MECHANISTIC DISSECTION OF ALTERNATIVE REACTIONS CATALYZED BY
IRON(II)- AND 2-(OXO)GLUTARATE-DEPENDENT OXYGENASES

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by
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ABSTRACT

Iron(II)- and 2-(oxo)glutarate-dependent (Fe/2OG) oxygenases catalyze an astonishing array of biochemical reactions with diverse and important physiological functions. Their manifold of primarily oxidative transformations arises from a conserved iron(IV)-oxo (ferryl) intermediate that forms upon the coupled activation of \( \text{O}_2 \) and decarboxylation of 2OG to succinate at the iron cofactor. In well-understood transformations of \( sp^2 \)-hybridized carbon centers, the ferryl intermediate abstracts a hydrogen atom (H•); the most common outcome, hydroxylation, then results from coupling (often termed "rebound") between the OH ligand and substrate radical produced by the H•-transfer (HAT) step. This investigation explores the alternative outcomes effected by two Fe/2OG enzymes: xanthine:2OG dioxygenase, XanA, which hydroxylates an \( sp^2 \)-hybridized carbon (C8) of xanthine to produce uric acid, and carbapenem synthase, CarC, which effects sequential redox-neutral (with respect to the substrate) epimerization and 1,2-dehydrogenation reactions. Members of this enzyme family could be used combinatorially for the construction of new drug compounds, but only with a fundamental understanding of how individual members direct different types of reactivity can we optimally exploit them for such a purpose.
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Chapter 1

Overview of Fe/2OG oxygenases
This chapter summarizes the canonical and alternative reaction mechanisms/outcomes catalyzed by the Fe/2OG oxygenases and the significance of this class of enzymes. This overview provides the backdrop for the subsequent chapters of the thesis, which focus on the advances made in the study of three alternative reactions catalyzed by two members of the Fe/2OG oxygenase family, XanA and CarC.

1.1 Background and Significance

Fe/2OG oxygenases catalyze numerous physiologically-relevant biochemical transformations through the coupled activation of O₂ at mononuclear non-heme iron(II) centers and decarboxylation of 2OG to succinate. This process generates a conserved Fe(IV)-oxo (ferryl complex), which has now been directly detected in several members of the class and characterized in detail in two of them. The reactions mediated by this ferryl intermediate are important to mammalian O₂ sensing, repair of DNA damage, antibiotic biosynthesis, xenobiotic degradation, and epigenetic inheritance. Oxidative decarboxylation of 2OG, a two-electron oxidation, is in most cases coupled to a two-electron oxidation of the primary substrate, balancing the net four-electron reduction of O₂. Typically, the outcome is hydroxylation of an sp³-hybridized carbon. However, the Fe/2OG enzymes are remarkably diverse mechanistically, as they can also catalyze hydroxylation and epoxidation of sp²-hybridized carbons, radical group transfers (e.g., halogenation) to sp³-hybridized carbons, 1,2-dehydrogenations, stereoinversions, cyclizations, and ring expansions upon their primary substrates (Scheme 1.1.1).
Scheme 1.1.1. Canonical and alternative reactions, known or thought to proceed through ferryl intermediates, that are catalyzed by the Fe/2OG oxygenases.

1.2 Mechanism and Reactivity

1.2.1 Consensus Mechanism and Canonical Hydroxylation Outcome

The Fe/2OG oxygenases, though very diverse functionally, share highly conserved active site features, most notably a ferrous ion coordinated by an octahedral ligand environment, in which a characteristic (His)$_2$-Asp/Glu facial triad Fe(II)-binding motif is usually (although not always) present.$^1$ A notable exception to this motif, the halogenase subclass, requires an additional substrate for catalysis (Cl- or Br-) and exhibits a conserved alanine or glycine residue at the position of the residue that normally provides the carboxylate ligand. Crystallographic and EXAFS data have shown that this substitution enables coordination of Cl- or Br- directly coordinate to the iron cofactor in both reactant [Fe(II)] and intermediate (ferryl) states.$^{4,5}$
A mechanism originally put forth by Hanauske-Abel and Günzler for prolyl-4-hydroxylase (P4H) established the foundation for ensuing experimental studies on many Fe/2OG oxygenases. In this consensus mechanism, 2OG coordinates to the ferrous ion in a bidentate fashion. This coordination mode was elucidated through a combination of crystallographic, absorption, CD, and MCD studies. An Fe(II)-to-2OG metal-to-ligand charge-transfer (MLCT) absorption band at ~500 nm is generally observed in these enzymes upon addition of 2OG to their Fe(II) complexes in the absence of O₂.

Many Fe/2OG enzymes react with O₂ much more rapidly in the presence of primary substrate compared to in its absence, a phenomenon termed substrate-triggering. Addition of the primary substrate and O₂ to the enzyme•Fe(II)•2OG complex results in formation of a conserved ferryl intermediate upon coupled activation of O₂ and oxidative decarboxylation of 2OG to succinate. In the well-understood transformations of sp²-hybridized carbon centers, reaction with the primary substrate is initiated by hydrogen atom (H•) transfer (HAT) from the substrate to the ferryl intermediate. The most common outcome, hydroxylation, then results from coupling (often termed “rebound”) between the OH ligand (reacting as HO•) and substrate radical. In-depth studies on TauD from Escherichia coli and P4H from Paramecium bursaria Chlorella virus have established these central tenets. Specifically, characterization of the ferryl intermediates by freeze-quench Mössbauer spectroscopy revealed small isomer shifts (~0.25-0.3 mm/s) and \( S = 2 \) electron-spin ground states, indicative of a high-spin ferryl species. Resonance Raman and EXAFS data on the TauD complex established the presence of the short, strong bond between the iron and oxygen expected for a ferryl intermediate. HAT to this high-spin ferryl was implied by the observations of large substrate
deuterium KIEs on ferryl decay ($k_H/k_D \approx 50$) in stopped-flow absorption and freeze-quench Mössbauer experiments.\textsuperscript{17,18}

