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Methodology for C-glycosylation and synthesis of probes for the detection of cysteine containing proteins

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Senior Honors Research

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I. Introduction

Throughout my research I have been able to expand my knowledge and gain experience in organic chemistry by working on projects related to biochemistry and hydrocarbons. The projects I have worked on were:

Project 1: C-glycosylation through photo irradiation with reductive halide atom transfer

Project 2: Synthesis of rhodamine and biotin probes for the detection of cysteine containing proteins

Project 3: Literature review on: Fluorescent probes for cysteine containing proteins

I have worked on the c-glycosylation through photo irradiation with reductive halide atom transfer for two semesters, with one semester focused mainly on understanding the project and presenting on it at meetings such as the Virginia academy of science. The other semester focused on bench work, it involved researching and pricing the chemicals for ordering as well as running the first reaction. During this semester I also presented on the project at the Hollins Science Seminar, as well as at the American Chemical Society undergraduate poster session.

I have worked on the synthesis of rhodamine and biotin probes for the detection of cysteine containing proteins for two and a half semesters. The first semester focused on the synthesis of the biotin probe. This synthesis required purification techniques that I had to learn how to preform, such has column chromatography. This semester was unfortunately cut short due to covid sending everyone home. The second semester focused again on the

synthesis of the biotin probe, during this semester I was able to complete the synthesis of the biotin probe however I was unable to test it to confirm that the product was the desired product due to not being able to return to campus after fall break. The compound was not tested on return in February due to the possibility of degradation of the product. During this semester I also presented on the project at the Rocky Mountain Regional meeting of the American Chemical Society. During the half semester I ran the first reaction of the synthesis of the rhodamine probe.

I have worked on the literary review during j-term and a spring semester. This time has been spend focused on reading articles and doing further research to ensure that I understand them. This has allowed me to have a further understanding of fluorescent probes for cysteine containing proteins. These projects have allowed me to explore interests in biochemistry and organic chemistry. I have learned many different synthetic purification techniques, such as separations and column chromatography. I have been able to use problem solving skills and apply it to the work being done.

II. Project 1: *C*-glycosylation through photo irradiation with reductive halide atom transfer

IIA. Background

There are different types of glycosylation including C, N, S, and O glycosylation. A glycosidic bond is a bond between the anomeric carbon on the sugar and the C, N, S, or O of another compound[1]. Compounds that contain these glycosidic bonds are termed complex carbohydrates. Complex carbohydrates contain many important biological processes and are found in natural products and drugs[2]. Compounds containing the C-glycosidic bond are hard to obtain from natural sources, this is because it is hard to obtain a homogeneous form of the compound and it is difficult to obtain the compounds in a large enough quantity. Therefore, synthesis is required to gain access to these kinds of compounds. Derivatives of the C-glycosyl contain importance in carbohydrate and natural products due to their biological and pharmaceutical activities.

Synthesis of these compounds can have many challenges due to stereoselectivity and regioselectivity, as well as the purification process[3]. Figure 1 shows the many ways that two identical monosaccharides can be linked together. The challenge demonstrated by this figure is the many different possibilities in the regio and stereoselectivity.

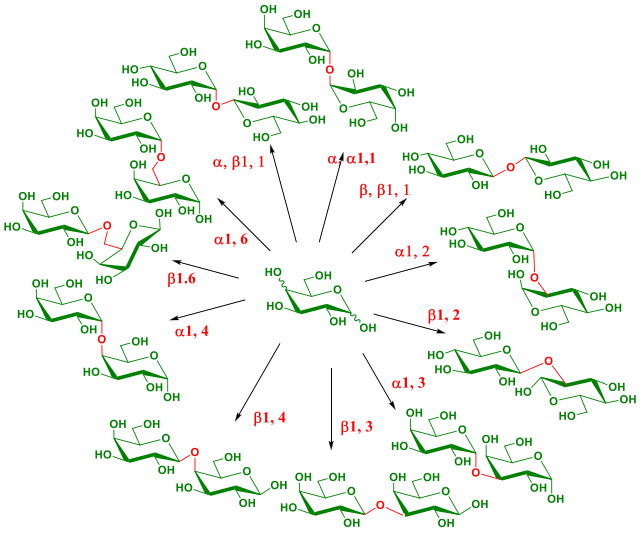


Figure 1: The different ways two identical monosaccharides can link together

Steric effect controls the regioselectivity since the free radicals preferentially attack less substituted, or in other words less bulky, carbon atoms on alkenes. The stereoselectivity of the α or β product depends on steric effects, anomeric effects, and protecting groups. With the main being the anomeric effect (Figure 2) since when forming the radical the interaction between the radical electron and the nonbinding electron pairs on the oxygen would make the α conformation to be more stable than the β confirmation; therefore, the α product is more favorable[4]. This is just one of the issues that needs to be tackled in the research. The purification process can cause challenges such as loss of product and being time consuming. These complications cause issues in gaining access to these compounds.

