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The synthesis of 3-formyltyrosine for the purpose of in virto fluorescent visualization of microtubules

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THE SYNTHESIS OF 3-FORMYLTYROSINE FOR THE PURPOSE OF *IN VITRO* FLUORESCENT VISUALIZATION OF MICROTUBULES

By

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THESIS

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Abstract:

Microtubules and the desire to visualize them has been of utmost importance in understanding the behavior of the cytoskeleton. Even further, scientists are using this knowledge to elucidate cancerous activities as they occur within cells. In order to acquire this knowledge, the following methods have been established: the expression of tubulin after it has been fused with a fluorescent protein, the insertion of tubulin that has been exogenously labeled, and the use of fluorescent-probe conjugated microtubule ligands, such as paclitaxel. These methods, however, tend to distort the protein's native function, thereby preventing an accurate analysis. This concern drives the need for an innovative approach that does not involve bulky attachments or genetic manipulation. One proposed approach is through the exploitation of specific, innate cellular processes, such as the post-translational modification responsible for the tyrosination of α-tubulin. By labeling α-tubulin with a tyrosine derivative capable of reacting with a fluorescent probe, the cellular activities pertaining to cancer can effectively be monitored.

In order to test this approach, 3-formyltyrosine (3FY) was synthesized and ligated *in vitro* to the α-tubulin subunit of microtubules in prostate cancer cells (PC3s) via the post-translational tyrosination facilitated by tubulin tyrosine ligase (TTL). A functionalized fluorophore, 3-trifluoromethyl-7-hydrazinyl coumarin (TFCH), was chosen as a means to optimize this technique which had previously been done with coumarin hydrazine. Coumarin hydrazine, although effective in labeling, mostly absorbs in the UV range and photo-decomposes easily. TFCH proves a better fluorophore for this process since it both absorbs at 405 nm and exhibits greater photostability.

Although there was no definitive conclusion regarding TFCH's effectiveness in

microtubule visualization, the conditions and experimental procedure were further elucidated in such a way that should hopefully eliminate possible pitfalls in the future. Thus, this study serves as a foundation for the following studies to explore TFCH, as well as other fluorophores, both *in vitro* and in living cells.

Introduction:

Microtubules are threadlike polymers of the globular protein tubulin that act as a foundational element of the eukaryotic cytoskeleton. Although mostly associated with the maintenance of the cellular structure, microtubules are also responsible for a myriad of other cellular processes. Amongst these processes are cell signaling, intracellular trafficking, cell division, and cell motility [1,2]. The clear significance of microtubules is further demonstrated by the intense impact that changes in its structural assembly and/or innate characteristics have. These changes have been linked to a number of diseases, most notably neurodegenerative diseases[3] and cancer[4]. As such, the study of these structures provides an exceptional insight into the biochemical processes. Currently, fluorescence microscopy is regarded as the most effective way to visualize microtubules as they perform their duties within the cell. This method, however, carries with it some limitations - of which, the most damning is that cells are most often fixed for better measurement accuracy. It is evident that there must be a push towards new techniques that can elucidate more than what fluorescent microscopy is capable of.

When attempting to study cellular structures in action, it is necessary to do so without impeding the inherent functions of said structures. It is for this reason that scientists have considered exploiting the naturally occurring enzymes and processes as a new means of noninvasive visualization. In terms of microtubule visualization, the most promising enzyme of study is known as tubulin tyrosine ligase (TTL). As mentioned previously, microtubules consist of a protein tubulin. This protein is a dimer composed of an α- and β-subunit that each have three domains[5]. In both subunits, there is the amino-terminal, the intermediate domain, and the carboxy-terminal (C-terminal). The latter is the focus of recent efforts in new visualization

techniques as it serves as a binding site for motor proteins and microtubule associated proteins[5]. The C-terminal domain contains glutamyl residues and is, therefore, highly acidic. It also undergoes post-translational modifications, such as tyrosination. This modification refers to the cyclic removal and addition of the C-terminal tyrosine on the α-subunit of tubulin by TTL, which is a particularly useful chemical process due to the nature of tyrosine and the specificity of TTL[6].

Being that it is an amino acid, tyrosine has great potential to be modified and adjusted to suit the needs of scientists. One such chemical modification is the addition of a reactive carbonyl group *ortho* to the alcohol functional group, making 3-formyltyrosine (3FY) - a molecule more readily available for a number of reactions (Figure 1)[7].

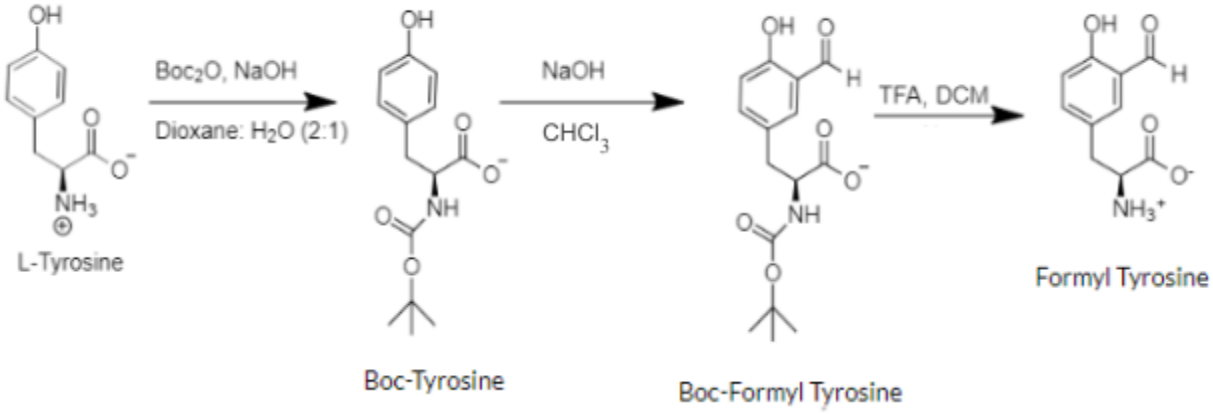


Figure 1. Reaction Scheme of the Conversion from L-Tyrosine to 3-Formyltyrosine.

Since 3FY is a good substrate for a varied set of reactions, it opens up the naturally occurring L-tyrosine up to two-step chemistry. It is in this way that the innate TTL chemistry is exploited to allow for a newer and hopefully, more effective method of microtubule visualization. To achieve said visualization, hydrazone formation is implemented as the second step in the two-step chemistry. Hydrazone chemistry has been studied in great detail and functions on the principle of reacting the hydrazine with a carbonyl group to form a hydrazone[8].

Some hydrazones exhibit fluorescence upon formation when the initial hydrazine does as well. In creating a hydrazone using the 3FY replacement in the α-tubulin subunit, one effectively creates an inducible fluorescent label of microtubules. Ultimately, it is the goal of Bane lab to label microtubules in live cells using a hydrazine-fluorophore analog. In an effort to help achieve this goal, it is my hope that I can show a viable option for a hydrazine-fluorophore analog by first showing its effective visualization *in vitro*.

Results and Discussion:

Retrosynthesis of 3FY

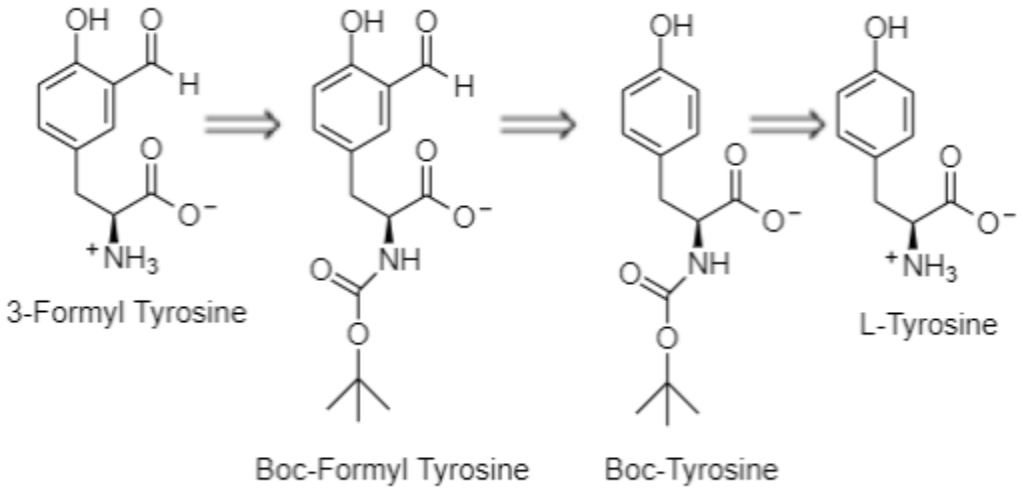


Figure 2. Retrosynthesis of 3-Formyltyrosine. Abbreviation: Boc → Tert-butoxycarbonyl

The retrosynthesis of 3FY is demonstrated above (Figure 2). The principal characteristic of this synthetic route is the addition of an aldehyde to L-tyrosine in the *ortho* position of the phenol so as to open the molecule up to two-step chemistry. In order to achieve this, it is pertinent that the N-terminal amine is protected in such a way that the integrity of the initial molecule is preserved.

Boc-Protection of the N-terminal Amine

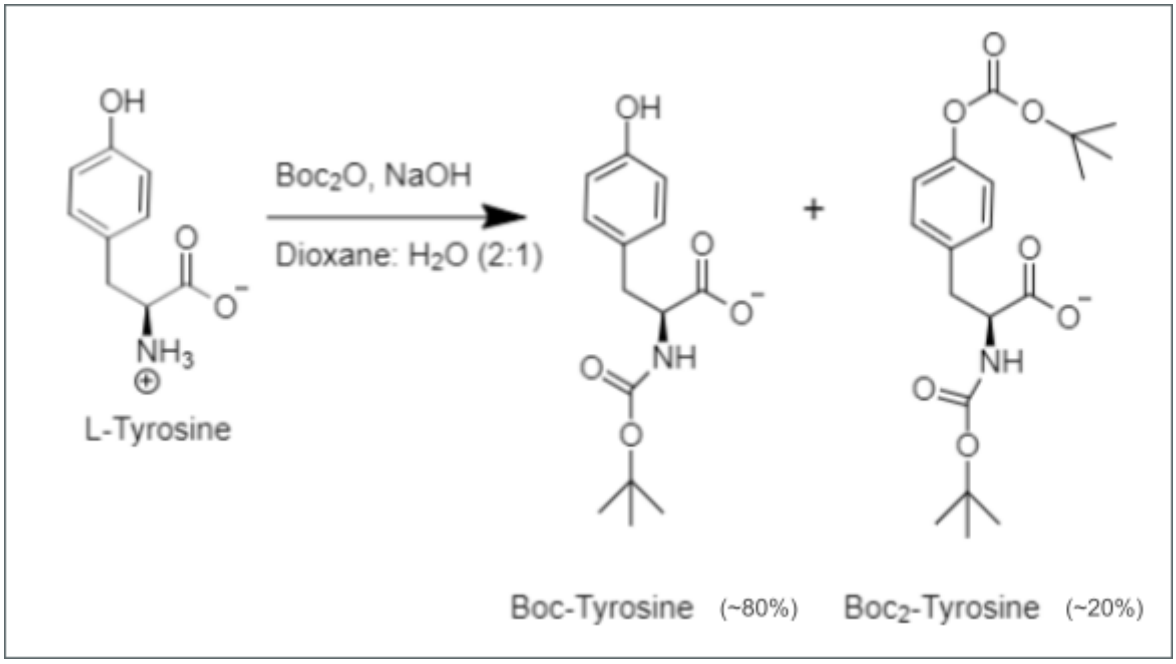


Figure 3. Boc-Protection of L-Tyrosine

As mentioned previously, the protection of the N-terminal amine must occur in order to obtain the final product, 3FY. Should the N-terminal amine remain unprotected, it will interfere with subsequent reactions in the pathway due to the high reactivity created by the reaction's basic conditions. Basic conditions neutralize the amine, allowing it to act more as a nucleophile. Boc anhydride (di-tert-butyl-dicarbonate) is the commonly used reagent for the protection of this amine[9]. The aforementioned basic conditions produce a nucleophilic amine which can attack the Boc anhydride, yielding the protected amine shown. To ensure that the protection had succeeded, a ^{1}H NMR spectra of the crude product in deuterated chloroform was taken and indicated the presence of a small impurity (Boc_2-Tyrosine in Figure 3). As can be observed, the following spectra illustrates the impurity, which is signified by the presence of

another doublet in the aromatic region slightly downfield of the expected aromatic peaks (Figure 4).