Incorporation of $^{18}$O from $^{18}$O\textsubscript{2} into the ferryl intermediate and subsequently into the oxidized product was verified in the aforementioned resonance Raman study on TauD and by later mass spectrometric characterization of products of several other Fe/2OG enzymes. However, incorporation of the $^{18}$O can be fractional as a result of exchange of the oxo group with solvent (washout), which can occur in competition with substrate oxidation.\textsuperscript{1,19} For a ferryl complex that decays by HAT, as in the hydroxylases, substitution of the target position of the substrate with deuterium should, by extending the lifetime of the ferryl complex, result in increased solvent exchange into the intermediate and diminished incorporation of $^{18}$O into the product. This prediction was verified for hydroxylation reactions mediated by the Fe/2OG halogenase, SyrB2, upon non-native substrates.\textsuperscript{19} The large substrate deuterium KIEs on HAT to the ferryl complexes can have two additional, mechanistically informative effects. In cases of substrates with multiple hydrons that can undergo HAT to the ferryl complex, deuterium substitution of the preferred site can redirect the ferryl complex to undergo HAT from an adjacent site. This scenario was encountered in studies on SyrB2 employing a substrate with the non-native amino acid, norvaline (Nva), tethered to the carrier protein, SyrB1. HAT from C5 of Nva was found to be favored over HAT from C4 (by a factor of 6), but substitution of C5 with deuterium reversed the regiochemical preference to C4 (by a factor of 10).\textsuperscript{19} Additionally, the large deuterium KIEs can result in failure in the HAT step, with the result that 2OG decarboxylation becomes uncoupled from substrate oxidation. This effect was observed in both TauD, which becomes ~ 30% uncoupled with C1-deuterium-labeled taurine,\textsuperscript{17} and carbapenem
syntase, which is already imperfectly (~ 80%) coupled with protium-containing substrate and becomes only 15% coupled in the reaction with the C5-deuterium-labeled substrate.²

Scheme 1.2.1.1. Consensus mechanism for Fe/2OG oxygenases.¹²

1.2.2 Alternative Reactions

Prior to the work detailed in this thesis, the Bollinger/Krebs group had dissected the chemical and kinetic mechanisms of several Fe/2OG oxygenases. In each case, the results could be understood in terms of a canonical HAT from the \( sp^3 \)-hybridized target carbon of the substrate to the characteristic ferryl intermediate, yielding Fe(III)-OH and substrate radical, followed by
transfer of an Fe-coordinated ligand (hydroxyl or halogen) as a radical to the substrate radical, yielding oxidized product.\textsuperscript{14,19,20}

The three alternative reactions detailed in this thesis, stereoinversion and 1,2-dehydrogenation of \textit{sp}^3-hybridized carbon centers and hydroxylation of an \textit{sp}^2-hybridized carbon, diverge from this paradigm and provide a new “mechanistic toolbox” useful for evaluating other alternative reactions. Notable alternative reactions that have yet to be explored in mechanistic detail include:

1) C-S-bond-formation by the enzyme OvoA, which couples cysteine and histidine in the biosynthesis of ovothiol A\textsuperscript{21–23}

2) Oxacyclization reactions catalyzed by (i) clavamine synthase (CAS) on the pathway to the \(\beta\)-lactamase inhibitor, clavulanic acid,\textsuperscript{24} (ii) hyoscyamine 6-hydroxylase (H6H) on the pathway to the plant-derived anesthetic, scopolamine,\textsuperscript{25} and \textit{N}-acetylnorloline synthase (LolO) on the pathway to the fungal insecticides known collectively as lolines and norlolines\textsuperscript{26}

3) Azacyclization by the enzyme GLOXY4, which forms 4\textit{S}-methyl-L-proline from L-leucine\textsuperscript{27}

4) Carbocyclization by the enzyme SnoK, on the pathway to the anthracycline, nogalamycin.\textsuperscript{28}

1.3 Conclusion

The Bollinger/Krebs group has contributed significantly to the mechanistic dissection of Fe/2OG hydroxylases and halogenases. Nevertheless, abundant opportunities still exist for the
discovery and examination of alternative reaction outcomes/mechanisms. This investigation used transient-state-kinetic and other biochemical/biophysical methods to explore the mechanistic diversity of the Fe/2OG enzymes by studying how they direct the conserved ferryl intermediate to yield different outcomes. Two alternative reactivities were examined in this work: (Chapter 2) the hydroxylation of an $sp^2$-hybridized carbon by xanthine hydroxylase (XanA), a reaction expected to deviate from the canonical substrate-to-ferryl HAT paradigm; and (Chapter 3) the redox-neutral (with respect to the substrate) epimerization and putative 1,2-dehydrogenation reactions catalyzed by carbapenem synthase (CarC), transformations required for production of carbapenem antibiotics in bioengineered systems.
Chapter 2

Mechanism of hydroxylation of the $sp^2$-hybridized carbon 8 of xanthine by XanA
This chapter examines the alternative reaction catalyzed by XanA, an Fe/2OG enzyme that hydroxylates C8 of xanthine to produce uric acid. HAT from C8 to the ferryl complex and HO• coupling to the C8 radical has been proposed for the XanA reaction but would require cleavage of a very strong C-H bond to the \( sp^2 \)-hybridized target carbon (homolytic bond dissociation energy of \( \sim 113 \) kcal/mol). By \(^{18}\)O-tracer experiments and analysis of outcomes with substrate analogs and isotopologues, we provide evidence that the XanA reaction instead proceeds by a polar mechanism initiated by the nucleophilic attack of the \( \pi \) system upon the electrophilic ferryl complex. This mechanism is consistent with those proposed for transformations of trigonal carbon centers by other \( O_2 \)-activating iron enzymes, including the pterin-dependent aromatic amino acid hydroxylases.

### 2.1 Introduction

An Fe/2OG hydroxylase, xanthine hydroxylase (XanA), from \textit{Aspergillus nidulans} catalyzes hydroxylation of C8 of xanthine to produce uric acid.\(^{29}\) Although a mechanism initiated by the canonical HAT from C8 to the ferryl complex has been proposed [denoted hereafter C(\( sp^2 \))-HAT],\(^{30}\) this mechanism seems unlikely in view of the very high homolytic bond dissociation energy of this C(\( sp^2 \))-H bond (approximately 113 kcal/mol) in comparison to typical C(\( sp^3 \))-H bonds (< 100 kcal/mol).\(^{31}\) We therefore formulated three alternative mechanisms through which the XanA-catalyzed reaction could potentially proceed (Scheme 2.1.1). These involve initiation by (i) attack upon C8 of xanthine by an enzyme or solvent nucleophile followed by HAT from the transiently \( sp^2 \)-hybridized C8 to the ferryl complex.
[denoted C(sp³)-HAT], (ii) HAT from N7 or N9 to the ferryl complex (N-HAT), and (iii) attack of the substrate π-system on the electrophilic Fe(IV)=O unit (EAS).

