ABSTRACT
Dicks, Justin Kyle. Molecular Oxygen Triggered Biocatalytic C-C Bond Formation: Probing Olefination Mechanism Catalyzed by a 2-Oxo-glutarate Dependent Non-Heme Enzyme: AsqJ. (Under the direction of Wei-chen Chang).

2-Oxo-glutarate dependent non-heme iron(II) enzymes are a large and complex family of enzymes capable of catalyzing a gamut of reactions. These enzymes are present in a number of life forms from humans and animals to bacteria and fungi. The enzyme family is interesting because of its ability to incorporate inert molecular oxygen into its reaction mechanism. While each enzyme mechanism will slightly differ based on the function, each biocatalyzed reaction is driven by the formation of a high-spin iron(IV) center within the enzyme active site. This iron(IV) center is what makes 2-oxoglutarate-dependent enzymes capable of catalyzing an array of reactions like halogenation, hydroxylation, desaturation, epoxidation, etc. While each enzyme has subtle differences in reaction, they are all share a common mechanism in the formation of the iron (IV) core.

Aspergillus nidulans (A. nidulans) is a fungus that is capable of producing viridicatin, a key 6,6-quinolone structural core found in a number of biologically active small molecules. Previous studies have revealed that AsqJ is a key enzyme involved in the formation of viridicatin. This particular 2-oxoglutarate-dependent enzyme is fascinating, due to its cascade mechanism that incorporates a desaturation, and an epoxidation that leads to the final product. Due to its relation to the 2-oxoglutarate-dependent enzyme family, AsqJ is thought to integrate the iron(IV) center into its mechanism. Previous studies have proposed an explanation to the desaturation of cyclopeptin, but remain experimentally untested. Herein describes the process to define olefination pathway of cyclopeptin to dehydro-cyclopeptin via AsqJ biocatalysis through implementation of mechanistic probes.
Molecular Oxygen Triggered Biocatalytic C-C Bond Formation: Probing Olefination Mechanism Catalyzed by a 2-Oxo-glutarate Dependent Non-Heme Enzyme: AsqJ

By
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A thesis submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Master of Science.

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Chair of Advisory Committee
DEDICATION

I dedicate this to my family. This experience would be meaningless without their love and support. I would like to specifically dedicate this to my nana, Peggy O’Neill, whom I know would be extremely proud.
BIOGRAPHY

Justin Dicks was born in Houston, Texas on April 27, 1993. His parents are John and Denise Dicks. He has one younger sister, Kaeley Dicks, who is currently enrolled as a sophomore at California Polytechnic State University and majors in mechanical engineering. In 1997, Justin and his family moved to Morgantown, West Virginia. He attended University High School and graduated in the spring of 2011. Justin then enrolled at Elmira College where he studied chemistry. While studying at EC, he was influenced to become involved in academic research by his mentor, Dr. Jared Baker. Justin graduated from Elmira College in 2015 with a Bachelor of Science degree in chemistry. After four years of undergraduate research, Justin decided to pursue further education. He enrolled in the Department of Chemistry at North Carolina State University where he studied under the supervision of Dr. Wei-chen Chang. Upon completion of his Master of Science degree in Chemistry, Justin will begin working at Burleson Research Technologies in Morrisville, North Carolina as a Senior Research Associate.
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TABLE OF CONTENTS

List of Figures.......................................................................................................................... vii
List of Tables................................................................................................................................. viii
List of Schemes............................................................................................................................... viii
Chapter 1: Introduction...................................................................................................................... 1
  1.1 Introduction of Mononuclear Non-Heme Iron(II) Enzymes....................................................... 1
  1.2 Introducing 2OG-Dependent Oxygenases................................................................................. 3
  1.3 C=C Bond Formation in Chemistry........................................................................................... 5
  1.4 C=C Bond Formation in Biology............................................................................................... 5
  1.5 A One-Pot Biocatalysis of Cyclopeptin to Viridicatin Using 2-OG dependent Oxygenase: AsqJ........................................................................................................................................... 7
  1.6 Chapter 1 References................................................................................................................ 11
Chapter 2: Materials and Methods.................................................................................................... 14
  2.1 Transformation of asqJ-containing Plasmid into E. coli Strains and Restriction Enzyme Digestion..................................................................................................................................................... 14
    2.2 Small Scale Over-Expression of AsqJ....................................................................................... 16
    2.3 Large Scale AsqJ Over-Expression......................................................................................... 19
    2.4 AsqJ Purification...................................................................................................................... 19
    2.5 Determining Iron Contamination via Ferrozine Assay............................................................ 22
    2.6 Optimizing a Liquid Chromatography/Mass Spectrometry (LC-MS) Method.............. 23
    2.7 AsqJ Biochemical Assay and Substrate Isotopic Distribution Determination................. 24
    2.8 AsqJ-Catalyzed One-Pot Epoxidation............................................................................... 26
    2.9 Chapter 2 References............................................................................................................ 28
Chapter 3: Results and Discussion..................................................................................................... 30
  3.1 Measuring Residual Fe(II) Concentration Using Ferrozine Assay........................................... 30
    3.1.1 Establishing Ferrozine Assay Standard Curve................................................................. 30
    3.1.2 Residual Fe(II) Concentration Measurement.................................................................. 31
  3.2 AsqJ-Catalyzed Olefination of both C3 Stereoisomers............................................................ 33
  3.3 Elucidate Desaturation Reaction Mechanism Using Substrate Isotopes............................ 34
3.4 AsqJ Substrate Specificity Confirmation of Both C3 Stereoisomers ...................... 37
3.5 Chapter 3 References ............................................................................................. 38
Chapter 4: Conclusions ............................................................................................. 40
  4.1 Summarizing of Mechanistic Findings .............................................................. 40
  4.2 Chapter 4 References ....................................................................................... 45
Appendix .................................................................................................................... 46
  Appendix A ............................................................................................................. 47
List of Figures

Figure 1.1: CAS and CarC Active Sites.......................................................... 3
Figure 1.2: AsqJ-Catalyzed Reaction of Cyclopeptin.................................... 7
Figure 1.3: Mechanistic Probes and Expected Products.............................. 9
Figure 2.1: AsqJ DNA Plasmid Map............................................................... 14
Figure 2.2: AsqJ-Plasmid Restriction Digestion........................................ 16
Figure 2.3: Small Scale AsqJ Over-Expression............................................. 18
Figure 2.4: Ni-NTA Affinity Column Purification Schematic.......................... 20
Figure 2.5: Purification of AsqJ from Large Scale Over-Expression............... 21
Figure 2.6: Optimized Retention Times of Compound Standards.................... 24
Figure 2.7: Schematic of AsqJ-Catalyzed Desaturation.................................. 26
Figure 2.8: Schematic of AsqJ-Catalyzed One-Pot Epoxidation...................... 26
Figure 2.9: LC-MS Chromatogram of Internal Standard, L-Tryptophan........... 27
Figure 3.1: Ferrozine Assay Standards......................................................... 31
Figure 3.2: Ferrozine Assay........................................................................... 32
Figure 3.3: LC-Chromatograms of Catalyzed Reactions using C3 Stereoisomers .................................................................................................................. 34
Figure 3.4: Isotopic Distributions of Deuterated Mechanistic Probes............. 35
Figure 3.5: Isotopic Distributions of Hydroxylated Products of Deuterated Probes ............................................................................................................. 36
Figure 3.6: AsqJ-Catalyzed One-Pot Epoxidation of Cyclopeptin................... 37
Figure 4.1: Snapshot of AsqJ Active Site....................................................... 41
List of Tables

