41, 42} The ²H-HYSCORE Q-band (34 GHz) spectra of NO-treated

LdoA•Fe(II)•2OG•substrate complexes were obtained at a magnetic field strength corresponding to the maximum intensity of the echo-detected field-swept EPR spectra at T = 3.7 K (effective *g* ≈ 3.96, see Figures S24 and 25 for CW X-band EPR spectra).

Experimental ²H-HYSCORE spectra together with simulations are shown in Figure 3. Each isotopolog exhibits a single signal on the main diagonal with coordinates corresponding to the ²H Zeeman frequency. The shape as well as the intensity of the ridge are determined by the strength of the corresponding hyperfine interactions (see Table S1). Deuteron at C5 is the closest to the iron. It is then followed by the C6 deuteron, and the deuteron at C4 is the most distant. By using a point-dipole approximation with respect to the electron-nuclear hyperfine interaction, the distances between the iron center and the hydrogens at C4, C5 and C6 of **2** were estimated to be approximately 4.3, 3.3, and 3.8 Å, respectively. Thus, these C–H bonds are in a close vicinity to the iron and, therefore, may interact with the ferryl species.

Considering the oxidative power of the Fe(IV)-oxo intermediate and the strength of the C_{sp2}-H bond,^{13, 15} hydroxylation involving C5-H or C6-H is less likely. In contrast, hydroxylation at allylic position (C4-H), as usually observed in P450s, is feasible. On the other hand, due to the proximity of sp²-hybridized C5 and C6 to the iron(IV)-oxo as suggested by HYSCORE analysis, epoxidation on the olefin moiety via OAT pathway could become competitive against the native hydroxylation.

In summary, when LdoA is challenged with the non-native substrate possessing an olefin moiety, the reactivity is redirected from hydroxylation to asymmetric epoxidation. Furthermore, the epoxide (*S*-**3**) undergoes a subsequent cyclization to generate (2*S*, 5*S*)-5-hydroxypipelicolic acid, which is a useful synthon in the preparation of functionalized chiral piperidines. Distinct from Cpd I and non-heme Fe-oxo complexes where mixtures of allylic hydroxylation and epoxidation are generally detected, these results imply that the ferryl species within the LdoA active site strongly prefers epoxidation over hydroxylation when presented with an unsaturated π-system. Namely, the change in the hybridization of the targeted carbon through introduction of an olefin moiety not only prevents the HAT by increasing the C-H bond strength, but also provides an alternative pathway that allows for non-native reactivity. One possible explanation for this behavior is offered by the

HYSCORE analysis, which implies that the olefinic carbon susceptible to OAT is in the close proximity to the iron. Thus LdoA represents an important system for exploring the chemo-selectivity of the ferryl species, a conserved intermediate used by Fe/2OG enzymes. This observation may lead to the discovery of new approaches and methodologies for the repurposing these enzymes for synthetic and other applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

This work was supported by North Carolina State University, Carnegie Mellon University, NSF CHE-1845913, NIH GM125924. HYSCORE experiments were supported by DE-FG02-02ER15354. EPR instrumentation was supported by NIH RR023614 and NSF CHE-0840501. We thank Dr. Rusczycky for helpful discussion and comments.

