ABSTRACT

ALI, DOMINICK LOUIS Identification of Synthetic Methodology to Elucidate Substrates for the Fe-dependent Oxidative Desaturation Enzyme PIsnB (Under the direction of Dr. Wei-chen Chang).

Isonitrile is a useful functional group to access in many different building blocks for polymers, synthesis of complex molecules, and natural products because of its unique reactivity. The reactivity is also medically-relevant to be used as anti-tumor, anti-fungal, anti-malarial, anti-tubercular, anti-biofouling, and anti-plasmodial agents. Biologically, vinyl isonitrile containing molecules are produced from many microbial organisms but the elucidation of how isonitrile functional groups are installed on these natural products has yet to be understood. Specifically, isonitrile synthase genes are believed to target terpenoid structures isolated from marine organisms and amino acid structures from terrestrial organisms. The interest in this biomachinery has increased due to the development of these functional groups on new drugs.

Synthetically, preparation of vinyl isonitriles has limitations but provide useful in certain applications. This study begins the foundation to further elucidate PIsnB catalysis by developing substrate probes to inspect this mechanism. Verification of the optimized protein overexpression is discussed along with the synthesized probes and the reasons for the established methodology.
Identification of Synthetic Methodology to Elucidate Substrates for the Fe-dependent Oxidative Desaturation Enzyme PIsnB

by
Dominick Louis Ali

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APPROVED BY:

__________________________________________
Dr. Wei-chen Chang  Dr. Christian Melander
(Committee Chair)

__________________________________________
Dr. Gavin Williams
BIOGRAPHY

Dominick was born and raised in Cecil, Pennsylvania. He moved to Erie, Pennsylvania in 2011 to attend Penn State Erie, The Behrend College for his undergraduate studies in Chemistry. He graduated in the spring of 2015 with a Bachelor of Science in Chemistry while knowing he wanted to pursue a graduate degree to advance himself in his career. He was excited to receive an offer from North Carolina State University to work with Dr. Wei-chen Chang in the Department of Chemistry. He moved to Raleigh, North Carolina in the fall of 2015 to continue his studies in Chemistry.
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I would first like to thank Dr. Wei-chen Chang for providing me with opportunities to grow as a scientist and allowing me to take on new and exciting projects in the Chang Lab. In addition, I would like to thank my committee members, Dr. Christian Melander and Dr. Gavin Williams, and Dr. Chang’s post-doctoral researcher, Dr. Jhih-Liang (Scott) Huang.

I would also like to recognize the continued support that I have received from the members of the Chang Lab as well as my family and friends. Having a strong support system through my professional and personal endeavors has helped further my success through the years as a graduate student.
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CHAPTER 1: Background of Vinyl-Isonitrile Functionality in Natural Products

1.1 Origins, Isolation, and Uses of Vinyl Isonitriles

Isonitrile functional group is a useful moiety due to its relevance in many disciplines (i.e. a versatile synthon, a warhead for its bioactivity). The unique properties associated with the isonitrile moiety is the isoelectronic stabilized resonance structures and its reactivity. The isoelectronic property makes the isonitrile viewed as a stabilized carbene (−N=C) or the isonitrile (−N≡C−) and expand its reactivity towards both electrophile and nucleophile. Thus, the isonitrile is a very useful functional group not only in synthetic applications but also in bioactivation.

Isonitrile moiety is a useful building blocks in organic chemistry due to their reactivity and versatility.¹⁻³⁻²² For example, Ugi and Passerini reactions are relevant representations of their importance as useful synthons to build complex molecules in a very efficient manner.¹,² In inorganic chemistry and chemical biology, the isonitrile moiety is a ligand that has a strong affinity for transition metals (such as Fe, etc.), that can be toxic by adding or removing specific metals from a system.³ For inorganic chemists, palladium and copper catalyzed coupling reactions utilize the stabilized carbene property of isonitrile to tune and expand the catalysts reactivity.³⁻⁴ In addition, it can be used in polymerization due to its ability to react with electrophiles and nucleophiles in material science application.⁵ Medically, isonitriles have been evaluated for their antitumor, antimalarial (i.e. diterpene isonitriles isolated from the Caribbean sponge *Pseudoaxinella flava*), antibiofouling (i.e. kalihinols isolated from *Acanthella cavernosa*), antifungal (i.e. Welwitindolinone A Isonitrile), antitubercular (i.e. isoneoamphilectane isolated from *Svenzea flava*), and antiplasmodial biological activity (Figure 1.1, 1.2, 1.3).⁶⁻⁹ The inhibition for these medicinal purposes has been observed to possibly disrupt the growth of cellulose.¹⁰ These secondary metabolites containing the isonitrile group have recently gained valuable insight into the biosynthetic origins due to the necessity of finding new biologically potent compounds. These purposes highlight the interest in accessing isonitriles and discovering how this unique moiety can be diversified from the limitations discussed in recent literature.
Figure 1.1. Diterpene compounds 1-4 isolated from *Pseudoaxinella flava* that show antitumor and antimalarial activity.\(^7\)

Figure 1.2. Kalihinols, O, Q, P, T, isolated from *Acanthella cavernosa* that show the antibiofouling.\(^8\)

Figure 1.3. Welwitindolinone A and its analogs Welwitindolinones C and D, that show antifungal activity.\(^9\)
1.2 Biological Studies of Isonitrile Containing Natural Products and Possible Biosynthetic Pathway

Biological studies of the biosynthetic origins in marine nature products which contain isonitriles suggest that the isonitrile-containing natural products are derived from terpenoids. Studies of terrestrial natural products suggest amino acids are the sources.\textsuperscript{1,2} For example, the original terrestrial isonitrile natural product of xanthocillin was isolated in 1950 from \textit{Penicillium notatum}, and the marine isonitrile natural product of axisonitrile-1 was isolated in 1973 from \textit{Axinella cannabina} (Figure 1-4).\textsuperscript{8} More recently, understanding of the biosynthetic logic has raised interest due to the unique synthesis of compounds that have limitations. My approach is to combine chemical biology with organic synthesis to inspect this unique vinyl isonitrile functional group biosynthesis.

Figure 1.4. Xanthocillin is likely to originate from naturally occurring amino acids (terrestrial organisms) and axisonitrile-1 is originated from terpenoid precursor (marine organisms).