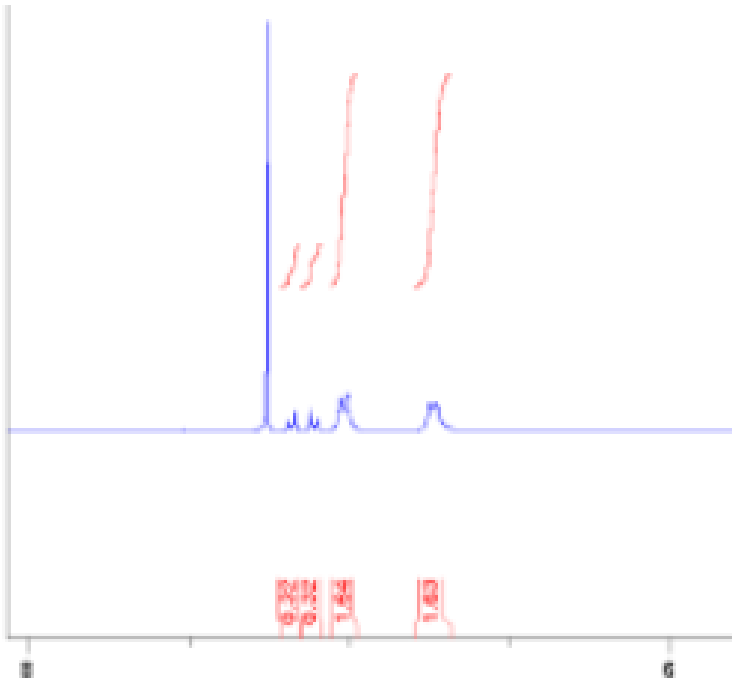


Figure 4. The Aromatic Region of the Proton NMR of L-Tyrosine Protection Crude

This impurity, although unexpected, does occur as shown by the additional aromatic peaks, which occur as a result of the additional Boc's effect on the benzene ring. This impurity, as indicated in Figure 3, lowers the yield of this reaction. However, this lowered yield is not significant enough to change the reaction pathway as commercially available BOC-L-Tyrosine is also shown to contain this impurity.

Although the reaction pathway is not changed to remove the impurity, the impurity must be removed for the next step to increase the yield of subsequent reactions. As such, a silica column purification was performed to separate the two products. The two products have similar polarities but can be separated via the aforementioned method using a gradient solvent condition of 1:4 ethyl acetate:hexane + 0.5% acetic acid to 1:1 ethyl acetate + 0.5% acetic acid. This gradient works because the impurity is more nonpolar than the desired product so it elutes first. The final

fractions of this purification were collected and a proton NMR was taken, showing that the additional aromatic doublets were no longer present and therefore, Boc-Tyrosine had been successfully synthesized and purified (Figure 5).

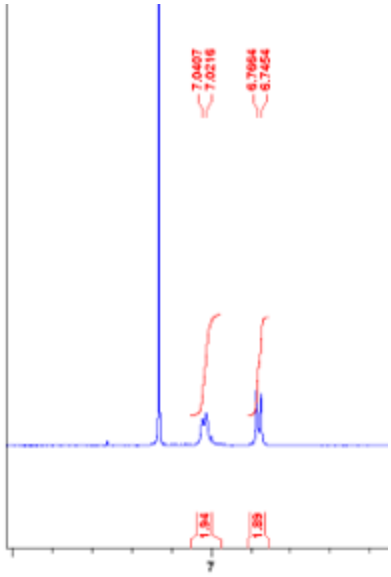


Figure 5. The Aromatic Region of the Proton NMR of Purified Boc-Tyrosine

This proton NMR also brought up a need for clarification as the CH peak illustrated in Figure 6.1 cannot be distinguished from the NH peak. To ensure that each peak was accounted for, a COSY was taken that would allow for the elucidation of the final two peaks (Figure 6.2).

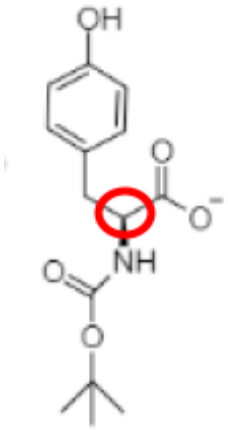
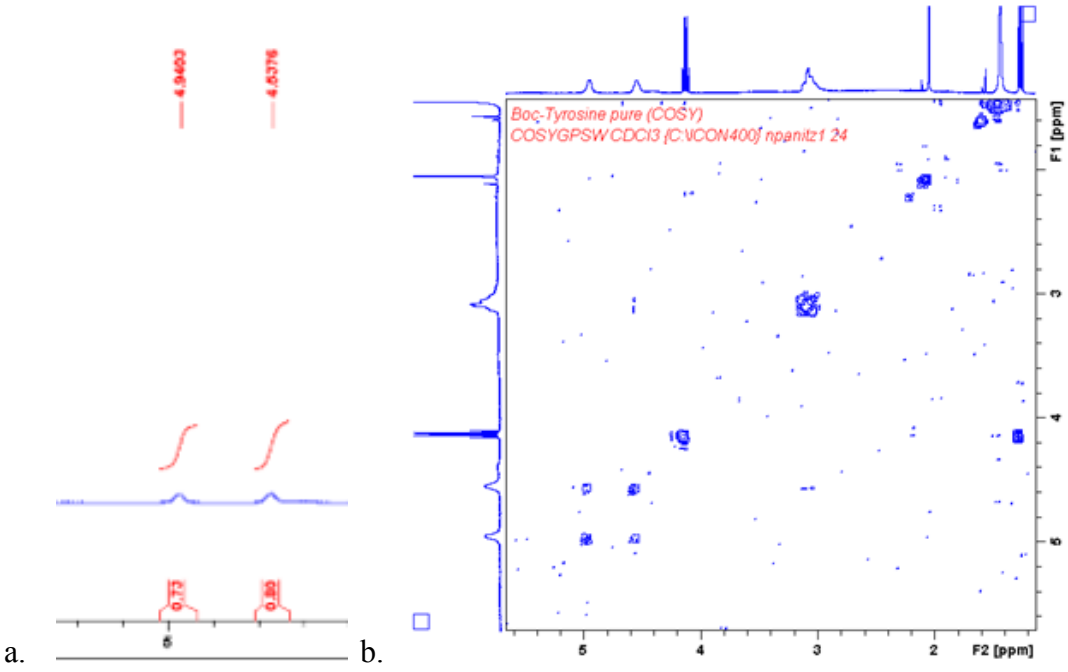


Figure 6.1. The CH Peak of Concern of Boc-Tyrosine



a. b.

Figure 6.2. The CH and NH peaks on the Proton NMR of Purified Boc-Tyrosine (a) and the Accompanying COSY (b)

The COSY shows that the peak at 4.54 ppm must be the CH peak since that peak does couple with the CH_2 peak, which is known to occur in Boc-Tyrosine. Therefore, the peak at 4.94 ppm must be the NH peak. It is important to note this distinction as the protection reaction

directly affects the expression of the amine.

This reaction was completed three times on a series of different scales to create a total of 6.9 g of Boc-Tyrosine with an average yield of 80%.

Synthesis of Boc- Formyl Tyrosine

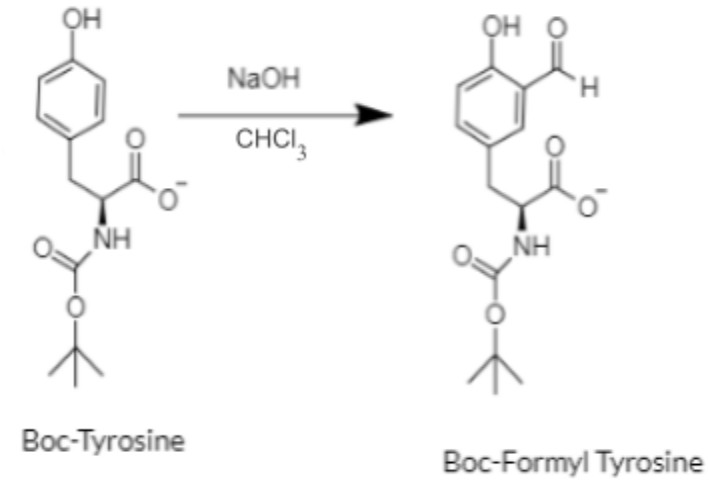


Figure 7. Synthesis of Boc-Formyl Tyrosine from Boc-Tyrosine

To add an aldehyde to Boc-Tyrosine, the Reimer Tiemann reaction is the best known method. This reaction relies on the creation of a carbene, a molecule whose empty *p*-orbital makes it susceptible to nucleophilic attack. The following schematic illustrates the mechanism of the Reimer Tiemann reaction (Figure 8).

