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Alternative Reactivity of Leucine 5-Hydroxylase Using an Olefin-Containing Substrate to Construct a Substituted Piperidine Ring

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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 sp^3 to sp^2. 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*-hydroxypipecolic 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-hydroxypipecolic acid (**1**), serve as key precursors in the preparation of piperidine-containing molecules.[5] Thus, chiral hydroxypipecolic acids represent an important synthetic target. Currently, a majority of approaches to prepare hydroxypipecolic acids require chiral building blocks, enzymatic resolution, or asymmetric syntheses.[6−10] An alternative approach to use enzymes for installing 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 hydroxypipecolic acids.[11,12] A drawback of such an enzymatic approach, however, is the specific reactivity of the enzymes. Therefore, identifying the key determinants by which the reactivity of an enzyme can be reprogrammed so it could be repurposed to catalyze non-native and synthetically useful reactions is desirable.

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 analogues and/or enzyme variants are employed.[20−22] In the past decade, tremendous effort has been devoted to understand the chemistry of Fe/2OG enzyme catalysis.[13−16] By tuning the regioselectivity of hydroxylation, researchers have applied Fe/2OG hydroxylases to the preparation of important molecules.[23−25] Given the importance of functionalized piperidines and aiming to develop a potentially generalizable method for controlling the Fe/2OG enzyme reactivities, we considered the plausibility of preparing hydroxypipecolic 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 sulfoxide formation.[26] Herein, we demonstrate that conversion of the targeted carbon hybridization from sp^3 to sp^2 through introduction of an olefin moiety redirects the LdoA reactivity to non-native epoxidation. It may then be possible to convert the resulting epoxide (**3**) to 5-hydroxypipecolic acid (**1**) via intramolecular cyclization (Scheme 1).

Hydroxylation and epoxidation are two common reactivities associated with reactive iron−oxo species. Hydroxylation proceeds with 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 the 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 to 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 chemoselectivity, e.g., HAT vs OAT, of Fe−oxo complexes is affected by a number of factors, including solvent, temperature, ligand, and the geometric and electronic nature of the supporting ligand and substrate.[34−38] Likewise, the ionization energy of the olefin and the strength of the targeted C−H bond also contribute to chemoselectivity.[39,40]

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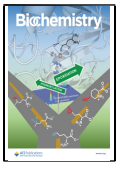

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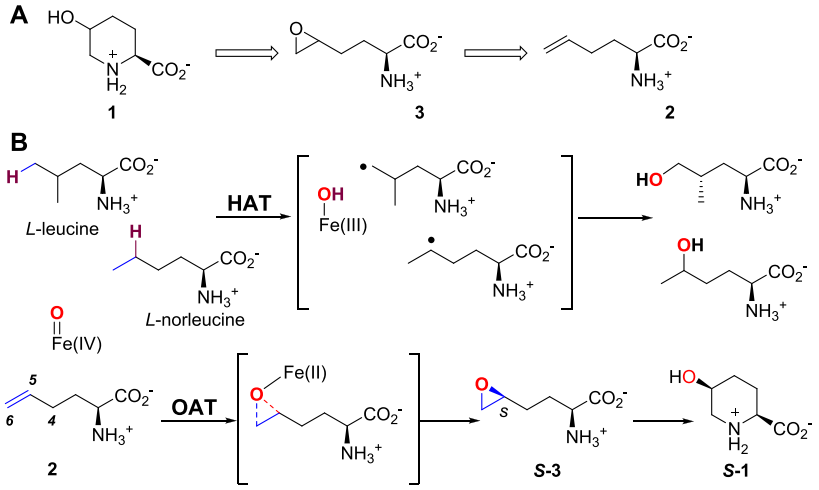
Scheme 1. Retrosynthesis and Redirecting of Fe(IV) Reactivity for the Preparation of 1



Although native hydroxylation and epoxidation are performed by Fe/2OG enzymes,[13,15,16] the chemoselectivity of a ferryl species remains to be elucidated when it is challenged with a substrate poised for both HAT and OAT. To investigate the possible factors governing the chemoselectivity and then be able to apply these principles in the preparation of hydroxypipecolic acids, a mechanistic probe (**2**) bearing both an olefin group and allylic C−H bonds was tested with LdoA (Scheme 1). With this substrate, the LdoA reaction proceeds entirely via OAT to produce the epoxide. Characterization by electron paramagnetic resonance (EPR) and hyperfine sublevel correlation spectroscopy (HYSCORE) along with nuclear magnetic resonance (NMR) and mass spectrometry (MS) reveals that converting the targeted carbon (C5) hybridization from sp^3 to sp^2 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 hydroxypipecolic acid synthon.