Scheme 2.1.1. Plausible mechanisms for reaction catalyzed by XanA. **Top left:** HAT from sp²-hybridized C8. **Bottom left:** nucleophilic attack on C8 of xanthine, yielding an C(sp³)-H, which then undergoes the canonical HAT to the ferryl complex. **Top right:** HAT from N7 or N9 to the ferryl complexes. **Bottom right:** attack of the substrate π-system on the electrophilic Fe(IV)=O.

In this study, we attempted to monitor directly the kinetics of formation and decay of the ferryl complex in order to test for a substrate deuterium KIE associated with cleavage of the C8-H bond (substrate labeling) or the solvent-exchangeable N7-H or N9-H bond (in ²H₂O). Observation of such an effect would have identified the H• donor to the ferryl complex in the case of a HAT-initiated mechanism. However, we obtained evidence that decay of the ferryl complex is fast relative to its formation, precluding direct detection in either stopped-flow absorption (SF-abs) or freeze-quench Mössbauer experiments. In addition, the fast reaction of the ferryl complex apparently prevents exchange of the oxo group with solvent in hydroxylation of
either all-protium xanthine or its $^2$H-containing isotopologues. $^2$H-substitution also did not significantly uncouple the XanA-catalyzed reaction. In addition, data obtained from alternative substrates studies suggested that XanA can hydroxylate 8-methylxanthine (8-CH$_3$-xan) and 7-methylxanthine (7-CH$_3$-xan). These results rule out the proposed C(sp$^2$)-HAT mechanism and, instead, are consistent with an electrophilic aromatic substitution (EAS) mechanism, in which the substrate π-system attacks the electrophilic ferryl intermediate. This mechanism, which is similar to that proposed for the pterin-dependent aromatic amino acid hydroxylases,$^{32,33}$ is quite distinct from that initiated by the canonical HAT step, thus expanding the known mechanistic repertoire of this versatile enzyme family and its potent high-valent-iron intermediate.

2.2 Experimental Results

2.2.1 $^2$H-substitution does not lead to observable $^{18}$O-washout

Previous studies on the Fe/2OG oxygenases have established large substrate $^2$H-KIEs on decay of the ferryl complexes. As two proposed mechanisms, C(sp$^2$)-HAT and C(sp$^3$)-HAT, involve HAT from C8 of xanthine to the ferryl species, it was anticipated that substitution of C8 with $^2$H would slow decay of the intermediate, should one of these two mechanisms be operant. To test for this effect, the reactions of XanA with natural-isotopic-abundance xanthine (referred to hereafter as xan) and 8-$[^2]$H-xanthine (referred to hereafter as 8-[$^2$H]-xan) in natural-abundance solvent were examined via stopped-flow absorption (SF-abs) experiments. The absorbance at 320 nm, used in previous studies to track the H•-abstracting ferryl intermediates,$^{13,14}$ showed no transient behavior in either reaction (Figure 2.5.1), implying that
the ferryl complex either does not accumulate or accumulates and subsequently decays too fast relative to the SF timescale to be detected.

In a complementary approach, $^{18}$O$_2$-tracer experiments were carried out to measure the effect of $^2$H-substitution on the lifetime of the ferryl complex. On the basis of the SyrB2 precedent, it was anticipated that a substrate $^2$H-KIE might significantly extend the lifetime of the intermediate, enhance solvent exchange of the ferryl oxygen, and thus diminish the fraction of $^{18}$O incorporated into the uric acid. The XanA reactions were initiated with xan, $[8$-$^2$H]-xan, or xanthine in ~85% deuterated solvent (effectively $^2$H-substituting the exchangeable N7 and N9 positions, referred to hereafter as $7/9$-$[^2$H]-xan) in the presence of excess $^{18}$O$_2$ (Figure 2.1.1.1). The extent of incorporation of the $^{18}$O-label into succinate and uric acid was quantified by liquid chromatography with mass-spectrometric detection (LC-MS). Because decarboxylation of 2OG to succinate proceeds with 100% incorporation of an $O_2$-derived O-atom, the ratio of $^{18}$O-enriched succinate ($m/z = 119$) to succinate ($m/z = 117$) reflects the initial isotopic purity of the $^{18}$O$_2$ in the reaction solution (which is diminished from 100% by both isotopic heterogeneity of the source gas and incomplete removal of atmospheric $^{16}$O$_2$). The ratio was found to be 0.99:0.01 for each substrate. Substrate oxidation (to produce uric acid) proceeds through ferryl-mediated chemistry and, therefore, washout would be detectable by a ratio of $^{18}$O-enriched uric acid ($m/z = 169$) to uric acid ($m/z = 167$) diminished from the value for succinate. However, the ratio measured was 0.98:0.02 for each substrate, signifying that washout of the ferryl oxo group does not occur to a significant extent under any of these three conditions. The observations do not permit a HAT mechanism to be ruled out, but rather imply that, even with the potentially H•-donating positions of the substrate substituted with $^2$H, substrate hydroxylation by the ferryl complex is still fast relative to exchange of its oxo group with solvent. This observation, together
with precedent showing that the large $^2$H-KIE often does permit solvent exchange to compete with substrate oxidation, weighs against the HAT mechanisms.

Figure 2.2.1.1. LC/MS traces from analysis of the XanA reaction under an $^{18}$O$_2$ atmosphere with a limiting quantity of xan, 8-$^{2}$H]-xan, or 7/9-$^{2}$H]-xan. Traces correspond to the $m/z$ values indicated and correspond to xanthine (green), $^{18}$O-enriched xanthine (blue), succinate (orange), and $^{18}$O-enriched succinate (red). The MS analysis used electrospray ionization in the negative mode.