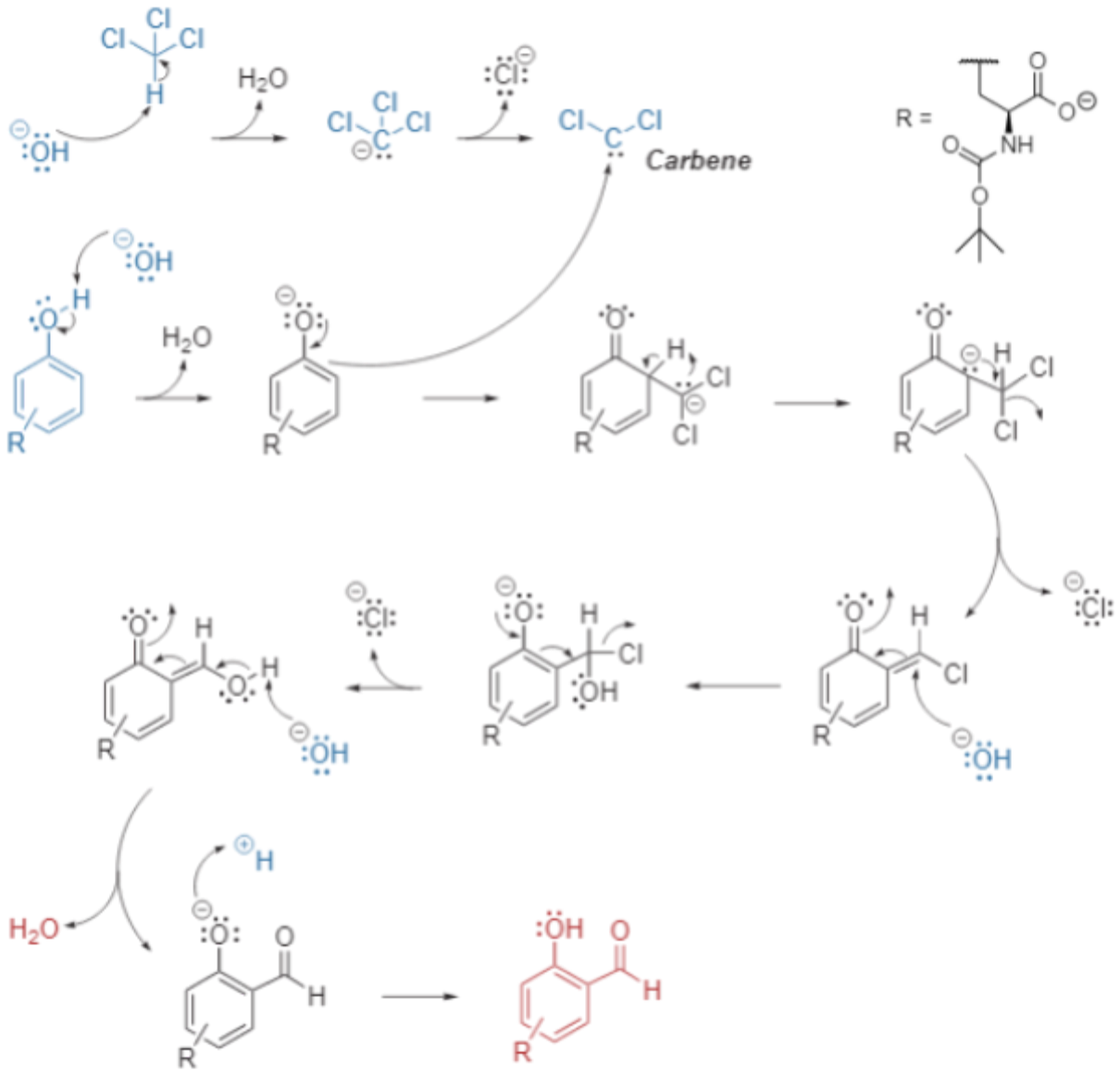


Figure 8. The Mechanism of the Reimer Tiemann Reaction[10]

To obtain the carbene, chloroform is deprotonated in the presence of a base. The *ortho*-directing, electron-donating properties of the phenol facilitate the electrophilic aromatic attack of the carbene by the *ortho* carbon[10]. Following the reaction, a proton NMR in deuterated chloroform was taken of the crude to ensure that the reaction was successful (Figure 9).

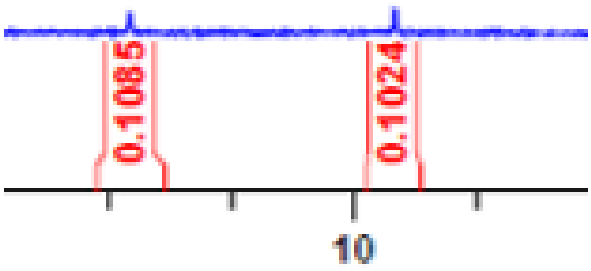


Figure 9. Proton NMR of Boc-Formyl Tyrosine in the Aldehyde Region

As shown in the above spectra, the presence of a peak in the typical region for aldehydes indicates that the aldehyde was successfully added. Although the integration would suggest otherwise being that it is less than one, which an aldehyde should integrate to for the one hydrogen it has. The yield of this reaction is exceptionally low, averaging 12-17% in the literature. This is a result of the side reactions that occur alongside the desired reaction[11]. This low yield explains the low integration. However, this low yield is of little concern since the materials are very cheap and plenty of the starting material was synthesized in the previous step. Overall, this reaction created a total of 104 mg of Boc-Formyl Tyrosine for an average yield of about 10%.

Deprotection of 3FY

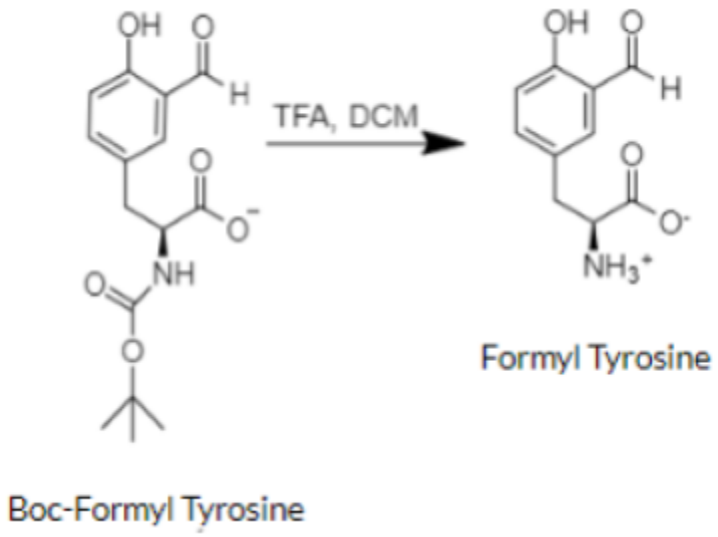


Figure 10. The Deprotection of 3FY from Boc-Formyl Tyrosine

The final step of the synthetic route of this pathway is the deprotection of 3FY, which is shown in Figure 10. This procedure was elucidated by former Bane lab member, Han Gu, who detailed the removal of a Boc group using trifluoroacetic acid (TFA) and N_2 gas to remove Boc and dry product, respectively - after having dissolved it in DCM[12]. Prior to the start of this reaction, the 104 mg of Boc-Formyl Tyrosine from the previous step was combined with other previously synthesized Boc-Formyl Tyrosine, bringing it to ~ 1.98g. This reaction was completed and evaluated using a proton NMR in $CDCl_3$ (Figure 11).

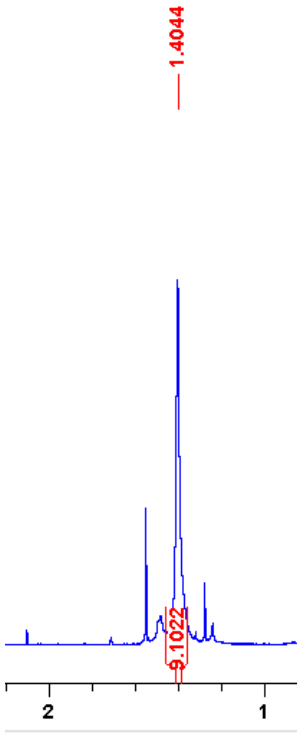


Figure 11. Proton NMR of 3FY Showing the Boc Peak After the Deprotection Process

This NMR indicates that the deprotection failed because the Boc peak is still very much intact. A thin-layer chromatography (TLC) was performed to gain further clarity by comparing the starting material with the final product. During the TLC, Boc-3-formyltyrosine is more nonpolar and travels further while 3FY does not travel as much since it is more polar. The TLC showed that the final product was not fully converted, leaving a significant amount of starting material still present.

To further delve into what occurred, the reaction was rerun. However, this time with a small caveat. This time, a TLC was used to track the progress of the reaction every 30 minutes and left to run until it reached completion. Unfortunately, the TLC tracking was not as successful as expected. According to the TLC plates, the reaction never quite progressed past the previous

material (a combination of Boc-3FY and 3FY). Another proton NMR of the product, in D_2O this time, as that is better suited for this molecule (Figure 12).

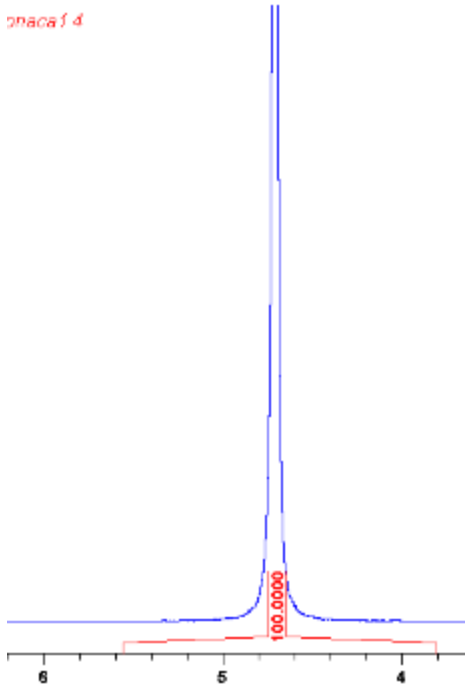


Figure 12. The Solution Peak of the Proton NMR of 3FY in NaOD

This spectrum did not give any information as the spectrum only showed a solution peak. This is due to the fact that 3FY is not soluble in water. NaOD was then used as the solvent and that was beneficial (Figure 13).

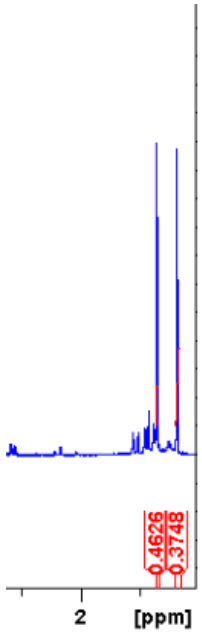


Figure 13. Proton NMR of 3FY Showing No Boc Peak After the Second Deprotection Process

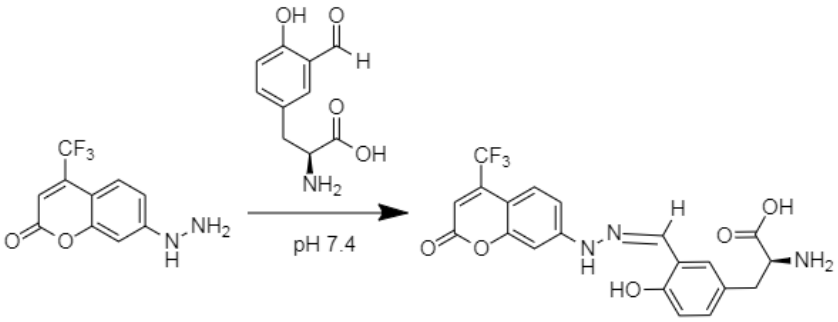
This spectrum shows that this NMR looks pure with no Boc peak. Although there is no clear reason as to why the reaction appeared to have not progressed, NMR is more discerning in its characterization than TLC. After the completion of this experiment, 1.21 g of 3FY was purified for a yield of about 90%.