First, enzyme activity was established by incubating LdoA with L-norleucine (see the Supporting Information for details). Formation of the hydroxylated product (m/z 132.1 → 148.1, ESI, positive ion mode) in the presence of O_2 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 16.0 (m/z 130.1 → 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-D_2-**2** and 5-D_1-**2** were used, the only observable product had m/z values 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 position C4 or C5 in the hydroxylated product (4-D_2-**2**, m/z 132.1 → 147.1, or 5-D_1-**2**, m/z 131.1 → 146.1; $\Delta m/z$ = +15.0). This result suggests that deuterons at C4 and C5 are retained and the reaction likely proceeds through the OAT pathway leading to epoxide formation.

To establish the reaction outcome, two regiospecifically ^{13}C-labeled substrates (5-^{13}C-**2** and 6-^{13}C-**2**) were prepared (see the Supporting Information for preparation) and the LdoA reaction was monitored by ^{13}C NMR as shown in Figure 2 and Figure S26. After LdoA had been incubated with the ^{13}C-labeled substrate in the presence of O_2 and 2OG, LdoA was removed and the reaction mixture was analyzed. When using 6-^{13}C-**2**, two signals with ^{13}C chemical shifts of 49.0 and 61.7 ppm were

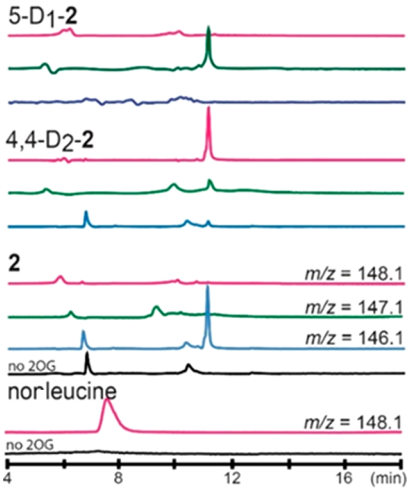


Figure 1. LC-MS analysis of the LdoA reaction. L-Norleucine is converted to the hydroxylated product. Compared to the reaction using **2** as the substrate, the corresponding product m/z value changes from 146.1 to 148.1 and 147.1 when employing 4-D_2-**2** and 5-D_1-**2**. The m/z values that correspond to 146.1, 147.1, and 148.1 are colored blue, green, and purple, respectively.

produced. Under the same conditions with 5-^{13}C-**2**, two signals were observed at 63.2 and 64.9 ppm. The requirement of both O_2 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 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 ^{13}C NMR resonances of the reaction products were also compared with those of the (2*S*,5*R*)- and (2*S*,5*S*)-5-hydroxypipecolic acid standards. Neither C5 nor C6 ^{13}C NMR resonances of LdoA reaction products were found to correlate with the corresponding signals in the (2*S*,5*R*)-5-hydroxypipecolic acid. In contrast, the C5 and C6 chemical shifts of the presumptive 5-hydroxypipecolic acid product were found to correlate very well with C5 and C6 in the (2*S*,5*S*)-5-hydroxypipecolic acid (Figure 2). This observation supports the hypothesis that the immediate product of the reaction of

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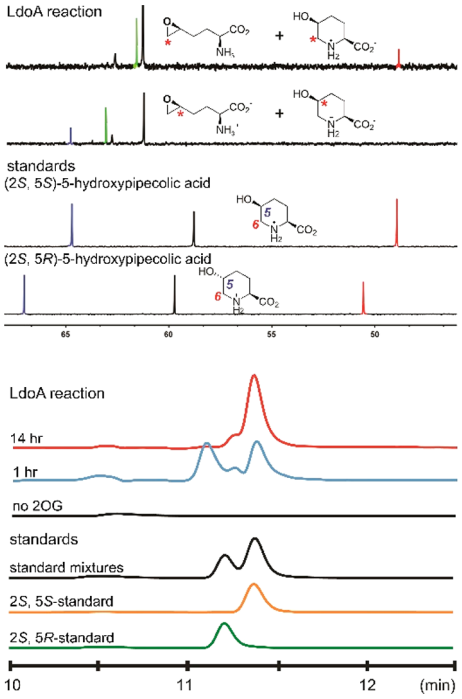

Figure 2. ^{13}C NMR and LC-MS analysis of the LdoA-catalyzed reaction. In the top panel, the ^{13}C peaks corresponding to the epoxide are colored green. The peaks correlating to C5 and C6 of 5-hydroxypipecolic acid are colored purple and red, respectively. The ^{13}C-enriched carbon is labeled with an asterisk. In the bottom panel upon comparison of the reaction from which 2OG was omitted, after 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-hydroxypipecolic acid was observed.

