ABSTRACT

SANYAL, DEV KUMAR. PaPvcB: Mechanistic Study of an Olefin-producing Mononuclear Iron Enzyme. (Under the direction of Dr. Wei-chen Chang)

Pyoverdine chromophore synthase B from *Pseudomonas aeruginosa*, a.k.a. PaPvcB, is a member of iron (II)-/ 2-oxoglutarate (2-OG) dependent enzyme family. The enzyme is proposed to catalyze a novel 4-electron oxidation producing a double bond and a direct C-O bond formation to form 2-Isocyano-7-hydroxy coumarin, a proposed key intermediate in the biosynthesis of the natural product paerucoumarin. This study provides a background and description of possible PaPvcB reaction pathways. In addition, the work carried out to study the mechanism of PaPvcB catalyzed reaction is documented in this thesis.

PaPvcB: Mechanistic Study of an Olefin-producing Mononuclear Iron Enzyme.

by
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DEDICATION

I would like to thank my family, first and foremost, for always supporting me throughout my educational journey. In addition, I would like to thank Dr. Mike Messina and Dr. Tom Coombs for their mentorship and advice during my time as an undergraduate research student at the University of North Carolina at Wilmington. I would especially like to thank Dr. Wei-chen Chang for selecting me to join his group in the fall of 2016 as well as Dr. Vincent Lindsay and Dr. Caroline Proulx for their guidance during my time as a graduate research student at North Carolina State University.

BIOGRAPHY

Dev Sanyal was born on February 27, 1988 to Kaya Sanyal and Arunava Sanyal at Rex hospital in Raleigh, North Carolina. He has one brother, Neil Sanyal. Dev attended Leesville Road High School in Raleigh where he was a state championship winning soccer player and president of the electronics club. Dev obtained his B.S. in chemistry and minor in mathematics from the University of North Carolina at Wilmington and joined Wei-chen Chang's lab at North Carolina State University to earn his M.S. in Chemistry. Dev plans to continue teaching at North Carolina State University before joining the chemical industry.

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CHAPTER 1: INTRODUCTION

1.1 Discovery of PaPvcB via Vinyl Isonitrile Group Biosynthetic Enzymes

Over a decade ago in an effort to explore the biosynthetic potential of bacteria, Brady and colleagues used a characterized isonitrile synthase, IsnA,[1-6] and a non-heme iron oxygenase, IsnB gene as probes to search bacterial genomes as well as DNA extracted directly from environmental soil samples.[1-6] Among identified genes, pyoverdine chromophore synthase B derived from *Pseudomonas aeruginosa* (*P. aeruginosa*), PaPvcB, the enzyme of interest in this study, is homologous to IsnB. The proposed natural product of PaPvcB involved pathway is predicted to be paerucoumarin, compound, in figure 1 below. It was found in cultures of P. *aeruginosa* that overexpresses PvcB containing genes.[1] The biosynthetic precursor to paerucoumarin, 2-isocyano-7-hydroxy coumarin, **6 (scheme 1)**, is unique, if it is in fact the enzymatic result of PaPvcB because it would represent a novel oxidative direct C-O bond cyclization catalyzed by Fe^{2+} and 2-oxoglutarate dependent enzymes.

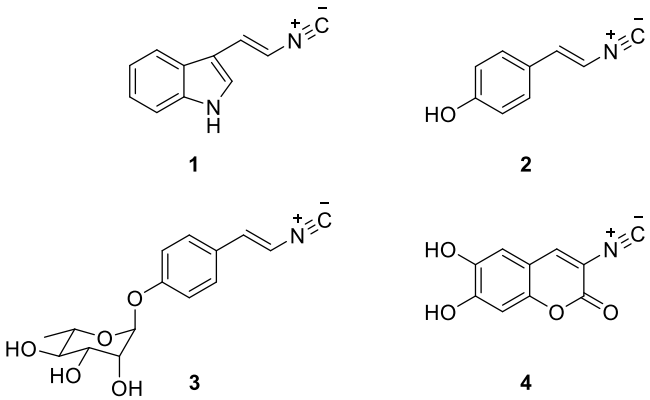
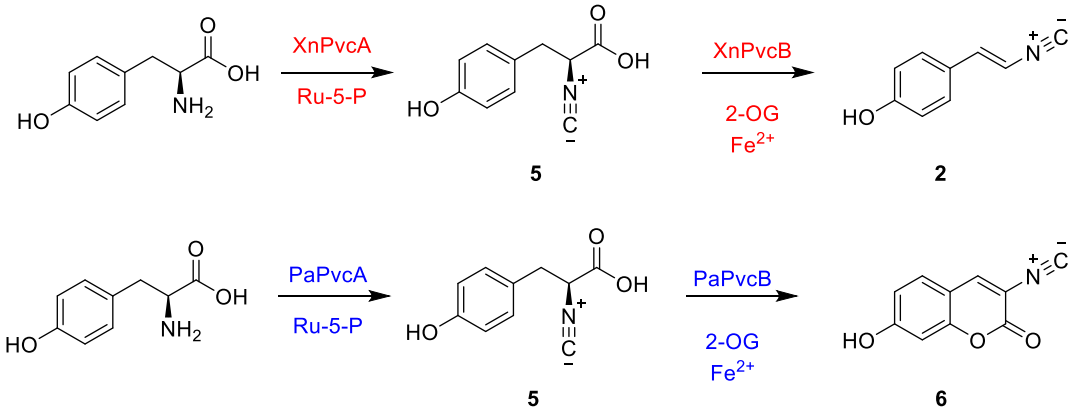


Figure 1. Metabolites isolated from the culture broths of bacteria transformed with predicted isnA, isnB-biosynthetic operons. Adapted from Brady et al.[1]

The enzymes responsible for the production of **2** and **3** from the corresponding isonitrile precursors, XnPvcB and PaPvcB, share a 41% sequence identity to one another.[7] While both enzymes catalyze olefination via a 2-electron oxidation using tyrosine isonitrile **5** as the

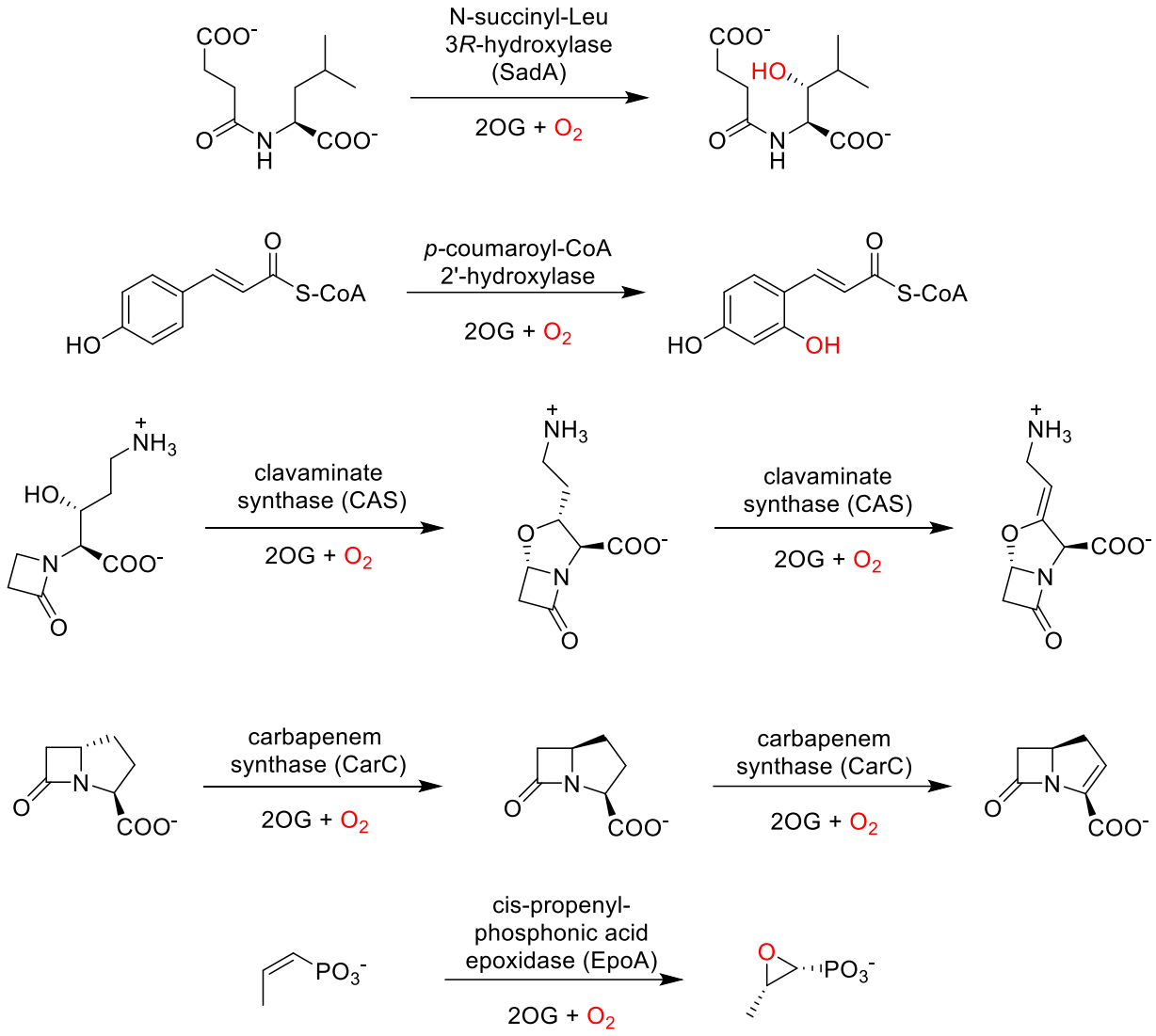
substrate, PaPvcB is predicted to carry out an additional 2-electron oxidation to catalyze a direct C-O bond formation to form the coumarin ring.[8] The product of sequential XnPvcA, XnPvcB reaction is then converted to compound **3** via o-glycosylation, where **3** can prevent an insect from regenerating its exoskeleton after invasion by the bacterial host nematode.[9] While the biological role of **3** is known in nature, the function of the proposed enzymatic product of the PaPvcABCD gene cluster, **4** is unknown. Although it was believed that PaPvcA/PaPvcB are involved in the biosynthesis of the pyoverdine chromophore, the function of each protein has not been established. In addition, compound **1** is the enzymatic product of isonitrile synthase A/B which utilizes tryptophan as a substrate whereas XnPvcA/B and PaPvcA/B utilize tyrosine as the substrate as shown in **scheme 1**.



Scheme 1. Proposed isonitrile synthase and desaturase biosynthetic pathway to account for **2** & **6** production.

1.2 Fe/2OG dependent oxygenases

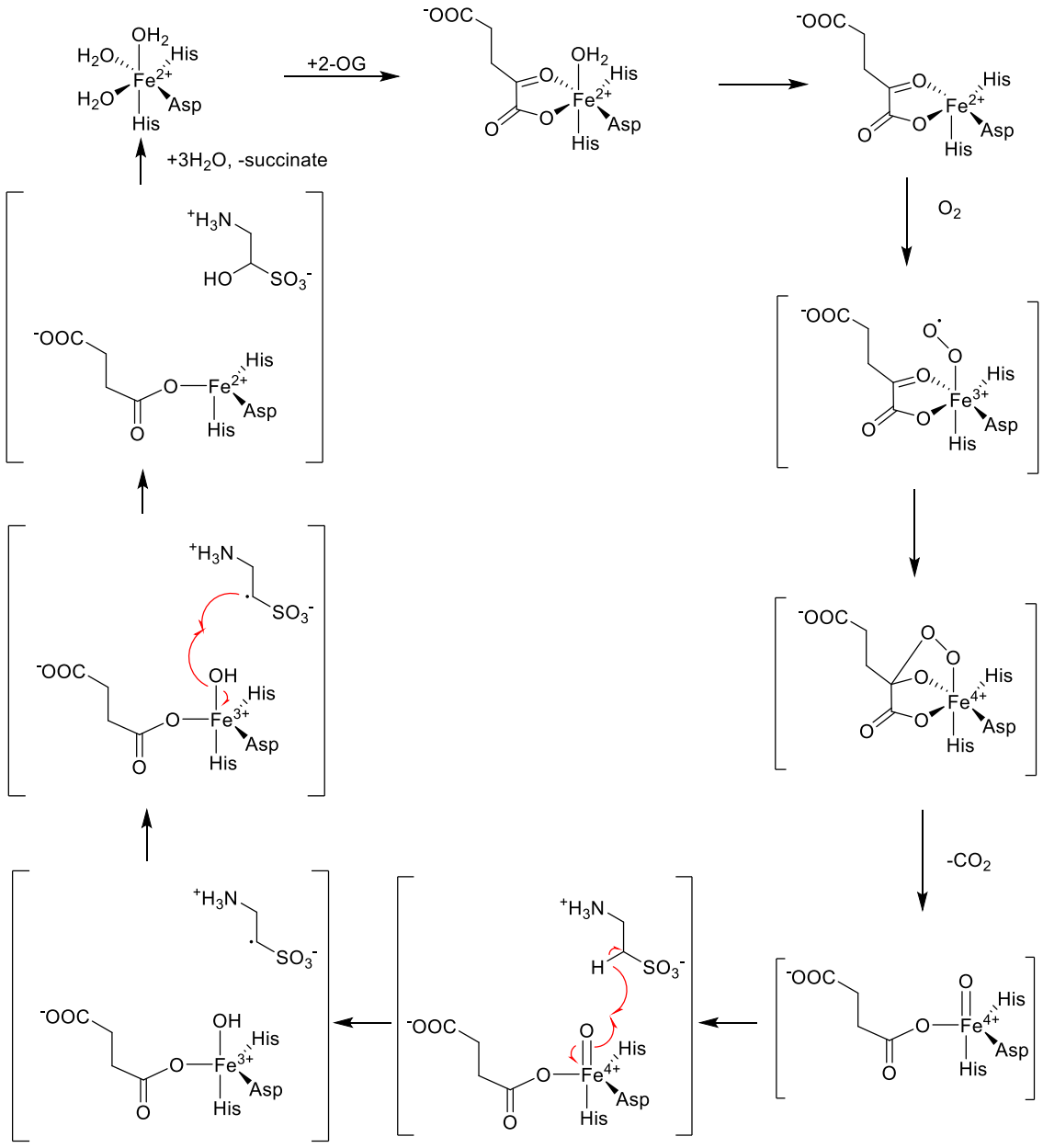
Mononuclear iron and 2-oxoglutarate (Fe/2OG) enzymes are well known in nature.[10] There is a plethora of published literature discussing various oxidation outcomes and plausible mechanisms of actions of catalysis.[10] Most of these enzymes coordinate the active site iron at one end of a double stranded β-helix fold using a 2-His-1-carboxylate motif, bind 2-OG along with the substrates using less conserved regions of the core and additional loops. The majority of enzymes in this family catalyze hydroxylation reactions, but desaturation, ring formation, ring expansion, halogenation, and several other types of chemistry are known.[10-15] Two known examples of hydroxylation on an aliphatic and aryl substrates are shown below in **scheme 2.** SadA from *Burholderia ambifaria* acts on several N-substituted amino acids with hydrophobic side chains, notably catalyzing regio- and stereospecific 3*R* hydroxylation of N-succinyl-L-Leu.[16] *P*-coumaroyl-CoA 2'-hydroxylase is involved in the biocatalysis of a coumarin ring.[17] As shown below it catalyzes hydroxylation at the C2 position of the aromatic ring. Clavaminate synthase, (CAS), is another remarkable trifunctional member in this family of enzymes, it is involved in the pathway for synthesis of clavulanic acid is *Streptomyces clavuligerus*.[18] CAS catalyzes a hydroxylation (not shown), followed by a cyclization and finally a desaturation.[18] Carbapenem synthase (CarC) is another oxygenase in the enzyme family proposed to catalyze two sequential atypical reactions: epimerization and desaturation to yield (5*R*)-carbapenem-3-carboxylate.[19] Rather than hydroxylation, cis-propenyl-phosphonic acid epoxidase (EpoA) of *Penicilium decumbens* catalyzes epoxidation using cis-propenylphosphonic acid during the synthesis of Fosfomycin.[20]