Evaluation of the TFCH Reaction for 3FY-Tubulin Labeling

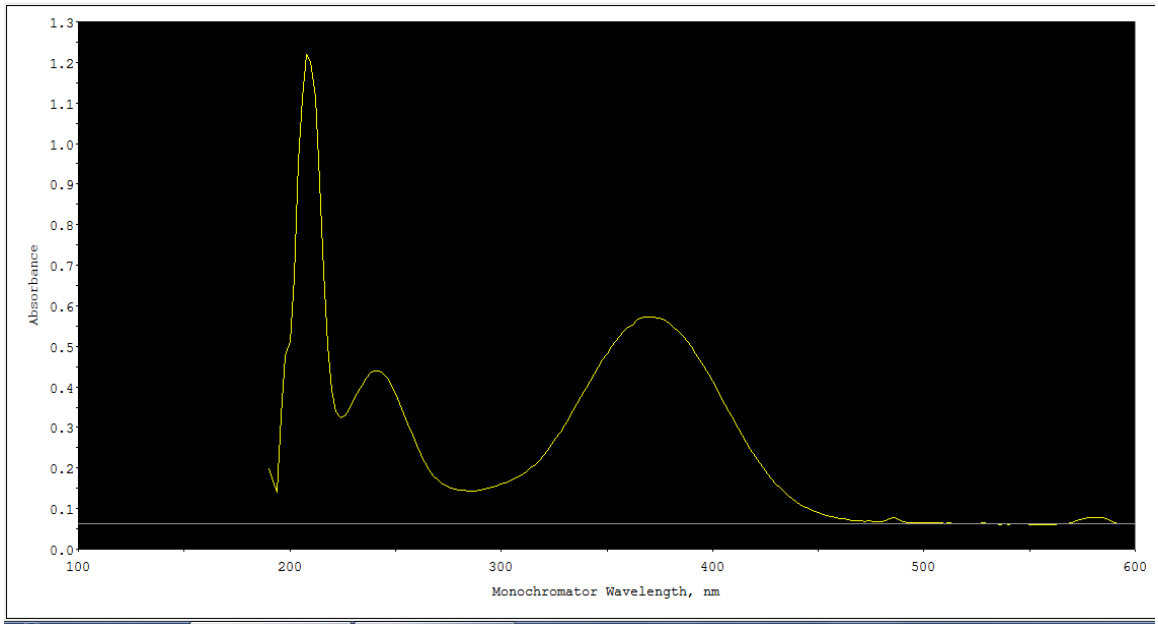
TFCH has been used to look at intracellular biomolecule carbonylation induced by oxidative stress[13]. The emission spectrum of TFCH undergoes a red shift and an increase in quantum yield when it forms a hydrazone with carbonylated biomolecules. These are aliphatic aldehydes, however, and 3FY is an aromatic aldehyde. Since the resulting hydrazone is conjugated to the aromatic ring, it would be expected to have different photochemical properties than the product from the aliphatic aldehyde. Therefore, a brief characterization of the hydrazone was performed.

The kinetics of hydrazone formation were measured under pseudo-first order conditions using absorption difference spectroscopy. Absorption difference spectroscopy displays the change in wavelengths absorbed by a molecule once it has undergone a chemical reaction. In this particular reaction, pseudo-first order conditions were utilized as this allows for the focus to be on the two main reactants, despite the reaction presenting as second-order overall. The difference spectrum shows formation of the hydrazone, which takes about 92 minutes under these conditions.

The effect of hydrazone formation on the absorption and emission spectra of TFCH is shown below. Panel A shows the absorption spectrum before and after the reaction with 3FY as well as the illustration of said kinetics reaction. There is a slight red shift in the absorption spectrum of the hydrazone compared to the hydrazine, which is expected because of the extended conjugation of the fluorophore after hydrazone formation. Panels C and D show the emission spectra of the solution before and after the chemical reaction. Panel C shows that the emission spectrum red shifts and increases in intensity after the reaction, as expected (at the absorbance maximum of 372 nm). The sample was also excited at 405 nm, which is the excitation wavelength available in the fluorescence microscope that would be used to visualize intracellular microtubules. The results of this were very similar to that of the excitation at 372 nm so they were not included here. In Panel D, the reaction was run and the change in fluorescence was monitored for its duration at 372 nm and 405 nm (Figure 14).


OH
O
H
O
OH
NH2
CF3
O
O
N
H
NH2
pH 7.4
CF3
O
O
N
H
N
H
OH
O
NH2
HO

A


Absorbance
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
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0.2
0.1
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100
200
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400
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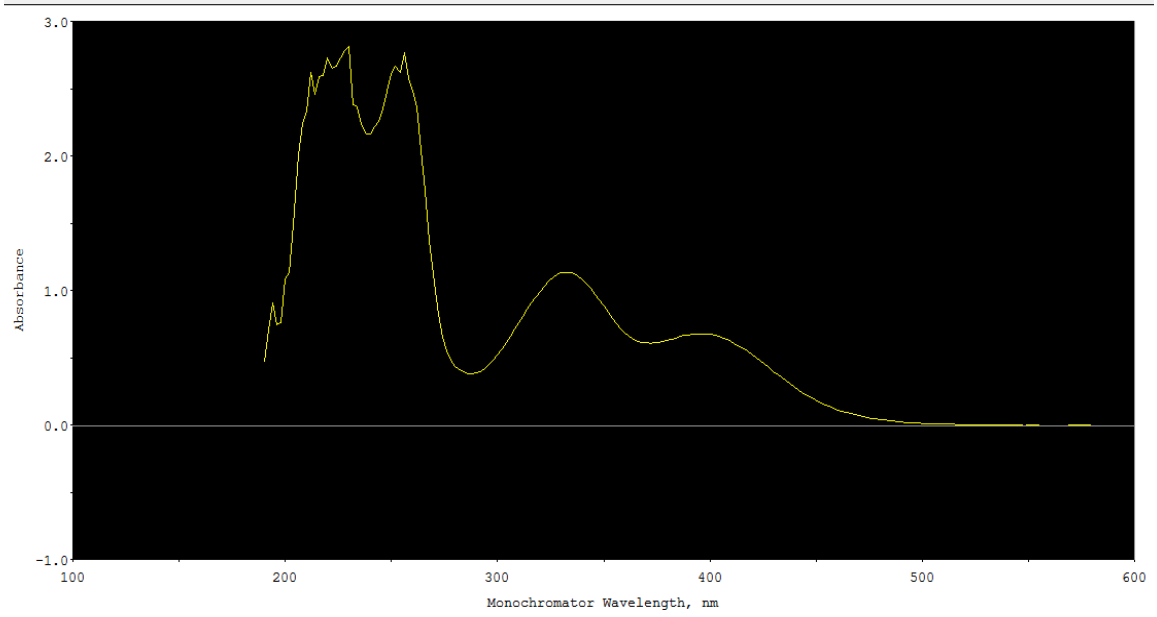
Monochromator Wavelength, nm


Absorbance
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2.0
1.0
0.0
-1.0
100
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Monochromator Wavelength, nm

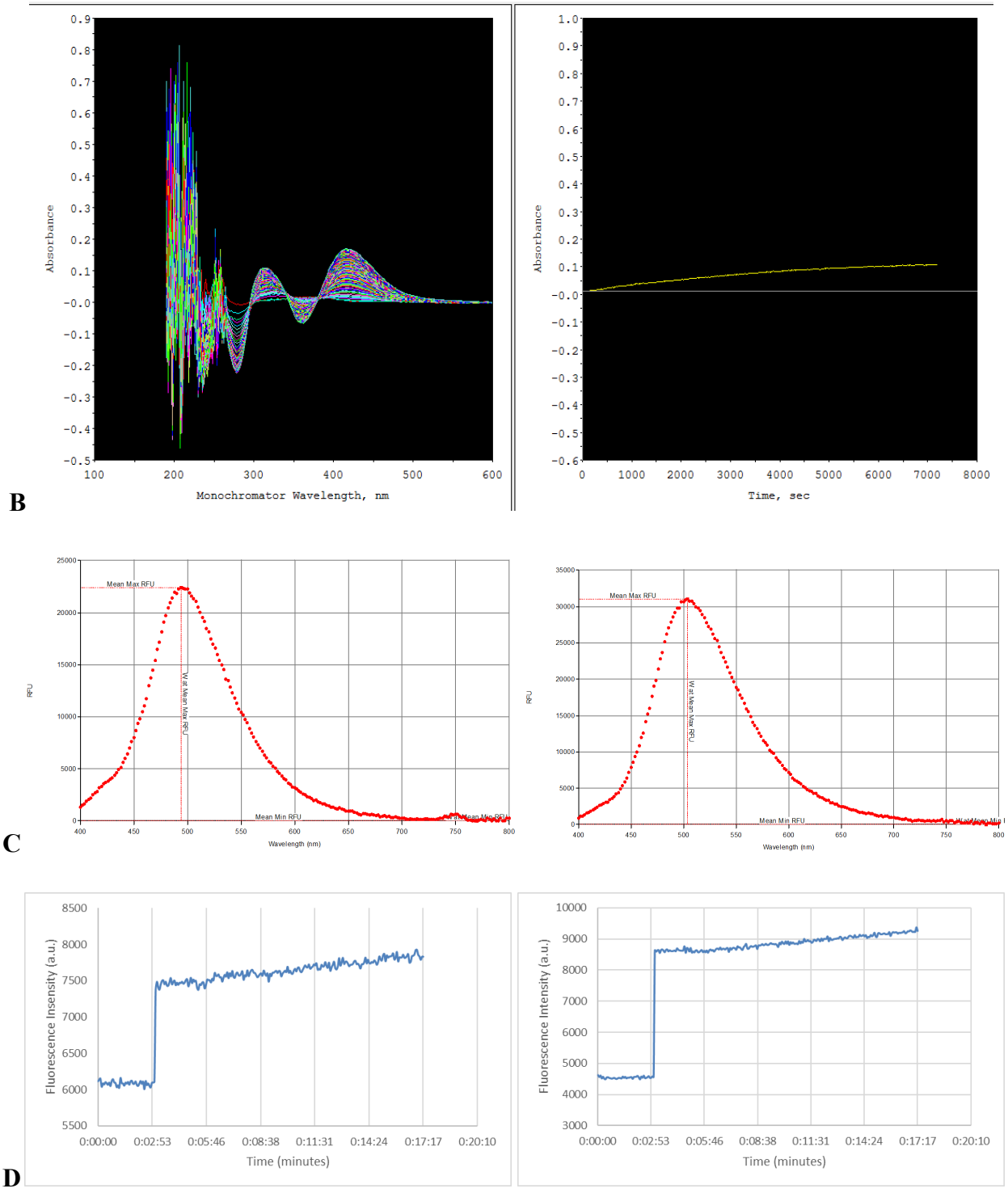

Figure 14. Evaluation of TFCH for *in vitro* Hydrazone Chemistry Via Absorbance and Emission Spectra.

A) The Absorbance Difference Spectrum of TFCH Before and After Reaction with 3FY B) The Kinetics Plot of the

Reaction as it Occurred C) The Emission Spectrum of TFCH Before and After Reaction with 3FY at 372 nm D) The Fluorescence Changes During the Reaction at 372 nm and 405 nm

It is therefore concluded that the hydrazone reaction between 3FY and TFCH is likely to occur under physiological conditions inside the cell and produce a product that can be seen with fluorescence microscopy.