To date, isonitrile synthases and the Fe (II)/\(\alpha\)-ketoglutarate-dependent oxygenases (IsnA and B and their homologues, i.e. AmbI1-3, WelI1-3, and XnPvcA and B) have been proposed to synthesize vinyl isonitriles from aromatic amino acids (i.e. \(L\)-Tryptophan and \(L\)-Tyrosine).\textsuperscript{10} The first study of vinyl isonitrile biosynthetic pathway suggest that the precursor is \(L\)-Tryptophan.\textsuperscript{11,12} Other studies using tyrosine as the precursor led to paerucumarin formation found in \textit{Pseudomonas aeruginosa} and rhabduscin production from \textit{Xenorhabdus nematophila} (Figure 1-5).\textsuperscript{12} Identification of various products through analogous approaches where isnA and isnB homologues are found in the corresponding biosynthesis pathways promoted the potential possibility to biochemically verify these genes substrate promiscuity and their applications in other areas.\textsuperscript{13,14}
In this study, p.IsnB gene obtained 

\textit{Pseudomonas aeruginosa} was overexpressed in 

\textit{E}. coli. The PlsnB will be used to evaluate vinyl isonitrile biosynthetic mechanism and substrate flexibility. Based on the previous biochemical studies, the isonitrile containing substrate will be activated by the Fe (IV)-oxo intermediate and lead to a vinyl group installation.\textsuperscript{11} The Fe (II)/α-ketoglutarate-dependent oxygenase, PlsnB, is proposed to facilitate the hydroxylation, but also the decarboxylation to complete C=C bond formation.\textsuperscript{11} To address the possibility of expanding the substrate scope, PlsnB must be isolated. Transformation of the gene, p.IsnB containing plasmid in \textit{E}. coli, and purify PlsnB will facilitate \textit{in-vitro} studies. Based on the recent biochemical studies, the PlsnB active site can accept both l-tryptophan and l-tyrosine. After substrate binging, the reaction may proceed through hydroxylation and removal of CO\textsubscript{2} to complete the desaturation.\textsuperscript{11} Due to the hydroxylation and decarboxylative desaturation reactions never being isolated or characterized, our approach is to synthesize the initial substrates and explore the reaction mechanism using purified PlsnB.

\section*{1.3 Synthetic Studies for the Preparation of Isonitriles}

Isonitrile formation can be dated back to Lieke (1859) when allyl iodide was reacted with silver cyanide.\textsuperscript{15} Since then, the synthetic preparation of isonitriles has been improved and applied in total synthesis, multi-component reactions, peptide synthesis, coordination, and carbohydrate chemistry research in recent literature.\textsuperscript{3,4} Preparation of isonitriles usually
happens by dehydration of the formyl group using phosphoryl chloride (POCl₃), Burgess reagent, and phosgene derivatives (Figure 3.3 and 3.4). For example, in the total synthesis of hapalindole, welwitindolinone A and D, fischerindoles, and ambiguine products, these reagents were used to introduce isonitrile moiety. On the other hand, preparation of vinyl isonitrile is limited to a very few reactions. Specifically, the Wittig approach has been commonly used to prepare vinyl isonitriles (Scheme 1.1).

Wittig reaction in making vinyl isonitrile has its limitations based on the following reasons: (i) The synthesis of the desired aldehyde moiety could be challenging with other functional groups present, (ii) The Wittig reagents cannot react efficiently when sterically hindered aldehyde is used. Our goal is to use our approach to catalyze vinyl isonitrile formation using PIßnB as the biocatalyst.

1.4 Scope of Thesis Project

This thesis serves as the foundation of the further study in elucidating PIßnB catalysis. Specifically, compounds prepared here will be used to evaluate and further improve PIßnB activity in catalyzing vinyl isonitrile formation (Scheme 1.2).
The goal of chapter 2 is to establish the synthetic methodology to prepare substrate probes: Isonitrile-L-Phenylalanine, isonitrile-D-phenylalanine, isonitrile-L-tyrosine, isonitrile-L-tryptophan, isonitrile p-methyl-L-phenylalanine, isonitrile p-trifluoromethyl-L-phenylalanine. The biological procedure to overexpress and purify protein will also be discussed. The goal of chapter 3 is to discuss the various synthetic methods as well as protein overexpression optimization.
REFERENCES


CHAPTER 2: Synthetic and Biological Experimental Section

2.1. General Materials

All chemicals (used without purification unless specifically mentioned) were purchased from Fisher Chemical (Fair Lawn, NJ), Acros Organics (Geel, Belgium), or Chem-Impex Int’l Inc. (Wood Dale, IL). Deuterated solvents were acquired by Cambridge Isotope Laboratories Inc. (Andover, MA). Analytical thin layer chromatography (TLC) was carried out on pre-coated TLC aluminum sheets (silica gel, grad 60, F254, 5 x 20 cm) acquired from EMD chemicals (Billevica, MA). Flash column chromatography was performed using silica gel (230-400 mesh, grade 60) obtained from Fisher Scientific (Hanover Park, IL). Nuclear magnetic resonance (NMR) spectra were recorded using Varian 300 or 400 MHz spectrometer at the nuclear magnetic resonance facility of the Department of Chemistry, North Carolina State University.

Enzymes, molecular weight marker, Isopropyl β-D-1-thiogalactopyranoside (IPTG), antibiotics, Luria-Bertani (LB), and E. coli BL21 star (DE3) are products of New England Biolabs (Beverly, MA), BioBasic (Ontario, Canada), or Bio-Rad (Hercules, CA). Protein concentrations were recorded by ultraviolet visible (UV-Vis) spectra on Agilent Technologies Cary UV-Vis system.

2.2. General Synthetic Method for Isonitrile Functional Group Installation

![Scheme 2.1. General synthetic scheme of functional group installation.](image)

2.2.1. Step 1: Methyl Ester Formation
To a solution of the amino acid (1.0 eq.) stirring in methanol (~ 0.1 M), trimethylsilyl chloride (4.0 eq.) was added slowly on ice. After the addition at 0°C, the reaction mixture was warmed to room temperature (r.t.) and kept stirring for 16 hours at that temperature. After 16 hour stirring, the solvent was removed under reduced pressure using rotatory evaporation the crude methyl ester product was used without further purification.

2.2.2. Step 2: Formylation of the Amine Group

To a solution of the crude methyl ester (1.0 eq.) produced in step 1 in acetonitrile (MeCN, ~ 0.1 M), ammonium formate (HCO₂NH₄, 4.0 eq.) was added at room temperature. After addition, the reaction was brought to refluxing by heating an oil bath to 95°C for 16 hours. After cooling the reaction mixture to room temperature, water (same amount as MeCN) was added to the reaction and the mixture was partitioned between ethyl acetate and water. The aqueous layer was extracted using ethyl acetate. The organic layers were combined and dried over magnesium sulfate. After removal of magnesium sulfate by filtration, the product was concentrated and the crude product was subjected to silca-gel column chromatography using ethyl acetate as the eluent. After removal of the solvent under reduced pressure, the formylated product was obtained in reasonable yield (79% to 87%).