Scheme 2. Several examples of hydroxylation, cyclization, olefination, epimerization and epoxidation catalyzed Fe/2OG enzymes. Adapted from Housinger et al.[10]

Through investigations of several Fe/2OG hydroxylases, the mechanism of hydroxylation, which involves the abstraction of the hydrogen atom by the high-spin (S=2) ferryl complex and 'rebound' of the Fe(III)-coordinated hydroxyl ligand to the substrate radical, has been established.[10-15] Important features of this mechanism include bidentate coordination of 2OG to the Fe(II) center, which the protein coordinates facially by one carboxylate (Asp or Glu) and two histidine ligands.[10-15] Addition of O_2 to the Fe(II) center to yield an Fe(III)-superoxo intermediate, where the reaction is followed by attack of the distal oxygen atom from O_2 on C2

of 2OG initiates O-O cleavage, decarboxylation and formation of a ferryl intermediate (**scheme 3**). The reaction is followed by abstraction of the target hydrogen atom from the substrate by the ferryl intermediate to yield a Fe(III)-OH and a substrate radical. Formation of the hydroxylated product by recombination of substrate radical and the Fe(III)-OH species complete the hydroxylation and reforms the Fe(II) species.[21]



Scheme 3. Proposed mechanism of Fe^{2+} activation and taurine hydroxylation catalyzed by TauD, an Fe/2OG enzyme. Adapted from Bollinger et al.[21]

1.3 PaPvcB

A stereo-representation of the active site of PaPvcB is shown below in **figure 2**. To reveal possible binding position of the protein-substrate interaction, computational docking was used to identify a possible position for the substrate, compound **7** in the active site.[7] According to the structure, His110, Asp112 and His259 are thought to be the triad, similar to the one shown previously in **scheme 3**, responsible for coordinating Fe^{2+}.[7] The pose of the docked tyrosine isonitrile is oriented such that hydroxylation occurs at the *pro-S* position, and subsequent anti-elimination upon abstraction of the α-proton, would result in the *E* configuration of the double bond, which is required for cyclization to form the coumarin ring in the PaPvcB-catalyzed reaction.[7]

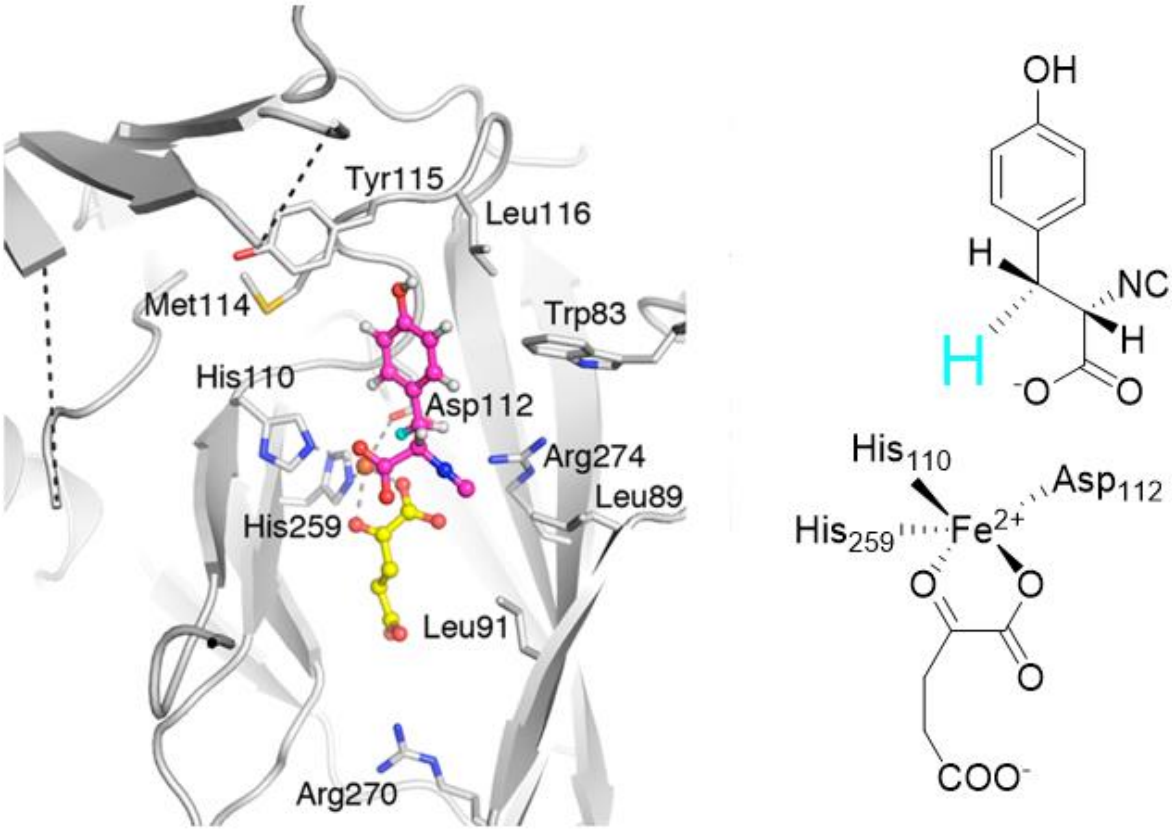
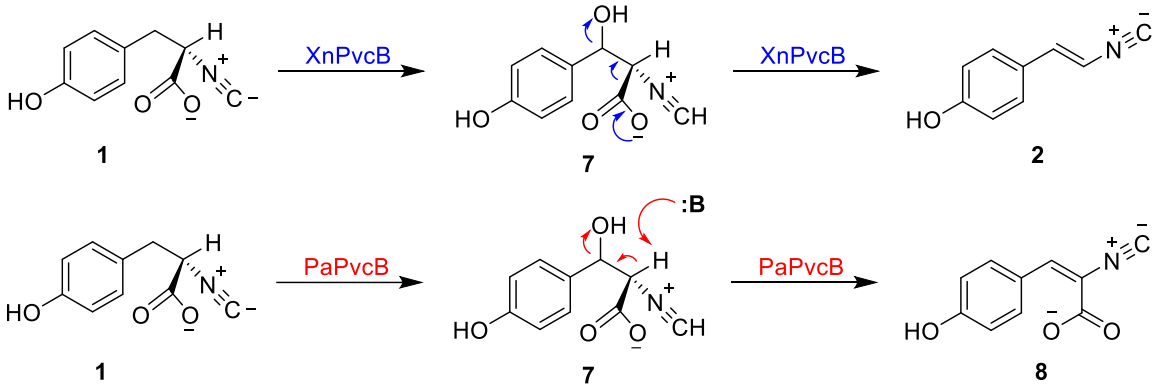


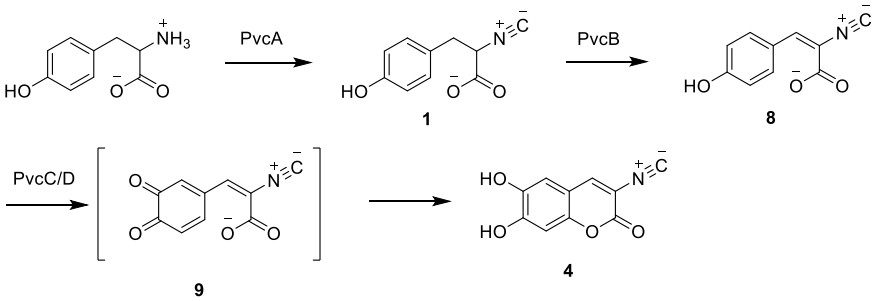
Figure 2. Stereo-representation of the model of tyrosine isonitrile docked into the active site of PaPvcB. Adapted from Zhu et al.[7]

In a study by Zhu and colleagues, 2-isocyano-7-hydroxy coumarin was not isolated in enzymatic experiments.[7] Instead, based on the analysis of the derivatized methyl ester, 3-(4-hydroxyphenyl)-2-isocyonacrylate (**8**) as the major product,[7] Zhu proposed that PaPvcB catalyzes desaturation through an active site general base to facilitate expulsion of the hydroxyl group as shown below in **scheme 4.**[7]



Scheme 4. Proposed Mechanism of XnPvcB & PaPvcB catalyzed desaturation. Adapted from Zhu et. Al.[7]

Based on the recent discovery,[7] the revised role of PaPvcB and the other proteins involved in paerucoumarin (**4)** biosynthesis is summarized in **scheme 5**. The PvcA is involved in isonitrile formation. PvcB introduces an olefin moiety onto the substrate. Subsequently, PvcC/D catalyzes sequential modifications, hydroxylation, C-O bond formation to complete the reaction.[22]



Scheme 5. Revised Pathway of PvcABCD involved in paerucoumarin (**4**). Adapted from Clarke-pearson et al.[22]

CHAPTER 2: RESULTS

2.1 Overexpression and Purification of PaPvcA & PaPvcB

In order to elucidate the mechanism of PaPvcB and confirm the product of the PaPvcA/B catalyzed reaction, both PaPvcA and PaPvcB need to be purified. PaPvcA and PaPvcB gene containing plasmids were first transformed into *Escherichia coli* (*E. coli BL 21*). The *E. coli* cells were then grown in Luria-Bertani rich (LB-rich) medium and shaken at 37 ˚C until the optical density of media solution reached a minimum value of 0.6 (OD_{600} = 0.6), and protein overexpression was induced with isopropyl β-D-1-thiogalactosidase (IPTG). After an additional 16-18 hours of shaking at 18 ˚C, the cells were centrifuged, lysed by sonication and the desired proteins were purified using a Nickel-nitrilotriacetic acid (Ni-NTA) agarose resin to isolate the histidine-tagged proteins. PaPvcA and PaPvcB with approximate molecular weights of 38 kDa and 41 kDa were isolated as with a final concentration of 2.8 mM and 1.5 mM respectively as shown below in **figure 3** and **figure 4**.

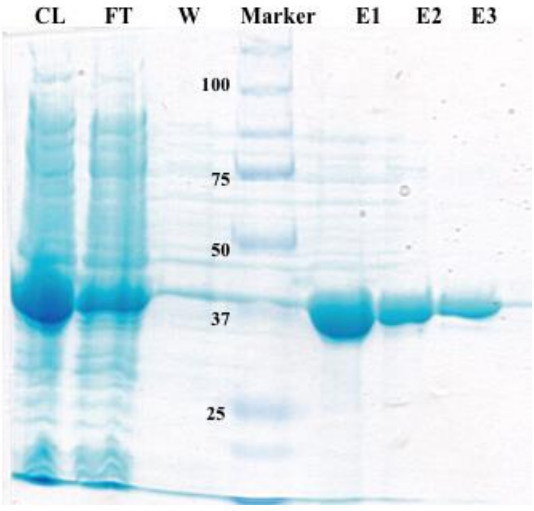


Figure 3. Sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE) analysis of PaPvcA, MW ≅ 38 kDa.CL = Cell lysate, FT = flow through, W = wash, Marker is a protein ladder (kDa), E = elution.

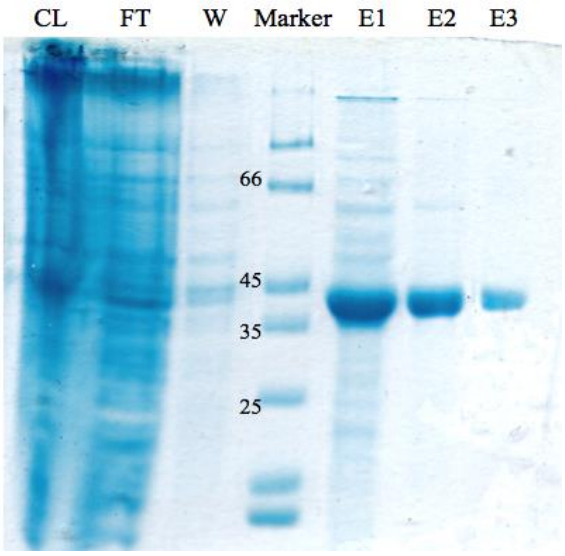


Figure 4. SDS-PAGE Analysis of PaPvcB, MW ≅ 41 kDa. CL = Cell lysate, FT = flow through, W = wash, Marker is a protein ladder (kDa), E = elution

2.2. Synthetic substrates, intermediates and product standards

In addition to purifying the enzymes of interest, substrates and proposed intermediates based on the natural substrates, intermediates and natural products were required to properly explore the mechanism of action. The compounds designed to elucidate the mechanism are shown below in **figure 5**.

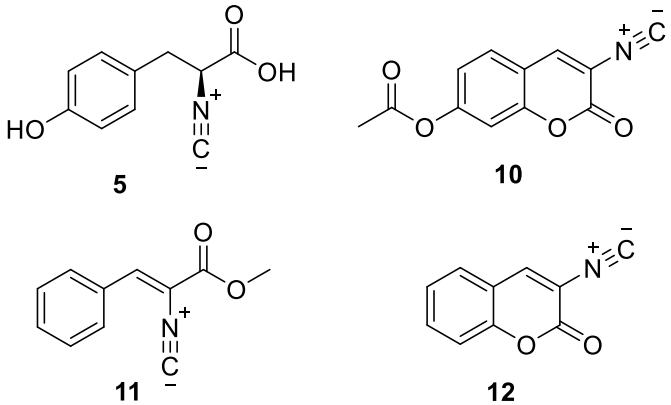
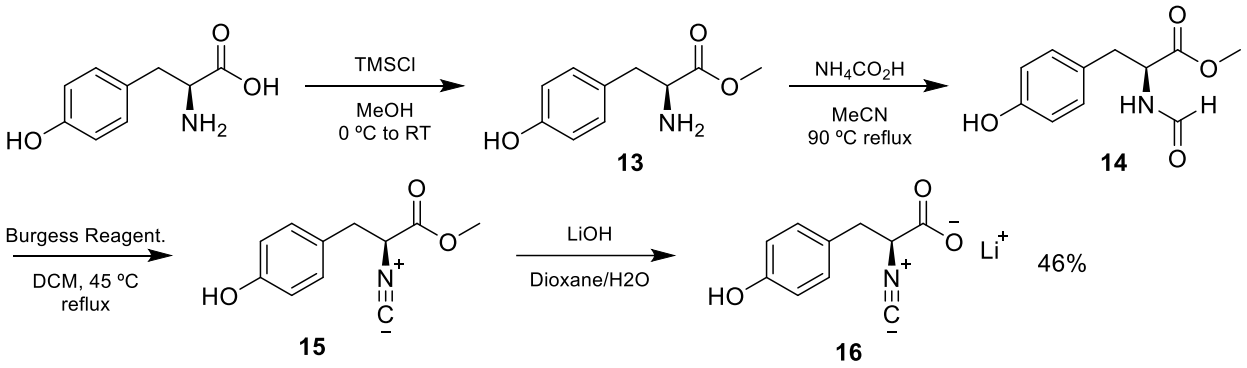


Figure 5. Substrates, probes, proposed intermediates and product standards synthesized to elucidate the mechanism of PaPvcB