Table 2.1: Contents of TB and LB Broth ................................................................................. 18
Table 2.2: Ferrozine Assay Standards Contents ...................................................................... 23
Table 2.3: AsqJ Biochemical Assay .......................................................................................... 25

List of Schemes

Scheme 1.1: Example 2OG-Dependent-Catalyzed Reactions .............................................. 4
Scheme 1.2: Formation of Fe(IV)-Oxo Center ........................................................................ 4
Scheme 1.3: Synthetic Olefination Mechanisms ..................................................................... 5
Scheme 1.4: Biosynthetic Olefination Schemes ....................................................................... 6
Scheme 1.5: Previously Proposed AsqJ-Catalyzed Desaturation Pathways ......................... 8
Scheme 4.1: Plausible AsqJ-Catalyzed C=C Pathways ......................................................... 40
Scheme 4.2: Possible AsqJ-Catalyzed HAT Events ................................................................. 43
Chapter 1

1.1 Introduction of Mononuclear Non-Heme Iron(II) Enzymes

Cytochrome P450 proteins have been of large focus in academic research in the last few decades, due to their ability to incorporate molecular oxygen onto inactive C-C bonds. More importantly, this class of enzymes has been found to catalyze oxyfunctionalization of medicinally important natural products such as terpenes and alkaloids. Because of its biomedical importance, much work has been done to push P450 enzymes from academia to commercial application. However, there are several restrictions associated with this family of heme enzymes. Some key issues are associated with low enzymatic activity, as well as they require electron transfer partners to regenerate the reactive iron center after each catalytic cycle using nicotinamide adenine dinucleotide (NADPH). While work has been done to troubleshoot many of these limitations, the ability to conduct commercial-sized reactions is still limited. Because of this, it is reasonable to investigate other options for catalyzing oxyfunctionalization.

One particular enzyme family is the mononuclear non-heme iron enzyme family. These enzymes are similar to P450 enzymes in their ability to incorporate molecular oxygen into compounds through C-H bond activation. Different from P450 enzymes where the iron is chelated by four pyrrolic groups, the non-heme iron enzymes contain a “free” iron in the center of their active sites. This family of enzymes are reported to be involved in key oxidative biological processes such as O₂ detection, DNA repair, etc. and are widely found in nature from bacteria and plants to humans.
While these enzymes have diverse reactivities, one commonality shared by a majority of enzymes in this family, is the iron in the active site is bound by three amino acid residues. These residues were identified to be 2 histidines and a carboxylated amino acid (aspartate or glutamate). These ligands are bound to the iron center and form what is commonly referred to as a 2-His-1-carboxyl facial triad. In some cases, the carboxylate residue can be substituted for a halogen, which is the conserved feature of halogenases in this family. The term facial triad is used because of the arrangement of the amino acid residues to one face of the octahedral complex that is the non-heme iron center (Figure 1.1). This then leaves three remaining sites on the active center that are proposed to be filled with water molecules when the enzyme is in the resting state but are quickly exchanged to bind co-substrate and molecular oxygen in the presence of substrate. One particularly interesting sub-class of this family is the 2-oxoglutarate-dependent enzymes. These enzymes require 2-oxoglutarate (2OG) as a co-substrate to initiate the activation of molecular oxygen (O₂). Figure 1.1 presents the active sites of two examples of 2OG-dependent enzymes, CAS and CarC. As previously described, the figure illustrates the peptide residues on one side of the octahedral complex, which leaves the other side of the complex open for the binding of 2OG and molecular oxygen.
Figure 1.1: Examples of 2-His-1-carboxyl facial triad. The arrangement of peptide residues (2-His-1-carboxylate) on one face of the octahedral complex leaves three remaining sites on the active iron center that can interact with substrates, co-substrates, and molecular oxygen. The above active sites are 2-oxoglutarate-dependent enzymes: CAS (PDB: 1DRT, left) and CarC (PDB: 4OJ8, right).

1.2 Introducing 2OG-Dependent Oxygenases

The 2OG-dependent oxygenases use a high-valent Fe(IV)-oxo species to carry out a variety of reactions\textsuperscript{16}; such reactions include, hydroxylation, desaturation, epimerization, ring expansion, epoxidation, and etc.\textsuperscript{17-23} Scheme 1.1 outlines the above reactions found in specific enzymes. While these reactions are diverse, all 2-OG dependent enzymes are likely to utilize an Fe(IV)-oxo intermediate to catalyze hydrogen atom transfer (HAT) prior to diverse reaction outcomes.\textsuperscript{16} Formation of the proposed Fe(IV)-oxo intermediate is outlined in Scheme 1.2. Subsequent to substrate addition, 2OG binds to the iron center at the C1 carboxylate and the ketone carbonyl groups.\textsuperscript{17} This leaves one site on the metal that allows for molecular oxygen addition. Upon binding of molecular oxygen, and forming the Fe(III)-superoxo species, oxidative decarboxylation of 2OG to form succinate generates the high-valent Fe(IV)-oxo species.
Scheme 1.1: Examples of diverse reactions catalyzed by Fe/2-OG-dependent enzymes. In addition to each illustrated product, all reactions produce succinate and CO₂ as co-products.

Subsequently, the Fe(IV)-oxo center catalyzes H⁺ abstraction, which reduces it back to Fe(III)-OH.¹⁶,¹⁷ At this point the enzymatic reactions branch out into the intended functionality. These enzymes are recognized for catalyzing hydroxylation reactions, but as Scheme 1.1 illustrates, some are capable of catalyzing many different reactions. This study investigates the desaturation mechanism of 2OG-dependent enzymes. Specifically, I am interested in Fe/2OG-dependent enzymes that can catalyze cascade reactions (i.e. AsqJ catalyzes desaturation and epoxidation reactions).²⁰-²²

Scheme 1.2: The proposed pathway for Fe(IV)-oxo formation in 2OG dependent non-heme enzyme.

X = Asp, Glu, or Halogen
1.3 C=C Bond Formation in Chemistry

Formation of carbon-carbon double bonds is a common, yet very important reaction in organic chemistry.\textsuperscript{24-26} Reagents and reactions like the Wittig Reaction, Peterson Olefination, etc. (Scheme 1.3) are well-understood and broadly used in organic synthesis.\textsuperscript{24-26} Generally, the formation of C=C bonds can be introduced via two approaches: (i) introduction of a leaving group, followed by C=C bond formation as a result of the removal of the leaving group, (ii) formation of a four-membered ring intermediate prior to C=C bond construction.\textsuperscript{24-26} In biology, many enzymes perform C=C bond creation to yield biologically active compounds using uncharacterized mechanisms.\textsuperscript{27}

**Wittig Reaction**

![Wittig Reaction Diagram]

**Peterson Olefination**

![Peterson Olefination Diagram]

Scheme 1.3: Some examples of commonly used reactions in preparing C=C bonds in organic synthesis.