2.2.2 $^2$H-substitution does not significantly uncouple XanA-catalyzed reaction

An Fe/2OG dioxygenase couples decarboxylation of 2OG (which produces succinate and CO$_2$) to oxidation of its primary substrate. For the case of the native substrate, these two processes are often tightly coupled, resulting in an experimental 2OG:primary-substrate consumption stoichiometry of precisely unity. However, when challenged by a poor, non-native
substrate or, for the case of reactions initiated by HAT, an appropriately deuterium-labeled primary substrate, its processing can become less efficient (uncoupled) as a result of events in which the ferryl fails to abstract $^2$H and instead decays by another pathway. For the latter case, observation of this uncoupling diagnoses HAT from the deuterium-substituted position to the ferryl complex. The coupling ratios for xan, N7/9-[2H]-xan, and 8-[2H]-xan were quantified by LC-MS analysis of XanA-catalyzed reactions carried out with varying and limiting 2OG and excess primary substrate and O$_2$ (Figure 2.2.2.1). This ratio is close to unity for all substrates (1.00 ± 0.09 for xan, 1.1 ± 0.1 for 8-[2H]-xan, and 0.96 ± 0.04 for N7/9-[2H]-xan). Thus, both 8-[2H]-xan and 7/9-[2H]-xan are consumed as efficiently as the native substrate. Again, this result does not rule out a HAT-initated mechanism, but provides no evidence in favor of one of these three possibilities.

![Figure 2.2.2.1](image)

Figure 2.2.2.1. Determination of coupling efficiencies for the substrates xan (red), 8-[2H]-xan (blue), and 7/9-[2H]-xan (green). In each case, the primary substrate and O$_2$ were in excess over 2OG, which was the limiting reactant. Data points were fit by the equation for a line. The
coupling ratios (slopes) were close to unity for all substrates (1.05 ± 0.09 for xan, 1.1 ± 0.1 for 8-[\textsuperscript{2}H]-xan, and 0.96 ± 0.04 for 7/9-[\textsuperscript{2}H]-xan).

2.2.3 XanA hydroxylates 8-methylxanthine at C8

XanA should not be able to hydroxylate the xanthine analog, 8-CH\textsubscript{3}-xan, by a C(sp\textsuperscript{2})-HAT or C(sp\textsuperscript{3})-HAT mechanism, because the methyl group on C8 replaces the H-atom that would have to be abstracted. By contrast, hydroxylation of C8 by N-HAT or EAS might still proceed in the presence of the methyl substituent. However, following oxygen transfer, the methyl group would be expected to prevent the final step of the normal hydroxylation reaction, deprotonation and rehybridization of the transiently sp\textsuperscript{3}-hybridized, hydroxylated C8. In the reaction with XanA, excess 2OG and O\textsubscript{2}, and limiting 8-CH\textsubscript{3}-xan, complete consumption of the xanthine analogue was observed. Quantitative analysis of substrate consumption in reactions with limiting 2OG showed that oxidation of the analogue is as tightly coupled (8-CH\textsubscript{3}-xan consumed:succinate produced = 1.03 ± 0.09) as for the native substrate, xanthine (Figure 2.5.2). Analysis by LC-MS (Figure 2.5.3) revealed that the major product of the reaction has m/z = 183, corresponding to addition of 18 amu to the substrate, rather than the +16 amu expected for a simple hydroxylation. A minor peak at m/z = 181 corresponds to addition of 16 amu to the substrate, but the associated product was seen to decompose during the LC/MS analysis. Analogously, upon reaction of XanA with 8-CD\textsubscript{3}-xan under the same conditions, the major product with m/z = 186 was stable over the timescale of the analysis, whereas the minor product with m/z = 184 was observed to decompose. The +3 shift in the m/z of both products upon use of the methyl-deuterated analogue confirms that both products are derived from the 8-CH\textsubscript{3}-xan and
also demonstrates that the methyl group remains entirely intact in both. High-resolution mass-spectrometric analysis by the Proteomics and Mass Spectrometry Core Facility at The Pennsylvania State University of the stable, major species afforded an accurate determination of its molecular mass, \([\text{M-H}]^+ = 183.0533 \pm 0.01\) amu. This mass matches that predicted for the molecular formula \(\text{C}_6\text{H}_8\text{N}_4\text{O}_3\), which reflects addition of one oxygen and two hydrogen atoms to the substrate analogue. This addition could most simply result from hydrolysis of the starting material; however, further characterization of the major product by a combination of \(^{18}\text{O}\)-tracer experiments and \(^{13}\text{C}\)-NMR spectroscopy (described below) revealed that it instead forms by a ring-opening oxygenation of C8 followed by a spontaneous reduction (formal hydrogenation) of the di-iminoquinone initial product.

To test whether the major (+18, \(m/z = 183\)) product results from a complex transformation initiated by oxygenation of 8-CH\(_3\)-xan, or, alternatively, by its simple hydrolysis, XanA reactions with the analogue were carried out in the presence of \(^{18}\text{O}_2\) (Figure 2.2.3.1). LC-MS analysis of this reaction revealed a prominent peak at \(m/z = 185.0\), corresponding to addition of 20 amu to the substrate, as well as a significantly less intense (relative intensities of 0.96:0.04) peak at \(m/z = 183.0\), corresponding to addition of 18 amu to the substrate. This +2 shift in \(m/z\) upon use of \(^{18}\text{O}_2\) proves that the 8-CH\(_3\)-xan analogue undergoes an authentic oxygenation reaction, with incorporation of one atom of O\(_2\) into the product, rather than a hydrolysis reaction.
Figure 2.2.3.1. LC-MS analysis of the XanA reaction with limiting 8-methylxanthine under an
$^{16}\text{O}_2$ or $^{18}\text{O}_2$ atmosphere in the absence or presence of excess 2OG. The traces correspond to 8-
methylxanthine (green), the $m/z = 181$ minor product (orange), the $m/z = 183$ major product
formed under $^{16}\text{O}_2$ (blue), and the $m/z = 185$ $^{18}\text{O}$-enriched product generated under $^{18}\text{O}_2$ (red).

To identify the site of oxygenation, [8-$^{13}\text{C}$]-8-methylxanthine (8-[${}^{13}\text{CH}_3$]-xan), was
prepared, and its transformation by XanA was monitored by $^{13}\text{C}$-NMR spectroscopy. A signal
corresponding to the 8-$^{13}\text{C}$ nucleus at 158.071 ppm was observed in the O$_2$-free reaction solution
containing 8-[${}^{13}\text{CH}_3$]-xan, excess 2OG, and XanA (Figure 2.2.3.2, blue). Upon exposure to
excess O$_2$, the signal corresponding to the 8-$^{13}\text{C}$ nucleus was observed to shift to 175.467 ppm
(Figure 2.2.3.2, red). This new chemical shift is consistent with known values for amide carbonyl
carbons, confirming that C8 undergoes oxygenation by XanA. In addition, heteronuclear
multiple bond correlation (HMBC) spectra showed that the correlation between the 8-$^{13}\text{C}$ nucleus
and protons on the 8-methyl group is retained in the product (Figure 2.2.3.2, red). This
observation implies that the 8-methyl group remains intact, consistent with the aforementioned 
LC-MS analysis of the products with the **8-CD₃-xan** isotopologue.