Culturing of Prostate Cancer Cells (PC3s)

For this experiment, PC3 cells were used according to the procedure delineated previously in former Bane lab member Kamalika Mukherjee's dissertation[8]. Firstly, a medium was chosen to grow the cells that would best foster their growth while allowing for the desired experimentation. In this experiment, HAM-F10 media was used because it is similar to the very common HAM-F12 media (2.62 mg/L and 7.81 mg/L, respectively). Since 3FY and tyrosine are both substrates for TTL, a lower concentration of tyrosine in the medium was hoped to improve the yield of the intracellular TTL reaction.

Introduction of 3FY to the PC3s

To test the proposed technique, a solution of 3FY must be prepared so that it can be added in a way that the cells can incorporate the tyrosine derivative. This solution is set to have a final concentration of 250 μM as this will allow the cells enough time to incorporate the 3FY without killing them. The cells are to be treated with 3FY for a total of 24 hours following a similar reason as the final concentration determination. Although TTL retains its preference for the original substrate (L-Tyrosine), it still incorporates 3FY with success at a moderately lower rate so long as 3FY is in excess[7].

The cells treated with 3FY will serve as the experimental group whereas the other cells will serve as the necessary control. The experimental conditions of the 6-well plate are shown below in Figure 15.

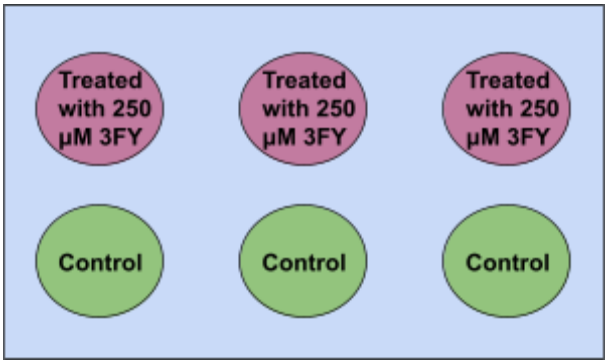

Figure 15. Experimental Conditions of 6-Well Plate

Treatment with TFCH

The choice of TFCH as opposed to the Kamalika's previously used coumarin hydrazine is the drive of this project. TFCH is a more desirable hydrazine-fluorophore analog than coumarin hydrazine because coumarin hydrazine barely absorbs in the 405 nm wavelength. Furthermore, coumarin hydrazine lacks photostability, a trait very necessary for the visualization of microtubules. TFCH has an excitation wavelength of 405 nm and is more stable, making it all the more capable of microtubule visualization[13].

SDS-PAGE

SDS-PAGE is a type of gel electrophoresis through which proteins are separated from other proteins by their molecular weight in solution. This is accomplished via an SDS detergent treatment, a porous gel, and an electrical current. Proteins are first denatured using SDS, which applies a negative charge to the proteins proportional to their molecular weight. The porous gel

allows proteins to sift through and travel in such a way that they are separated by charge when an electrical current is applied. The proteins are loaded in wells at the top of the gel, which is negative. The bottom of the gel is positive. Thus, when an electrical current is applied, the smaller proteins travel further down the gel whereas larger proteins cannot travel as much through the gel sieve. Following the gel procedure, the gel can either be imaged under UV light to evaluate fluorescence or stained with coomassie blue dye to view the proteins as bands[14].

First Attempt

PC3 cells were thawed and incubated in the freshly prepared HAM-F10 media. After the original growth date, the cells had achieved 50% confluency in a week's time, before contamination occurred (Figure 16).

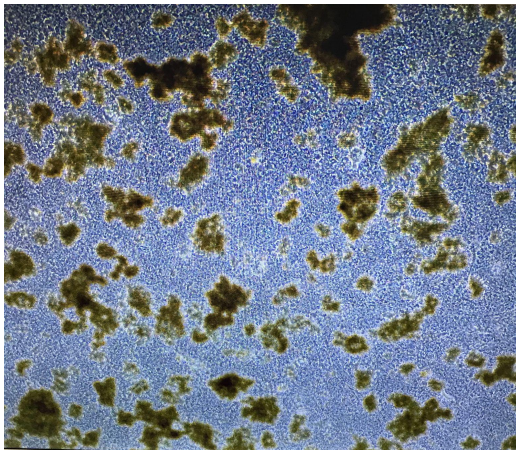

Figure 16. PC3s Cells After Contamination

After clear contamination, the next step was to investigate the cause of this before continuing any further. The cause could have been one of three things: the media, FBS, or mycoplasma. The media could be a source of contamination since it was made in the lab instead of purchasing it, and the filtering process was not done immediately due to lab access technicalities. To verify if this concern was valid, the media was incubated by itself to see if a

color change occurred. A color change would indicate possible contamination since the color change is indicative of a transformation. There was a slight color change so the media was highly suspected as the culprit. To be thorough, all other possible sources of contamination were delved into deeper still. Since the FBS was aliquoted many years ago, there was no previous knowledge of contamination. To check for that, store-bought media (McCoy's 5A) was supplemented with FBS from the same batch used for the experiment and incubated along with a non-supplemented flask of that media. There was no perceivable change. The last concern, mycoplasma, was quite alarming as mycoplasma is extremely pervasive. This concern was quelled as a possibility since other experiments were also occurring in the same facility and did not experience any contamination.

Second Attempt

Hopeful that the contamination was remedied, the growth process of the PC3s was restarted with store-bought HAM F-12 media. The change from HAM-F10 to HAM-F12 was made because HAM-F10 is not easily available in liquid form, whereas HAM-F12 is. Although it has a greater tyrosine concentration, it will not be so high as to overpower the benefits of it being store-bought. The cells were incubated with media and changed according to their 48-hour incubation mark. Since the cells had reached 70% confluency, the cells were passaged once before ultimately seeding them on a six-well plate.

Once the cells were again confluent enough, three of the wells were treated with 3FY for 48 hours. The cells are typically only treated for 24 hours, but unfortunately, circumstances prevented the reaction from beginning at the appropriate time. It is also important to note that the 3FY had not been syringe filtered so that could lead to contamination.

Luckily, the cells were not contaminated and the hydrazine reaction was performed. The 3FY treatment was removed and those three wells were lysed then treated with TFCH. The lysate was collected and prepared for an SDS-PAGE gel electrophoresis (Figure 17).

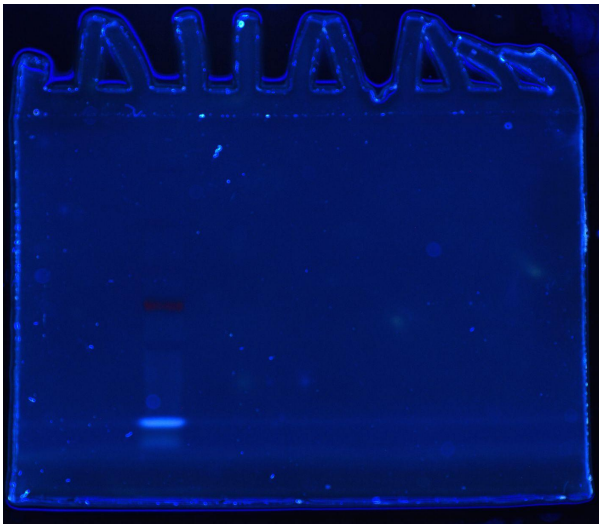

Figure 17. UV-Imaged SDS-PAGE Gel of Untreated PC3s (Lane 5) and 3FY-Treated PC3s (Lane 7)

As seen in the above gel, the 3FY-treated lysate did not express any level of fluorescence. This may be the result of the reaction between TFCH and 3FY not occurring. In this instance, it is likely that the reaction did not occur since the 3FY was not syringe filtered. Although no direct contamination was observed, it is still possible that this altered the ability of 3FY to react with TFCH. Another possible reason is that the 3FY did not react with the α-tubulin. Again, the lack of syringe filtration could be responsible for this as well. To determine it the 3FY never actually bound to α-tubulin, a coomassie stain of the previous gel was completed (Figure 18).

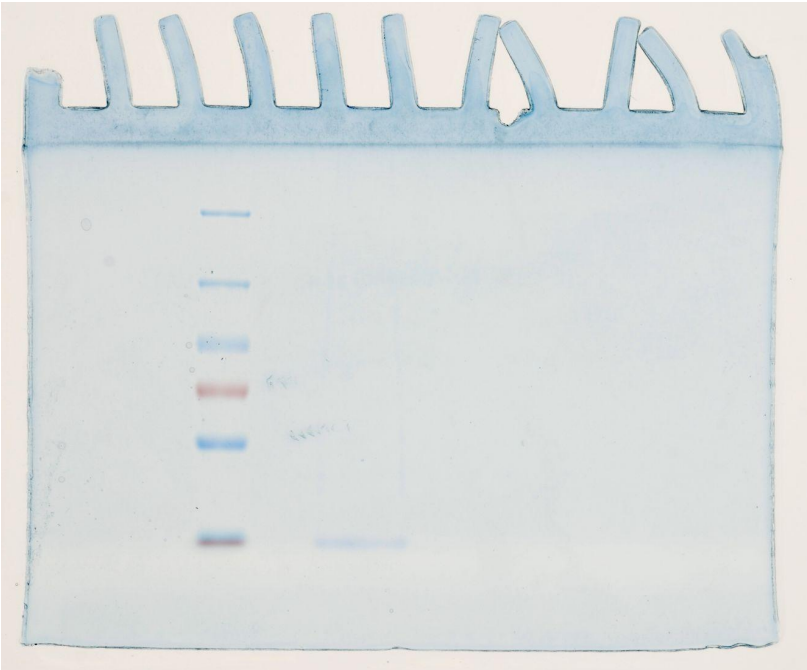

Figure 18. Coomassie-stained SDS-PAGE Gel of Untreated PC3s (Lane 5) and 3FY-Treated PC3s (Lane 7)

This gel is inconclusive due to the fact that the control lysate seemingly has leaked into its neighboring well. Even further, no bands are showing up which is extremely unexpected since both lysates should show the characteristic band smearing that occurs with this type of cell sample. As such, the experiment was repeated in order to acquire conclusive results.

Third Attempt

Again, the PC3s were thawed and grown to 70% confluency using HAM-F12 media and the same procedure as the first two attempts. This time, however, the cells were passaged twice. After their second passage, the cells were treated with 3FY, that had been syringe filtered, for the correct time of 24 hours. The cells were lysed and incubated with TFCH for two hours before the lysates were collected and subjected to an SDS-PAGE gel. The gel was first imaged under UV light to inspect for fluorescence (Figure 19).