LdoA with **2** is a 5,6-epoxide that undergoes intramolecular cyclization to yield a 5-hydroxypipecolic acid.

The LdoA-catalyzed reaction was also monitored by LC-MS in a time-dependent manner. Specifically, a reaction mixture containing LdoA, Fe(II), 2OG, and **2** was prepared anaerobically. After exposure to air for 1 h, along with substrate consumption, two peaks with different retention times (11.1 and 11.4 min) but the same m/z value (146.1) and the same isotope distribution were detected (Figure 2). Following a longer incubation time (14 h at room temperature), in a filtrate from which the protein had been removed using a centrifugal filter (10K, VWR) after the enzymatic reaction (1 h), the peak with a 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 ~7.6). One possibility is that upon formation of an epoxide product, it may undergo intramolecular cyclization to generate 5-hydroxypipecolic acid. In comparison with the standards (2*S*,5*S*)- and (2*S*,5*R*)-5-hydroxypipecolic acid, only the (2*S*,5*S*)-5-hydroxypipecolic 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 an *S*-configuration at C5. Subsequently, it undergoes spontaneous cyclization to yield stable (2*S*,5*S*)-5-hydroxypipecolic acid.

To provide further insights into the observations discussed above, pulsed EPR measurements were carried out using isotopologues that contain deuterium at position C4, C5, or C6 [4-D_2-**2**, 5-D_1-**2**, or 6-D_2-**2**, respectively (see the Supporting Information for preparation)] to estimate the distances between the iron and the hydrogens susceptible to HAT. Nitric oxide (NO) was used as the O_2 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 $g \sim 3.96$ (see Figures S24 and 25 for continuous-wave X-band EPR spectra)]. Experimental ^2H HYSCORE spectra together with simulations are shown in Figure 3. Each isotopologue exhibits a single signal

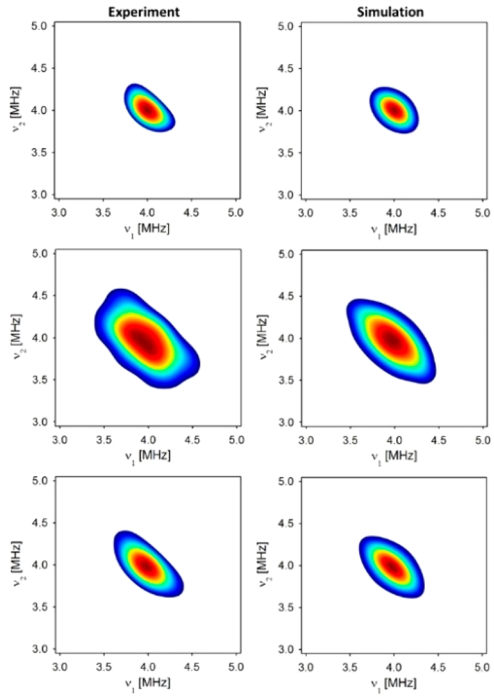

Figure 3. Q-Band (34 GHz) ^2H HYSCORE spectra of NO-treated LdoA·Fe(II)·2OG·substrate (4-D_2-**2**, 5-D_1-**2**, and 6-D_2-**2**) complexes obtained at a magnetic field corresponding to the $g \sim 3.96$ resonance in the echo-detected field-swept EPR spectrum at T = 3.7 K. Spectra are limited to the region showing features resulting from ^2H and FeNO interactions (left column) and the corresponding simulations (right column).

on the main diagonal with coordinates corresponding to the ^2H Zeeman frequency. The shape and intensity of the ridge are determined by the strength of the corresponding hyperfine interactions (see Table S1). Analysis of the HYSCORE spectra reveals that the 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−C6 of **2** were estimated to be approximately 4.3, 3.3, and 3.8 Å, respectively. Thus, these C−H bonds are close to the iron and, there, may interact with the ferryl species. Considering the oxidative power of the Fe(IV)−oxo intermediate and the strength of the C_{sp^2}−H bond,[13,15] hydroxylation involving C5−H or C6−H is less likely. In contrast, hydroxylation at the allylic

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position (C4−H), as usually observed in P450s, is feasible. On the other hand, due to the proximity of sp^2-hybridized C5 and C6 to the iron(IV)−oxo intermediate as suggested by HYSCORE analysis, epoxidation on the olefin moiety via the 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 subsequent cyclization to generate (2*S*,5*S*)-5-hydroxypipecolic 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 close to the iron. The LdoA system represents an important system for exploring the chemoselectivity 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 of these enzymes for synthetic and other applications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biochem.0c00289.

Experimental methods, Figures S1−26, Tables S1 and S2, and references (PDF)

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Notes

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