2.2.3. Step 3: Isonitrile Functional Group Installation

The formylated product (1.0 eq.) was dissolved in dichloromethane (~ 0.1 M) and Burgess reagent (1.5 eq.), methyl N-(triethylammonium sulfonyl) carbamate, was added at room temperature. The reaction mixture was brought to reflux at 45°C. After kept refluxing for 2 hours, the reaction was cooled to room temperature and then quenched by adding water. The aqueous layer was extracted using ethyl acetate and the organic layers were combined, and purified by silca-gel column chromatography using hexanes (hex) and ethyl acetate (EA). The product was obtained after removal of the solvent under reduced pressure.

2.2.4. Step 4: Hydrolysis of Methyl Ester

The final product was obtained by dissolving the corresponding methyl ester (1.0 eq.) in 1,4-dioxane. The solution was then cooled on ice followed by addition of water, and then lithium hydroxide (LiOH, 1.2 eq., ~ 2M) was added. After addition of LiOH, the reaction was
monitored by TLC (EA/Hex = 7/3). Once the reaction reached completion, 1,4-dioxane was removed under reduced pressure. The reaction mixture was then warmed to room temperature and stirred for 2 hours. The aqueous layer was washed using ethyl acetate and the aqueous layer was concentrated to obtain the final product in reasonable yield (89% to 95%).

2.3. Isonitrile $\text{L-Phenylalanine}^{14}$

\[
\text{\text{L-Phenylalanine}}
\]

Compound 1a ($\text{L-Phenylalanine}$, 823.7 mg, 4.99 mmol), methanol (40 mL), and trimethylsilyl chloride (2.5 mL, 19.94 mmol). $\text{L-Phenylalanine Methyl Ester}$ (~ 97% yield) as a white solid. $^1$H NMR (300 MHz, D$_2$O): $\delta$ = 7.41 (t, $J$ = 9.0 Hz, 3H), 7.27 (d, $J$ = 9.0 Hz, 2H), 4.41 (t, $J$ = 9.0 Hz, 1H), 3.82 (s, 3H), 3.32 (dd, $J$ = 6.0 Hz and 15.0 Hz, 1H), 3.21 (dd, $J$ = 6.0 Hz and 15.0 Hz, 1H) (Appendix A.1).

\[
\text{\text{L-Phenylalanine Methyl Ester}}
\]

Compound 1b ($\text{L-Phenylalanine Methyl Ester}$, 866.9 mg, 4.84 mmol), acetonitrile (40 mL), and ammonium formate (1.221 g, 19.34 mmol). Formyl-$\text{L-Phenylalanine Methyl Ester}$ (84% yield) as a yellow oil. $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ = 8.07 (s, 1H), 7.28 (m, 3H), 7.15 (d, $J$ = 9.0 Hz, 2H), 6.81 (broad s, 1H), 4.94 (q, $J$ = 6.0 Hz, 1H), 3.72 (s, 3H), 3.17 (dd, $J$ = 9.0 Hz and 12.0 Hz, 1H), 3.06 (dd, $J$ = 6.0 Hz and 12.0 Hz, 1H) (Appendix A.2).

\[
\text{\text{Formyl-L-Phenylalanine Methyl Ester}}
\]

Compound 1c (Formyl-$\text{L-Phenylalanine Methyl Ester}$, 841.9 mg, 4.062 mmol), dichloromethane (30 mL), and Burgess reagent (1.45 g, 6.096 mmol). Isonitrile-$\text{L-Phenylalanine Methyl Ester}$
Phenylalanine Methyl Ester (43% yield) as a pale yellow oil (extracted with EA/hex = 4/1). $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ = 7.27 (t, $J$ = 6 Hz, 3H), 7.21 (d, $J$ = 6 Hz, 2H), 4.41 (q, $J$ = 6 Hz, 1H), 3.72 (s, 3H), 3.20 (dd, $J$ = 9.0 Hz and 12.0 Hz, 1H), 3.11(dd, $J$ = 6.0 Hz and 12.0 Hz, 1H) (Appendix A.3).

![Phenylalanine Methyl Ester](image)

Compound 1d (Isonitrile-\(\alpha\)-Phenylalanine Methyl Ester, 331 mg, 1.75 mmol), H$_2$O and 1,4-dioxane (1:2, 3 mL), and LiOH•H$_2$O (88.1 mg, 2.01 mmol). Compound 1e (Isonitrile-\(\alpha\)-Phenylalanine Carboxylic Acid, 91% yield) as a pale yellow solid. $^1$H NMR (400 MHz, D$_2$O): $\delta$ = 7.37-7.34 (m, 3H), 7.29 (t, $J$ = 8.0 Hz, 2H), 4.41 (t, $J$ = 4.0 Hz, 1H), 3.17-3.14 (m, 1H), 3.03 (m, 1H). $^{13}$C NMR (100 MHz, D$_2$O): $\delta$ = 172.65, 153.27 (t, $J$ = 7.0 Hz), 136.06, 129.34, 128.53, 127.26, 60.7 (t, $J$ = 7.0), 38.36 (Appendix A.4 and A.5).

2.4. Isonitrile \(\beta\)-Phenylalanine

![Isonitrile \(\beta\)-Phenylalanine](image)

Compound 2a (\(\beta\)-Phenylalanine, 2.014 g, 12.19 mmol), methanol (120 mL), and trimethylsilyl chloride (6.2 mL, 48.77 mmol). \(\beta\)-Phenylalanine Methyl Ester (92% yield) as a white solid. $^1$H NMR (300 MHz, D$_2$O): $\delta$ = 7.40 (t, $J$ = 6.0 Hz, 3H), 7.28 (d, $J$ = 6.0 Hz, 2H), 4.41 (t, $J$ = 6.0 Hz, 1H), 3.82 (s, 3H), 3.32 (dd, $J$ = 6 Hz and 15.0 Hz, 1H), 3.21 (dd, $J$ = 9.0 Hz and 15.0 Hz, 1H) (Appendix A.6).
Compound 2b (\(\alpha\)-Phenylalanine Methyl Ester, 2.01 g, 11.21 mmol), acetonitrile (110 mL), and ammonium formate (2.83 g, 44.84 mmol) Formyl-\(\alpha\)-Phenylalanine Methyl Ester (81% yield) as a pale yellow oil. \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta = 8.16\) (s, 1H), 7.28 (t, \(J = 6.0\) Hz, 3H), 7.10-7.09 (m, 2H), 6.13 (broad s, 1H), 4.96 (q, \(J = 6.0\) Hz, 1H), 3.74 (s, 3H), 3.19 (dd, \(J = 6.0\) Hz and 15.0 Hz, 1H), 3.10 (dd, \(J = 9.0\) Hz and 15.0 Hz, 1H) (Appendix A.7).

\[
\text{\begin{center}
\includegraphics[width=0.2\textwidth]{compound2b.png}
\end{center}}
\]