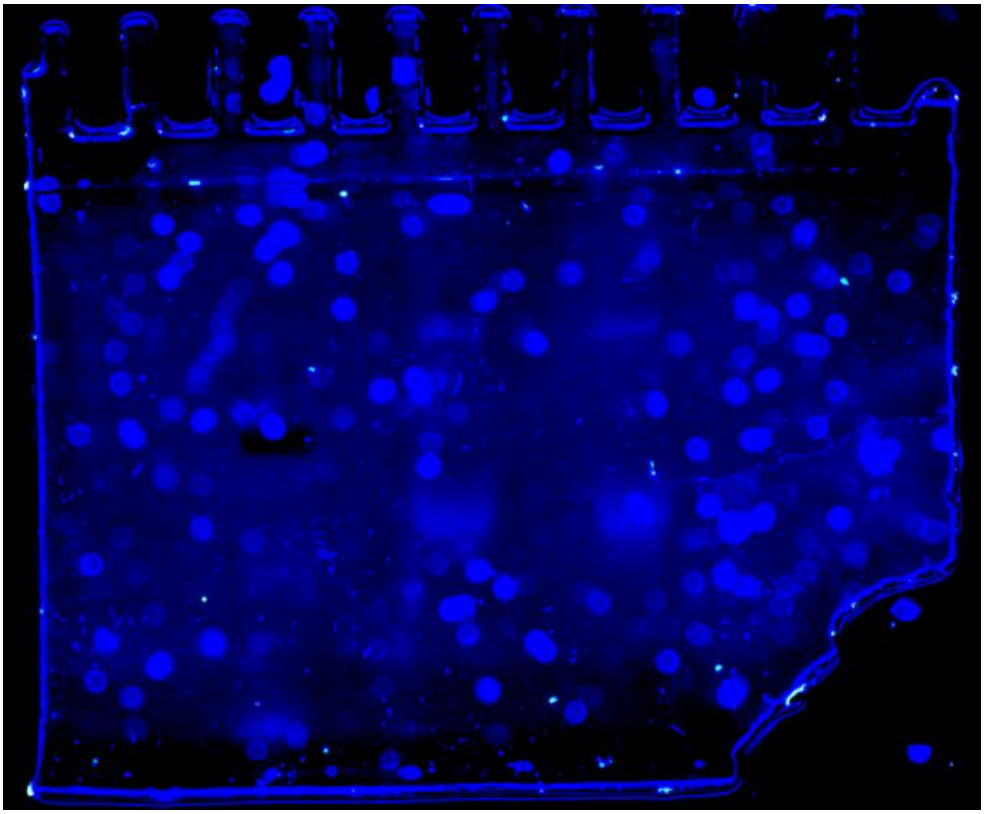

Figure 19. UV-Imaged SDS-PAGE Gel of Untreated PC3s (Lane 5) and 3FY-Treated PC3s (Lane 7)

The above gel is hard to interpret for a number of reasons, most notably the very prominent noise created by dust on the imaging fabric. Secondly, the fluorescence is altogether very dull despite being exposed to UV light for 15 seconds. Although, there is still some fluorescence. The trouble lies in the fact that this fluorescence appears in both the control and experimental lysates. Even further, it appears at two distinct bands in each lysate. A coomassie-blue dye stain was also completed on this gel for further elucidation (Figure 20).

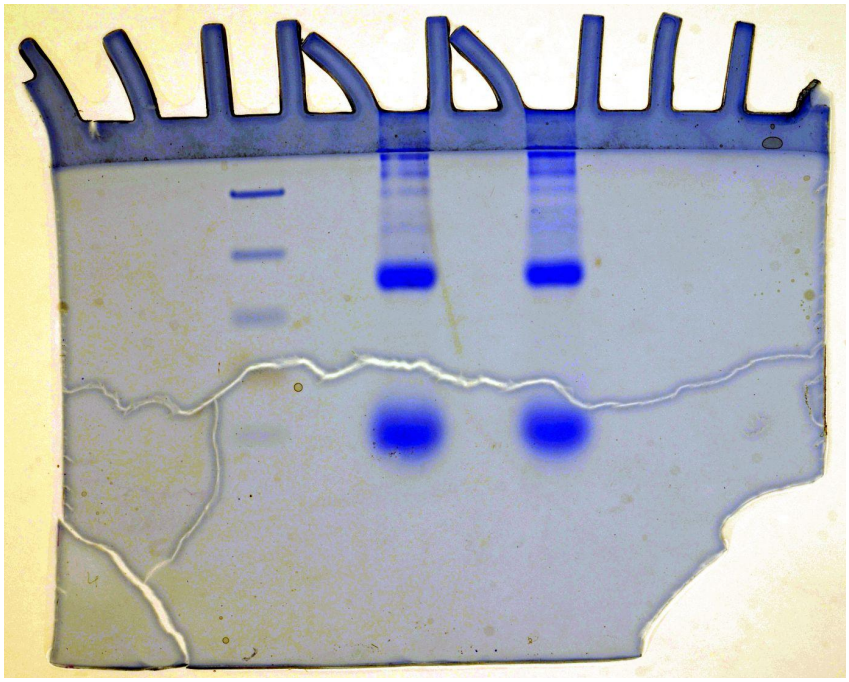

Figure 20. Coomassie-Stained SDS-PAGE Gel of Untreated PC3s (Lane 5) and 3FY-Treated PC3s (Lane 7)

This gel continues to be worrisome since it does not resemble a characteristic lysate in that it has two very distinct protein bands. According to the molecular weight marker, the lower band is about 60 kDa, which could correspond to α-tubulin. Although, the intensity of this band does not corroborate this notion since it is unlikely that this native protein would show up as such a distinct band in the lysate. Upon viewing its intensity, it is likely that that band actually corresponds to serum albumin, which also has a weight close to 60 kDa. Serum albumin should not be present at all in this lysate, led alone as intensely as it is. However, serum albumin may be present if supplemented media was used to store the lysate instead of serum-free media (SFM). As for the upper band, it appears to be 120 kDa and its identity remains unknown.

An additional reason to suspect that the 60 kDa band might be serum albumen is the fact that it is fluorescent in both gels. Commercial bovine serum albumin contains a small amount of oxidized (carbonylated) protein, which will also form a hydrazone with coumarin hydrazine derivatives[13].

Fourth Attempt

The same procedure as the third attempt was again completed with everything the same to see if the same results would occur. The samples were collected by the same means and submitted to another set of SDS-PAGE analysis experiments to gain greater insight into the results of the reaction (Figure 21).

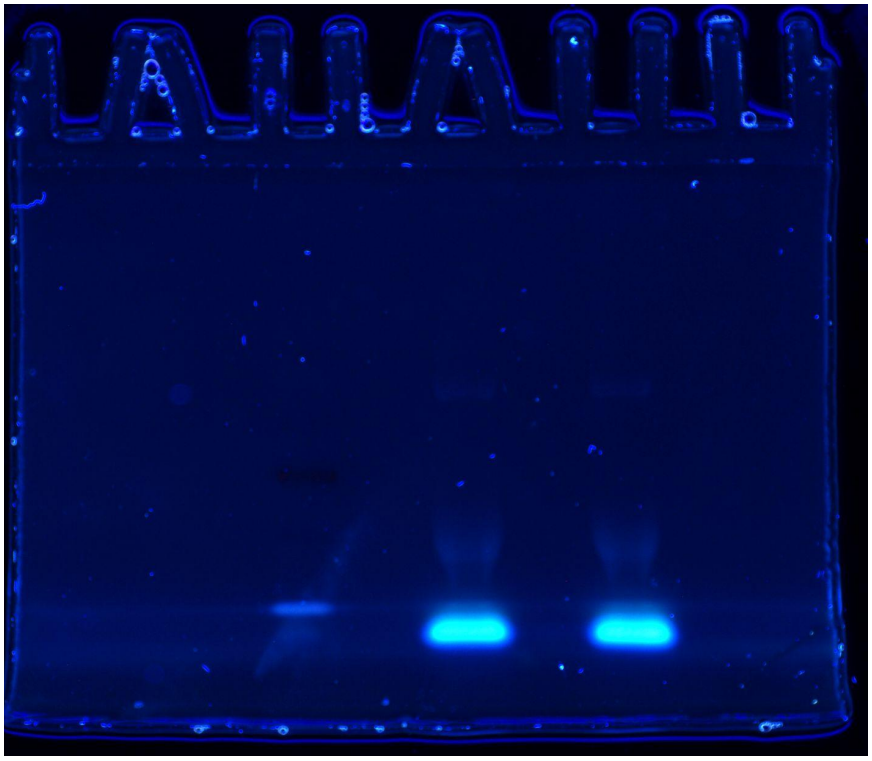

Figure 21. UV-Imaged SDS-PAGE Gel of Untreated PC3s (Lane 6) and 3FY-Treated PC3s (Lane 8)

This gel still has very little fluorescence after being exposed to UV light for 8 seconds. The extreme fluorescent bands at the bottom are just the loaded samples as they had not traveled entirely through the gel. The previous two bands are still present in the same position and experiencing the same fluorescence. Again, this gel was then coomassie-stained and re-imaged (Figure 22).

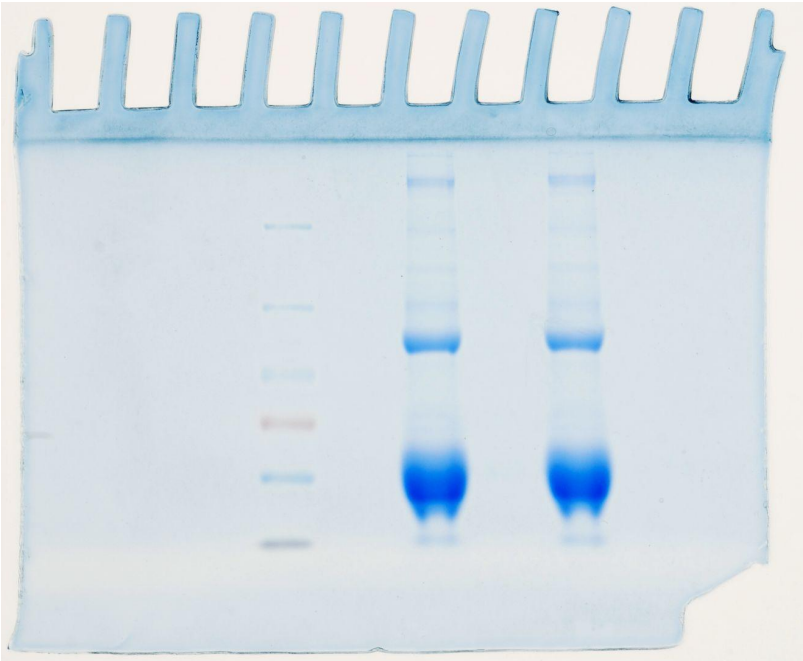

Figure 22. Coomassie-Stained SDS-PAGE Gel of Untreated PC3s (Lane 6) and 3FY-Treated PC3s (Lane 8)

The same issues are still observed. However, this time the bottom band appears even more intense. Again, the gel indicates that the lower band is about 60 kDa, which could correspond to either serum albumin or α-tubulin as discussed previously. The upper band is still an unknown protein at about 120 kDa. However, it could possibly be the dimer of the 60 kDa protein.

Conclusion:

Ultimately, this project had two main focuses: the synthesis of 3FY and the optimization of the fluorophore used in the innovative approach to microtubule visualization. These projects are best evaluated as separate functions of the same overarching goal. As such, the former will be discussed first.