REFERENCES

- [1]. Lamarre D, Croteau G, Wardrop E, Bourgon L, Thibeault D, Clouette C, Vaillancourt M, Cohen E, Pargellis C, Yoakim C, and Anderson PC (1997) Antiviral properties of palinavir, a potent inhibitor of the human immunodeficiency virus type 1 protease, *Antimicrob. Agents Chemother* 41, 965–971. [PubMed: 9145853]
- [2]. Beaulieu PL, Lavallee P, Abraham A, Anderson PC, Boucher C, Bousquet Y, Duceppe JS, Gillard J, Gorys V, GrandMaitre C, Grenier L, Guindon Y, Guse I, Plamondon L, Soucy F, Valois S, Wernic D, and Yoakim C (1997) Practical, stereoselective synthesis of palinavir, a potent HIV protease inhibitor, *J. Org. Chem* 62, 3440–3448.
- [3]. Ojima I, and Vidal ES (1998) Rhodium-catalyzed cyclohydrocarbonylation: Application to the synthesis of (+)-protopinine and (–)-deoxoprosophylline, *J. Org. Chem* 63, 7999–8003.
- [4]. Jourdan A, and Zhu JP (2000) An efficient stereoselective and stereodivergent synthesis of (2R,3R)- and (2R,3S)-3-hydroxypipicolinic acids, *Tetrahedron Lett.* 41, 7033–7036.
- [5]. Miller SP, Zhong YL, Liu Z, Simeone M, Yasuda N, Limanto J, Chen Z, Lynch J, and Capodanno V (2014) Practical and cost-effective manufacturing route for the synthesis of a beta-lactamase inhibitor, *Org. Lett* 16, 174–177. [PubMed: 24329136]
- [6]. Cohen LA, Irreverre F, Piez KA, Witkop B, and Wolff HL (1956) Synthesis of 5-hydroxypipicolinic acid and separation of its diastereoisomers, *Science* 123, 842–843. [PubMed: 13324096]
- [7]. Botman PN, Dommerholt FJ, de Gelder R, Broxterman QB, Schoemaker HE, Rutjes FP, and Blaauw RH (2004) Diastereoselective synthesis of (2S,5R)-5-hydroxypipicolinic acid and 6-substituted derivatives, *Org. Lett* 6, 4941–4944. [PubMed: 15606105]
- [8]. Sun C-S, Lin Y-S, and Hou D-R (2008) Asymmetric Synthesis of 4- and 5-Substituted Pipicolinic Esters by Ring-Closing Metathesis and Palladium-Catalyzed Formate Reduction, *J. Org. Chem* 73, 6877–6880. [PubMed: 18662030]
- [9]. Scarpi D, Bartali L, Casini A, and Occhiato EG (2013) Complementary and Stereodivergent Approaches to the Synthesis of 5-Hydroxy- and 4,5-Dihydroxypipicolinic Acids from Enantiopure Hydroxylated Lactams, *Eur. J. Org. Chem* 2013, 1306–1317.
- [10]. Krishnamurthy S, Venkataprasad J, Vagvala TC, Moriguchi T, and Tsuge A (2015) alpha-Chymotrypsin and L-acylase aided synthesis of 5-hydroxypipicolinic acid via Jacobsen's hydrolytic kinetic resolution of epoxy amino acids, *Rsc Adv* 5, 52154–52160.
- [11]. Hara R, Uchiumi N, Okamoto N, and Kino K (2014) Regio- and stereoselective oxygenation of proline derivatives by using microbial 2-oxoglutarate-dependent dioxygenases, *Biosci. Biotechnol. Biochem* 78, 1384–1388. [PubMed: 25130741]