1.4 C=C Bond Formation in Biology
Nature has evolved various approaches to allow for construction of C(sp\(^2\))-C(sp\(^2\)) bonds.\textsuperscript{27,28} Importantly, through enzyme catalysis, it is possible to install a C=C bond across two consecutive inactive C-H bonds. Previous studies have discovered that enzymes such as P-450 and 2OG-dependent enzymes, are capable of creating the C=C bond through cleavage of two C-H bonds.\textsuperscript{6,15-22}

![Scheme 1.4:](image)

**Scheme 1.4:** Selected examples of enzyme-catalyzed desaturation reactions. A) the desaturation of P-450 enzyme. B) The Fe/2OG-dependent enzyme (AsqJ, CarC, CAS) catalyzed desaturations.

While the P450 mechanism has been studied and proposed, mechanistic understanding of enzymes like CarC, CAS, and AsqJ remain to be elucidated and have the potential to be applied to the production of bioactive compounds.\textsuperscript{15-22} Previous studies have begun to elucidate these systems, but to my knowledge, no properly tested enzymatic mechanisms for these systems
have been proposed to date. During AsqJ catalysis, a C=C bond is installed between the C3 and C10 sites of the substrate. Subsequent to desaturation, the addition of molecular oxygen across the double bond gives way to an epoxide product. In addition to its ability to catalyze desaturation and epoxidation steps, AsqJ is also potentially capable of catalyzing a rearrangement to form the final 6,6 quinolone structure, viridicatin. This rearrangement is interesting, because there have been no reported 2OG-dependent enzymes capable of catalyzing redox-neutral rearrangement reactions.

1.5 A One-Pot Bioconversion of Cyclopeptin to Viridicatin via 2OG-Dependent Oxygenase: AsqJ

*Aspergillus nidulans* is a fungus that was found to produce viridicatin, a key 6,6-quinolone structural core found in a number of biologically active small molecules (Figure 1.3).

Previous studies have revealed that AsqJ is a key enzyme involved in the formation of viridicatin. This enzyme catalyzes desaturation, epoxidation, and perhaps rearrangement reactions to obtain final product (Figure 1.2). Recent studies have attempted to elucidate plausible pathways for both the desaturation and epoxide transformations. Previously proposed desaturation mechanisms (Scheme 1.5) were based on crystallographic data that shows close proximity of both the C3-H and the C10-H to the iron center (4.3 Å and 4.7 Å,
respectively), as well as previous calculations performed on a similar enzymatic system (CAS). However, while desaturation mechanisms have been proposed, to my knowledge, they have not been experimentally tested. The two proposed mechanisms are similar in that they suggest a di-radical pathway (Scheme 1.5), but they differ in how desaturation is initiated. In the top panel, desaturation is initiated through C10-HAT, while in the bottom panel, it is initiated through C3-HAT.

**Scheme 1.5:** The di-radical pathway proposed by Ishakawa et. al. (top) and Brauer, et. al. (bottom). Ishikawa proposed that AsqJ-catalyzed desaturation initiates by breaking the C10-H. Brauer proposed that desaturation is initiated through C3-H abstraction.

To summarize both reactions pathways, desaturation is initiated through C3 or C10 HAT using a presumptive Fe(IV)=O, which then forms a substrate radical. The resulting Fe(III)-OH species is alleged to perform the second HAT to form a di-radical species. This is subsequently followed by the combination of di-radicals to form the desaturated product.

Proposing a di-radical intermediate mechanism postulates the following mechanistic implications. First, it suggests that the high-valent Fe(IV)-oxo center and the resulting Fe(III)-
OH center are capable of abstracting hydrogen atoms.\textsuperscript{16} Although, it has been previously calculated that the C-H bond dissociation energy is reduced in the presence of the adjacent carbon radical, no experimental studies are able to support this conclusion. Therefore, it remains a question as to whether Fe(III)-OH is capable of performing the second HAT.\textsuperscript{16} On the other hand, the crystallographic data presented by Brauer, et. al. illustrates that both C3 and C10 face towards the iron center (Chapter 4, Figure 4.1). This does support the di-radical mechanism, but is not sufficient evidence to confirm the mechanism.

Herein, I present my results used to elucidate a plausible desaturation pathway. In order to study the AsqJ-catalyzed desaturation, four mechanistic probes were designed and tested with AsqJ. The probes were synthesized by a colleague. All reaction samples were analyzed via LC-MS. Figure 1.3 presents the 4 designed mechanistic probes (1, 2, 3, and 4).

![Figure 1.3: The mechanistic probes (1-4). 1 and 2 are C3 stereoisomers of each other, and 3 and 4 are the deuterated isotopes of L-cyclopeptin at the C3 and C10 positions.](image-url)
While no previous studies have proposed AsqJ to be capable of hydroxylation, it is reasonable to believe that hydroxylation could occur as many 2OG-dependent enzymes are known to catalyze hydroxylation reactions.\textsuperscript{12,17,18}

Mechanistic probe 1 represents the native substrate, L-cyclopeptin and was used as a positive control to confirm reactivity of over-expressed AsqJ \textit{in vitro}. Probe 2 is the C3 stereoisomer of 1, and I hypothesize that if AsqJ-catalyzed desaturation occurs via a di-radical pathway, then mechanistic probe 2, D-cyclopeptin, will show no activity or another reaction product (e.g., hydroxylation) will be detected. Probes 3 and 4 are deuterated compounds at the C3 and C10 carbons, respectively. These compounds will help determine where the initial C-H activation occurs by quantifying the isotopic distribution for the product. All methods are presented in chapter 2, all experimental results are discussed in chapter 3, and conclusions are offered in chapter 4.
1.6 References


Chapter 2

2.1 Transformation of \textit{asqJ}-containing Plasmid into \textit{E. coli} Strains and Restriction Enzyme Digestion

The \textit{asqJ} gene from \textit{Aspergillus Nidulans} was ligated to a pET-28a vector (Gene Art, Regensburg, Germany) at the multiple cloning sites between the BamHI and the NdeI restriction sites (Figure 2.1).

![Diagram of AsqJ DNA plasmid]

**Figure 2.1:** The AsqJ DNA plasmid. The \textit{asqJ} gene (green) was inserted between BamHI and NdeI of the multiple cloning site of the pET-28 vector. This plasmid contains kanamycin resistance (red), which allows for selective growth of only transformed \textit{E. coli}. Protein over-expression is controlled by a lac operon, which can be triggered by removal of the lac inhibitor (lacI) via Isopropyl β-D-1-thiogalactopyranoside (IPTG).