The synthesis of 3FY was successful for two main reasons. The first of which is very clearly expected: the desired molecule was completely purified. The proton NMR showed that the molecule is as expected. The second point of success lies in the fact that not only was the molecule synthesized and pure, but it was also made in abundance. This is particularly helpful for the continuation of this project as well as many others in Bane lab. For most experiments, only a few milligrams of 3FY are needed so the more than one gram that was synthesized in this experiment is enough to supply the lab for a multitude of projects.

As for the second focus of this project, its success is not as clear. To begin, the results obtained throughout tend to be either hard to interpret or simply confusing. However, this does not necessarily mean that the new approach with this fluorophore does not work. The materials or human error may be the source of these issues. Regardless, this research still provides insight into establishing a new approach to microtubule visualization as it either shows the need for a different fluorophore or demonstrates the lack of universality of this procedure. Further studies will be needed in order to build off the foundation created by this research.

This project has many potential routes for future study. The first and foremost being to attempt this procedure with new materials to eliminate if it's the procedure that does not work or just the materials being used. If the former is found to be true, then the next area of study would

be to investigate what differences exist between coumarin hydrazine and TFCH that would allow this experiment to work for one of the fluorophores but not the other. This could also lend itself into a greater expansion of this project by sampling different fluorophores and determining which are best suited for the purpose of microtubule visualization. If the latter is found to be the cause of error, then the preference of TTL for 3FY can be quantified. With this quantified, a beneficial concentration for the reaction can be calculated and the project can progress further to the next stage of labeling live cells, further validating this novel approach to visualization and opening it up to clinical studies.

Materials and Methods:

Boc Tyrosine: 2-((tert-butoxycarbonyl)amino)-3-(4-hydroxyphenyl)propanoic acid

5.1 g of store-bought L-tyrosine was dissolved in a 2:1 mixture of dioxane (128 mL) and water (64 mL) in a 500 mL round bottom flask. Subsequently, aqueous NaOH (1 M, 70 mL,) and Boc_2O (6.8 g, 31.2 mmol) were added. The reaction was stirred at room temperature for 3 hours. Upon completion, the product was acidified (pH ~ 2) with 5M HCl and extracted with EtOAc (3 x 60 mL). The EtOAc layers were combined after each extraction and finally washed with NaCl (60 mL). Solution was dried with $MgSO_4$, filtered, and concentrated via rotary evaporation.

The product was purified using a gradient solvent condition of 1:4 ethyl acetate:hexane + 0.5% acetic acid to 1:1 ethyl acetate + 0.5% acetic acid on a silica column. Purification yielded 10.8 g of the Boc-Tyrosine with an ~80% yield.

Boc-Formyl Tyrosine: 2-((tert-butoxycarbonyl)amino)-3-(3-formyl-4-hydroxyphenyl)propanoic acid

5.8 g of NaOH powder (~145.0 mmol) was added to a round-bottom flask containing 6.9 g of Boc-Tyrosine (~24.5 mmol). 867 µL of ddH2O (~48.1 mmol) and 117.1 mL of chloroform (~ 1.4 mol) were then added to the flask. The contents were refluxed for 4.5 hours, with addition of 1.7 g of NaOH powder (~41 mmol) 1 hour into the reflux reaction. The reaction was quenched

after 4.5 hours with 50 mL EtOAc and 50 mL H2O. The aqueous phase was acidified (pH ~ 1) with 5 M HCl. The aqueous phase was then extracted with EtOAc (3 x 50 mL) and the combined EtOAc layers were washed using an NaCl brine (50 mL). The solution was dried with $MgSO_4$, filtered, and concentrated via rotary evaporation. The product was purified with a silica column using a gradient solvent condition of 1% MeOH in DCM to 8% MeOH in DCM (with 0.5% acetic acid) until all product was eluted from the column. Purification yielded 104 mg of Boc-formyltyrosine at ~10% yield.

3-Formyltyrosine: 2-amino-3-(3-formyl-4-hydroxyphenyl)propanoic acid

1.98 g of Boc-Formyltyrosine (~8.00 mmol) was dissolved in 3.4 mL of DCM and stirred at 180 rpm. While the solution was stirring, 222.5 μL of trifluoroacetic acid (TFA) was added to the flask and the stirring was increased to 300 rpm. Flask was left to stir for 2 hours with a TLC (conditions: 10% MeOH in DCM + 0.5% acetic acid) being run every 30 minutes to check for progress. After 2 hours, 1 mL of DCM and 1 mL of TFA were added and the reaction was left to stir for another hour. Following this hour, N_2 was continuously pumped into the flask overnight. No purification procedure was needed since the reaction results in TFA salt, which is removed during the concentration process via rotary evaporation The reaction yielded 1.21 g of 3-formyltyrosine at ~90% yield.

Absorbance and Emission Spectroscopy of TFCH

800 μL of 20 μM TFCH (244.2 g/mol) was prepared by first creating a 20 mM stock, which consisted of 147 mg in 3 mL of phosphate buffer (pH = 7.02). The stock was then diluted on a 1:10 scale to yield a 2 mM stock solution. 8 μL of the 2 mM stock was added to 792 μL of phosphate buffer. 800 μL of 200 μM 3FY (209.2 g/mol) was prepared by first creating a 20 mM stock, which consisted of 126 mg in 3 mL of phosphate buffer (pH = 7.02). The stock was then diluted on a 1:10 scale to yield a 2 mM stock solution. 80 μL of the 2 mM stock was added to 720 μL of phosphate buffer.

The phosphate buffer absorbance was taken as a reference and then, the absorbance of each solution was taken separately. Subsequently, the solutions were added to separate chambers of the quartz cuvette and it was covered with parafilm. It was referenced, mixed, and the reaction was monitored at 500 scans for 7200 seconds. After the reaction, a phosphate buffer reference was taken once again and a single-scan absorbance was taken of the final product.

Following the kinetics experiment, 100 μL of 20 μM TFCH was subjected to an excitation wavelength of 372 nm and an excitation wavelength of 405 nm. This was noted as the emission spectrum before the reaction. 100 μL of 200 μM 3FY was then added to the TFCH and the reaction was allowed to run to completion before another emission spectrum was collected using the same parameters as before.

Prostate Cancer Cells

Previously passaged (P=32) and frozen PC3 cells were obtained and thawed. Ham-F10K media (pH = 7.4) was prepared and filtered followed by supplementation with 10% (v/v) FBS and 1% penicillin-streptomycin. The media is warmed and added to the cells, which are then centrifuged to remove the DMSO that is used in the storage of cells. Resuspension of the cells in fresh supplemented media and incubation of the cells at 37 ℃ in 5% CO_2 allows the cells to grow. The cells have an incubation period of 48 hours before the media must be changed. For PC3 cells to be functional, they must reach 70% confluence which can take about two weeks of media changes.

Cells were then trypsinized using 3 mL of 0.25% (w/v) trypsin and underwent two rounds of passaging before ultimately being plated on a 6-well plate up to 70% confluence and grown for the stated time periods. After the first experiment, PC3s were grown in store-bought Ham-F12K following the same procedure.

3-Formyltyrosine Addition to α-Tubulin

PC3 cells in F12K medium were grown overnight in a 6-well plate and the solution of 3FY that would be added to the cells was prepared by adding 3 mg of 3FY to 5.71 mL of DMSO, resulting in a final concentration of 250 μM. The following solution must then be syringe filtered through a 0.22 micron filter to avoid contamination. The cells were then treated with 3FY for a total of 24 hours.

Hydrazone Reaction via TFCH

In order to treat the cells with TFCH, the cells must first be rinsed with 0.01% triton X in medium and incubated for 3 minutes. Then, the media is decanted and the cells are rinsed with fresh media, before being incubated once more in another 2 mL of fresh media for 5 minutes. From there, the cells are washed two times with PBS and lysed using a lysis buffer consisting of 980 µL PBS, 10 µL of 10% SDS, and 10 µL of Protease inhibitor followed by three 3 minute-cycles of vortexing, heating (100℃), and quickspining. 2.2 mg of TFCH was dissolved in 1 mL of DMSO, of which 0.04 mL was added to each lysate, along with 1.96 mL of fresh media, for a final concentration of 250 µM TFCH and allowed to react for two hours. The lysates and any subsequent handling of TFCH requires concealment from light so as to maintain the fluorescence of the fluorophore. This is achieved by covering the vials and apparatuses with tin foil.

SDS-PAGE Analysis

To prepare the SDS-PAGE analysis, a gel was made such that it contained an 8% resolving layer and a 6% stacking layer according to known recipes. From there, a running buffer (3.04g Tris-OH, 14.42g glycine, 1g 10% SDS, and 1 L ddH_2O) was added to the gel apparatus. 5 µL of PageRuler Plus (ThermoFisher) molecular weight marker and 30 µL of each sample solution was loaded after they were boiled for 5 minutes. Each sample solution consisted of 20 µL of sample and 10µL of previously prepared 3x sample buffer. The gel was run at 140 V for 45-60 minutes. While the gel ran, the apparatus was stored under a cardboard box so as to prevent the fluorescent samples from being exposed to light.

When the gel finished, it was stored in excess running buffer and covered with tin foil until it was imaged under UV light. After the UV image was obtained, the gel was stained using Coomassie Brilliant Blue dye for 3 hours followed by a 2-day destaining process and imaging.