Compound 2c (Formyl-\(\alpha\)-Phenylalanine Methyl Ester, 1.88 g, 9.08 mmol), dichloromethane (90 mL), and Burgess reagent (3.24 g, 13.62 mmol). Isonitrile-\(\alpha\)-Phenylalanine Methyl Ester (41% yield) as a pale white oil (extracted with EA/hex = 4/1). \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta = 7.28\) (t, \(J = 9.0\) Hz, 3H), 7.21 (d, \(J = 9.0\) Hz, 2H), 4.40 (dd, \(J = 6.0\) Hz, 1H), 3.73 (s, 3H), 3.19 (dd, \(J = 6.0\) Hz and 15.0 Hz, 1H), 3.07 (dd, \(J = 9.0\) Hz and 15.0 Hz, 1H) (Appendix A.8).

\[
\text{\begin{center}
\includegraphics[width=0.2\textwidth]{compound2c.png}
\end{center}}
\]

Compound 2d (Isonitrile-\(\alpha\)-Phenylalanine Methyl Ester, 704 mg, 3.72 mmol), H\(_2\)O and 1,4-dioxane (1:2, 5 mL), and LiOH\(\bullet\)H\(_2\)O (188 mg, 4.47 mmol). Compound 2e (Isonitrile-\(\alpha\)-Phenylalanine Carboxylic Acid, 93% yield) as a pale white solid. \(^1\)H NMR (400 MHz, D\(_2\)O): \(\delta = 7.34\) (t, \(J = 8.0\) Hz, 3H), 7.39 (d, \(J = 8.0\) Hz, 2H), 4.42 (t, \(J = 4.0\) Hz, 1H), 3.19-3.14 (m, 1H), 3.07-3.02 (m, 1H). \(^1^3\)C NMR (100 MHz, D\(_2\)O): \(\delta = 172.66, 153.20\) (t, \(J = 7.0\) Hz), 136.05, 129.34, 128.53, 127.25, 60.69 (t, \(J = 7.0\) Hz), 38.36 (Appendix A.9 and A.10).
2.5. Isonitrile \( \alpha \)-Tyrosine\(^3\)

![Chemical structure](image)

Compound 3a (\( \alpha \)-Tyrosine, 1.12 g, 6.15 mmol), methanol (60 mL), and trimethylsilyl chloride (3.1 mL, 24.62 mmol). \( \alpha \)-Tyrosine Methyl Ester (91% yield) as a white solid. \(^1\)H NMR (300 MHz, D\(_2\)O): \( \delta = 7.03 \) (d, \( J = 9 \) Hz, 2H), 6.80 (d, \( J = 9.0 \) Hz, 2H), 4.28 (t, \( J = 6.0 \) Hz, 1H), 3.75 (s, 3H), 3.05 (dd, \( J = 6.0 \) Hz and 15.0 Hz, 2H) (Appendix A.11).

![Chemical structure](image)

Compound 3b (\( \alpha \)-Tyrosine Methyl Ester, 1.09 g, 5.60 mmol), acetonitrile (60 mL), and ammonium formate (1.41 g, 22.40 mmol). Formyl-\( \alpha \)-Tyrosine Methyl Ester (83% yield) as a yellow solid. \(^1\)H NMR (300 MHz, CDCl\(_3\)): \( \delta = 8.17 \) (s, 1H), 6.96 (d, \( J = 9.0 \) Hz, 2H), 6.74 (d, \( J = 9.0 \) Hz, 2H), 6.09 (broad s, 1H), 5.49 (s, 1H), 4.94 (q, \( J = 9.0 \) Hz, 1H), 3.76 (s, 3H), 3.13 (dd, \( J = 6.0 \) Hz and 15.0 Hz, 1H), 3.04 (dd, \( J = 9.0 \) Hz and 15.0 Hz, 1H) (Appendix A.12).

![Chemical structure](image)

Compound 3c (Formyl-\( \alpha \)-Tyrosine Methyl Ester, 1.04 g, 4.65 mmol), dichloromethane (60 mL), and Burgess reagent (1.66 g, 6.97 mmol). Isonitrile-\( \alpha \)-Tyrosine Methyl Ester (42% yield) as a light orange oil (extracted with EA/hex = 3/2). \(^1\)H NMR (300 MHz, CDCl\(_3\)): \( \delta = 7.08 \) (d, \( J = 8.0 \) Hz, 2H), 6.78 (d, \( J = 8.0 \) Hz, 2H), 6.38 (s, 1H), 4.45 (t, \( J = 8.0 \) Hz, 1H), 3.79 (s, 3H), 3.18 (dd, \( J = 8.0 \) Hz and 16.0 Hz, 1H), 3.07 (dd, \( J = 12.0 \) Hz and 16.0 Hz, 1H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \( \delta = 166.83, 159.69 \) (broad), 155.75, 130.68, 126.13, 115.90, 58.52 (broad), 53.63, 38.21 (Appendix A.13 and A.14).
Compound 3d (Isonitrile-\(\text{\textit{L}}\)-Tyrosine Methyl Ester, 401 mg, 1.952 mmol), \(\text{H}_2\text{O}\) and 1,4-dioxane (1:2, 3mL), and LiOH•\(\text{H}_2\text{O}\) (98.29 mg, 2.342 mmol). Compound 3e, (Isonitrile-\(\text{\textit{L}}\)-Tyrosine Carboxylic Acid, 95% yield) as a red solid. \(^1\)H NMR (400 MHz, \(\text{D}_2\text{O}\)): \(\delta = 7.07\ (d, J = 8.0\ \text{Hz}, 2H), 6.64\ (d, J = 8.0\ \text{Hz}, 2H), 4.35\ (q, J = 4.0\ \text{Hz}, 1H), 3.07-3.04\ (m, 1H), 2.95-2.89\ (m, 1H). \(^{13}\)C NMR (100 MHz, \(\text{D}_2\text{O}\)): \(\delta = 175.67, 163.79, 155.50\ (t, J = 7.0\ \text{Hz}), 133.15, 126.61, 119.89, 63.73\ (t, J = 7.0\ \text{Hz}), 40.30\) (Appendix A.15 and A.16).

2.6. Isonitrile \(\text{\textit{L}}\)-Tryptophan\(^{15}\)

Compound 4a (\(\text{\textit{L}}\)-Tryptophan, 6.14 g, 30.04 mmol), methanol (100 mL), and trimethylsilyl chloride (15.3 mL, 120.2 mmol). \(\text{\textit{L}}\)-Tryptophan Methyl Ester (96% yield) as a white solid. \(^1\)H NMR (400 MHz, \(\text{D}_2\text{O}\)): \(\delta = 7.53\ (d, J = 8.0\ \text{Hz}, 1H), 7.48\ (d, J = 8.0\ \text{Hz}, 1H), 7.24-7.21\ (m, 2H), 7.14\ (t, J = 8.0\ \text{Hz}, 1H), 4.37\ (t, J = 8.0\ \text{Hz}, 1H), 3.75\ (s, 3H), 3.43-3.32\ (m, 2H)\) (Appendix A.17).