The optimized gene sequence can be found in appendix I. The \textit{asqJ} plasmid was used to transform the Top10 \textit{E. coli} strain purchased from New England Biolabs (Ipswich, MA) to amplify plasmid and confirm proper gene insertion. Transformation was done by mixing 1 µL of 100 ng/mL of \textit{asqJ} plasmid with 100 µL of Top10 \textit{E. coli}. This solution was then mixed and placed on ice for 30 minutes. The mixture was placed on a 42 °C heat block for 45 seconds,
then placed on ice for 10 minutes. After transforming, 900 µL of super optimal broth (SOC), purchased from Bio Basic Canada (Markham, Ontario), was added to the solution. The cells were incubated at 37 °C for an hour. Once the cells had been grown, the solution was centrifuged at 10,000 rpm for 1 minute and the cell pellet was re-suspended with 100 µL of luria broth (LB). This solution was spread on an agar plate containing 50 µg/mL kanamycin. The plate was incubated at 37 °C for 16 hours, then stored at 4 °C before use.

A single colony was picked and added to 10 mL of LB containing 50 µg/mL of kanamycin. This culture was grown overnight (14-16 hours) at 37 °C shaking at 220 rpm. After growth, cells were concentrated into a pellet by centrifuging at 10,000 rpm. The DNA was then purified using a miniprep kit (Bio Basic Canada, Markham, Ontario). Specifically, the cell pellet was re-suspended into 100 µL of a solution containing RNase, and incubated for 1 min at room temperature. Subsequently, 200 µL of a 2% Sodium dodecyl sulfate solution (SDS) was added to lysis the cell wall. After a minute of incubation at room temperature, the solution was neutralized with a guanidine hydrochloride solution and centrifuged at 10,000 rpm for 5 min. The resulting supernatant was loaded on an EZ-10® column (Bio Basic Canada, Markham, Ontario) and centrifuged for 2 min at 10,000 rpm. The column was then washed with a 4:1 ethanol-water solution, which was centrifuged at 10,000 rpm for 2 min. The residual ethanol was removed by an additional centrifuge process under the same conditions. Subsequently, 50 µL of DNase free water was loaded onto the column and incubated at room temperature for 2 minutes. This solution was then centrifuged at 10,000 rpm for 1 min. The purified DNA was stored at -20 °C.
DNA digestion was carried out by mixing a 40 µL solution containing 1 µL of HindIII, 1 µL NdeI, 5 µL NEBuffer 2.1, and 36 µL of DNA (3 µg). The restriction enzymes used for DNA digest were purchased from New England Biolabs (Ipswich, MA). The solution was incubated for 14 hrs at 37 °C. After digestion, samples were loaded onto a 1% agarose gel stained with 10 µg/mL of ethidium bromide and run with 100 V for an hour. DNA digestion was performed to confirm successful insertion of asqJ into the DNA vector. The results of the digestion are displayed in Figure 2.2.

![Figure 2.2: 3 µg of asqJ-containing plasmid was digested using HindIII (AGCTT) and NdeI (TATG) restriction enzymes. The reaction was incubated for 14 hours. Reactions were loaded onto an ethidium bromide containing 1% agarose gel. The double digest shows two bands, one at ~6000 bp and another at ~1000 bp.](image)

2.2 Small Scale Over-Expression of AsqJ
The *asqJ*-containing plasmid was then transform into BL21 *E. coli* cells (New England Biolabs, Iswich, MA) using the same method described in Section 2.1. The BL21 strain is capable of over-expressing protein, because it is lacking Lon and OmpT proteases, which are responsible for the degradation of proteins found within bacteria like *E. coli*. This deficiency allows for the accumulation of over-expressed protein.

In order to test whether AsqJ could over-express, small scale protein over-expression was performed. Over-expression began by picking a single transformed colony and incubating in 10 mL of a LB solution with 50 µg/mL of kanamycin, shaken at 210 rpm and incubated at 37 °C for 16 hours. This stock solution was then added to 250 mL of LB solution with 50 µg/mL of kanamycin, and placed on a shaker at 37 °C and 210 rpm. The optical density at 600 nm (O.D.\(_{600}\)) was monitored (~4 hours) until the optimal optical density (~0.6 – 1.0) was obtained. The cells were placed on ice for 30 minutes and 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the solution to induce AsqJ over-expression. Following IPTG addition, 4.5 mL aliquots were taken at time intervals of t = 0 hr, 1 hr, 2 hrs, and 3 hrs. The aliquots were spun down and the supernatant was removed. The pellet was then re-suspended in 100 mL of deionized water and 50 mL of SDS-containing protein dye. The solution was then boiled at 90 °C for 10 minutes, and centrifuged at 10,000 rpm for 15 minutes. Each aliquot was then loaded onto a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), and the gel was developed using 180 V for an hour. This process was repeated, but instead of using LB broth, LB-rich broth was used to compare expression efficiency. The resulting gels are compared in Figure 2.3.
Figure 2.3: (A) the resulting small scale over-expression of AsqJ using LB, the size of AsqJ is ~38 kDa. (B) the resulting small scale over-expression of AsqJ using LB-Rich. The increased concentration of nutrients available and the differences in the buffer used to pH the solution shows a distinct difference in the protein expression.

The reagents used in both LB and LB-rich are outlined in Table 2.1

Table 2.1: The amounts of reagents used in 1 L of a LB or LB-rich solution.

<table>
<thead>
<tr>
<th></th>
<th>LB</th>
<th>LB-rich</th>
</tr>
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<tbody>
<tr>
<td>Yeast Extract</td>
<td>5 g</td>
<td>24 g</td>
</tr>
<tr>
<td>Tryptone</td>
<td>10 g</td>
<td>12 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>10 g</td>
<td>5 g</td>
</tr>
<tr>
<td>Base used to pH</td>
<td>NaOH</td>
<td>2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)</td>
</tr>
</tbody>
</table>
2.3 Large Scale AsqJ Over-Expression

It was determined from small scale over-expression that LB-rich was a better media for AsqJ preparation. Therefore, LB-rich was used for large scale over-expression. Analogous to previous small scale over-expression, a single colony was picked for large scale over-expression to grow in 250 mL a LB-rich solution with 50 µg/mL of kanamycin and was allowed to grow for 16 hours in a shaker at 37 °C and 200 rpm. A 30 mL aliquot of the cell media was introduced to 1 L of LB-rich solution with 50 µg/mL of kanamycin. The 6 L of solution were then placed in a shaker at 37 °C and 210 rpm and was allowed to grow for ~4 hours until reaching the optimal O.D.₆₀₀ measurement (~0.6). The solutions were placed on ice for 30 minutes and then 0.5 mM IPTG was added. After growing at 18 °C shaking at 200 rpm for 16 hours, the cellular solutions were centrifuged at 3200 rpm for 20 minutes at 4 °C. The cell pellet was frozen and stored at -20 °C. Typically, ~8g/L of cells can be obtained.

2.4 AsqJ Purification

While there are numerous purification methods, e.g. ammonium sulfate precipitation, size-exclusion, column chromatography, etc., it was decided to use affinity column purification as it is a simplistic approach that produces large amounts of desired protein. The realization of affinity column purification comes from the advent of modifying recombinant DNA to include specific affinity tags such as a polyhistidine tag (6xHis-tag). As described in Figure 2.4, the specific affinity column purification chosen for AsqJ purification was a nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography.
nickel column (purchased from Qiagen, Hilden, Germany), which is normally stored in 4 °C fridge in a 30% ethanol solution, was washed with about 30 mL of lysis/wash buffer that consisted of 100 mM Tris, 250 mM sodium chloride, 10 mM imidazole with a pH = 7.6. During column equilibration, the flash frozen cell pellets were re-suspended in 100 mL of lysis/wash buffer.