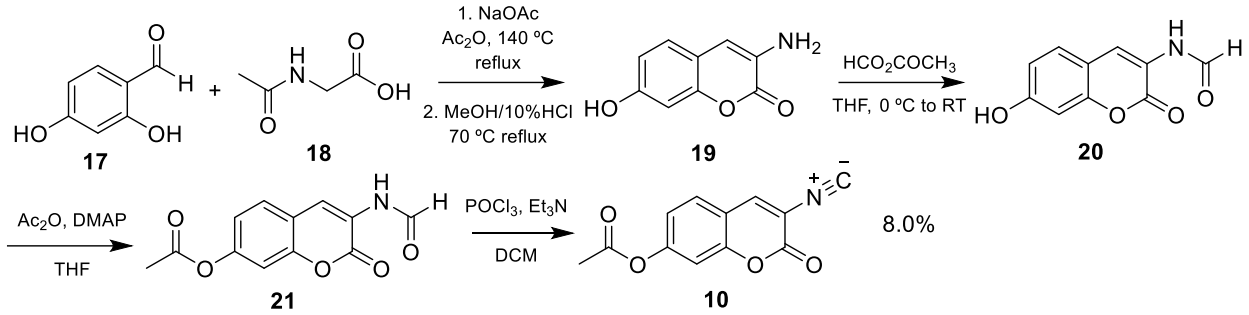
First of all, the native substrate of PaPvcB, L-tyrosine isonitrile, **5**, was synthesized to test purified PvcB activity and to probe the mechanism of PaPvB. The compound (**5)**, was completed

utilizing a synthetic method in which L-tyrosine was first methyl protected as the carboxylic acid methyl ester. Next, formylation of the amine was carried out using ammonium formate under mild conditions. After formylating the amine, subsequent dehydration to install the isocyanide group was carried out using Burgess's reagent. Saponification using lithium hydroxide was used to produce L-tyrosine isonitrile, **5**, as the lithium salt, **16**.[24]



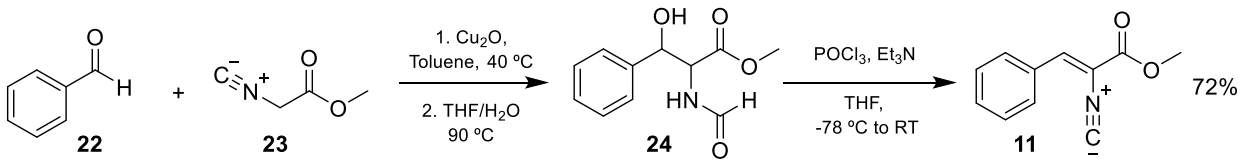
Scheme 6. Synthesis of L-tyrosine isonitrile lithium salt (**16**).

Next, to uncover PaPvcB biocatalysis, a PaPvcB reaction product derivative, 2-isocyano-7-acetyl coumarin **10** was attempted. There are many known procedures to obtain 2-amino-7-hydroxy coumarin (**19)**,[25,26] however reaction conditions needed to be explored and optimized to obtain N-formyl coumarin (**20)**. Attempts to dehydrate **20** to obtain 2-isocyano-7-hydroxy coumarin (**6**) proved unsuccessful. Thus, the hydroxyl group was protected to facilitate dehydration without plausible interference. Specifically, the hydroxyl group at C7 was protected using an acetyl (Ac) group to give N-formyl-7-acetyl coumarin (**21)**, followed by dehydration using $POCl_3$, to obtain 2-Isocyano-7-acetyl coumarin (**10)**, can also be used as a precursor to elucidate the reaction product of PaPvcB reaction. Although the protected isocyanide containing compound was isolated, I was unable to successfully isolate 2-isocyano-7-hydroxy coumarin (**6**). Alternatively, I plan to carry out acetyl group deprotection in situ by using lipases or esterases.



Scheme 7. Synthesis of 2-isocyano-7-acetyl coumarin (**10**).

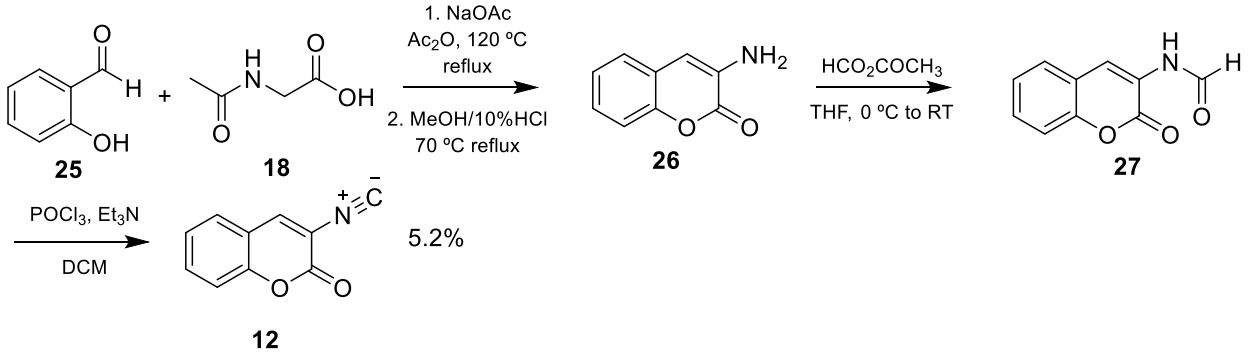
Due to the difficulty to isolate compound **6**, and the idea that phenylalanine should be accepted as a substrate by PaPvcA, two additional compounds, **11** and **12**, were prepared. A potential biosynthetic product precursor **11**, was prepared by coupling benzaldehyde with isocyano-glycine methyl ester, the resulting five-membered ring is then decomposed under THF/water heated conditions to afford compound **24**. The vinyl isonitrile phenylalanine methyl ester (**11**) (**scheme 8**), was then synthesized by a one pot dehydration/elimination condition utilizing triethylamine as the base in the elimination reaction. Deprotection conditions proved unsuccessful as I was unable to isolate the deprotected methyl ester. We plan to hydrolyze the methyl ester bond in situ by using esterase or lipase.



Scheme 8. Synthesis of vinyl isonitrile phenylalanine methyl ester (**11**).

Even though the plausible intermediate could not be obtained, one could incubate **11** with PaPvcB to investigate the possible product. Similar methods have been found to be successful in obtaining the IsnA/IsnB reaction product.[23] In this case, the anticipated product would be **12**. The conditions used to obtain 2-isocyano-7-H coumarin **12**, were developed using the same conditions used in **scheme 7**. Compound **12** was obtained by coupling salicylic acid (**25**), with N-acetyl glycine (**18**), under mild condensation conditions to afford compound **26**. 2-Amino-6,7-

hydro coumarin (**26**) was formylated to achieve N-formyl-6,7-hydro coumarin (**27**), using mixed formic acetic anhydride[27] and dehydrated using $POCl_3$ to obtain 2-Isocyano-6,7-hydro coumarin, **12**. If PaPvcB really does perform catalysis via a 4-electron oxidation, **12** would serve as the product standard if the pathway accepts phenylalanine. If PaPvcA was able to install the isonitrile on phenylalanine, then its plausible that PaPvcB will install the olefin and possibly catalyze the proposed C-O bond formation.

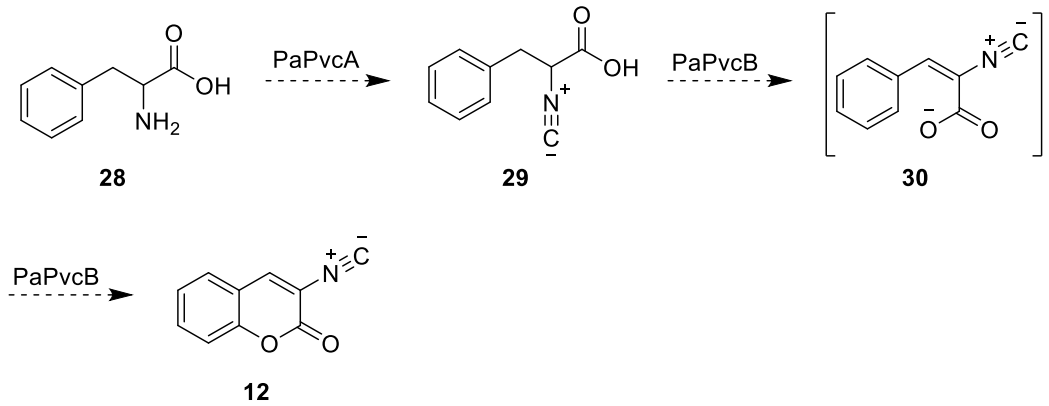


Scheme 9. Synthesis of 2-isocyano-6,7-hydro coumarin (**12**).

CHAPTER 3: CONCLUSIONS AND FUTURE DIRECTIONS

3.1 Synthetic Probes to elucidate PaPvcA/PaPvcB biotransformations

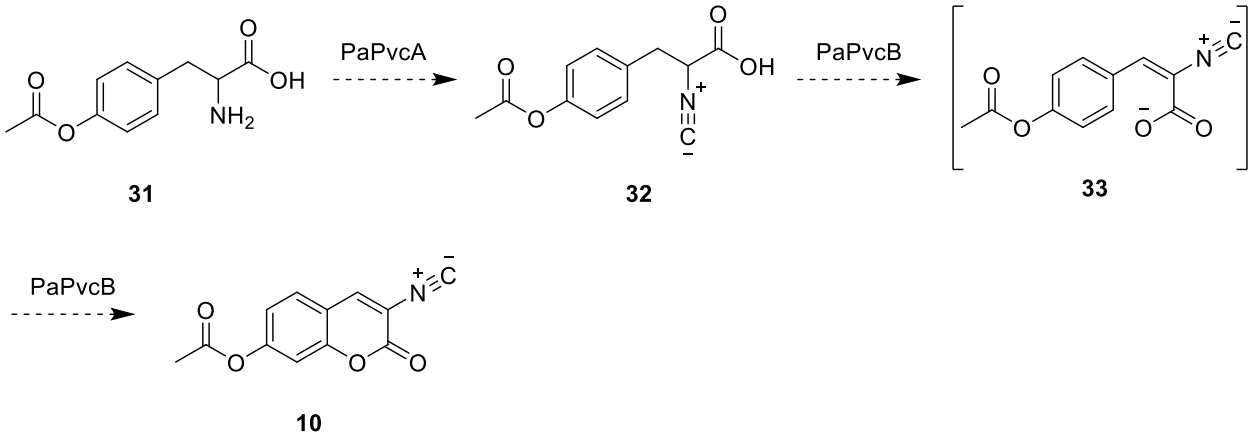
While L-tyrosine is the natural substrate for PaPvcA, due to structure similarity it's likely that phenylalanine could also be accepted by PaPvcA to generate isocyano phenylalanine (**29**) as depicted in **scheme 10**, will be generated. Subsequently PaPvcB could use it as the substrate to produce the proposed cyclized product **12**. In addition, it was discovered that compound **12** was soluble in organic extracts, e.g. ethyl acetate and dichloromethane, which would facilitate simple preparation from enzymatic assay and accurately determine if cyclization occurs as a result of PaPvcB catalysis. In addition, the deprotected vinyl isonitrile L-phenylalanine, **30** can be assayed to investigate its possible role in PaPvcB catalyzed reaction. Liquid chromatography coupled mass spectrometry analysis will be used to establish PaPvcB reaction pathway. Specifically, we will compare the retention time and m/z value of the PaPvcB reactions mixtures with those of the synthetically generated compounds.



Scheme 10. Mechanistic probe L-phenylalanine vinyl isocyanide methyl ester (**11**) used to elucidate plausible mechanism.

Similarly, a new synthetic probe **10** can be used to elucidate the mechanism. Comparing to the native substrate, it is possible that the acetylated tyrosine can be accepted as the substrate

by PaPvcA. If cyclization were to occur through PaPvcA/PvcB reactions, **10** would be readily available as a product standard

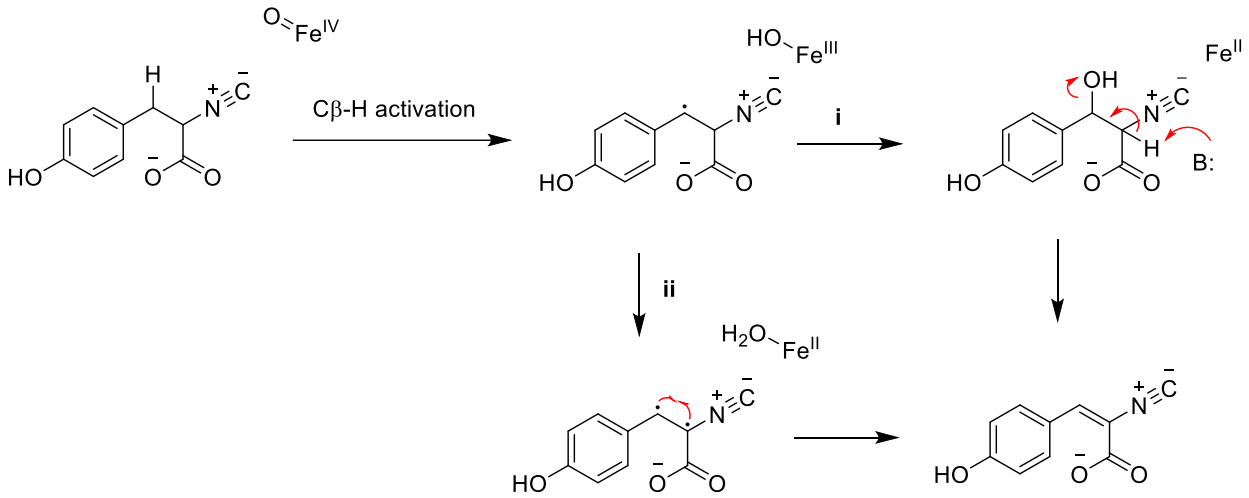


Scheme 11. Mechanistic probe 2-Isocyano-7-acetyl coumarin used to elucidate plausible mechanism.

3.2 Plausible mechanisms of PvcB catalysis

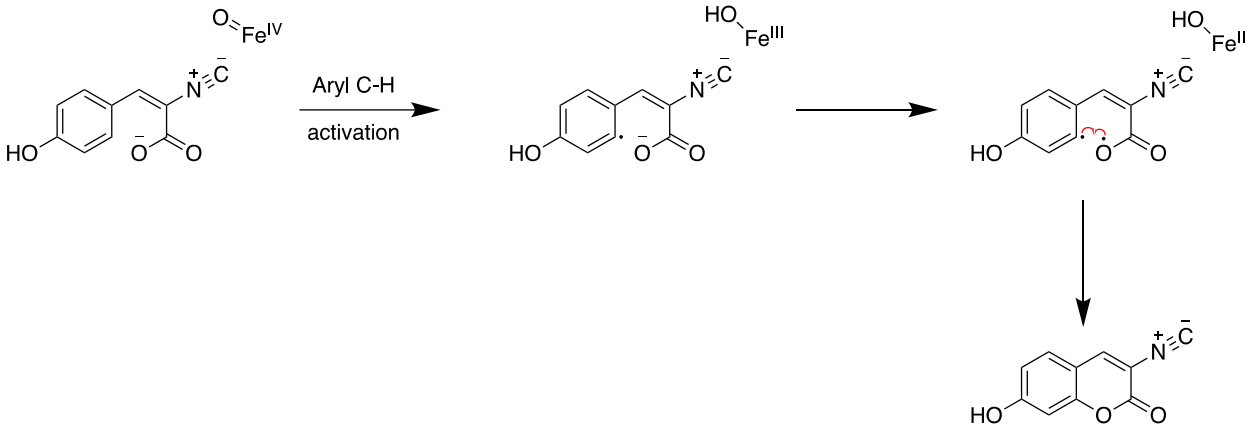
It has been suggested that PaPvcB carries out 2 sequential 2 electron oxidation reactions in its role in the paerucoumarin pathway.[8] However, based on recent publication by Zhu et. Al., only a single 2 electron oxidation occurs to yield the olefin containing tyrosine derivative as shown below in **scheme 12**.[7] Even though the reaction has been demonstrated, the detailed mechanism for the PaPvcB catalyzed olefination has not been elucidated. There are two pathways to account for this transformation. In pathway i, after β-hydrogen abstraction by the iron (IV) oxo species,[21] subsequently, the hydroxyl group rebound would result in beta hydroxylation. Subsequent deprotonation of the α-proton would facilitate the removal of water to generate the double bond and complete the reaction. Alternatively, as evidenced by other Fe^{2+} 2-OG enzymes,[10] the catalysis could proceed via a di-radical as shown in pathway ii (**scheme 12**). In this pathway, after the first hydrogen atom base abstraction, the α-proton is abstracted by the Fe(III)-OH species. The two radicals generated could combine to form the double bond. It is

of fundamental interest to understand how PaPvcB controls the reaction outcome and the stereochemistry of the olefination.