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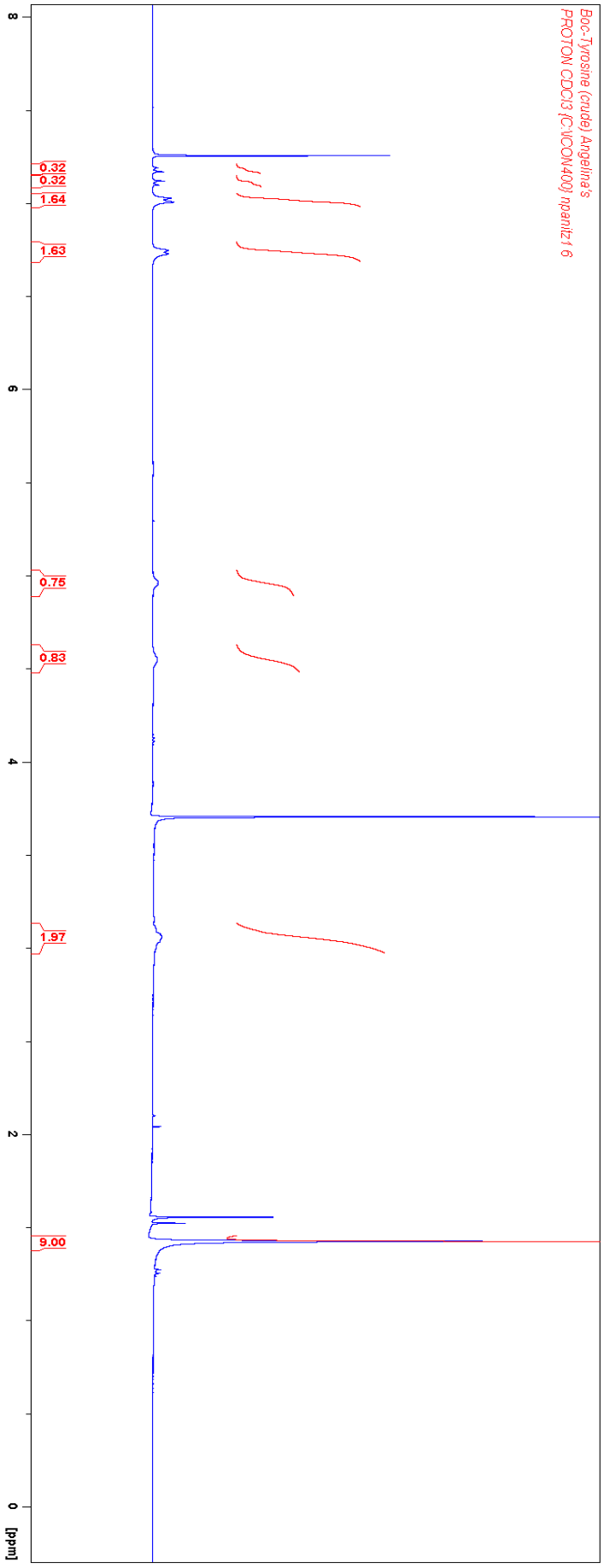
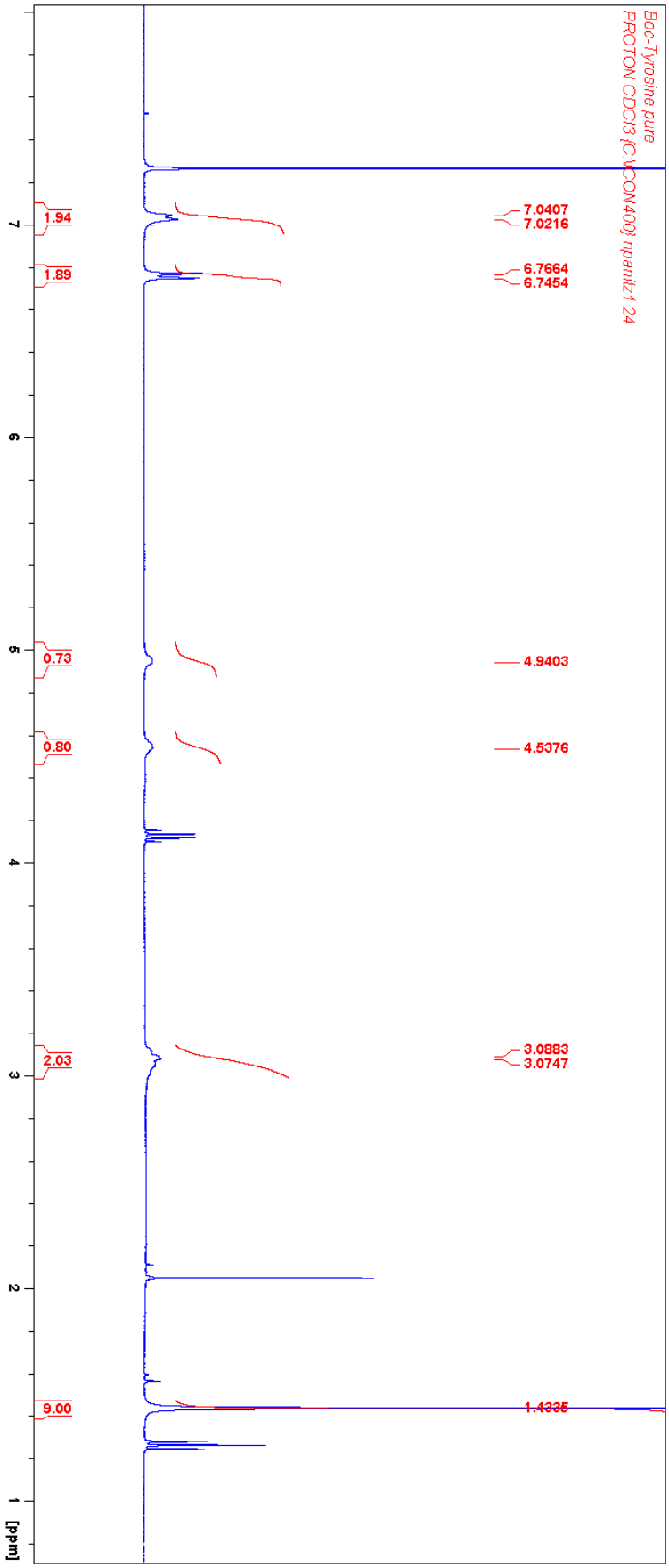
Appendix:

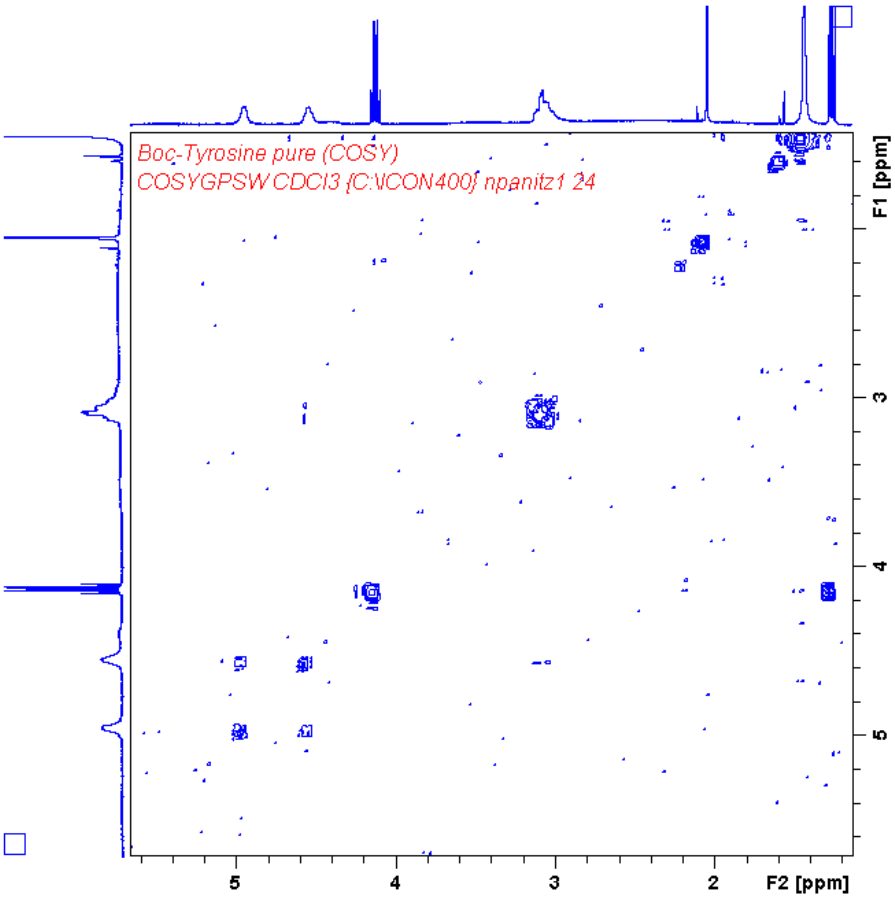
Crude Boc-Tyrosine Proton NMR



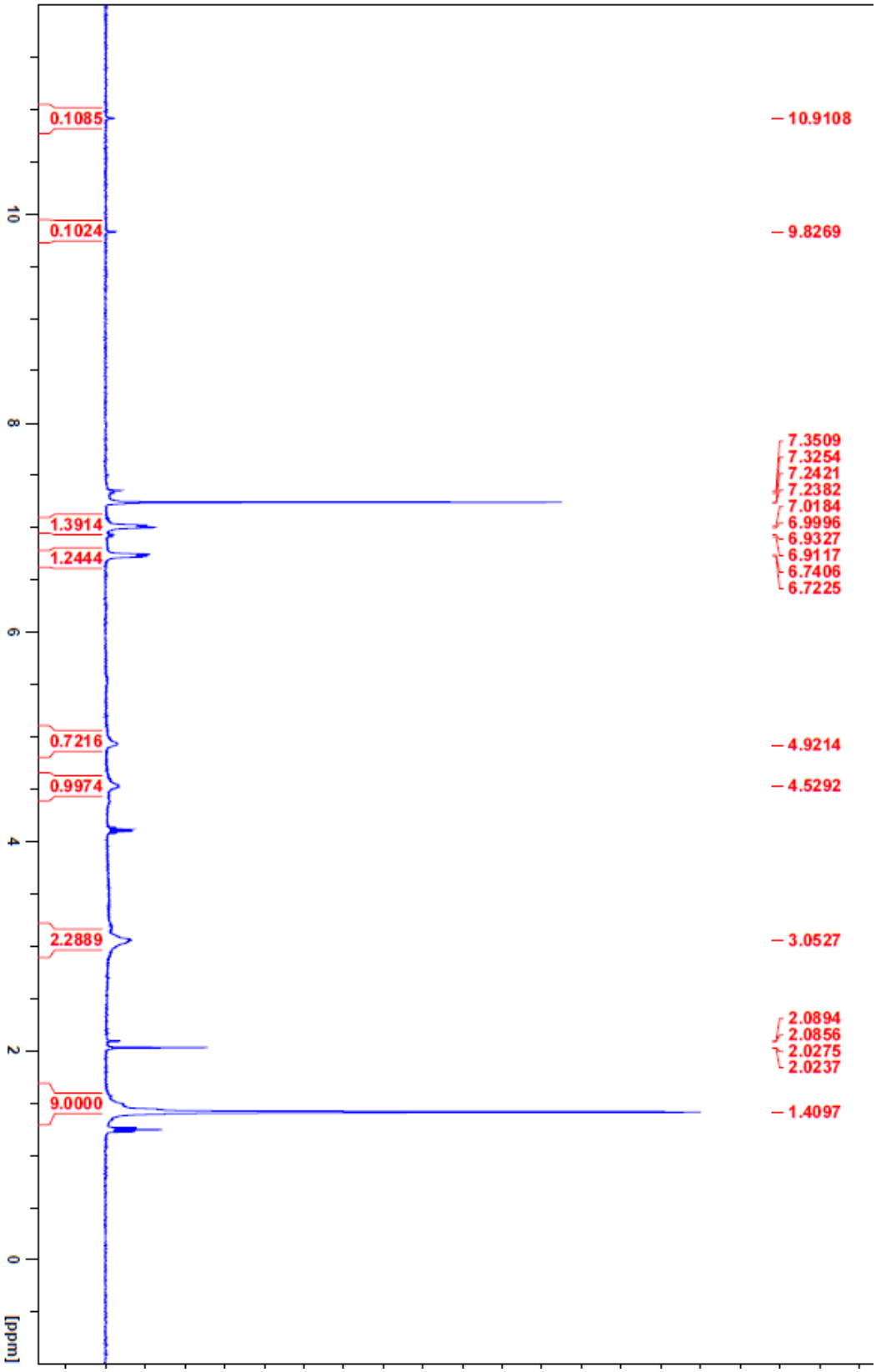
Boc-Tyrosine Proton NMR



Boc-Tyrosine COSY



Boc-Formyltyrosine Proton NMR



3-Formyltyrosine Proton NMR