**Figure 2.4:** A schematic representation of the Ni-affinity column chromatography. The 6-Histidine tagged proteins (green) along with endogenous proteins (red) are suspended in cellular lysate, when this lysate is poured onto the column, the 6-His proteins bind to the Ni-NTA column while untagged protein and debris remained unbound. The column is then washed with wash buffer to remove non-target protein. This leaves only the His-tagged AsqJ on the column, which is then eluted with elution buffer.

Once fully re-suspended, penylmethylsulfonyl fluoride (PMSF) was added to the solution with a final concentration of 1.0 mM. The solution was sonicated at 30 second increments for 3.5 minutes. The sonicated solution was centrifuged at 36000 rcf at 4 °C for 30 minutes. The supernatant was then loaded onto the Ni-NTA column and incubated for 15 minutes before eluting. After eluting the flow-through, the column was washed with 160 mL of the wash
buffer. Subsequent to the washing step, AsqJ was eluted using 160 mL of the elution buffer (100 mM Tris, 250 mM NaCl, 250 mM imidazole, pH = 7.6). Aliquots that were taken from the collected flow-through, wash, and elution fractions were loaded onto a 10% SDS-PAGE gel. The gel was developed using the same conditions previously described in section 2.2. The stained gel is presented in figure 2.5.

![Figure 2.5: The resulting AsqJ purification SDS-PAGE. Fractions containing target protein were combined and concentrated via centrifugation filtering tubes.](image)

The fractions containing AsqJ were concentrated using a centrifuge filter purchased from Pall Life Sciences (Radnor, PA) and centrifuging at 3200 rpm at 4 °C. The concentrated solution (~4 mL) was then placed in dialysis buffer. The first dialysis buffer contained 10 mM EDTA, 100 mM Tris, and 250 mM NaCl (pH = 7.6). The last two dialysis buffers contained 100 mM Tris and 250 mM NaCl (pH = 7.6). After dialysis, the protein concentration was measured using an Agilent Technologies Cary 8454 UV-Vis spectrometer (Santa Clara, CA). The protein concentration was calculated using Beer’s Law which is shown below:
A is the absorbance, \( \varepsilon \) is the molar absorptivity of AsqJ, which is 27,960 \( M^{-1} \text{cm}^{-1} \), \( b \) is the path length (cm), and \( C \) is the concentration (M). The protein was then degassed to remove oxygen. The degassed solution was aliquoted in an anaerobic chamber, the proteins were stored at -80 \(^\circ\)C for biochemical assays.

2.5 Determining Iron Contamination via Ferrozine Assay

A ferrozine assay is used to measure the residual iron concentration after dialysis.\(^{15,16}\) This is done by comparing a protein sample against a concentration curve and calculating the percentage of iron remaining based on the generated calibration curve.\(^{15}\) The iron concentration is quantitatively measured by detecting absorption at 562 nm (Abs\(^{562}\)) and calculating the concentration using Beer’s Law, knowing that the molar absorptivity of iron (II) when bound to ferrozine is 27900 \( M^{-1} \text{cm}^{-1} \).\(^{15}\) The iron content of the protein sample was obtained by comparing the Abs\(^{562}\) against a standard curve generated by mixing ammonium iron(II) sulfate (\( \text{Fe}_2(\text{NH}_4)_2(\text{SO}_4)_3 \)) with ferrozine.\(^{15}\)

In this assay, different volumes of 1 mM \( \text{Fe}_2(\text{NH}_4)_2(\text{SO}_4)_3 \) (Bio Basic Canada, Markham, Ontario) was mixed with 20 \( \mu \text{L} \) of 10 mM 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p-p’-disulfonic acid, disodium salt hydrate, referred to as ferrozine, (Acros Organics, Geel, Belgium), to form the Fe(II)-ferrozine complex used to create the standard curve. Additionally, 75 mM ascorbic acid (Sigma-Aldrich St. Louis, MO) and a saturated ammonium acetate (Bio Basic Canada, Markham, Ontario) were added to each standard. Fe(II) standards ranging from 0 nmol to 25 nmol were prepared according to Table 2.2.
Table 2.2: The amounts of reagents used in each standard. All standards and protein samples were prepared in duplicate.

<table>
<thead>
<tr>
<th>Standard</th>
<th>Fe(II) added</th>
<th>Ferrozine added</th>
<th>Ascorbic acid</th>
<th>Saturated NH$_4$OAc</th>
<th>dH$_2$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 µL</td>
<td>20 µL</td>
<td>20 µL</td>
<td>120 µL</td>
<td>495 µL</td>
</tr>
<tr>
<td>2</td>
<td>10 µL</td>
<td>20 µL</td>
<td>20 µL</td>
<td>120 µL</td>
<td>490 µL</td>
</tr>
<tr>
<td>3</td>
<td>15 µL</td>
<td>20 µL</td>
<td>20 µL</td>
<td>120 µL</td>
<td>485 µL</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>120</td>
<td>480</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>20</td>
<td>20</td>
<td>120</td>
<td>475</td>
</tr>
</tbody>
</table>

The A$_{562}$ was measured for each standard. Once a satisfactory curve ($r^2 > 0.995$) was obtained, protein samples were prepared by denaturing 12 nmol of protein with 2 µL of 50% v/v trichloroacetic acid (Fisher Scientific, Waltham, MA). The denatured protein was centrifuged at 10,000 rpm for one minute. The supernatant was added to 498 µL of deionized water and prepared according to Table 2.2. It was then possible to calculate the percent contamination using the equation derived from the calibration curve.

2.6 Optimizing a Liquid Chromatography/Mass Spectrometry (LC-MS) Method

In my study, a reverse-phase LC-MS column method was used to elucidate AsqJ-catalyzed olefination reaction mechanism. A reverse-phase C18 (4.6x50 mm, 1.8-micron) column purchased from Agilent (Santa Clara, CA) was attached to an Agilent Technologies 12000 HPLC system coupled to a 6410 single quadrupole mass spectrometer. Before performing biochemical assays, it was necessary to develop a LC-MS method that could detect
each product resulting from the reaction of cyclopeptin with AsqJ. Specifically, 100 µM standards of 1, 5, and 7 were prepared along with a 25 mM ammonium formate aqueous mobile phase. The ratio of organic and aqueous mobile phases was adjusted where all standards could be detected with a 7 minute method. The optimal methanol-to-ammonium formate ratio was determined to be 60:40. Compounds 1, 5, and 7 were all properly separated (Figure 2.6).

**Figure 2.6:** LC chromatograms of substrate (orange), desaturated product (grey), and epoxide product (blue). The m/z of each compound is also reported.

In addition, the limit of detection of 1 was determined. This was done by diluting the 100 µM standard to concentrations of 50 µM, 25 µM, 15 µM, and then 5 µM. It was found that at the concentration of 1 needed to at least be 5 µM in order to produce a reproducible signal.