Scheme 12. Proposed mechanisms of PaPvcB catalyzed double bond formation.

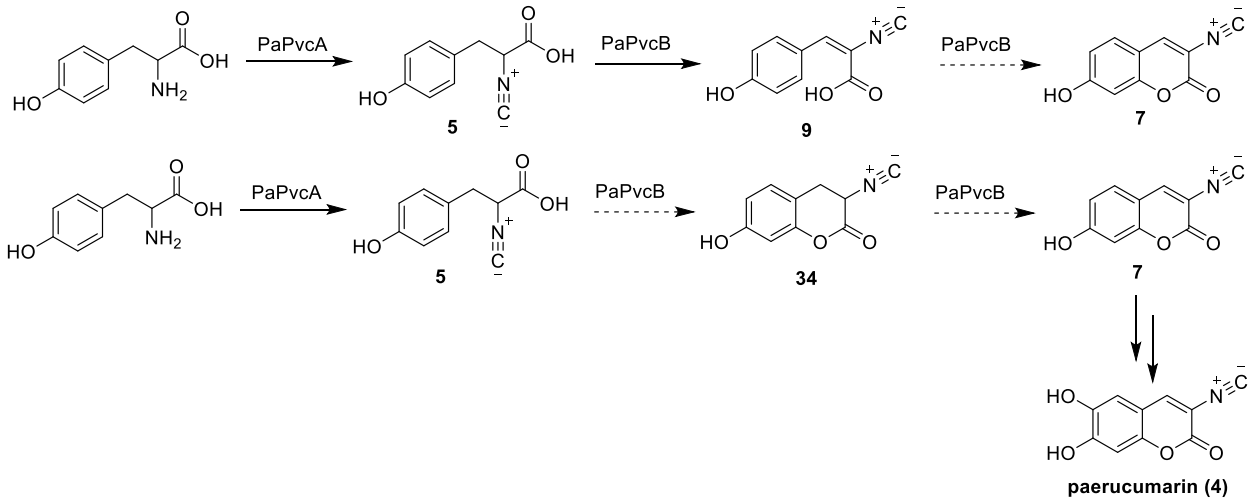
Even though the current literature[7,20] suggests that PaPvcB catalyzes double bond formation, it is still under debate whether the C-O bond formation can be catalyzed by PaPvcB. It is still plausible that C-O bond formation could be performed, if not by PaPvcB as shown in **scheme 13**, but PaPvcC or PaPvcD. If it were to occur by PaPvcB, one would expect that the aryl carbon C-H bond at ortho position to the vinyl ligand would be activated through hydrogen abstraction by the Fe(IV)=O species to yield the aryl radical species. Similar to the radical mechanism proposed above the Fe(III)-OH species could then carry out electron transfer to form the radical species oxyanion and the C-O bond could be formed via di-radical combination. A further examination of PaPvcB, PaPvcC and PaPvcD are required to truly understand the paerucoumarin biosynthetic pathway.



Scheme 13. Proposed Mechanism of coumarin cyclization via C-O bond formation.

3.3 Possible Reaction catalyzed by PaPvcB

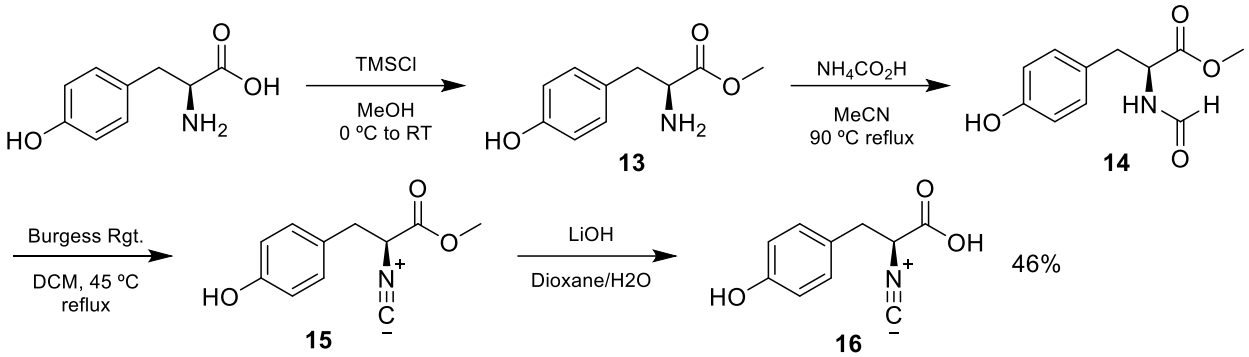
It is of fundamental interest to elucidate PaPvcB's reaction mechanism. There are two plausible routes by which, if 2-isocyano-7-hydroxy coumarin, **7**, is formed, can occur as shown below in **scheme 14**. Olefination could occur first via biocatalytic elimination followed by cyclization, as shown in the top pathway. Alternatively, cyclization could occur first via a biocatalytic direct C-O bond formation followed by elimination to yield compound **7**.



Scheme 14. Plausible routes to account for paerucoumarin (**4**) biosynthesis.

CHAPTER 4: EXPERIMENTAL

4.1 L-Tyrosine isonitrile



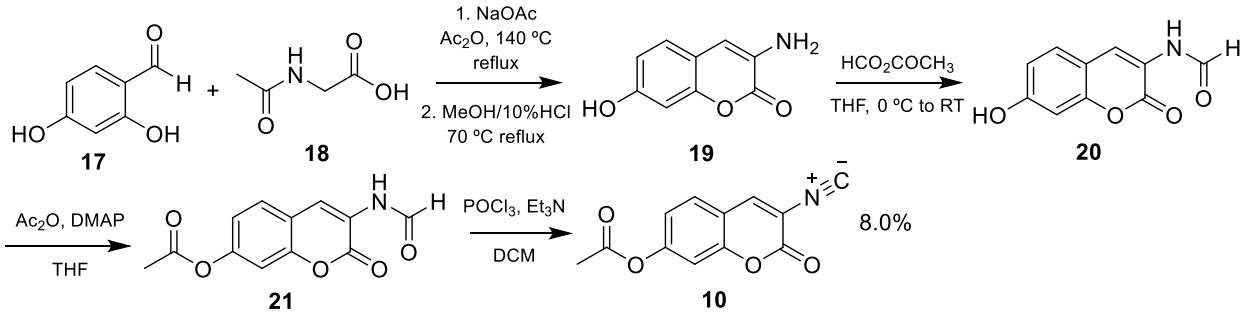
Scheme 15. Preparation of L-Tyrosine Isonitrile lithium salt (**16**).

1.0 g of L-tyrosine (5.5 mmol) was dissolved in 10 mL of methanol and cooled to 0 ºC. 3 mL of trimethylsilyl chloride (TMSCl) was added dropwise via syringe at 0 ºC and the solution was warmed to room temperature and stirred for 12 hours. The solvent was removed under reduced pressure. ^{1}H NMR (MeOD) (400 MHz), δ 7.11 (d, *J* = 8.0 Hz, 2H), 6.85 (d, *J* = 8.0 Hz, 2H), 4.33 (dd, *J* = 16.0, *J* = 8.0 Hz, 1H), 3.2 (dd, *J* = 16.0 Hz, 8.0 Hz, 1H), 3.1 (dd, *J* = 16.0 Hz, 8.0 Hz, 1H)

The crude product was dissolved in 16 mL MeCN, 2.0 g ammonium formate (32.0 mmol) was added to the mixture and the reaction was heated to reflux at 90 ºC for 16 hours. The solvent was removed under reduced pressure and the crude product was purified by column chromatography using 3:1 ethyl acetate:hexanes as eluent. Next, 0.20 g of N-formyl-L-Tyrosine (0.80 mmol) methyl ester and 0.43 g (1.8 mmol) of Burgess reagent were dissolved in 4.0 mL of dichloromethane and allowed to stir for 0.5 hours. The solvent was removed under reduced pressure and the crude product was purified by column chromatography using 1:1 EA:Hex as eluent. ^{1}H NMR ($CDCl_3$) (400 MHz), δ 7.10 (d, *J* = 12.0 Hz, 2H), 6.89 (d, *J* = 12.0 Hz, 2H), 5.85 (s, 1H) 4.44 (dd, *J* = 16.0, *J* = 8.0 Hz, 1H), 3.19 (dd, *J* = 16.0 Hz, 8.0 Hz, 1H), 3.09 (dd, *J* = 16.0 Hz, 8.0 Hz, 1H).

The purified tyrosine isonitrile methyl ester was dissolved in 2:1 dioxane:water mixture followed by the addition of 2 equivalents of lithium hydroxide. After 1 hour the solvent was removed under reduced pressure to give compound **16** in 46% yield. ^{1}H NMR ($CDCl_3$) (400 MHz), δ 7.04 (d, *J* = 8.0 Hz, 2H), 6.61 (d, *J* = 8.0 Hz, 2H), 4.33 (dd, *J* =16.0 Hz, *J* = 8.0 Hz, 1H), 3.02 (dd, *J* = 16.0 Hz, 8.0Hz, 1H), 2.89 (dd, *J* = 16.0 Hz, 8.0Hz, 1H). ^{13}C NMR, (CDCl3) (100 MHz), δ 172.6, 163.3, 153.2, 136.1, 129.3, 129.2, 128.5, 128.4,127.3,126.8

4.2 2-isocyano-7-acetyl coumarin



Scheme 16. Preparation of 2-isocyano-6,7-dihydro coumarin (**10**).

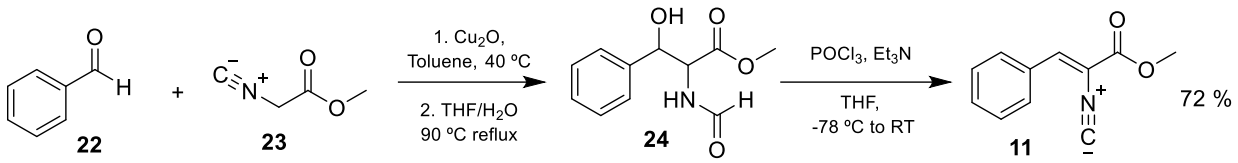
5.52 g of 2,4-dihydroxybenzaldehyde (40 mmol), 4.68 g of N-Acetyl glycine (40 mmol), and 8.20 g of Sodium acetate (100 mmol) were dissolved in 13 mL of Ac_2O. The reaction was brought to reflux at 140 ºC and allowed to stir for 12 hours. After stirring the reaction mixture was poured over ice, filtered, and washed with dichloromethane to give 2.97 g in 35 % yield. Next 2.97 g of diacetyl coumarin (11.2 mmol) was dissolved in 60 mL 10% methanolic HCl. The solution was heated to reflux at 65 ºC and after 4 hours of stirring the solvent was removed under reduced pressure and the crude solid was purified by column chromatography with 10% methanol/saturated ammonia in dichloromethane to produce 1.06 g of 2-amino coumarin in 45 % yield. ^{1}H-NMR, (DMSO) (600 MHz), δ 7.53 (d, *J* = 8.0 Hz, 1H), 6.68 (m, 3H), 5.24 (s, 2H), 3.38 (s, 3H). ^{13}C-NMR, (DMSO) (150 MHz), δ 159.5, 156.6, 149.8, 130.8, 126.3, 114.0, 113.5, 110.3, 102.4.

1.06 g of amino-coumarin (6.0 mmol) was dissolved in 30 mL THF. The solution was cooled to 0 ºC and 3.3 mL of mixed anhydride, that was prepared immediately before the reaction,[27] was added dropwise via syringe. The reaction mixture was allowed to stir for 4 hours. The solvent was removed under reduced pressure and the resulting solid was washed with methanol to give 0.86 g of N-formyl coumarin in 70% yield. ^{1}H-NMR, (DMSO) (600 MHz), δ 10.37 (s, 1H), 10.04 (s, 1H), 8.55 (s, 1H), 8.35 (s, 1H), 7.53 (d, *J* = 12.0 Hz, 1H), 6.80 (d, *J* = 12.0 Hz, 1H), 6.73 (s, 1H). ^{13}C-NMR, (DMSO) (150 MHz), δ 161.5, 160.1, 158.1, 152.0, 129.5, 125.7, 120.8, 114.1, 111.7, 102.5.

0.86 g of N-formyl coumarin (4.2 mmol) was dissolved in 250 mL of tetrahydrofuran. 1.1 mL of Ac_2O was added to the reaction mixture via syringe. 0.3 g of dimethyl amino pyridine (2.4 mmol) was added to reaction mixture, and after 1 minute the reaction was quenched with 100 mL of H_2O and 10 mL of Brine and dried with magnesium sulfate. The crude reside was purified by column chromatography with 2:1 EA:Hex to give 0.7 g of N-formyl O-acetyl coumarin in 70% yield. ^{1}H-NMR, (DMSO) (600 MHz), δ 10.25 (s, 1H), 8.64 (s,1H), 7.77 (d, *J* = 12.0 Hz, 1H), 7.28 (s, 1H), 7.14 (d, *J* = 12.0 Hz, 1H), 2.30 (s, 3H).

0.63 g N-formyl O-acetyl coumarin (2.5 mmol) was dissolved in 15 mL DCM. 3mL Et_3N was added via syringe. 0.3 mL $POCl_3$ was added dropwise via syringe. The reaction mixture was stirred for 30 minutes and then washed with 20 mL of H_2O and 10 mL of brine and dried with magnesium sulfate. The crude product was purified with by column chromatography using silica gel and dichloromethane as eluent to give 0.120 g of 2-Isocyano 7-acetyl coumarin in 36 % yield and 8.0 % overall yield. ^{1}H-NMR, (DMSO) (600 MHz), δ 8.56 (s, 1H), 7.80 (d, *J* = 12.0 Hz, 1H), 7.38 (s, 1H), 7.25 (d, *J* = 12.0 Hz, 1H), 2.31 (s, 3H). ^{13}C-NMR, (DMSO) (150 MHz), δ 170.3, 169.1, 155.9, 154.6, 153.6, 141.0, 130.7, 120.2, 115.9, 114.8, 110.9, 21.3.