Compound 4b (\(\text{\textit{L}}\)-Tryptophan Methyl Ester, 6.29 g, 28.84 mmol), acetonitrile (100 mL), and ammonium formate (7.27 g, 115.36 mmol). Formyl-\(\text{\textit{L}}\)-Tryptophan Methyl Ester (87% yield) as a pale purple solid (extracted with EA/hex = 3/2). \(^1\)H NMR (400 MHz, \(\text{CDCl}_3\)): \(\delta = 8.35\ (\text{broad s, 1H}), 8.11\ (s, 1H), 7.53\ (d, J = 8.0\ \text{Hz}, 1H), 7.34\ (d, J = 8.0\ \text{Hz}, 1H), 7.19\ (t, J = 8.35\ (\text{broad s, 1H}), 8.11\ (s, 1H), 7.53\ (d, J = 8.0\ \text{Hz}, 1H), 7.34\ (d, J = 8.0\ \text{Hz}, 1H), 7.19\ (t, J =
8.0 Hz, 1H), 7.12 (t, \( J = 8.0 \) Hz, 1H), 6.96 (s, 1H), 6.24 (broad s, 1H), 5.01 (q, \( J = 4.0 \) Hz and 12.0 Hz, 1H), 3.70 (s, 3H), 3.39-3.29 (m, 1H) (Appendix A.18).

Compound 4c (Formyl-\( \tau \)-Tryptophan Methyl Ester, 6.18 g, 25.09 mmol), dichloromethane (100 mL), and Burgess reagent (8.97 g, 37.64 mmol). Isonitrile-\( \tau \)-Tryptophan Methyl Ester (47% yield) as a light orange oil. \(^1\)H NMR (400 MHz, CDCl\(_3\)): \( \delta = 8.30 \) (broad s, 1H), 7.59 (d, \( J = 8.0 \) Hz, 1H), 7.36 (d, \( J = 8.0 \) Hz, 1H), 7.25-7.15 (m, 3H), 4.57 (q, \( J = 4.0 \) Hz, 1H), 3.77 (s, 3H), 3.47 (dd, \( J = 4.0 \) Hz and 16.0 Hz, 1H), 3.36 (dd, \( J = 8.0 \) Hz and 16.0 Hz, 1H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \( \delta = 167.19, 160.04 \) (broad), 136.17, 136.17, 126.78, 123.94, 122.39, 119.84, 118.17, 111.64, 108.38, 57.63 (broad), 53.52, 29.55 (Appendix A.19 and A.20).

Compound 4d (Isonitrile-\( \tau \)-Tryptophan Methyl Ester, 2.69 g, 11.79 mmol), H\(_2\)O and 1,4-dioxane (1:2, 20 mL), and LiOH\( \cdot \)H\(_2\)O (593.7 mg, 14.15 mmol). Compound 4e (Isonitrile-\( \tau \)-Tryptophan Carboxylic Acid, 94% yield) as a pale orange solid. \(^1\)H NMR (400 MHz, D\(_2\)O): \( \delta = 7.69 \) (d, \( J = 8.0 \) Hz, 1H), 7.44 (d, \( J = 8.0 \) Hz, 1H), 7.26 (s, 1H), 7.20 (t, \( J = 8.0 \) Hz, 1H), 7.11 (t, \( J = 8.0 \) Hz, 1H), 4.44 (t, \( J = 8.0 \) Hz, 1H), 3.36 (dd, \( J = 4.0 \) Hz and 16.0 Hz, 1H), 3.23 (dd, \( J = 8.0 \) Hz and 16.0 Hz, 1H). \(^{13}\)C NMR (100 MHz, D\(_2\)O): \( \delta = 173.21, 152.79 \) (t, \( J = 7.0 \) Hz), 135.83, 126.69, 124.50, 121.71, 119.07, 118.56, 111.66, 108.96, 60.30 (t, \( J = 7.0 \) Hz), 28.79 (Appendix A.21 and A.22).
2.7. Isonitrile p-Methyl-l-Phenylalanine

Compound 5a (p-Methyl-l-Phenylalanine, 861.2 mg, 4.46 mmol), methanol (30 mL), and trimethylsilyl chloride (2.3 mL, 17.83 mmol). p-Methyl-l-Phenylalanine Methyl Ester (90% yield) as a white solid. $^1$H NMR (300 MHz, D$_2$O): $\delta = 7.23$ (d, $J = 9.0$ Hz, 2H), 7.16 (d, $J = 9.0$ Hz, 2H), 4.38 (t, $J = 6.0$ Hz, 1H), 3.81 (s, 3H), 3.28 (dd, $J = 6.0$ Hz and 18.0 Hz, 1H), 3.17 (dd, $J = 9.0$ Hz and 18.0 Hz, 1H), 2.30 (s, 3H) (Appendix A.23).

Compound 5b (p-Methyl-l-Phenylalanine Methyl Ester, 775 mg, 4.01 mmol), acetonitrile (30 mL), and ammonium formate (1.01 g, 16.04 mmol). Formyl p-Methyl-l-Phenylalanine Methyl Ester (79% yield) as a yellow oil. $^1$H NMR (300 MHz, CDCl$_3$): $\delta = 8.12$ (s, 1H), 7.08 (d, $J = 9.0$ Hz, 2H), 6.97 (d, $J = 9.0$ Hz, 2H), 6.27 (broad s, 1H), 4.90 (q, $J = 6.0$ Hz, 1H), 3.73 (s, 3H), 3.12 (dd, $J = 6.0$ Hz and 15.0 Hz, 1H), 3.05 (dd, $J = 6.0$ Hz and 15.0 Hz, 1H), 2.30 (s, 3H) (Appendix A.24).

Compound 5c (Formyl p-Methyl-l-Phenylalanine Methyl Ester, 701 mg, 3.17 mmol), dichloromethane (30 mL), and Burgess reagent (1.13 g, 4.75 mmol). Isonitrile p-Methyl-l-Phenylalanine Methyl Ester (45% yield) as a clear oil (extracted with EA/hex = 4/1). $^1$H NMR (300 MHz, CDCl$_3$): $\delta = 7.26$-7.12 (m, 4H), 4.44 (q, $J = 6.0$ Hz, 1H), 3.80 (s, 3H), 3.26 (dd, $J = 6$ Hz and 15.0 Hz, 1H), 3.10 (dd, $J = 9.0$ Hz and 15.0 Hz, 1H), 2.34 (s, 3H) (Appendix A.25).
Compound 5d (Isonitrile p-Methyl-L-Phenylalanine Methyl Ester, 299 mg, 1.43 mmol), H₂O and 1,4-dioxane (1:2, 3 mL), and LiOH•H₂O (72 mg, 1.71 mmol). Compound 5e (Isonitrile p-Methyl-L-Phenylalanine Carboxylic Acid, 89% yield) as a white solid. ¹H NMR (400 MHz, D₂O): δ = 7.17-7.13 (m, 4H), 4.39 (t, J = 6.0 Hz, 1H), 3.13-3.09 (m, 1H), 3.01-2.97 (m, 1H), 2.26 (s, 3H). ¹³C NMR (100 MHz, D₂O): δ = 172.74, 153.11 (t, J = 6.0 Hz), 137.29, 132.98, 129.28, 129.06, 60.78 (t, J = 6.0 Hz), 37.96, 19.98 (Appendix A.26 and A.27).