### 2.7 AsqJ Biochemical Assay and Substrate Isotopic Distribution Determination

The isotopic distributions for 3 and 4 were measured using the LC-MS method developed in section 2.6. Specifically, 100 µM samples of 3 and 4 were injected into the LC-MS and distributions were measured. Figure 3.1 displays the collected data of the standards.
To study the AsqJ-catalyzed desaturation mechanism, an assay was developed. Specifically, AsqJ is only allowed to catalyze olefination step, but not the subsequent epoxidation.\textsuperscript{1,17,18} This was achieved by using limited co-substrate, 2-oxoglutarate (2OG) in the reaction mixture (Figure 2.7). In this study, six separate reaction mixtures were prepared in an anaerobic glove box. Reactions contained final concentrations of 120 µM, AsqJ, 200 µM of selected substrate, 50 µM 2OG, and 100 µM Fe(II). Controls were also made that were either lacking the substrate or 2OG. The six reaction mixtures are summarized in table 2.3 and 2.7.

**Table 2.3:** Describes each prepared reaction mixture. There were 4 experimental mixtures created and each experimental contains one of four mechanistic probes (1, 2, 3, 4).

<table>
<thead>
<tr>
<th></th>
<th>AsqJ (µM)</th>
<th>Substrate (µM)</th>
<th>Fe(II) (µM)</th>
<th>2OG (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental</td>
<td>120</td>
<td>100</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>Control 1</td>
<td>120</td>
<td>0</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>Control 2</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>50</td>
</tr>
</tbody>
</table>

Once the reactions were prepared in the box, they were exposed to air for 20 minutes. The reactions were halted by loading the reaction mixture onto a VWR PES 10K centrifugal filter (Radnor, PA) and centrifuging them at 10,000 rpm for 30 minutes. The mixtures were then analyzed using the previously described LC-MS method. These findings are presented in Chapter 3.
Figure 2.7: Represents the outcome of every experimental reaction performed in section 7. The hydroxylation of all substrates is shown first, followed by the creation of (5) or (6).

2.8 AsqJ-Catalyzed One-Pot Epoxidation

After analyzing a single turnover reaction, a multi-turnover reaction was designed to support initial findings. This was done by adding sufficient 2OG into the reaction. The final concentrations of each reactant were 120 µM AsqJ, 100 µM Fe(II), 250 µM ascorbic acid, 250 µM 2OG, and 10 µM substrate (Figure 2.8). Reaction mixtures were halted as described in section 2.7. A control that was missing 2OG was also prepared.
Figure 2.8: The reaction of 1 and 2 to the epoxide. In the presence of 2 equivalents of 2OG, it is possible for AsqJ to catalyze a second reaction that involves the addition of oxygen across the C=C bond. A potential mechanism of epoxidation has already been previously proposed.\textsuperscript{1}

To accurately depict the consumption of analyte, an internal standard was used. It was decided that either L-phenylalanine or L-tyrosine would serve as a suitable internal standard for the reaction. Standards containing 100 µM of L-tyrosine or L-phenylalanine were prepared. However, while making the solutions, it was found that prepared L-tyrosine was not readily soluble in the aqueous Tris solution at a pH = 7.6. Therefore, phenylalanine was used as the internal standard. Additionally, it was determined that the limit of detection for the standard was 25 µM. The chromatogram of the standard can be found in figure 2.9.

Figure 2.9: LC-MS chromatogram of the internal standard, L-Tryptophan. The internal standard was added to confirm that it was possible to quantify epoxide product.
2.9 References


Chapter 3

3.1 Measuring Residual Fe(II) Concentration Using Ferrozine Assay

3.1.1 Establishing Ferrozine Assay Standard Curve

After dialysis, it was necessary to determine the residual iron concentration present in AsqJ samples.\(^1\) While there are a variety of different methods for measuring protein-bound iron,\(^2,3\) these methods involve using harsh chemicals and complex methods in order to remove iron from the protein. I chose to use the ferrozine assay, because it is a simplistic colorimetric assay capable of measuring nanoscale amounts of iron present in protein.\(^1\) Specifically, it involves the generation of a standard curve through standards of ammonium iron(II) sulfate dissolved in distilled water with ferrozine and ascorbic acid.\(^1,4\) Ferrozine is a compound that binds specifically to iron, which is ideal because there is less interference resulting from non-specific binding of the ligand to other metals that may be present in solution.\(^1\) When ferrozine binds to iron(II), it generates a purple color in natural light, larger amounts of Fe(II) present results in a darker purple solution. Figure 3.1 illustrates the colorimetric assay. The iron-ferrozine complex can be measured by using absorption spectroscopy, it has an absorbance maxima at 562 nm (\(A_{562}\)).\(^1\) Once the standard curve had been established, AsqJ was denatured using trichloroacetic acid (TCA) as described by the procedure in chapter 2. Denaturing protein releases the iron from the active site.\(^1\) The free iron will bind ferrozine, and the concentration of iron can be determined based on the previously generated curve. Specifically, the ferrozine assay was used in order to determine the amount residual iron present in over-expressed AsqJ.
Figure 3.1: The ferrozine assay standards. Standards ranged from 0 nmol to 25 nmol of Fe(II). Ferrozine binds to iron, which creates a ferrozine-iron complex, which produces a purple color in natural light and is measurable with UV-Vis methods. A dark purple is seen when more iron is available to form the complex with ferrozine.

3.1.2 Residual Fe(II) Concentration Measurement

Typically when Fe(II)/2OG-dependent protein are purified, the iron is present in the active site of the protein. This is problematic, because over time the iron center will eventually be oxidized to Fe(III). This oxidative process decreases protein activity, and can potentially lead to protein degradation. While the oxidation of iron can be reversed with the addition of a reducing agent, ultimately the loss of enzyme functionality cannot be restored due to protein degradation. In order to extend protein shape, I decided to remove the iron from AsqJ to yield the inactive “apo” form of the protein. To conclude the purification process, EDTA was added to the dialysis buffer to chelate iron, which removes it from AsqJ. This creates the “iron-free” apo AsqJ. In order to measure the residual amount of Fe(II), ferrozine was used. The ferrozine-iron complex was measured using UV-Vis spectroscopic
The amount of residual iron present within over-expressed AsqJ was calculated using the equation generated in Figure 3.2.