4.3 L-phenylalanine vinyl isocyanide methyl ester

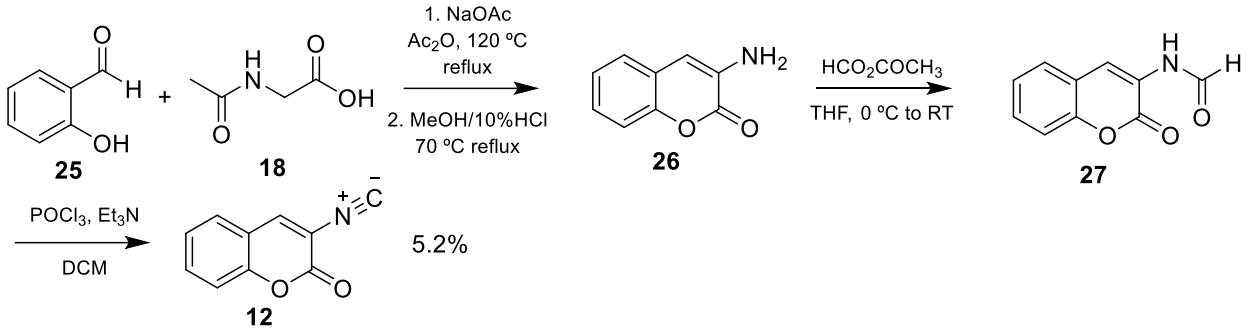


Scheme 17. Preparation of L-Phenylalanine vinyl isonitrile methyl ester (**11**).

0.054 g Cu_2O (0.37 mmol) and 1.2 g glycine isonitrile methyl ester (12.1 mmol) was dissolved in 32 mL toluene, followed by the addition of 1.0 g benzaldehyde (9.4 mmol). The reaction mixture was heated to 40 ºC and allowed to stir for 4 hours. The reaction mixture was concentrated under reduced pressure and purified by column chromatography with 1:1 EA:Hex. 1.0 g of the purified intermediate compound (4.5 mmol) was dissolved in 15 mL 2:1 THF:H_2O and heated at 90 ˚C for 6 hours. The reaction mixture was purified by column chromatography using 9:1 dichloromethane:methanol as eluent to give 1.1 g in quantitative yield.

1.03 g of compound **24** (6 mmol) was dissolved in 20 mL of THF. 1.92 mL of Et_3N was added and the reaction mixture was cooled to -78 ºC. 0.35 mL of $POCL_3$ was diluted in 10 mL of THF and the mixture was added dropwise at -78 ºC. The reaction mixture was allowed to stir for 21 hours and the organic layer was extracted with 40 mL of ethyl acetate and washed with 20 mL of sodium carbonate, and 20 mL brine. The organic extract was dried with magnesium sulfate and purified by column chromatography with 9:1 EA:Hex as eluent to give 0.62 g of vinyl isonitrile phenylalanine methyl ester in 72% yield. ^{1}H-NMR, ($CDCl_3$) (400 MHz) δ 7.87 (m, 2H), 7.66 (s, 1H), 7.49 (t, J = 4.0 Hz, 3H), 3.96 (s, 3H).

4.4 2-isocyano-6,7-hydro coumarin



Scheme 18. Preperation of 2-isocyano-6.7-hydro coumarin (**12**).

3.05 g of 2-hydroxybenzaldehyde (25 mmol), 2.92 g of N-Acetyl glycine (25 mmol), and 8.20 g of Sodium acetate (100 mmol) were dissolved in 13 mL of acetic anhydride. The reaction was brought to reflux at 120 ºC and allowed to stir for 12 hours. After stirring the reaction mixture was poured over ice, filtered, and washed with dichloromethane to give 1.12 g in 22 % yield. 1.12 g of N-acetyl coumarin (5.4 mmol) was dissolved in 30 mL of 10% methanolic HCl. The solution was heated to reflux at 65 ºC and after 4 hours of stirring the solvent was removed under reduced pressure and the crude solid was purified with 10% methanol/saturated ammonia in dichloromethane as eluent to produce 0.7 g of amino-coumarin in 80 % yield.

1.06 g of amino-coumarin (6.6 mmol) was dissolved in 30 mL of THF. The solution was cooled to 0 ºC and 1.3 mL of mixed anhydride, that was prepared immediately before the reaction,[27] was added dropwise via syringe. The reaction mixture was allowed to stir for 4 hours. The solvent was removed under reduced pressure and the resulting solid was washed with methanol to give 0.63 g of N-formyl coumarin in 51% yield.

0.63 g of N-formyl coumarin (3.3 mmol) was dissolved in 15 mL of dichloromethane. 3 mL of Et_3N was added via syringe. 0.3 mL of $POCl_3$ was added dropwise via syringe. The reaction mixture was stirred for 30 minutes and then washed with 20 mL of H_2O and 10 mL of Brine and dried with magnesium sulfate. The crude product was purified by column chromatography with

silica gel and dichloromethane as eluent to give 0.331 g of 2-isocyano coumarin in 58 % yield and 5.2 % overall yield. ^{1}H-NMR, (DMSO) (600 MHz), δ 8.57 (s, 1H), 7.73 (m, 2H), 7.46 (m, 2H). ^{13}C-NMR, (DMSO), (150 MHz), δ 170.3, 156.1, 153.0, 141.5, 134.2, 129.8, 125.9, 118.0, 117.0, 114.7. DEPT135 ^{13}C-NMR, (DMSO) (150 MHz), δ 141.5, 134.2, 129.8, 125.9, 116.9.

4.5. Expression and Purification of PaPvcA/PaPvcB

The PaPvcB and PaPvcB expression vectors were used to separately transform *E. Coli* BL21 (DE3) cells. Transformants were selected by growth on Luria-Bertani plates containing kanamycin. Individual colonies were picked and used to inoculate 250 mL cultures, which grew overnight at 37 ˚C, and were then used to inoculate flasks containing 1 L of Luria-Bertani rich medium supplemented with 100 μg/mL kanamycin. The cultures were grown at 37 ˚C with rotary shaking (220 rpm) to an optical density of 0.6 and then the temperature was lowered to 18 ˚C, IPTG was added to a final concentration of 0.5 mM to induce recombinant protein expression and the growth was continued at 18 ˚C for 16-20 hours. The cells were harvested by centrifugation at 16 k rpm for 30 minutes, yielding 8-10 g cell paste per liter, which was stored at -80 ˚C. Frozen cell paste was thawed in 80-120 mL of buffer. The cells were lysed by sonication and the cell debris were removed by centrifugation at 30 k rpm for 30 min. The clarified supernatant was loaded on a 40 to 50 mL column pre-equilibrated in the same lysis buffer the protein was thawed in. Lysis buffer with 250 mM imidazole was used to elute the protein. The purity of the proteins was assayed by SDS-PAGE. Protein concentrations were determined spectrophotometrically using extinction coefficients for each specific protein at 280 nm.

Chapter 5: Conclusions

While I was able to derive and provide a plausible mechanism for the olefin-forming 2-electron oxidation catalyzed by PaPvcB based on published literature and previous research in our group, the enzymatic assays and liquid chromatography mass spectrometry analyses remain to prove the mechanism of catalysis. Although Zhu and colleagues provided a hypothetical mechanism, I believe that our lab has a fundamentally better understanding of how to probe the mechanism of the enzyme by generating a library of compounds to test with the enzyme rather than merely the substrate. Furthermore, if this project were to continue, a better understanding of the other enzymes in the gene cluster PaPvcC and PaPvcD need to be overexpressed and purified to ascertain their role in paerucoumarin biosynthesis.

In addition, the next steps to drive the project forward would be further optimizing the syntheses of the reported compounds **5**, **10**, **11** and **12**. Not only could the synthetics methods be improved but the inability to produce compound **6**, the anticipated natural product of PaPvcB catalysis, leaves room for future work on the project. While I found conditions to deprotect the hydroxyl functional group at C7, perhaps they were too harsh to leave the isonitrile intact. Also, further derivatization of compound **11**, utilizing 4-hydroxy benzaldehyde in the synthesis shown in **scheme 17**, would provide an additional synthetic intermediate to using in enzymatic assay studies. Ideally, to aid in our understanding of PaPvcB, we would also require a crystal structure of the enzyme with substrate in the active site. I believe only a small amount of labor is necessary to fulfill the requirements to accurately study and elucidate a plausible mechanism for PaPvcB catalyzed reaction and I hope that a future student in our lab group drives the project forward.

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APPENDIX

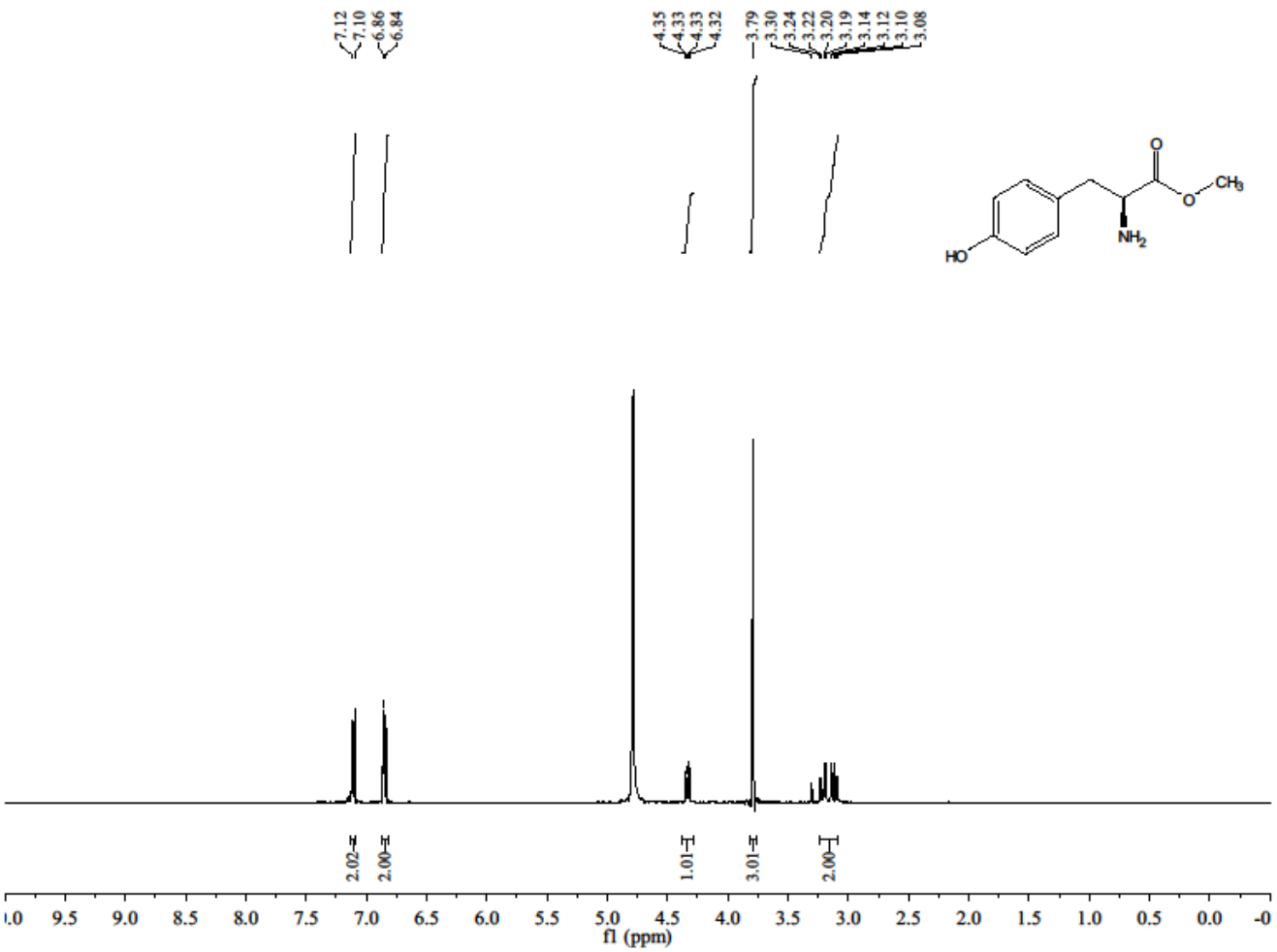

Figure A1. ^{1}H-NMR spectrum of L-tyrosine methyl ester.

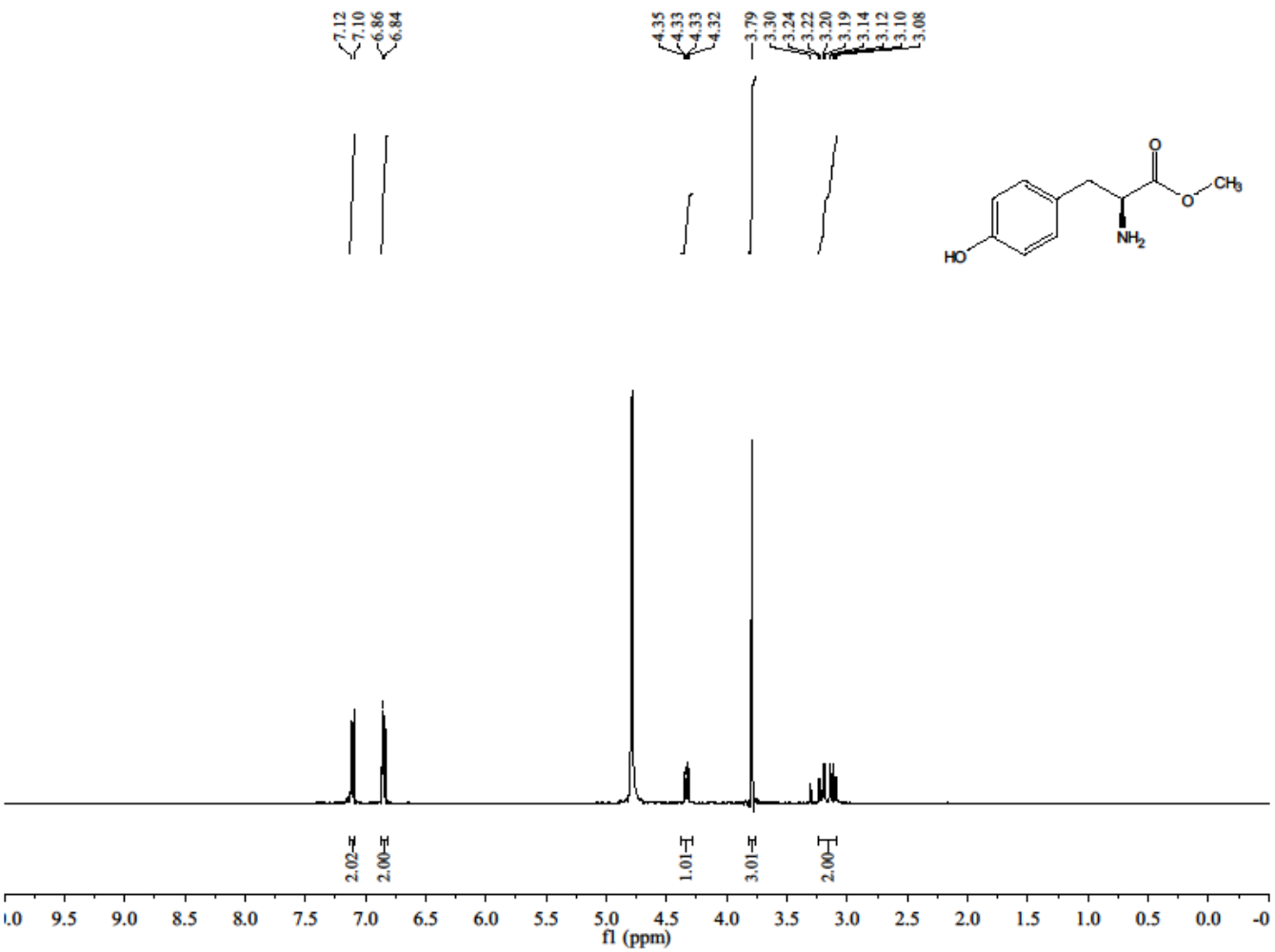

Figure A2. [1]H-NMR spectrum of L-tyrosine isocyanide methyl ester.