![Diagram](image)

Figure 2.2.3.2. Heteronuclear multiple bond correlation (HMBC) spectra of the XanA reaction 
with excess [8-¹³C]-8-methylxanthine and 2OG prior to (blue) and after (red) exposure to O₂.

### 2.2.4 The initial oxygenation product of 8-methylxanthine undergoes reduction

Formation of the ring-opened, amide-containing product following oxygenation of **8-CH₃-xan** at C8 requires a subsequent two-electron reduction, formally a hydrogenation, after the 
initial two-electron oxidation. The effect on the outcome of including the reductant, ascorbate, 
in the reaction is consistent with this pathway. The presence of ascorbate was observed to 
diminish accumulation of the minor m/z = 181 product and enhance accumulation of the major 
m/z = 183 product (Figure 2.5.4). As ascorbate can provide two reducing equivalents, this
observation is consistent with a major product that results from hydroxylation following by reduction.

### 2.2.5 XanA hydroxylates 7-methylxanthine

The above results strongly disfavor both C\(sp^2\)-HAT and C\(sp^3\)-HAT mechanisms. To distinguish which of the two remaining mechanisms (N-HAT or EAS) is operant, the reaction of XanA with 7-CH\(_3\)-xan was interrogated by LC-MS product analysis. This analogue lacks an abstractable hydrogen at either the N7 or N9 position; it should therefore be incompetent for hydroxylation by an N7/9-HAT mechanism. In the complete reaction with excess 2OG and O\(_2\), full consumption of the analogue was observed, and a primary product with \(m/z = 181.0\), +16 with respect to the value for 7-CH\(_3\)-xan, was detected. The result suggested that the analogue is oxidized by XanA, most likely to 7-methyluric acid (Figure 2.2.5.1).
Figure 2.2.5.1. LC/MS traces (scaled for clarity) of XanA reaction with a limiting amount of 7-methylxanthine in the presence and absence of excess 2OG. Traces correspond to specific m/z values and are assigned to specific compounds as follows: 7-methylxanthine (red), 7-methyluric acid (green), xanthine (orange), and uric acid (blue).

Indeed, this enzymatic product co-eluted with a commercial 7-methyluric acid standard obtained from Toronto Research Chemicals (Figure 2.5.5). In addition to the primary 7-methyluric acid product, two additional products were observed in this reaction. The m/z values of the minor products suggested that they are xanthine and uric acid, likely arising from sequential N-demethylation of 7-CH₃-xan to xanthine followed by hydroxylation of xanthine to uric acid (i.e., the native XanA reaction). Quantification of 7-methyluric acid produced in reactions with limiting 7-CH₃-xan revealed that more than 50% of substrate consumed is converted to 7-methyluric acid. The reaction is reasonably well-coupled (0.73 ± 0.06) albeit somewhat less so that the native xanthine substrate (Figure 2.5.2).
2.3 Discussion

The Fe/2OG oxygenase, XanA, catalyzes hydroxylation of the \( sp^2 \)-hybridized C8 of xanthine to produce uric acid. A mechanism involving HAT from C8 \([C(sp^2)\text{-HAT}]\) was proposed, but we considered it to be unlikely in view of the relatively high homolytic bond dissociation energy (~113 kcal/mol) of the C8-H bond. Envisioning three other mechanisms \([C(sp^3)\text{-HAT, N-HAT, and EAS}]\) that could also account for the XanA reaction, we sought to determine which one is operant.

A large substrate \(^2\text{H}\)-KIE on ferryl decay is expected for \( C(sp^2)\text{-HAT, C(sp^3)\text{-HAT, and N-HAT mechanisms, on the basis of previous studies on other mononuclear non-heme-iron enzymes that cleave C-H bonds.}^{2,14,17-20} \)

It was thus anticipated that deuterium-substitution of xanthine might elicit one or more of three well-precedented effects on ferryl-mediated, HAT-initiated oxidations: promotion of ferryl accumulation,\(^2,13,14,17\) enhanced incorporation of solvent-derived oxygen into the hydroxylated product as a result of the increased time for washout of the ferryl oxo ligand,\(^19\) and uncoupling of 2OG decarboxylation from substrate oxidation.\(^2,17\) None of these effects was observed. Attempts to detect the intermediate by its expected UV absorption feature at ~320 nm, a feature seen for all analogous complexes detected to date, were unsuccessful, suggesting that the presumptive ferryl intermediate fails to accumulate, even in reactions with deuterium present at any of the possible target positions. Reaction of XanA with \( \text{xan, 7/9-[}^2\text{H}\text{-xan, or 8-[}^2\text{H}\text{-xan under an atmosphere of } \sim99\% \text{ }^{18}\text{O}_2 \text{atmosphere gave nearly complete incorporation of }^{18}\text{O-label into the uric acid product (i.e., no washout). Lastly, coupling ratios for } \text{xan, 7/9-[}^2\text{H}\text{-xan, and 8-[}^2\text{H}\text{-xan were all within experimental error of unity, } 1.0 \pm 0.09, 1.1 \pm 0.1, \text{ and } 0.96 \pm 0.04, \text{ respectively. The absence of these three anticipated} \)}
effects was considered to weigh against a HAT-initiated mechanism and to favor the EAS mechanism. However, an alternative possibility remained – that XanA is simply unusually efficient in the HAT step, even when challenged by the heavier deuterium.