Based on the results, an accurate standard curve was created with a $R^2 = 0.999$.

\[(\text{Eq. 1}) \quad y = 0.0406x\]

Where $x$ is the amount of residual iron present in solution and $y$ is the $A_{562}$. It was determined that the amount of residual iron remaining in purified AsqJ was ~0%. This is also shown in Figure 3.2. A lack of residual iron indicates that the over-expressed protein is in its inactive apo form when stored at -80°C.\textsuperscript{1-5}

![Figure 3.2](image_url)

**Figure 3.2:** The resulting UV-Vis spectra from the ferrozine assay. Top right: The generated standard curve and the derived equation. The standards range from 0 nmol to 25 nmol of Fe(II). AsqJ samples were also analyzed and are also presented. The amount of residual iron in over-expressed AsqJ was calculated to be ~0%.
3.2 AsqJ-Catalyzed Olefination of both C3 Stereoisomers

Chapter 2 describes biochemical assay development and the analytical method that I performed to elucidate the AsqJ-catalyzed olefination reaction mechanism (chapt. 2, section 2.6-2.8). In order to dissect possible olefination pathways, four mechanistic probes were designed and tested. The native substrate, 1, was used as a positive control to ensure reactivity of purified AsqJ *in vitro*.\(^6\)-\(^8\) Furthermore, the reaction products generated using 1 can serve as standards and allows for comparison to other mechanistic probes.\(^6\)-\(^8\) Previous biochemical and structural studies suggest that AsqJ olefination is likely to proceed through a double hydrogen atom transfer (HAT) mechanism where the iron center is at close proximity to both C3 and C10, with distances of 4.3 and 4.7 Å, respectively (chapter 4).\(^6\),\(^7\),\(^9\),\(^10\) Based on structural information, it was proposed that the reaction initiates through C3-HAT. Following this mechanistic hypothesis, the C3-stereoisomer of 1, mechanistic probe 2, would not be capable of undergoing AsqJ-catalyzed olefination because the C3-H is now pointed away from the iron center. On the other hand, if the reaction starts from the C10-H, probe 2 is likely to be hydroxylated. Therefore, probe 2 was used to distinguish the initial site of HAT, specifically whether initial abstraction begins at C3 or C10, as well as aid in proposing an alternative olefination mechanism. The results of AsqJ-catalyzed reactions of 1 and 2 (Figure 3.3) show AsqJ converts not only 1, but also 2. LC-MS chromatograms illustrate that both 1 and 2 were converted to the presumptively hydroxylated product (m/z = 297.1), the desaturated product (5, m/z = 279.1), and surprisingly, a small amount of the epoxide product (m/z = 295.1).\(^11\)-\(^14\) Studies of several non-heme iron enzymes suggest that hydroxylation occurs as side
Therefore, it is possible that AsqJ could also catalyze a hydroxylation reaction as a side reaction. On the other hand, the hydroxylation product could be the key intermediate, which is further converted to desaturated product by dehydration (chapter 4). Additionally, the detection of epoxide product, 7, implies a substrate preference for the desaturated product over the saturated substrate (1 and 2). Namely, under this reaction condition, with an excess amount of substrate, the formation of the epoxide (7) suggests the desaturation product (5) is likely a tightly bound product in the active site.

**Figure 3.3:** LC-MS Chromatograms of AsqJ-catalyzed reactions using 1 or 2 under limited 2OG conditions. Substrate 2 is the C3 stereoisomer of substrate (1). The right panel displays the resulting products detected.

### 3.3 Elucidate Desaturation Reaction Mechanism Using Substrate Isotopes
Based on the results shown in Figure 3.3, it is very likely that the olefination initiates through a C10-HAT. This is further confirmed when reacting AsqJ with substrate isotopes (3 and 4), and analyzing the LC-MS results for the product isotopic distribution. First, the initial isotopic distribution of 3 and 4 was characterized using $^1$H-NMR, and quantified using the LC-MS method previously described in chapter 2, section 2.6. Figure 3.4 and 3.5 display the isotopic enrichment of the substrate isotopologues (3 and 4). Specifically, the mono-deuterium enrichment at the C3 position of 3 was calculated to be 32%, and the di-deuterium enrichment at the C10 position of 4 was found to be 95%. By comparing the retained deuterium level between the substrate isotopologues and the hydroxylated product, it was possible to determine the initial C-H breakage site.

![Figure 3.4: The isotopic distributions of 3 and 4. The LC-MS chromatograms represent the measured isotopic ratio. The percent deuteration is listed for each compound.](image)

AsqJ was challenged with 3 and 4 separately. While both 3 and 4 were converted to the desaturated product, characterization of the hydroxylated product made it possible to determine the position of the initial HAT site (Figure 2.7). There was substantial hydroxylation products which has the same retention time as the hydroxylated product formed using 1, with different m/z values can be obtained. The results of this hydroxylation-event are shown in figure 3.3.
Studies of non-heme enzyme-catalyzed hydroxylation reactions clearly suggests that the hydroxyl rebound mechanism where the –OH group is attached at the resulting radical center by reacting with the Fe(III)-OH species. Thus, it is possible to follow the removal of hydrogen based on the formation of the hydroxyl product that is thought to form prior to olefination (Figure 2.7). Figure 3.5 shows the isotopic distribution of the protonated, mono-deuterated, and di-deuterated product when 3 and 4 are tested with AsqJ.

**Figure 3.5**: LC-MS chromatogram of the hydroxylated product during AsqJ catalysis. (Top) When 3 is used as the substrate, the calculated isotopic distribution was found to be around 35%, which is similar to the initial isotopic distribution of 32% (see figure 3.3). This suggests that hydroxylation at C10. This statement is further supported by the data obtained when 4 was used. (Bottom) 4 is used as the substrate, no obvious signal correlating to m/z of 299.1 (red) can be detected. Which indicates that both deuteriums are retained. The results of (A) and (B) suggests the hydroxylation at the C10 center.

When compound 3 is used, the isotopic distribution of the mono-deuterated to protonated hydroxylated product was found to be ~35%. This suggests that the deuterium on C3 is retained during hydroxylation process. If the reaction had removed the deuterium, the
deuterium enrichment would not be comparable to substrate isotope ratio as shown in Figure 3.4. Furthermore, should the initial HAT proceed through C3-HAT, it would be expected that for the product of the reaction using 4 would produce a signal at m/z = 299.1. Namely, where both deuterium are retained (Figure 3.4B). However, with the absence of this signal (m/z = 299.1), and accumulation of a peak with m/z = 298.1, it is strongly suggested that C3-HAT/hydroxylation is unlikely.

3.4 AsqJ Substrate Specificity Confirmation of Both C3 Stereoisomers

In addition to the previous assay performed in 3.2, a second assay where AsqJ was mixed with substrates 1 and 2 with an abundance of 2OG was conducted. With an abundance of 2OG present (Substrate: 2OG = 1: 10), AsqJ was allowed to catalyze not only desaturation, but also epoxidation. The purpose of this experiment was to test whether AsqJ prefers 1 over 2 as a starting substrate and to determine the possible product distribution. The LC-MS chromatograms are shown in figure 3.6. Both 1 and 2 were converted to 7, with no presence of the desaturated product detected. The epoxide signals formed in the far right panel for both substrates appear to be equal in abundance. This observation and the equivalence in abundance suggests that AsqJ has no starting substrate preference for the C3 stereoisomer. To firmly establish this conclusion, the reaction was performed repeatedly with the addition of an internal standard (Chapter 2, section 2.8). With the aid of the internal standard (Figure 2.9), it was possible to quantify epoxide product and suggests no substrate preference for AsqJ.
Figure 3.6: To further inquire if AsqJ will accept either the D- or L-substrate (1 and 2), LC-MS chromatograms of an assay that allows full conversion of 1 and 2 to 7 with ratio between substrate and co-substrate being ~10:1 are displayed.

3.5 References


Chapter 4

4.1 Summation of Mechanistic Findings:

Scheme 4.1: Plausible AsqJ-catalyzed C=C pathways: Top panel: the reaction is initiated by C10-H abstraction. The di-radical pathway proposed by Ishikawa, et. al is shown (III). Bottom panel: reaction is initiated by C3-H abstraction. Analogous pathways (I'), (II'), and (III') are presented.