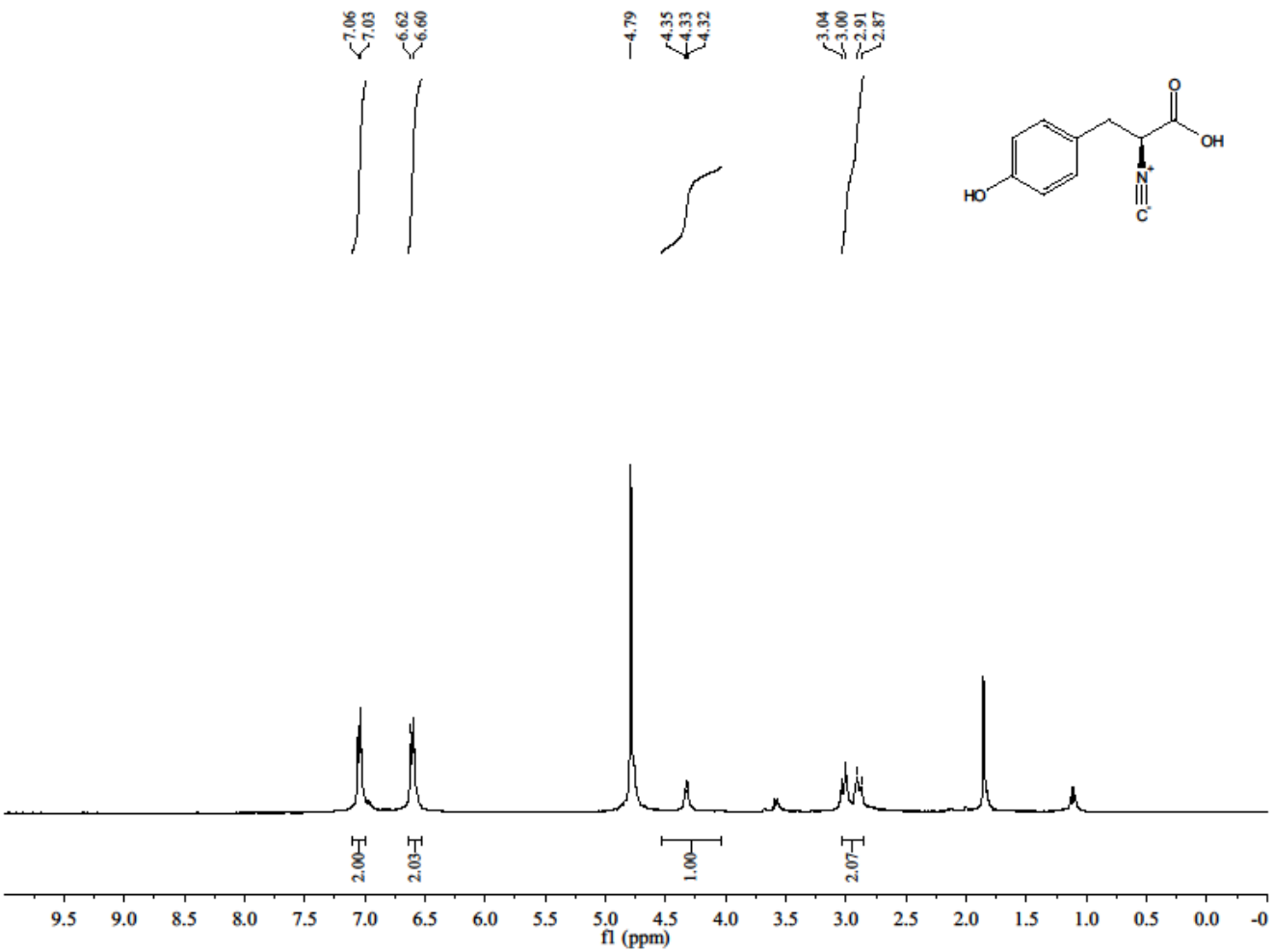

Figure A3. ^{1}H-NMR spectrum of L-tyrosine isocyanide.

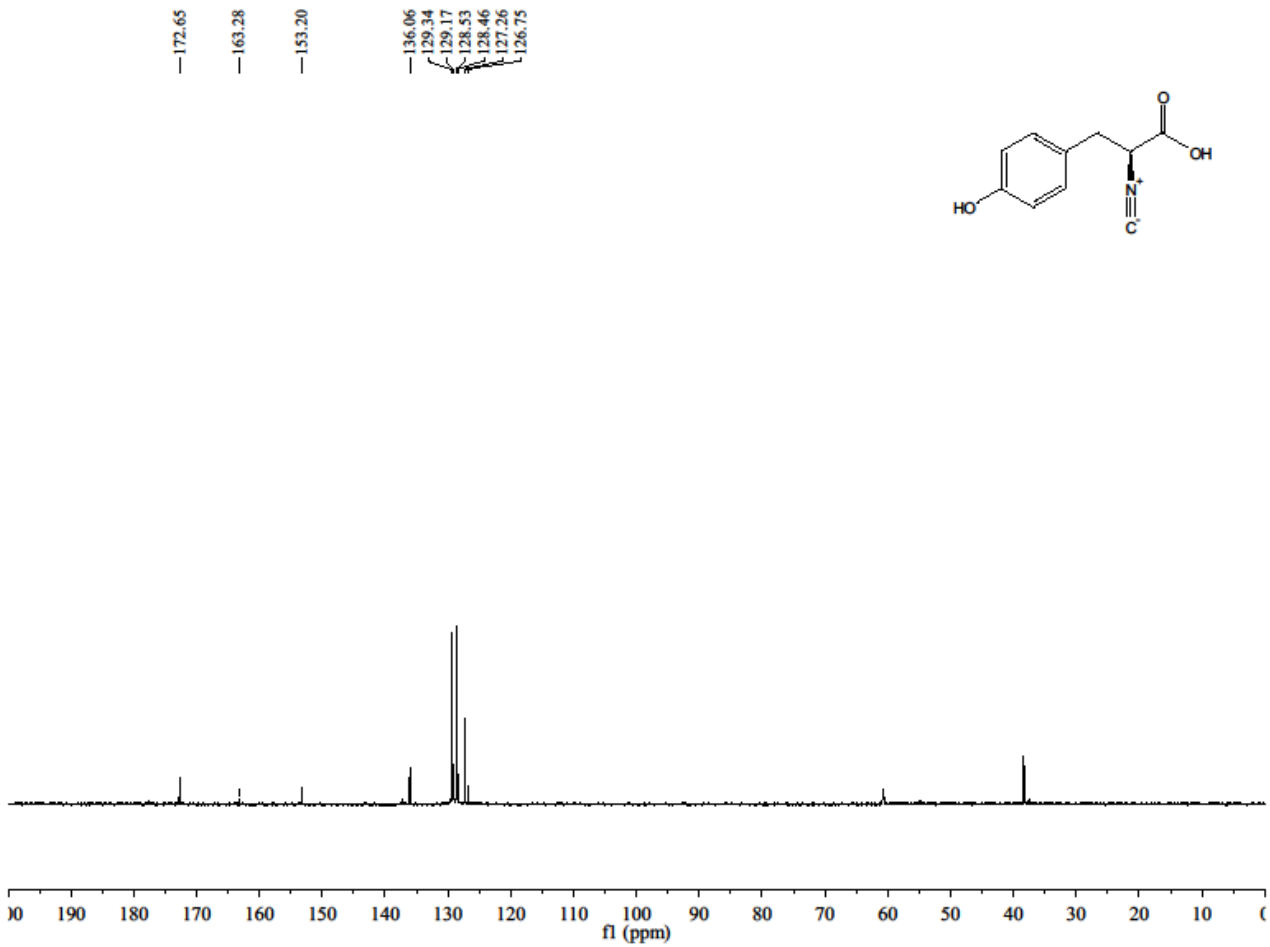

Figure A4. ^{13}C-NMR spectrum of L-tyrosine isocyanide.

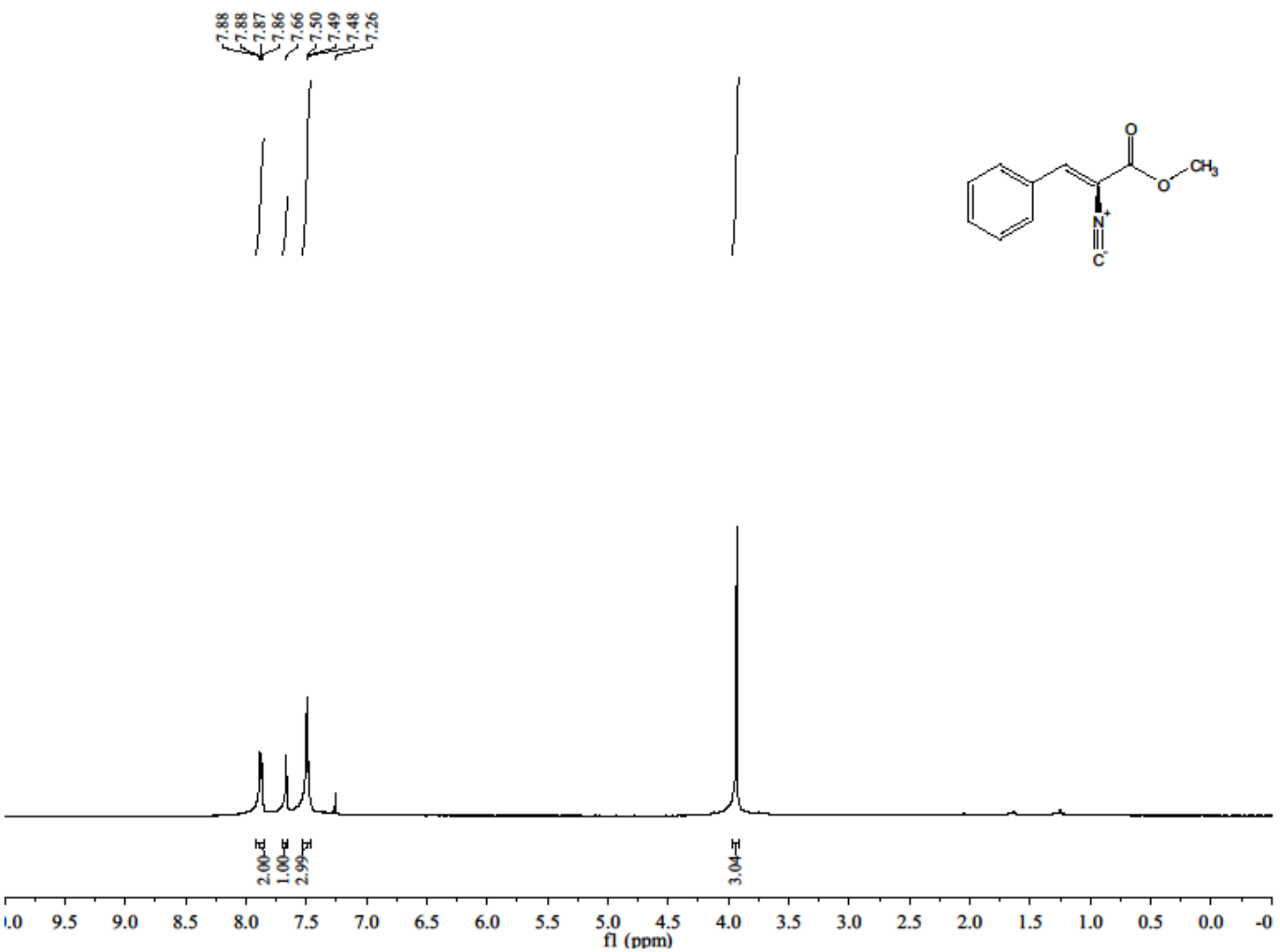

Figure A5. ^{1}H-NMR spectrum of vinyl isonitrile phenylalanine methyl

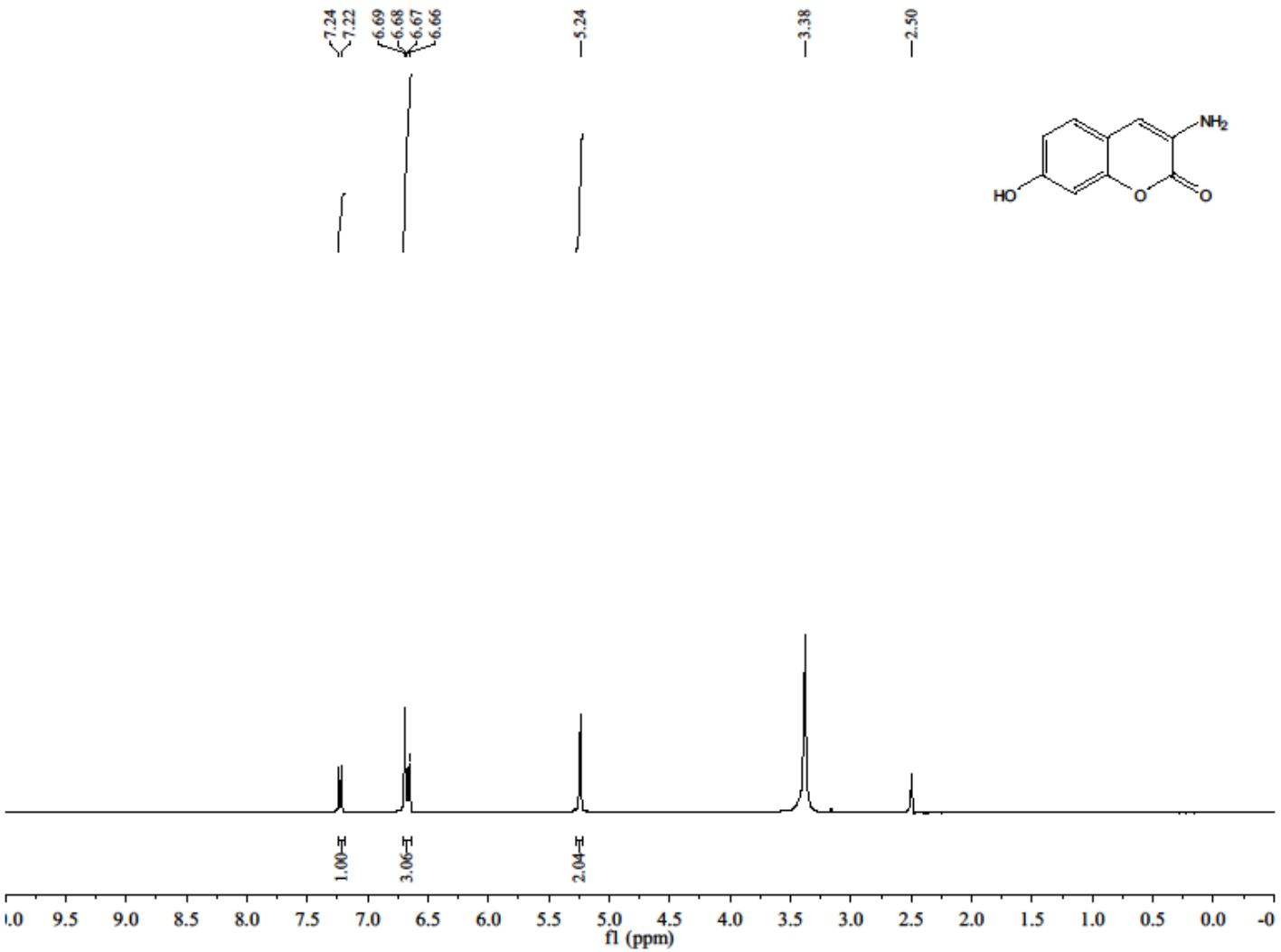

Figure A6. [1]H-NMR spectrum of 2-amino-7-hydroxy coumarin.