To provide clearer evidence for the EAS mechanism, we turned to two substrate analogues, 8-CH₃-xan and 7-CH₃-xan, which are both expected to be incompetent for a subset of the conceivable, HAT-initiated mechanisms. Hydroxylation of the C8 position of 8-CH₃-xan by XanA cannot conceivably occur through an C(sp²)-HAT or C(sp³)-HAT mechanism, because the methyl group replaces the target hydrogen. Similarly, 7-CH₃-xan lacks an abstractable N-bonded hydrogen required for the N-HAT mechanism. The major product of the reaction of XanA with 8-CH₃-xan results, formally, from addition of H₂O to the substrate. However, result of the ¹⁸O-tracer experiment with ¹⁸O₂ proved that this outcome emerges from an initial oxygenation event. As with the native xanthine, oxygen incorporation occurs at C8, as established by the ¹³C-NMR results. The chemical shift of the ¹³C₈ nuclei in the product matches the value expected for an amide species, and the HMBC data and LC-MS result on the methyl-deuterium-labeled isotopologue establish that the 8-methyl group is intact in the product. The amide likely results from hydroxylation followed by ring-opening of the oxygenated intermediate, which cannot, unlike the corresponding intermediate of the native hydroxylation, re-hybridize by simple C8 deprotonation. The resulting ring-opened, di-iminoquinone species is expected to be oxidizing and, accordingly, undergoes two-electron reduction from an unknown source. The effect of the reductant, ascorbate, which suppresses the m/z = +16 species and enhances the +18 product, confirms the involvement of a reduction step after the initial oxidation event. The fact that oxidation of 8-CH₃-xan by XanA is tightly coupled to 2OG decarboxylation (coupling ratio of 1.03 ± 0.09) implies that the presence of the non-native methyl substituent
does not impede the oxygenation step enough to allow unproductive pathways for ferryl decay to compete, providing argument that the analogue and native substrate react by a common mechanism and ruling out the C($sp^2$)-HAT and C($sp^3$)-HAT mechanisms. The observation of still relatively efficient (coupling ratio of ~0.7) oxidation of 7-CH$_3$-xan to the expected primary product as well as two readily understood side products (which complements a previous report that 9-methylxanthine is also an active substrate) allows the third possible HAT mechanism (N-HAT) also to be ruled out. Together, the results thus converge on the EAS mechanism as the only consistent possibility.

2.4 Conclusion

The results of isotope-tracer experiments and quantitative analysis of products with substrate analogues and isotopologues contradict the previously proposed C($sp^3$)-HAT mechanism for XanA and imply, instead, that an electrophilic (EAS) mechanism is operant. This mechanism is analogous to that proposed for the pterin-dependent aromatic amino acid hydroxylases$^{32,33}$ in its use of a ferryl intermediate as a potent electrophile. The defining variation between these enzymes is the identity of the cosubstrate (2OG in the case of XanA and tetrahydrobiopterin in the case of the aromatic amino acid hydroxylases). The EAS mechanism deviates from the HAT-initiated mechanisms of the more well-studied Fe/2OG oxygenases, thus expanding the known repertoire of this versatile enzyme class. The principal finding of this study speaks to the general problem of functionalizing $sp^2$-hybridized carbon centers, which in most cases would yield radical intermediates too high in energy to be accessible even to the very potent reactivity associated with high-valent metal-oxo complexes. The results leave open the
question of whether such transformations ever involve direct generation of a substrate radical by HAT from an $sp^2$-hybridized carbon center.

### 2.5 Supporting Information

![Figure 2.5.1. Absorbance (320 nm) versus time traces obtained after O$_2$-saturated buffer (100 mM Tris, pH 7.5, 4 °C) was mixed with an equal volume of an O$_2$-free solution containing 1mM XanA, 0.8 mM Fe(II), 5 mM 2OG, and 5 mM xan (blue) or 8-[${^2}$H]-xan (red).](image)
Figure 2.5.2. Efficiencies of coupling of succinate production and substrate oxidation
determined with excess xan (red), 8-CH₃-xan (blue), or 7-CH₃-xan (green) and varying limiting
concentrations of 2OG. Data points were fit by the equation for a line. The coupling ratios
(slopes) were close to unity for all substrates (1.05 ± 0.09, 1.03 ± 0.09, and 0.73 ± 0.06,
respectively).
Figure 2.5.3. LC/MS traces of XanA reaction with a limiting amount of 8-CH$_3$-xan or 8-CD$_3$-xan in the absence or presence of excess 2OG. Traces correspond to specific m/z values and are assigned to specific compounds as follows: 8-CH$_3$-xan (red), 8-CD$_3$-xan (purple), m/z = 183 product (green), and m/z = 186 product (red).

Figure 2.5.4. LC/MS traces of XanA reaction with a limiting amount of 8-CH$_3$-xan in the presence or absence of excess ascorbate and/or 2OG. Traces correspond to specific m/z values
and are assigned to specific compounds as follows: \( m/z = 181 \) product (red) and \( m/z = 183 \) product (blue).

Figure 2.5.5. LC/MS trace of product (\( m/z = 181 \)) yielded upon reaction of XanA with a limiting amount of 7-CH₃-xan and excess 2OG (blue). LC/MS trace (\( m/z = 181 \)) of coinjection of aforementioned product with ~2 equivalents of authentic 7-methyluric acid standard (red).
Chapter 3

Mechanistic dissection of

CarC, epimerase and putative 1,2-dehydrogenase
This chapter examines the alternative reactions catalyzed by the Fe/2OG enzyme, CarC. The experimental results presented and discussed herein constitute significant progress towards elucidating the mechanism by which CarC catalyzes epimerization and 1,2-dehydrogenation. Initial characterization of CarE, a ferredoxin in the (S)-carbapenem biosynthesis pathway is also described.

3.1 Introduction

Carbapenems are members of the β-lactam class of antibiotics, arguably the most important class in clinical use today. These antibiotics have a very broad spectrum of effectiveness and are also resistant to most clinically-relevant β-lactamases. Carbapenem synthase (CarC) catalyzes the C5 epimerization of the simplest carbapenam (which must occur prior to further elaboration). This transformation is necessary because the CarC substrate, (3S,5S)-carbapenam, has opposite stereochemistry at the C5 position to that of any known carbapenem drug compounds.

Although carbapenem natural products have been isolated from bacteria such as *Streptomyces cattleya* and *Pectobacterium carotovorum*, large-scale isolation for drug production remains plagued by low yields and lengthy purification processes. As a result, the current clinically-relevant carbapenems are produced by total synthesis. However, the introduction of multiple chiral centers by chemical synthesis can be both challenging and costly (in terms of both time and money). Given the challenges associated with fermentation and synthetic preparation, bioengineering could be the key to development of time- and cost-efficient production of known carbapenems, as well as engendering routes to new non-natural derivatives.
with potential as anti-microbial agents. However, such biotechnological exploitation will only be possible with a robust understanding of the natural biosynthetic pathways of carbapenems.

In 1976, the first carbapenem compound, thienamycin, was isolated from *Streptomyces cattleya*. Six years later, the simplest carbapenem, (5R)-carbapenem was isolated from *Pectobacterium carotovorum* and, in 1996, the car biosynthetic gene cluster was cloned and sequenced (Figure 3.1.1). Additional studies confirmed that this cluster is conserved in all (5R)-carbapenem-producing strains.