Ishakawa et. al. and Brauer et. al. suggested di-radical pathways to form the dehydro-cyclopeptin product (Scheme 4.1 III and III'). Their proposed mechanisms are based on the x-ray structure of the substrate (I), bound AsqJ as depicted in Figure 4.2. Within the active site, both C3 and C10 carbons are in close proximity (4.3 and 4.7 Å, respectively) to the iron center.
Thus, both the C3 and C10 hydrogens are proposed to interact with the iron center.\textsuperscript{1,2} This accessibility, along with calculations reported for similar systems, such as CAS,\textsuperscript{3} are potentially what drove previous studies to conclude that a double hydrogen atom transfer (HAT) is preferred.\textsuperscript{1-3} According to the structural information of substrate 1 and bound AsqJ, the two carbon centers, C3 and C10, are both accessible to iron for HAT.\textsuperscript{2,3} The reaction is presumptively initiated by either C3 or C10 HAT using Fe(IV)=O.\textsuperscript{1,2} The resulting Fe(III)-OH is proposed to be used to perform the second HAT.\textsuperscript{1-3} Subsequent to the di-radicals formation, the di-radicals are recombined to form the C=C bond.\textsuperscript{4} This mechanistic possibility is postulated in scheme 4.1. However, figures 3.3 and 3.6 show conversion of D-cyclopeptin
(substrate 2) to 5. This observation suggests that the both 1 and 2 bind similarly in AsqJ, and that both substrate isomers undergo similar reaction mechanisms.

If the di-radical pathway occurs, it suggests that the high-valent Fe(IV)-oxo center and the resulting Fe(III)-superoxo center are able to break inactive C-H bonds. Although, it has been calculated that the C-H bond dissociation energy reduces due to the formation of the radical species at the adjacent carbon, to my knowledge, no experimental evidence has been provided that this actually occurs.

In addition to desaturation, Figures 3.3 and 3.5 clearly show the formation of a hydroxylate product (m/z = 297.1 and 298.1). This observation implies either hydroxylation occurs as a separate pathway, or the double HAT mechanism does not occur. If radicals form, the di-radicals recombination will quickly result in the creation of 5. Taking this into consideration, it is important to carefully evaluate the C=C bond formation mechanism. Alternative to a second HAT event, I propose mechanisms that begin with C-H bond breakage at C3 or C10, and subsequently followed by hydroxyl rebound or an electron transfer to form the carbocation intermediate. These proposed intermediates will be further converted to the desaturated product (Scheme 4.1 I, II, I’, and II’).

Following this mechanistic hypothesis, the C3 stereoisomer of 1, compound 2, can be used to distinguish the initial HAT site. If the reaction proceeds through a C3-HAT, compound 2 will not be converted to product. This is the result of the C3-H now moved away from the iron center. However, if the reaction proceeds through a C10-HAT, the hydroxylated product would be expected. The C3-H at the opposite orientation will prevent the second HAT, and thus facilitate the hydroxylation (Scheme 4.2).
Scheme 4.2: Possible AsqJ-catalyzed HAT events. C3-HAT is not possible for 2 as the result of the hydrogen pointing away from the iron center. If C10-HAT occurs, the formation of the hydroxylated product is expected.

LC-MS analysis of AsqJ catalyzed reaction of 1 and 2 suggest that the olefination begins with breakage of the C-H bond at the C10 position (Figures 3.3 and 3.6). Moreover, this is further suggested by product isotopic distribution analysis when substrate isotopes 3 and 4 were used (Figures 3.4 and 3.5). The initial deuterium enrichment distributions of 3 and 4 were calculated to be 32%, and 95%, respectfully. Specifically, for compound 3, 32% of C3-proton was replaced by deuterium. For compound 4, ~95% of protons at C10 were replaced by deuterium. After reacting 3 with AsqJ, it was found that the hydroxylated product had an isotopic distribution of ~35%. This indicates that the C3-deuterium remains attached to the product following AsqJ-catalysis, and the hydroxylation occurs at the C10 position. This is supported by the result when 4 was reacted with AsqJ. If hydroxylation occurs at the C3 position, there would be a detection of the hydroxylated product with two deuterium. However,
if the hydroxylation occurs at the C10 position, the hydroxylated product is expected to have only one remaining deuterium attached (Scheme 4.3).

Reviewing the data presented in the bottom panel of Figure 3.5 shows that no hydroxylated product with two deuterium (m/z = 299.1) signal was detected. Instead, the detected m/z value corresponds to one deuterium remaining on the hydroxylated product (m/z = 298.1). This indicates that one deuterium is removed from C10, thus supporting the reaction initiates through a C10-HAT event.

![Scheme 4.3: Plausible hydroxylated products when 3 and 4 are reacted with 2OG and AsqJ.](image)

Lastly, AsqJ shows substrate preference between the desaturated product over the saturated substrate. This was discovered after reacting 1 and 2 with a limited amount of 2OG and AsqJ. It was found that even with less than 1 equivalent of co-substrate, the presence of 7 as a signal at m/z 295.1 was detected (Figure 3.3). This was consistently seen after repeating the mentioned experiment.

AsqJ is a fascinating enzyme that performs both desaturation and epoxidation, and the epoxide is further converted to the final 6,6-quinolone core found in many biologically active
molecules.\textsuperscript{1,2,5,6} Understanding this enzyme’s mechanistic pathway provides molecular insight for biosynthesizing important and valuable molecules using AsqJ as a biocatalyst.
4.2 References:


Appendix
Appendix A

Below is the optimized DNA gene sequence for \textit{asqJ}. The sequence was inserted into a pET-28+ DNA vector in the multiple cloning sites between the BamHI and NdeI sites.

\begin{verbatim}
ATCATCACAGCAGGCCCTGGTGCCGCGGCAGCCATATGATGACCAGCAAATGGATCTGCAATAAA
TCTGGCCGACCAGTTGAAAGACATGGTCAGCAATTATATGAAAGTTTCTGGAGCT
GGATATTGTGCGTCGTCTGAATGAAAGAATTTGATCCGTTTTTGTTTTTAAACTGAGCC
ATTCCGGCAGCAAAAAACAAAGATCATCCGAATCATGTTCTGGAGCACCACCACC
CGTCTGGTTAATGTGCTGGCACCAGATTAGCAAAACCATATCGTAGAAGATGTTTCTG
ACAGCAAAAGTCTGCACTCGTTATTTGATGGATGGCCTTTTATGTGTATGGTGAAT
TTGGGTATCTGATGGTGCACTTTATGGAAACTGAGCACCCTCAAATCCGGCACAGCC
GCTGCACTCGTAGATCGGTATTATAGCCATCCGATTGTGGAAATATCTGAAACCC
ATGGGTCAACCCATGTTCTGGTTAGCCATAAATGGCAGAATCTGAGCAATGGTGAACCC
CCATCGTACATTACATGATGGTATTTAGCCAGCTGGAAAATGGTGAACCC
CTGGCAAGCTGACTGGCCAGTGATATTGGCCTGCTGCAAC
\end{verbatim}