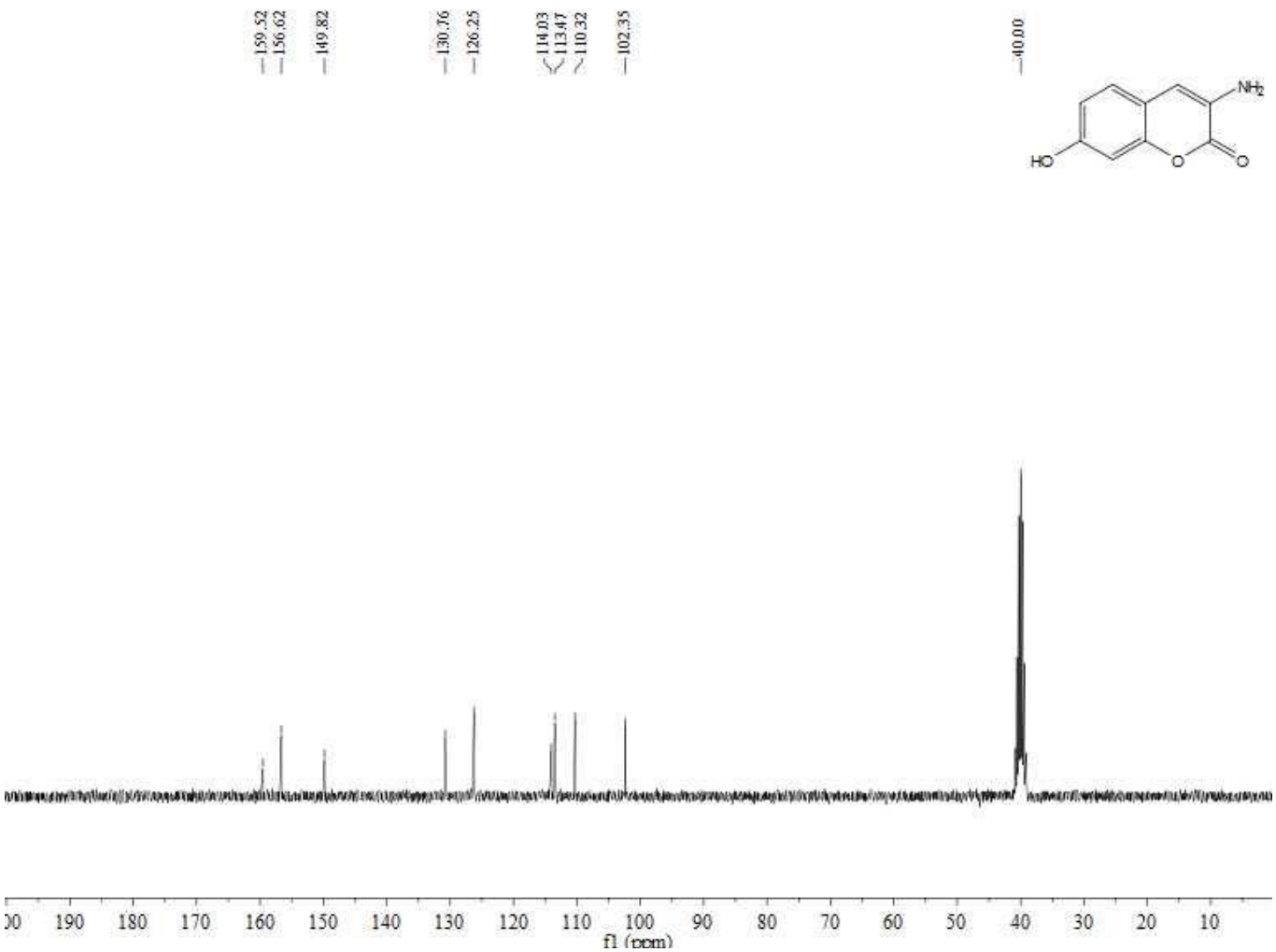

Figure A7. ^{13}C-NMR spectrum of 2-Amino-7-hydroxy coumarin.

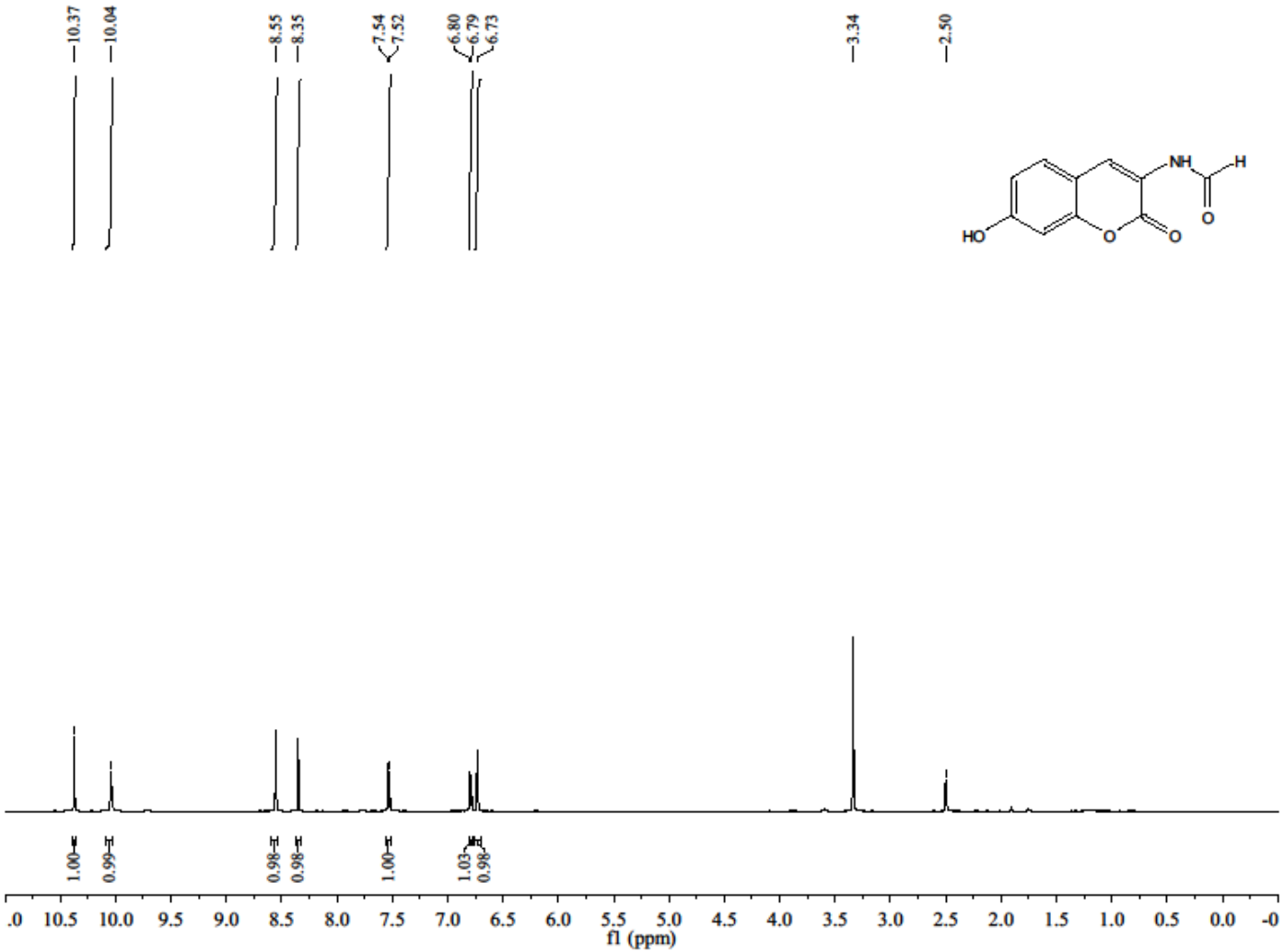

Figure A8. ^{1}H-NMR spectrum of N-formyl-7-hydroxy coumarin.

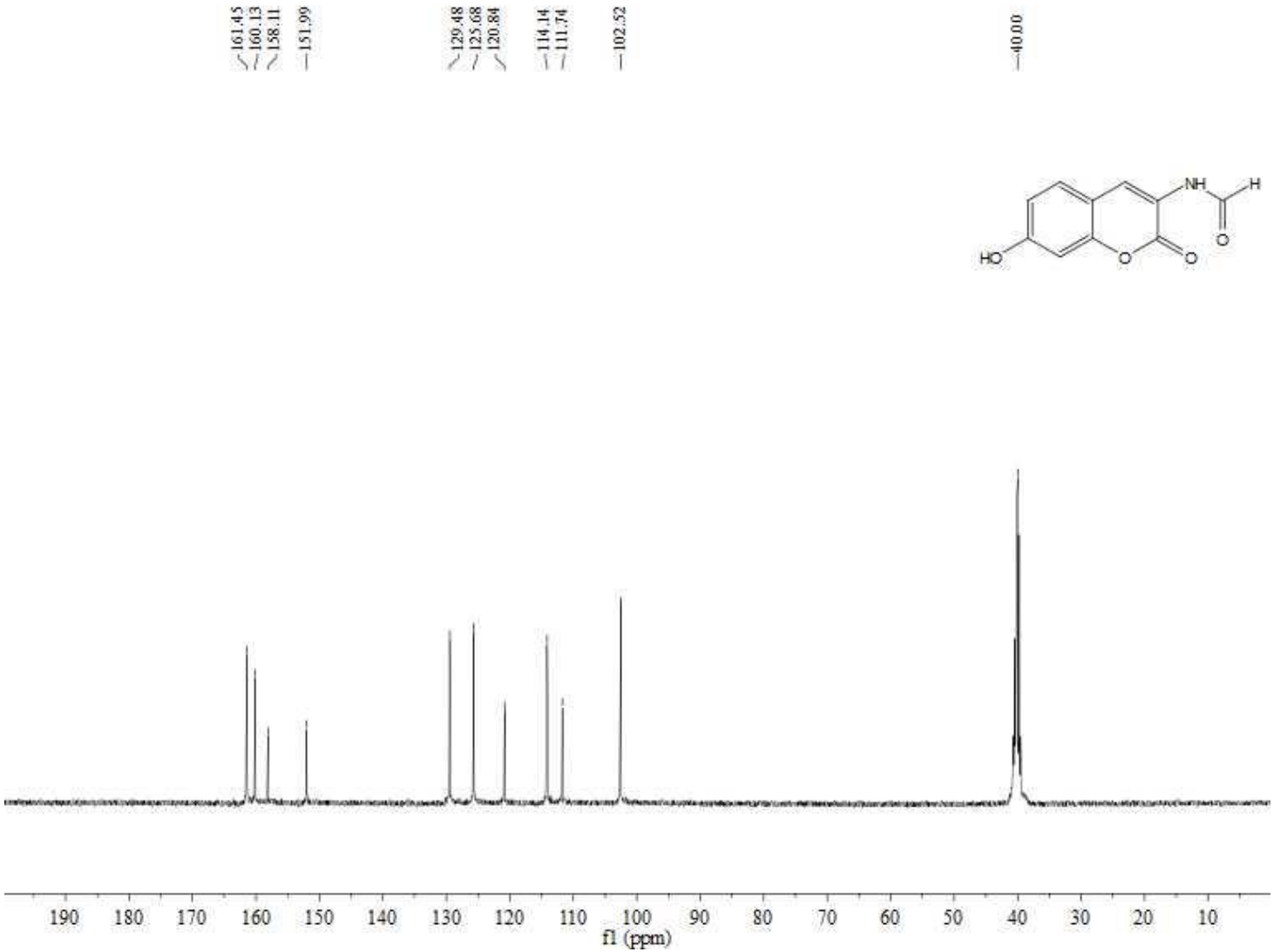

Figure A9. ^{13}C-NMR spectrum of N-formyl-7-hydroxy coumarin.

Figure A10. ^{1}H-NMR spectrum of N-formyl-7-acetyl coumarin.

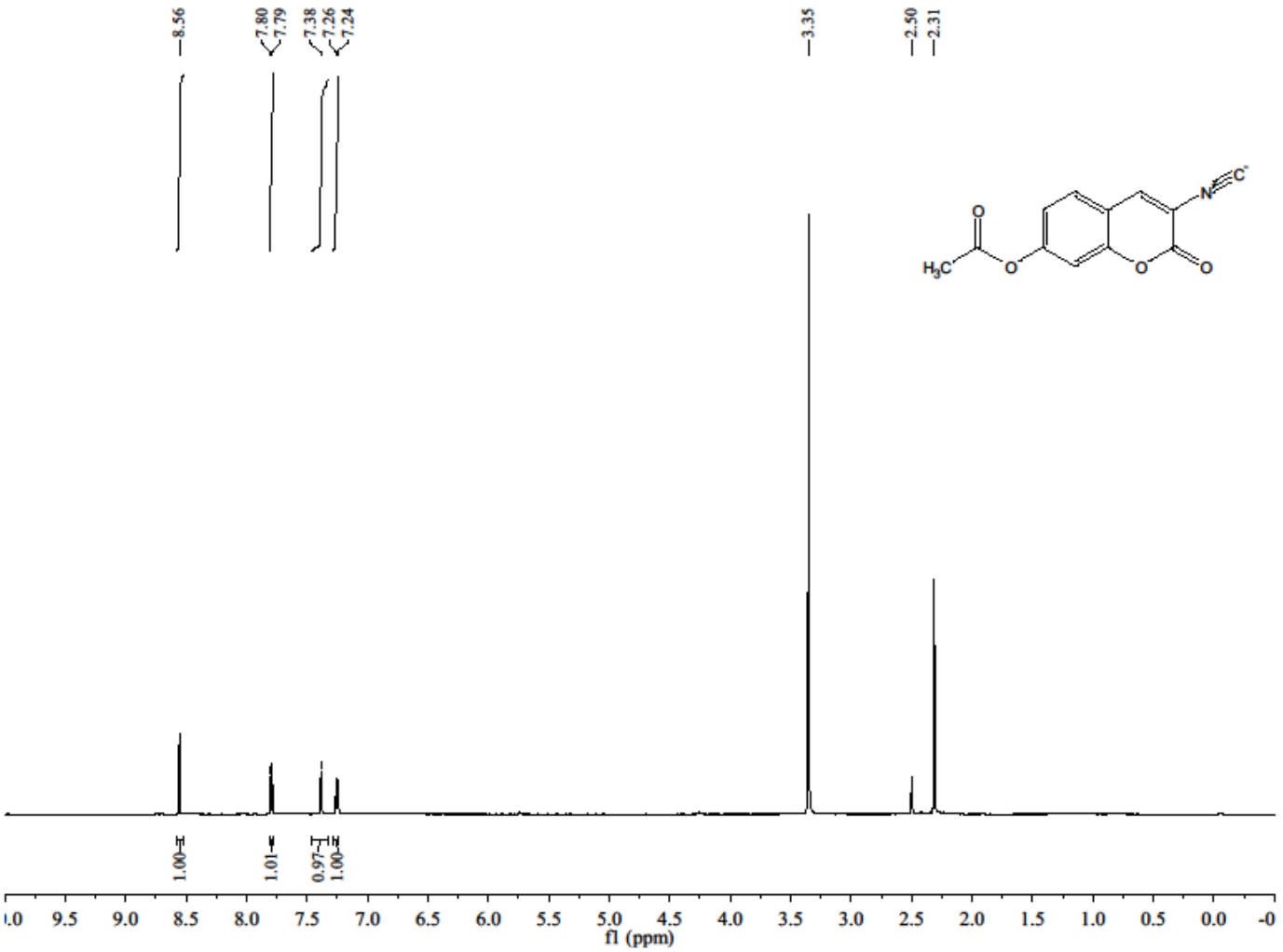

Figure A11. ^{1}H-NMR spectrum of 2-isocyano-7-acetyl coumarin.