![Figure 3.1.1. The car gene cluster present in Pectobacterium carotovorum.](image)

In 2000, Townsend and coworkers transformed the entire car gene cluster into *Escherichia coli* and observed production of (5R)-carbapenem. Mutation of *carA*, *carB*, or *carC* eliminated (5R)-carbapenem production, while disruption of *carD* or *carE* still allowed for production of (5R)-carbapenem, albeit at substantially diminished levels. This study prompted *in vitro* studies on the three enzymes necessary for (5R)-carbapenem production. CarB, using malonyl–CoA as cosubstrate, attaches a carboxymethylene unit to 1-pyrroline-5-carboxylate to form trans-5-carboxymethyl-L-proline. CarA then uses adenosine 5′-triphosphate hydrolysis to drive β-lactam ring closure, generating (3S,5S)-carbapenam (Scheme 3.1.1).
However, the stereochemistry established at C5 by CarB is opposite to that of all natural carbapenem antibiotics isolated to date. Therefore, further elaboration of (3S,5S)-carbapenam requires C5 stereoinversion by CarC, which produces (3S,5R)-carbapenam as its initial product.

3.2 Experimental Results

3.2.1 CarC epimerizes (3S,5S)-carbapenam

The mechanism of C5-stereoinversion was definitively established using a combination of rapid kinetic and spectroscopic techniques including: stopped-flow absorption spectroscopy, freeze quench-Mössbauer, freeze quench-EPR, chemical-quench LC-MS, and crystallography. Overall, these results established that CarC employs a ferryl intermediate for HAT from C5 of
(3S,5S)-carbapenam. Following abstraction, a tyrosine residue (Y165) donates a hydrogen atom to the opposite face of the stereocenter, producing the stereoinverted product and a tyrosyl radical. In addition, the reaction oxidizes the Fe(II) cofactor to Fe(III), limiting wild-type CarC to one turnover (Scheme 3.2.1.1).²

Scheme 3.2.1.1. Mechanism of the CarC-mediated stereoinversion reaction, involving HAT from the C5 of (3S,5S)-carbapenam followed by hydrogen atom donation by Y165 to the substrate radical.

3.2.2 Progress towards characterizing the effect of chemical and biological reductants

Our prior study² revealed that the reaction of the CarC with (3S,5S)-carbapenam and O₂ is stoichiometric, rather than catalytic. The CarC enzyme is arrested in the two-electron-oxidized state having a high-spin Fe(III) center and the Y165• tyrosyl radical. To initiate a second turnover, CarC must return to the O₂-reactive, Fe(II)- and Y165 tyrosine-containing form, which requires two reducing equivalents.

Therefore, we evaluated the effect of reductants on the reaction. First, we investigated the effect of the chemical reductant, ascorbate, which is routinely included in steady-state assays of many other Fe/2OG oxygenases. Its role is thought to be the ability to reduce the catalytically inactive Fe(III) center, which can be formed in the uncoupled reaction of the enzyme in the
absence of the primary substrate, to the active Fe(II) form. When ascorbate is included in the reaction of the CarC complex with (3S,5S)-carbapenam and O₂, the kinetics of tyrosyl radical formation and decay are not perturbed, although the amplitude of the signal associated with the Y165• is diminished by approximately 15-20%. We tentatively concluded that ascorbate cannot function as the reductant to regenerate the active form of CarC and that the active form of CarC is regenerated by another reductant.

Bioinformatic analysis of the car gene cluster revealed a potential candidate for the reductant. The carE gene encodes a protein (CarE) that is annotated as a [2Fe-2S]-containing ferredoxin. Prior to this work, CarE had never been expressed, purified, or characterized in any manner. We obtained the synthesized carE gene, which was codon-optimized for expression in E. coli and appended with an N-terminal His₆-tag, overexpressed it in E. coli, and purified it by immobilized metal affinity chromatography using Ni(II) NTA resin.

Upon purification of CarE under strict anaerobic conditions, it retains ~1.4 Fe per CarE (70% of the theoretically expected 2 Fe/CarE) and displays a UV/visible absorption spectrum reminiscent of a [2Fe-2S] cluster, a broad band at ~400 nm and a shoulder at ~460 nm (Figure 3.2.2.1). It should be noted that cluster content in this case was estimated based on correlation of protein concentration (measured by a Bradford assay) and iron content (measured by a ferrozine assay). We have not verified the cluster type and stoichiometry; therefore, these values were used mainly as a means of method development rather than analysis.
Figure 3.2.1.1. UV/Visible absorption spectrum of CarE, exhibiting features at ~400 nm and ~460 nm.

Upon exposure of [2Fe-2S]-containing CarE to O₂, the hallmark features associated with the [2Fe-2S] cluster disappear, suggesting that the cluster is not stable in the presence of O₂. In order to further confirm this result, a small fraction of the elution from the Ni(II) NTA resin (purified anaerobically) was divided into two Eppendorf tubes. One sample was exposed to air for 10 min while the other sample tube remained sealed. After incubation overnight at 4 °C, an obvious color change was observed (Figure 3.2.2.2). The sample that had been exposed to air was colorless while the sealed sample remained brown, indicating that oxygen exposure likely leads to degradation of the CarE cluster.
Figure 3.2.2.2. On the left, anaerobically purified CarE. On the right, anaerobically purified CarE either exposed to air for 10 min or kept anaerobic. Both samples were then incubated overnight at 4 °C.

3.2.2.1 Effect of CarE on carbapenem production: summary and future work

Unfortunately, much of our analysis to date has been thwarted by CarE instability. CarE, upon exposure to oxygen (an essential cosubstrate in the CarC-catalyzed reaction), releases iron into solution, which can be scavenged by CarC for additional turnovers (thereby obscuring experimental results). This instability impedes studies of the effect of reduced CarE on the ability of CarC to perform multiple turnovers, but future work is aimed at circumventing this issue by studying the effect of CarE on the CarC reaction under single turnover conditions. Specifically, we will perform a “double-mix” stopped-flow absorption-spectroscopic experiment, in which the CarC:Fe(II):2OG:(3S,5S)-carbapenam complex will be mixed first with a limiting amount of O₂ for ~10 s (the time of maximum accumulation of the Y165•) and then mixed with reduced CarE.