- [12]. Mattay J, and Huttel W (2017) Pipecolic Acid Hydroxylases: A Monophyletic Clade among cis-Selective Bacterial Proline Hydroxylases that Discriminates l-Proline, *Chembiochem* 18, 1523–1528. [PubMed: 28489306]
- [13]. Hausinger RP (2015) Biochemical Diversity of 2-Oxoglutarate-Dependent Oxygenases, In *2-Oxoglutarate-Dependent Oxygenases* (Hausinger RP, and Schofield CJ, Eds.), pp 1–58, Royal Society of Chemistry, London.
- [14]. Bollinger JM Jr., Chang W.-c., Matthews ML, Martinie RJ, Boal AK, and Krebs C (2015) Mechanisms of 2-Oxoglutarate-Dependent Oxygenases: The Hydroxylation Paradigm and Beyond, In *2-Oxoglutarate-Dependent Oxygenases* (Hausinger RP, and Schofield CJ, Eds.), pp 95–122, Royal Society of Chemistry London.
- [15]. Martinez S, and Hausinger RP (2015) Catalytic Mechanisms of Fe(II)- and 2-Oxoglutarate-dependent Oxygenases, *J. Biol. Chem* 290, 20702–20711. [PubMed: 26152721]
- [16]. Gao SS, Naowarojna N, Cheng R, Liu X, and Liu P (2018) Recent examples of alpha-ketoglutarate-dependent mononuclear non-haem iron enzymes in natural product biosyntheses, *Nat. Prod. Rep* 35, 792–837. [PubMed: 29932179]
- [17]. Hollenhorst MA, Bumpus SB, Matthews ML, Bollinger JM Jr., Kelleher NL, and Walsh CT (2010) The nonribosomal peptide synthetase enzyme DdaD tethers N(beta)-fumaramoyl-l-2,3-diaminopropionate for Fe(II)/alpha-ketoglutarate-dependent epoxidation by DdaC during daptamide antibiotic biosynthesis, *J Am Chem Soc* 132, 15773–15781. [PubMed: 20945916]
- [18]. Hashimoto T, Matsuda J, and Yamada Y (1993) Two-step epoxidation of hyoscyamine to scopalamine is catalyzed by bifunctional hyoscyamine 6 beta-hydroxylase, *FEBS Lett* 329, 35–39. [PubMed: 8354403]
- [19]. Ishikawa N, Tanaka H, Koyama F, Noguchi H, Wang CC, Hotta K, and Watanabe K (2014) Non-heme dioxygenase catalyzes atypical oxidations of 6,7-bicyclic systems to form the 6,6-quinolone core of viridicatin-type fungal alkaloids, *Angew Chem Int Ed Engl* 53, 12880–12884. [PubMed: 25251934]
- [20]. Li J, van Belkum MJ, and Vederas JC (2012) Functional characterization of recombinant hyoscyamine 6beta-hydroxylase from *Atropa belladonna*, *Bioorg Med Chem* 20, 4356–4363. [PubMed: 22705021]
- [21]. Matthews ML, Chang WC, Layne AP, Miles LA, Krebs C, and Bollinger JM Jr. (2014) Direct nitration and azidation of aliphatic carbons by an iron-dependent halogenase, *Nat Chem Biol* 10, 209–215. [PubMed: 24463698]
- [22]. Mitchell AJ, Dunham NP, Bergman JA, Wang B, Zhu Q, Chang W. c., Liu X, and Boal AK (2017) Structure-Guided Reprogramming of a Hydroxylase To Halogenate Its Small Molecule Substrate, *Biochemistry* 56, 441–444. [PubMed: 28029241]
- [23]. Lukat P, Katsuyama Y, Wenzel S, Binz T, König C, Blankenfeldt W, Bronstrup M, and Müller R (2017) Biosynthesis of methyl-proline containing griselimycins, natural products with anti-tuberculosis activity, *Chem. Sci* 8, 7521–7527. [PubMed: 29163906]
- [24]. Zwick CR, and Renata H (2018) Remote C-H Hydroxylation by an alpha-Ketoglutarate-Dependent Dioxygenase Enables Efficient Chemoenzymatic Synthesis of Manzacidin C and Proline Analogs, *J. Am. Chem. Soc* 140, 1165–1169. [PubMed: 29283572]
- [25]. Zwick CR, Sosa MB, and Renata H (2019) Characterization of a Citrulline 4-Hydroxylase from Nonribosomal Peptide GE81112 Biosynthesis and Engineering of Its Substrate Specificity for the Chemoenzymatic Synthesis of Enduracididine, *Angew. Chem. Int. Ed* 58, 18854–18858.
- [26]. Hibi M, Kawashima T, Sokolov PM, Smirnov SV, Koder T, Sugiyama M, Shimizu S, Yokozeki K, and Ogawa J (2013) L-leucine 5-hydroxylase of *Nostoc punctiforme* is a novel type of Fe(II)/alpha-ketoglutarate-dependent dioxygenase that is useful as a biocatalyst, *Appl. Microbiol. Biotechnol* 97, 2467–2472. [PubMed: 22584432]
- [27]. Ortiz de Montellano PR (2015) *Cytochrome P450 Structure, Mechanism, and Biochemistry*, 4th ed, Springer International Publishing, Cham.
- [28]. Groves JT, and McCluskey GA (1976) Aliphatic Hydroxylation Via Oxygen Rebound - Oxygen-Transfer Catalyzed by Iron, *J. Am. Chem. Soc* 98, 859–861.
- [29]. Thibodeaux CJ, Chang W. c., and Liu HW (2012) Enzymatic chemistry of cyclopropane, epoxide, and aziridine biosynthesis, *Chem. Rev* 112, 1681–1709. [PubMed: 22017381]