2.8. Isonitrile p-Trifluoromethyl-L-Phenylalanine

Compound 6a (p-Trifluoromethyl-L-Phenylalanine, 209 mg, 0.89 mmol), methanol (15 mL), and trimethylsilyl chloride (0.45 mL, 3.58 mmol). p-Trifluoromethyl-L-Phenylalanine Methyl Ester (91% yield) as a white solid. ¹H NMR (300 MHz, D₂O): δ = 7.67 (d, J = 9.0 Hz, 2H), 7.42 (d, J = 9.0 Hz, 2H), 4.44 (t, J = 9.0 Hz, 1H), 3.77 (s, 3H), 3.31 (m, 2H) (Appendix A.28).

Compound 6b (p-Trifluoromethyl-L-Phenylalanine Methyl Ester, 201 mg, 0.81 mmol), acetonitrile (15 mL), and ammonium formate (205 mg, 3.26 mmol). Formyl p-Trifluoromethyl-L-Phenylalanine Methyl Ester (81% yield) as a yellow oil. ¹H NMR (300 MHz, CDCl₃): δ = 8.16 (s, 1H), 7.53 (d, J = 9.0 Hz, 2H), 7.24 (d, J = 9.0 Hz, 2H), 6.28 (broad
s, 1H), 4.97 (q, J = 6.0 Hz, 1H), 3.74 (s, 3H), 3.25 (dd, J = 6.0 Hz and 15.0 Hz, 1H), 3.15 (dd, J = 6.0 Hz and 15.0 Hz, 1H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ = 171.36, 160.85, 139.95, 129.73 (d, J = 25 Hz), 129.69, 125.25 (q, J = 4.0 Hz), 124.13 (q, J = 270.0 Hz), 52.62, 51.69, 37.51 (Appendix A.29 and A.30).

![Chemical structure](image)

Compound 6c (Formyl p-Trifluoromethyl-$\alpha$-Phenylalanine Methyl Ester, 181 mg, 0.66 mmol), dichloromethane (10 mL), and Burgess reagent (237 mg, 0.99 mmol). Isonitrile p-Trifluoromethyl-$\alpha$-Phenylalanine Methyl Ester (50% yield) as a yellow oil (extracted with EA/hex = 3/1). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ = 7.62 (d, J = 4.0 Hz, 2H), 7.39 (d, J = 4.0 Hz, 2H), 4.50 (dd, J = 4.0 Hz, 1H), 3.82 (s, 3H), 3.32 (dd, J = 4.0 Hz and 12.0 Hz, 1H), 3.21 (dd, J = 8.0 Hz and 12.0 Hz, 1H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ = 166.20, 161.59, 138.45, 129.90 (d, J = 32.0 Hz), 129.82, 125.25 (q, J = 4.0 Hz), 124.07 (q, J = 270.0 Hz), 57.55, 53.60, 38.39 (Appendix A.31 and A.32).

![Chemical structure](image)

Compound 6d (Isonitrile p-Trifluoromethyl-$\alpha$-Phenylalanine Methyl Ester, 85 mg, 0.33 mmol), H$_2$O and 1,4-dioxane (1:2, 1 mL), and LiOH•H$_2$O (17 mg, 0.39 mmol). Compound 6e (Isonitrile p-Trifluoromethyl-$\alpha$-Phenylalanine Carboxylic Acid, 91% yield) as a pale yellow solid. $^1$H NMR (400 MHz, D$_2$O): $\delta$ = 7.63 (d, J = 8.0 Hz, 2H), 7.42 (d, J = 8.0 Hz, 2H), 4.47 (t, J = 8.0 Hz, 1H), 3.23-3.20 (m, 1H), 3.15-3.10 (m, 1H). $^{13}$C NMR (100 MHz, D$_2$O): $\delta$ = 172.21, 153.52 (t, J = 7.0 Hz), 140.19, 129.76, 128.62 (q, J = 32.0 Hz), 125.25 (q, J = 4.0 Hz), 124.15 (q, J = 270.0 Hz), 60.25 (t, J = 7.0 Hz), 38.02. $^{19}$F NMR (282.4 MHz, D$_2$O): $\delta$ = -62.34 (Appendix A.33, A34, and A.35).
2.9. Burgess Reagent\textsuperscript{7-11}

\[
\begin{align*}
\text{Cl} \quad \text{N} = \text{C} \quad \text{O} \quad \text{S} \quad \text{O} \\
\text{Cl} \quad \text{N} \quad \text{C} \quad \text{O} \quad \text{S} \\
\text{MeOH} \quad \text{toluene} \quad \text{r.t.}, 1 \text{ hour} \quad \text{TEA} \quad \text{toluene} \quad \text{r.t.}, 1 \text{ hour}
\end{align*}
\]

To a solution of chloro-sulfonyl-isocynoate (0.87 mL, 10.02 mmol) in toluene (15 mL), methanol (0.46 mL, 11.37 mmol) was added dropwise on ice. After addition, the solution was warmed to room temperature and kept stirring for an additional 1 hour. After stirring 1 hour at r.t., methyl (chlorosulfonyl)carbamate was obtained as a white solid under reduced pressure. The crude product was immediately resuspended into toluene and triethylamine (3.15 mL, 22.55 mmol) was added dropwise at room temperature to the reaction mixture. The reaction solution was stirring for 1 hour at r.t. The side product, triethylammonium chloride, the solid was further washed using dried THF. The combined filtrate was cooled 0°C and the Methyl (carboxysulfamoyl)triethylammonium (Burgess reagent) precipitated out of solution as an orange crystal. Burgess reagent was further dried to produce Methyl (carboxysulfamoyl)triethylammonium as a light orange solid (82% yield). \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}): \( \delta = 3.67 \) (s, 3H), 3.44 (q, 6H), 1.38 (t, 9H) (Appendix A.36).