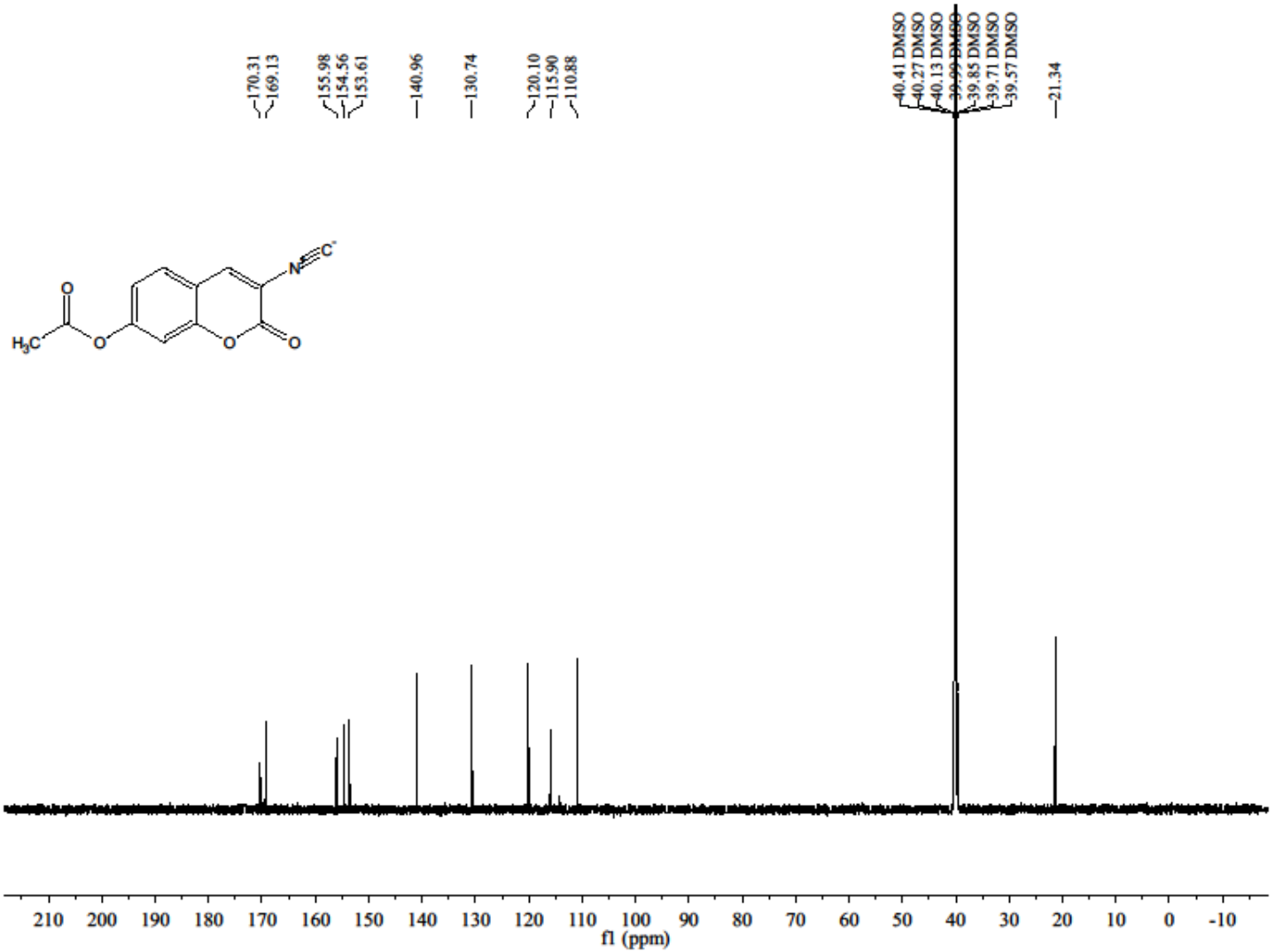

Figure A12. ^{13}C-NMR spectrum of 2-isocyano-7-acetyl coumarin.

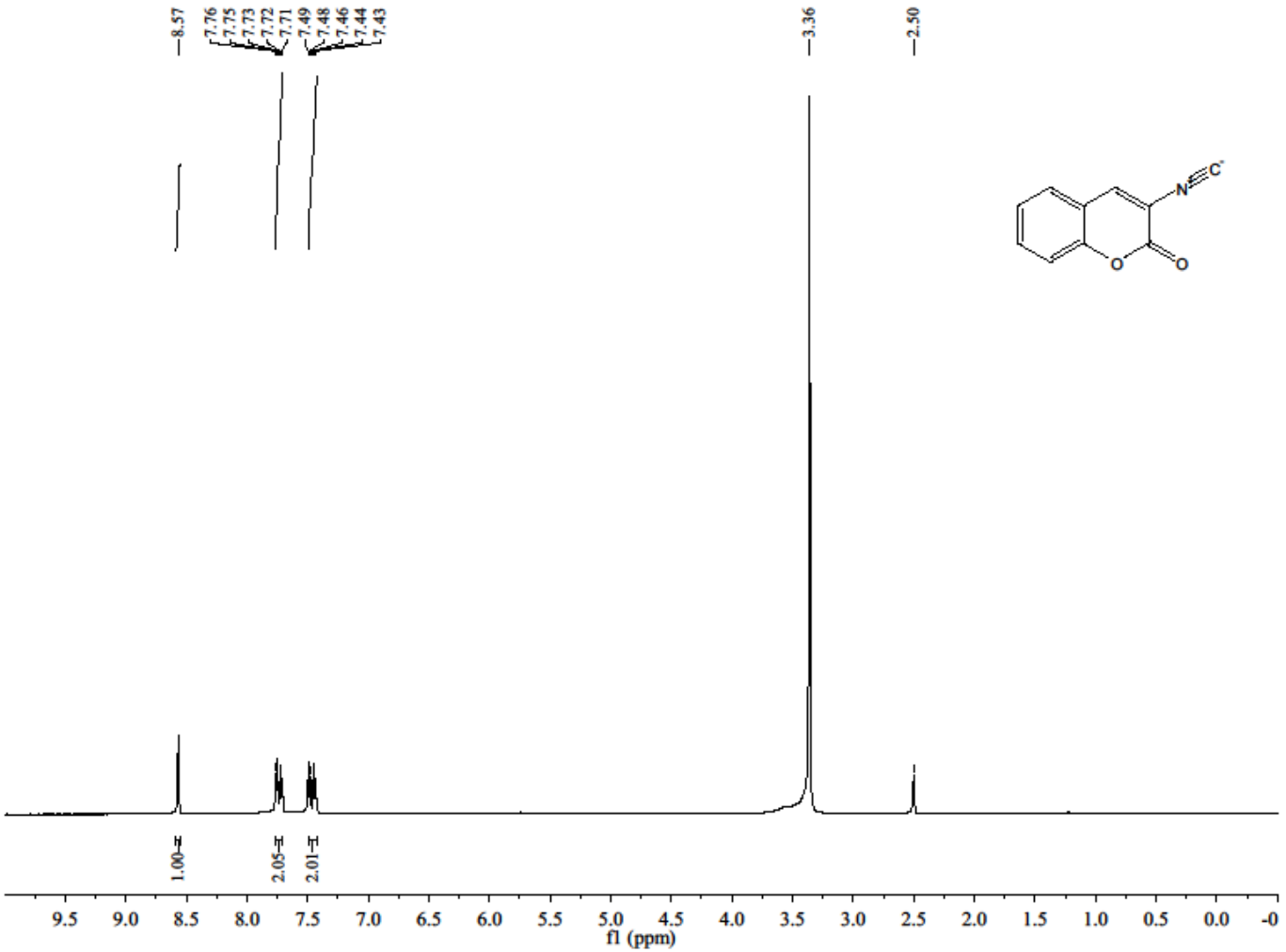

Figure A13. ^{1}H-NMR spectrum of 2-isocyano-6,7-hydro coumarin.

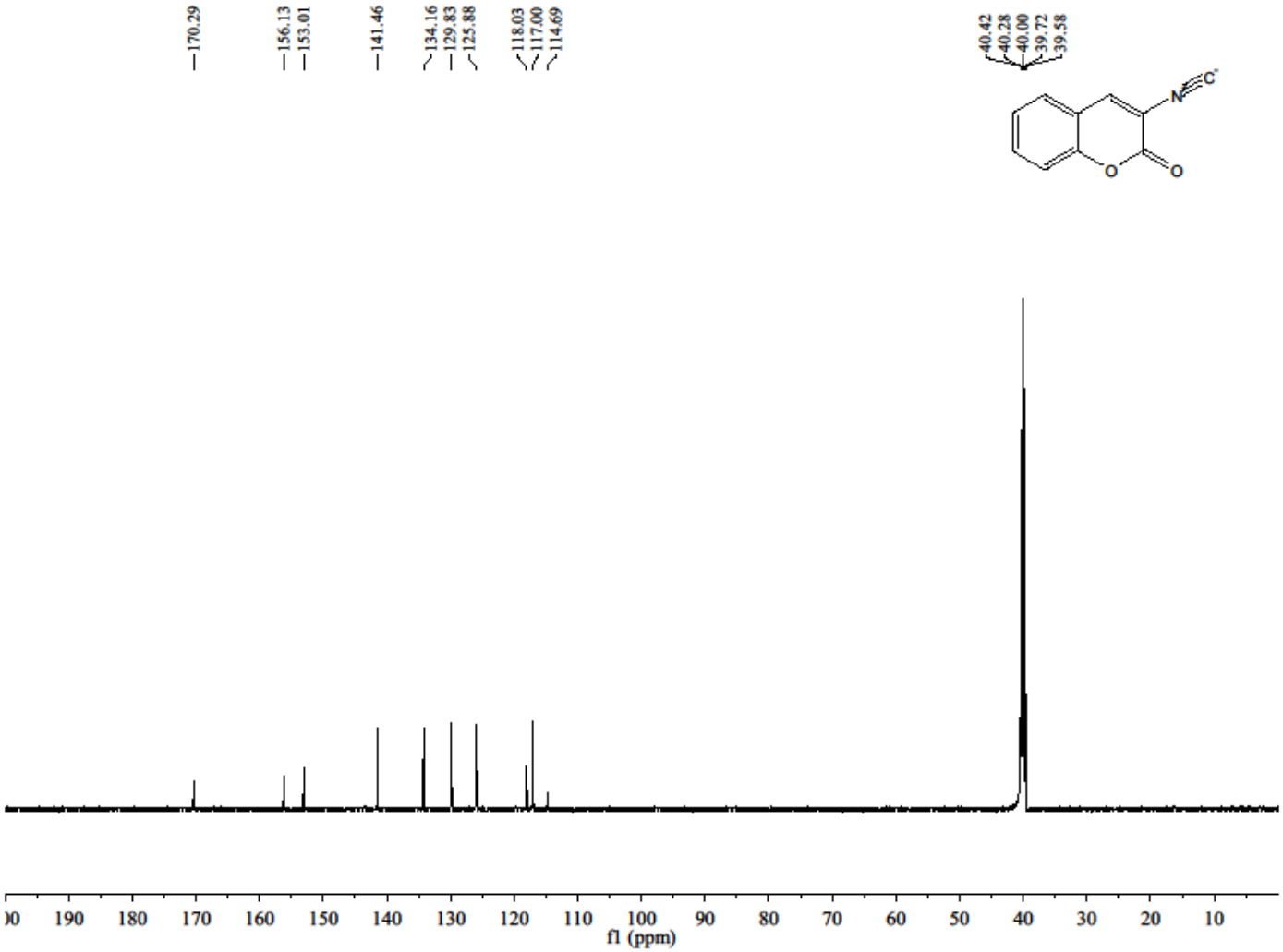

Figure A14. ^{13}C-NMR spectrum of 2-isocyano-6,7-hydro coumarin.

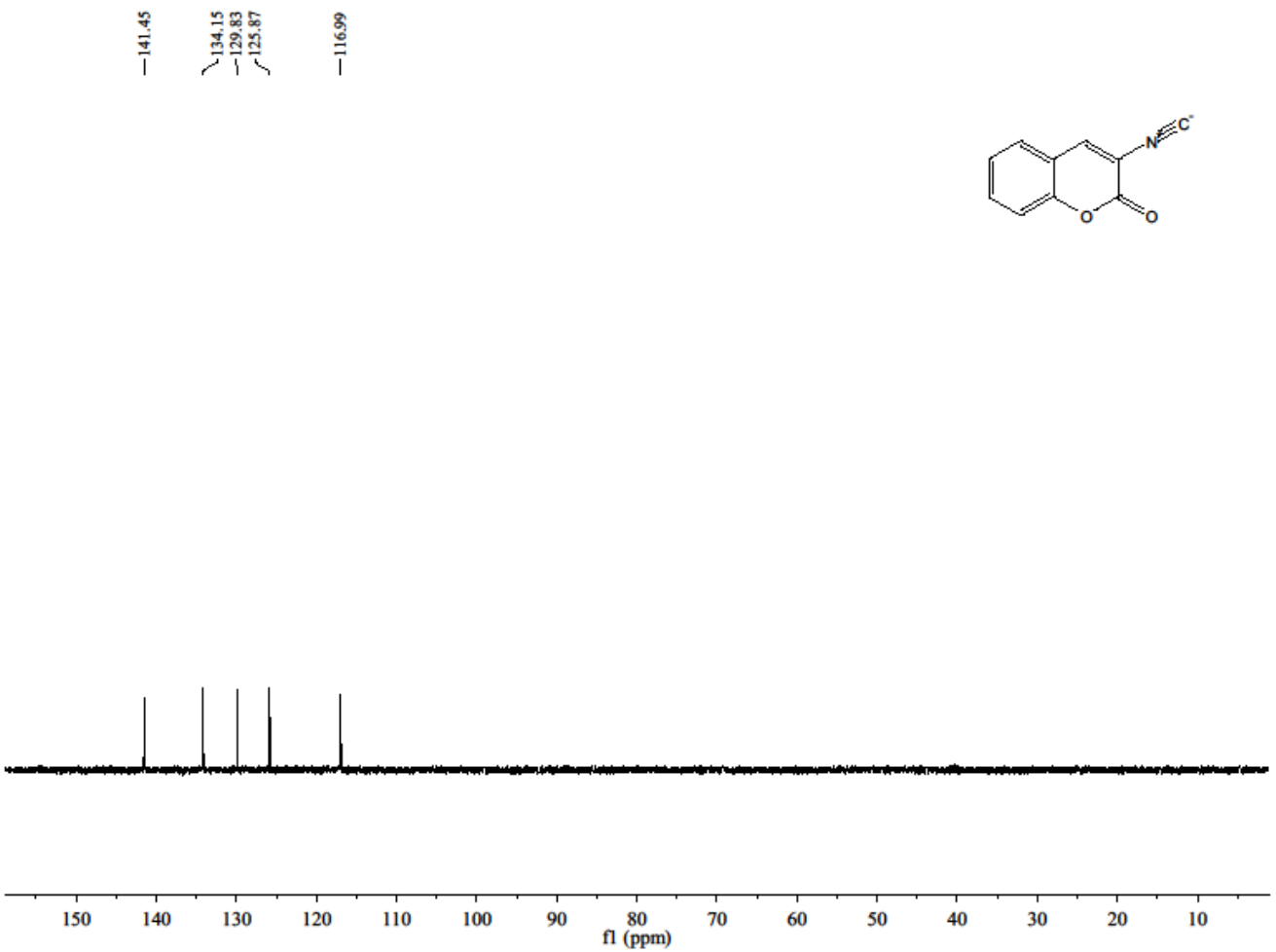

Figure A15. DEPT 135 ^{13}C-NMR spectrum of 2-isocyano-6,7-hydro coumarin.