- [30]. Groves JT, Avarianeisser GE, Fish KM, Imachi M, and Kuczkowski RL (1986) Hydrogen-Deuterium Exchange during Propylene Epoxidation by Cytochrome-P-450, *J. Am. Chem. Soc* 108, 3837–3838.
- [31]. Fruetel JA, Collins JR, Camper DL, Loew GH, and Demontellano PRO (1992) Calculated and Experimental Absolute Stereochemistry of the Styrene and Beta-Methylstyrene Epoxides Formed by Cytochrome-P450(Cam), *J. Am. Chem. Soc* 114, 6987–6993.
- [32]. Allain EJ, Hager LP, Deng L, and Jacobsen EN (1993) Highly Enantioselective Epoxidation of Disubstituted Alkenes with Hydrogen-Peroxide Catalyzed by Chloroperoxidase, *J. Am. Chem. Soc* 115, 4415–4416.
- [33]. Vaz AD, McGinnity DF, and Coon MJ (1998) Epoxidation of olefins by cytochrome P450: evidence from site-specific mutagenesis for hydroperoxo-iron as an electrophilic oxidant, *Proc. Natl. Acad. Sci. U.S.A* 95, 3555–3560. [PubMed: 9520404]
- [34]. de Visser SP, Ogliaro F, Sharma PK, and Shaik S (2002) What factors affect the regioselectivity of oxidation by cytochrome p450? A DFT study of allylic hydroxylation and double bond epoxidation in a model reaction, *J. Am. Chem. Soc* 124, 11809–11826. [PubMed: 12296749]
- [35]. Song WJ, Ryu YO, Song R, and Nam W (2005) Oxoiron(IV) porphyrin pi-cation radical complexes with a chameleon behavior in cytochrome P450 model reactions, *J. Biol. Inorg. Chem* 10, 294–304. [PubMed: 15827730]
- [36]. Mandal D, Ramanan R, Usharani D, Janardanan D, Wang B, and Shaik S (2015) How does tunneling contribute to counterintuitive H-abstraction reactivity of nonheme Fe(IV)O oxidants with alkanes?, *J. Am. Chem. Soc* 137, 722–733. [PubMed: 25513834]
- [37]. Chatfield DC, and Morozov AN (2018) Proximal Pocket Controls Alkene Oxidation Selectivity of Cytochrome P450 and Chloroperoxidase toward Small, Nonpolar Substrates, *J. Phys. Chem. B* 122, 7828–7838. [PubMed: 30052045]
- [38]. Engelmann X, Malik DD, Corona T, Warm K, Farquhar ER, Swart M, Nam W, and Ray K (2019) Trapping of a Highly Reactive Oxoiron(IV) Complex in the Catalytic Epoxidation of Olefins by Hydrogen Peroxide, *Angew. Chem. Int. Ed* 58, 4012–4016.
- [39]. Sainna MA, Kumar S, Kumar D, Fornarini S, Crestoni ME, and de Visser SP (2015) A comprehensive test set of epoxidation rate constants for iron(iv)-oxo porphyrin cation radical complexes, *Chem. Sci* 6, 1516–1529. [PubMed: 29560240]
- [40]. Kwon YH, Mai BK, Lee YM, Dhuri SN, Mandal D, Cho KB, Kim Y, Shaik S, and Nam W (2015) Determination of Spin Inversion Probability, H-Tunneling Correction, and Regioselectivity in the Two-State Reactivity of Nonheme Iron(IV)-Oxo Complexes, *J. Phys. Chem. Lett* 6, 1472–1476. [PubMed: 26263154]
- [41]. Orville AM, Chen VJ, Kriauciunas A, Harpel MR, Fox BG, Munck E, and Lipscomb JD (1992) Thiolate ligation of the active site Fe²⁺ of isopenicillin N synthase derives from substrate rather than endogenous cysteine: spectroscopic studies of site-specific Cys----Ser mutated enzymes, *Biochemistry* 31, 4602–4612. [PubMed: 1316153]
- [42]. Muthukumaran RB, Grzyska PK, Hausinger RP, and McCracken J (2007) Probing the iron-substrate orientation for taurine/alpha-ketoglutarate dioxygenase using deuterium electron spin echo envelope modulation spectroscopy, *Biochemistry* 46, 5951–5959. [PubMed: 17469855]

Figure 1.

LC-MS analysis of LdoA reaction. L-Norleucine is converted to the hydroxylated product. Comparing to the reaction using **2** as the substrate, the corresponding product m/z value changes from 146.1 to 148.1 and 147.1 when employing 4-D₂-**2** and 5-D₁-**2**. The m/z values correlate to 146.1, 147.1 and 148.1 are colored with blue, green and purple, respectively

Figure 2.

^{13}C -NMR and LC-MS analysis of the LdoA catalyzed reaction. In the top panel, the ^{13}C peaks corresponding to the epoxide are colored green. The peaks correlating to C5 and C6 of 5-hydroxyproline are colored purple and red, respectively. The ^{13}C -enriched carbon is labelled with an asterisk. In the bottom panel, upon comparison of the reaction where 2OG was omitted, reaction for 1 h, two major peaks with the same m/z value were produced. Following a longer incubation time (14 h), a major peak that has the same retention time and m/z value as (2*S*, 5*S*)-5-hydroxyproline was observed.

Figure 3.

Q-Band (34 GHz) ^2H -HYSCORE spectra of NO-treated LdoA•Fe(II)•2OG•substrate (4-D₂-2, 5-D₁-2 and 6-D₂-2) complexes obtained at a magnetic field corresponding to $g \approx 3.96$ resonance in the echo-detected field-swept EPR spectrum at $T = 3.7$ K. Spectra are limited to the region showing features resulting from ^2H and FeNO interactions (left column) and the corresponding simulations (right column).