2.10 – Plasmid Transformation/Extraction

Codon-optimized Isonitrile synthase B from \textit{Pseudomonas aeruginosa} (pIsnB) was cloned into pET-28a(+) via EcoRI and NdeI restriction cutting sites. The resulting plasmid was transformed into two different types of \textit{E. coli} cells: BL21 (DE3) for protein overexpression and Top10 for plasmid extraction. The transformations were performed via heat shock and plated on Kan-selective LB agar media. A single colony from each type of cell was picked and used to inoculate two 10 mL LB media (kanamycin, 50 µg/µL) and incubated in a shaker for \( \sim 16 \) hours at 37°C and 220 rpm.

To obtain the pIsnB plasmid for identification, 10 mL of top 10 cell culture was centrifuged. The DNA concentration was identified using Nanodrop by measuring the absorbance at 260 nm. The plasmid purification was performed using the EZ-10Spin Column DNA Plasmid Miniprep Kit. The isolated pIsnB concentration was 205.6 ng/µL and the purity was identified by A260/A280 value of 1.87.
2.11. Small Scale Overexpression of PI\textit{snB}

BL21 culture was used to overexpress the protein, PI\textit{snB}. After inoculation of 10 mL starting culture, a 250 mL LB Broth solution with 50 µg/µL kanamycin, the flask was placed in the shaker at 37°C and 220 rpm. With OD\textsubscript{600} at ~ 0.6, 0.5 M IPTG was added to induce PI\textit{snB} overexpression. Samples were collected at 0, 1, 2, 3, and 4 hours to have PI\textit{snB} overexpression. SDS-PAGE analysis (10% Coomassie Blue Staining) confirm expression did occur (Figure 3.1).

2.12. Large Scale Overexpression of PI\textit{snB}

For large scale growth, a single cell colony was used to inoculate 250 mL LB Broth solution with 50 µg/µL Kan to prepare the start culture and incubated in a shaker for ~ 16 hours. After inoculation, the cell LB (1 L x 6) was placed into the incubator 37°C and shaking at 200 rpm. When OD\textsubscript{600} reaches ~0.6, the cells were chilled on ice for 1 hour and 0.5 M IPTG was added to induce PI\textit{snB} overexpression. After IPTG induction, the cell culture was kept shaking at 18°C and 200 rpm for ~16 hours. The cells were harvested by centrifuged for 30 minutes at 4°C and 3200 rpm.

The resulting cell paste was then lysed via sonication (3 cycles with 1 cycle being 30 sec induction and 90 sec delay) for and centrifuged for 30 minutes at 16000 rpm 4°C. The supernatant was loaded to a nickel-nitrilotriacetic acid (Ni-NTA) column and washed with 3 column equivalents of lysis buffer (100mM Tris(hydroxymethyl)aminomethane (Tris) at pH 7.25). The N-His\textsubscript{6}-tagged proteins were eluted with the elution buffer containing 250mM imidazole and 100mM Tris at pH 7.25. Elution fractions containing the proteins were confirmed by SDS-PAGE analysis with 10% Coomassie Blue Staining (Figure 3.2) and concentrated using Macrosep Advance Centrifugal Devices (Pall, NY). The protein solution concentrated to a volume of 5 mL and dialysis with 2 L buffer containing 10mM EDTA, 5% glycerol, 200mM sodium chloride, and 100mM Tris (pH =7.25) overnight followed by twice with 2 L buffer containing 5% glycerol, 200mM sodium chloride, 100mM Tris (pH =7.25) overnight. The dialyzed protein solution was then degassed and aliquoted and frozen in liquid nitrogen. Protein concentration was determined by UV absorption at 280 nm using the
calculated PlsnB molar absorptivity 43890 M$^{-1}$cm$^{-1}$ (calculated from http://ca.expasy.org). The protein concentration was 1.44 mM.
REFERENCES


CHAPTER 3: Results and Discussion for Synthetic and Biological Methods

3.1 – Synthetic Methods Discussion

To successfully introduce an isonitrile functional group onto any amino acid and further use them as mechanistic probes to evaluate the PIsnB desaturation mechanism. The synthetic plan began with identifying a general method used to formylate a primary amine of amino acids and then convert the formylate functional group into the corresponding isonitrile. In the literature, a few of the commonly used methods are known to formylate a primary amine. Three approaches were evaluated. First, acetic formic anhydride was prepared to formylate the given amine of the amino acid (Scheme 3.1). In this synthetic plan, the acetic formic anhydride, prepared in situ, was used immediately.

![Scheme 3.1. Formylation of the primary amine using acetic formic anhydride.](image)

After monitoring the reaction using thin layer chromatography (TLC), I found that the acetic formic anhydride did not result in a consistent product. It is possible that the acetic formic anhydride was not prepared successfully in the first place. Next, I tried to formylate the amine using I₂ and formic acid (Scheme 3.2). Under I₂ and formic acid reaction conditions and column chromatography, the formylated product with impurities was obtained. Although formylation did work considering the product yield, it would not be an efficient method to work with the other substrates. After both previous methods were evaluated, the third approach
was to try using ammonium formate (HCO₂NH₄) as a formylation reagent in acetonitrile which is outlined in the synthetic method in chapter 2.1. Indeed, the formylation using HCO₂NH₄ gave moderate to great yields (79%-87%) with all amino acids tried in this thesis. It is worth mentioning that formylation can be achieved through formic esters activated using electron withdrawing groups (EWG). In this case, the presence of EWG will activate the nucleophilic attack on the formic carbonyl carbon center.

![Scheme 3.2. Formylation reaction using formic acid in the presence I₂ (with impurities).](image)

After formylation could be achieved in efficient yield with all probes (~ 82%), the common methods to convert the formyl group into an isonitrile group was examined. Initially, I tried phosphoryl chloride (POCl₃) in the presence of triethylamine, Et₃N (Scheme 3.3). Although I had success with this method on some products (i.e. L-Phenylalanine and D-Phenylalanine), I was not able to purify a tyrosine product when this method was applied. Furthermore, the yield was moderate and varied batch by batch. From practical perspective, this reaction can’t be amplified due to the inconsistencies.

With these issues, a milder dehydrating agent, Burgess Reagent (Methyl (carboxysulfamoyl)triethylammonium), was tried. This was the method outlined in chapter 2.1. When this method was used to all probes, all products can be obtained in ~ 50% yield after column chromatography. With this product purified in ~ 50% yields, I
Scheme 3.3. Isonitrile formation using phosphoryl chloride in the presence of Et$_3$N.

moved on to try the final reactions to complete the synthesis for all substrates using a single method for all compounds. In the literature, another option was to use phosgene (carbonyl dichloride) as the dehydrating reagent which has been used in the total synthesis of Welwitindolinone A.$^{10}$

Scheme 3.4. Phosgene reaction with triethylamine to install the isonitrile functional group.