The effect of CarE on the reaction will be monitored by stopped-flow absorption spectroscopy (increase in the absorption due to oxidation of the [2Fe-2S]⁺ to the [2Fe-2S]²⁺ form and decay of Y165•), freeze-quench EPR spectroscopy (disappearance of the signatures of the
[2Fe-2S]$^+$ cluster on CarE and of the Y165• and Fe(III) cofactors on CarC), and Mössbauer spectroscopy (reduction of Fe(III) to Fe(II) on CarC). In addition to functional characterization, future work may also include characterization of a potential protein-protein interaction between CarC with CarE by gel filtration chromatography, native gel electrophoresis, and crystallography.

**3.2.3 Progress towards characterization of the 1,2-dehydrogenation reaction**

Published data have suggested that, after inverting C5, CarC then undergoes a second round of O$_2$ activation and ferryl formation to effect C2-C3 1,2-dehydrogenation. This second turnover is necessary because both the configuration at C5 and the C2=C3 double bond of (5R)-carbapenem have been demonstrated to be crucial for optimal antibiotic activity.$^{42,43}$

The (3$S$,5$R$)-carbapenam intermediate was used to probe the putative 1,2-dehydrogenation reaction to yield (5R)-carbapenem. Analysis of the products of the reaction of the CarC complex with (3$S$,5$R$)-carbapenam and O$_2$ were analyzed by LC/MS. The anticipated $\Delta(m/z)$ shift of -2 indicative of 1,2-dehydrogenation was not observed. Rather, a product with $\Delta(m/z)$ of +16 was detected. At first glimpse, this observation might indicate the canonical hydroxylation outcome. However, this $\Delta(m/z)$ shift is also consistent with 1,2-dehyrogenation, followed by spontaneous hydrolysis of the beta-lactam ring (Scheme 3.2.3.1). Indeed, Townsend and co-workers, as well as others reported that the (5R)-carbapenem product is unstable.$^{37,44}$
Scheme 3.2.3.1. Potential pathways to generate an $\Delta(m/z)$ of +16 product: canonical hydroxylation or 1,2-dehydrogenation, followed by spontaneous beta-lactam ring hydrolysis.

Experiments aimed at distinguishing between hydroxylation and 1,2-dehydrogenation/$\beta$-lactam hydrolysis involving specifically labeled reagents ($^{18}\text{O}_2$ as cosubstrate and $\text{H}_2^{18}\text{O}$ as solvent) were performed. Analysis of the products of the reaction of the CarC complex with limiting (3S,5S)-carbapenam and excess ascorbate under ~98% $^{18}\text{O}_2$ atmosphere were analyzed by LC/MS. A large peak ($m/z$ 170) corresponding to $^{16}\text{O}$-enriched product was observed, whereas no appreciable peak corresponding to $^{18}\text{O}$-enriched product ($m/z$ 172) was observed (Figure 3.2.3.1). This indicates that either the product observed does not result from ferryl-mediated hydroxylation or that 100% washout of the $^{18}\text{O}$-label occurs over the timescale of hydroxylation.
Figure 3.2.3.1. LC/MS traces of CarC reaction initiated in ~98% \(^{18}\)O\(_2\) atmosphere with limiting amount of (3S,5S)-carbapenam and excess ascorbate. Traces correspond to specific \(m/z\) values and are assigned to compounds as follows: \(^{18}\)O-enriched product (green) and \(^{16}\)O-enriched product (orange).

A complimentary experiment was carried out upon reaction of the CarC complex with (3S,5S)-carbapenam, ascorbate, and \(O_2\) in ~80% H\(_2\)\(^{18}\)O solvent. A major peak (\(m/z\) 172) corresponding to \(^{18}\)O-enriched product and a minor peak corresponding to \(^{16}\)O-enriched product (\(m/z\) 170) were observed, relative ratio 2:1 (Figure 3.2.3.2). This result indicates that the observed product likely results from 1,2-dehydrogenation/\(\beta\)-lactam hydrolysis, rather than hydroxylation, although additional experiments are needed to confirm this result.
Figure 3.2.3.1. LC/MS traces of CarC reaction initiated in ~80% H$_2^{18}$O with limiting amount of (3S,5S)-carbapenam and excess ascorbate. Traces correspond to specific $m/z$ values and are assigned to compounds as follows: $^{18}$O-enriched product (orange) and $^{16}$O-enriched product (green).

3.2.3.1 Characterization of the 1,2-dehydrogenation reaction: summary and future work

Experiments aimed at characterizing the product observed upon reaction of CarC complex with (3S,5S)-carbapenam, ascorbate, and O$_2$ initially suggest a 1,2-dehydrogenation/β-lactam hydrolysis, rather than hydroxylation, pathway. Future work is aimed at supporting this characterization, as well as mechanistic evaluation of the 1,2-dehydrogenation reaction.

Initially, $^{13}$C NMR studies will be carried on the reaction of CarC complex with (3S,5S)-carbapenam specifically labelled at C6 and C7 of the β-lactam ring. Under limiting substrate
conditions, the $^{13}$C chemical shifts observed should indicate hydrolysis (or lack thereof) of the β-lactam ring. Spectral analysis should yield information concerning the number of species formed.

In addition, future work may include synthesis and analysis of specifically C2- and C3-deuterated substrates. A (3S,5S)-carbapenam substrate deuterium-labelled at both C2 and C3 should, upon reaction with CarC complex and O$_2$ produce either a hydroxylated product Δ($m/z$) +16 and/or a 1,2-dehydrogenation/β-lactam hydrolyzed product Δ($m/z$) +14. This simplifies LC/MS product analysis because these two potential products are separated by one or two $m/z$ units (Scheme 3.2.3.1.1).

Scheme 3.2.3.1.1. Potential products of reaction of CarC complex with (3S,5S)-carbapenam deuterium-labelled at both C2 and C3: canonical hydroxylation or 1,2-dehydrogenation/hydrolysis.

Synthesis of the specifically C2- and C3-deuterated substrates shown in Figure 3.2.3.1.1 should also inform the mechanism of CarC-mediated 1,2-dehydrogenation by allowing for
determination of \(^2\text{H}\)-KIEs, as well as coupling ratios. We have recently shown in a related Fe/2OG 1,2-dehydrogenase, termed NapI, that such a detailed “coupling analysis” provides detailed insight into the steps leading to 1,2-dehydrogenation (unpublished work).

Figure 3.2.3.1.1. \((3S,5R)\)-carbapenam specifically deuterium-labelled at C2 (right) or C3 (left).
References