Based on the NMR spectra (Appendix A.1-A.36), six isonitrile-containing amino acid derivatives were successfully synthesized in good yields (Table 3.1). It is worth to mention that the isonitrile functional group could be hydrolyzed and formed the formylated product during prolong exposure to water. If stored as the dry salt, all amino acid mechanistic probes are stable.
<table>
<thead>
<tr>
<th></th>
<th>Step 1</th>
<th>Step 2</th>
<th>Step 3</th>
<th>Step 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Isonitrile l-Phenylalanine</strong></td>
<td>92%</td>
<td>84%</td>
<td>43%</td>
<td>91%</td>
</tr>
<tr>
<td><strong>Isonitrile d-Phenylalanine</strong></td>
<td>92%</td>
<td>81%</td>
<td>41%</td>
<td>93%</td>
</tr>
<tr>
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<td>91%</td>
<td>83%</td>
<td>42%</td>
<td>95%</td>
</tr>
<tr>
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<td>87%</td>
<td>47%</td>
<td>94%</td>
</tr>
<tr>
<td><strong>Isonitrile p-Methyl-l-Phenylalanine</strong></td>
<td>90%</td>
<td>79%</td>
<td>45%</td>
<td>89%</td>
</tr>
<tr>
<td><strong>Isonitrile p-Trifluoromethyl-l-Phenylalanine</strong></td>
<td>91%</td>
<td>81%</td>
<td>50%</td>
<td>91%</td>
</tr>
<tr>
<td><strong>Burgess Reagent</strong></td>
<td></td>
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<td>82%</td>
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</table>

Table 3.1. Product yields for steps 1-4 of all isonitrile amino acids and the final yield of Burgess reagent.

All the compounds made using this general method are considered as potential probes. Specifically, we can test the possible factors that control PIsnB catalysis; i.e. electron withdrawing groups (p-trifluoro), electron donating groups (p-methyl), and stereospecificity (L vs D).

### 3.2 – Biological Methods Discussion

The *pisn*B gene was inserted into the pET-28a vector using NdeI and EcoRI cutting sites (Figure 3.1) in *E. coli*. The key features of the plasmid are (i) the T7 promoter and terminator, (ii) lac operon, and (iii) antibiotic resistance marker (kanamycin). The T7 promoter is used as the RNA polymerase marker to facilitate transcription. The lac operon is used as the inducer targeting gene overexpression when IPTG is added. The antibiotic resistance marker is used to maintain the plasmid in *E. coli*. Finally, the T7 terminator will cease the RNA polymerase transcription that facilitates the overexpression process.
Figure 3.1. Plasmid map of pET-28a with pisnB gene insertion. The amino acid sequence is known (Appendix A.37).

To evaluate the substrate scope PIsnB, the protein, needed to be obtained effectively and efficiently. To achieve this goal, overexpression using E. coli as the host was performed. The small scale (~ 250 mL) overexpression was carried out first to showcase that protein is properly overexpressed.

To start, a small scale overexpression test in 250 mL using LB broth was performed from the extended procedure described in chapter 2.11. This was performed to ensure protein overexpression and its solubility before scaling up. Specifically, once the cells were grown (OD$_{600}$ at ~ 0.6) and induced with IPTG (with the final concentration of 0.5 mM), samples were taken at hour 0, 1, 2, 3, and 4 after induction. A SDS-PAGE gel electrophoresis experiment was carried out to observe the level of overexpression when time increases (Figure 3.2). PIsnB, at 35 kD along with other host proteins, were seen. Only the protein with the corresponding dye share the positive correlation after the IPTG induction.
Figure 3.2. The SDS-PAGE gel of the small scale overexpression of PIsn B. Lane 1 is the molecular ladder. Lane 2 is at time = 0 hr after induced with 0.5 mM IPTG. Lane 3 is at time = 1 hr after induced with IPTG. Lane 4 is at time = 2 hr after induced with IPTG. Lane 5 is at time = 3 hr after induced with IPTG. Lane 6 is at time = 4 hr after induced with IPTG.

When the protein was observed to successfully overexpress in the small scale overexpression test, a 6L scale overexpression using LB broth was performed using the experimental procedure detailed in chapter 2.12 (as shown in Figure 3.3). Samples were taken from the cell lysate, flow through, wash, elution 1-4. PIsn B protein is observed in elutes 1-4 and hardly any desired protein is observed in the flow through or wash. The protein was concentrated to the final volume of 5 mL. The concentration of PIsnB was determined using UV-Vis spectroscopy (Figure 3.4). The concentration of purified PIsnB is calculated to be 1.44 mM using the absorbance at A_{280nm} and extinction coefficient of 43890 M⁻¹cm⁻¹.
Figure 3.3. The SDS-PAGE gel of the large scale overexpression of PI3n B. Lane 1 is the cell lysate. Lane 2 is the flow through of cell lysate before Ni-NTA the column. Lane 3 is the wash of the column. Lane 4 is the molecular ladder. Lane 5 is elution 1. Lane 6 is elution 2. Lane 7 is elution 3. Lane 8 is elution 4.

Figure 3-4. UV-Vis spectrum of the background (100 mM Tris pH = 7.25) and the protein (100x dilution in 100 mM Tris pH = 7.25).
3.3 – Experimental Methods Conclusion

The protein, $p$-Isn B, can be overexpressed and purified using Ni-NTA affinity column at 35 kD using the procedures described in chapter 2.11 and 2.12. The concentration of PI$n$B protein has been evaluated using UV-Vis spectroscopy. Toward this end, I have successfully prepared 6 mechanistic probes, and overexpressed and purified PI$n$B in a decent yield (1.44 mM, 5 mL). My work provides a good foundation ready for mechanistic investigation of PI$n$B catalysis.
REFERENCES

A.1 (1b)
A.2 (1c)
A.3 (1d)
A.6 (2b)
A.7 (2c)
A.9 (2e)
A.10 (2e)
A.11 (3b)
A.12 (3c)
A.13 (3d)
A.15 (3e)
A.19 (4d)
A.21 (4e)
A.22 (4e)
A.24 (5c)
A.25 (5d)
A.29 (6c)
A.30 (6c)
A.31 (6d)
A.33 (6e)
A.35 (6e)
A.36

[Chemical structure diagram]

[Graphical representation of a spectrum with labels and values]
p. IsnB amino acid sequence (5’-NdeI (His-tag) and 3’-EcoRI)

MGSSHHHHHHHHSSGLVPRGSHMMTELENSFQTEEITPFLKIPQYSDQYIDTLPEQLKELARKHHLILRGFKSDLSDHEKYEKYARNWGEIMMWPGAILDVRHQQDATDHFNDSMPLHWDGMYKPIPEFIFHCAMPSQGGRRTTFFNTRRVANATQQQLEQWKNISITYRINKVTHGGEVHSPLEEHPRDRNGFVIRYNEPAVDGKFLNKHAEYHNINPDQVAEFQQDFINILYDKRHLYAHAWKKSVPDLVIVDNFSLHLHGREGFTSKSERHLQRQIHSNPAFNNQALRSSLSTOP