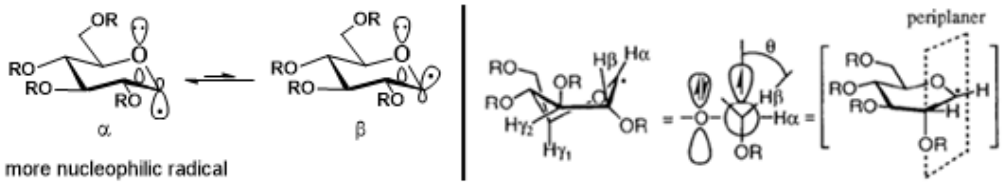
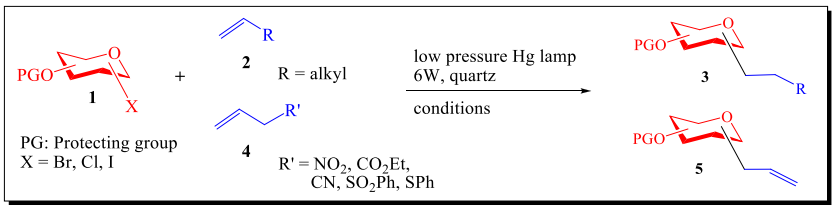


Figure 2: The anomeric effect

There are current methods out there that can allow access to these compounds, the C glycosidic bond can be formed from glycosyl anions or radicals. A current widely used method is the radical addition of a glycosyl bromide to activated alkenes. This reaction is typically catalyzed by a chemical radical initiator. One of the most frequently used radical initiator is tributyltin hydride[2]. This complex can have harmful effects on the researcher's health as well as environmental implications due to the metal. These methods also have typically low yields, sensitivities to air, difficulties in phase separating, and purification.

Combined this leads to an undesirable method for the formation of these products. Therefore, there is desire to discover a new method which is capable of producing a high yield and stereoselectivity without using the toxic chemical initiator.

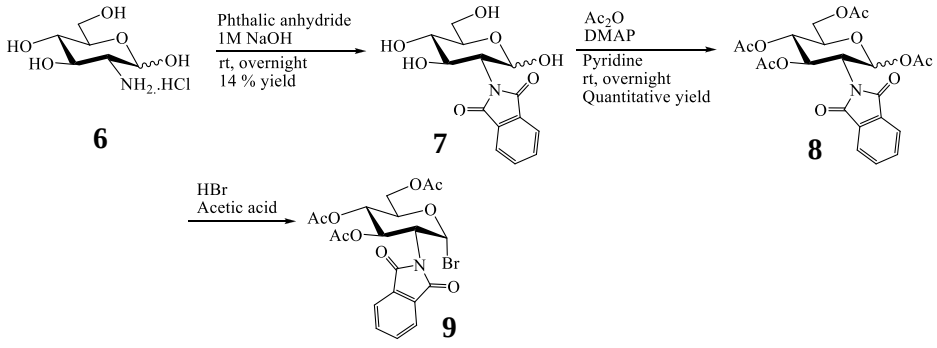
Our method for C-glycosidic bond formation was developed from the work of the Ryu group in Japan that used a low-pressure Mercury lamp to form the radical[5]. They formed a C-C bond from the reaction of an alkene and a bromo ester and ketone in a radical fashion with moderate to high yields without the use of the chemical initiator. This project plans to apply these conditions for the use of the formation of the C glycosidic bond to remove the use of the toxic tin complex and increase the stereoselectivity and yield.



Scheme 1:Overall reaction of C-glycosylation method

IIB. Experimental section

The glycosyl halide which is used for the reaction, scheme 1, was synthesized in three steps as detailed in scheme 2. The first step is to add the protecting group onto the nitrogen, the second step is to add the protecting group onto the OH groups and the third step is to replace the OH at the anomeric C with the halide Br. The use of protecting groups is to control which carbon the bromide adds to. I have worked on the synthesis of the glycosyl bromide **9** by using glucosamine **6** as the starting material. It has presented us with many challenges such as low yield and difficulties in the separation. Unfortunately, the complete synthesis of the glycosyl bromide was not able to be run to completion due to time constraints and malfunctioning equipment.



Scheme 2: Synthesis of the glycosyl bromide

The synthesis of compound **7** was done in several attempts and the most yield was achieved in the third attempt. To set up the reaction, 10.775g of the β-D-glucosamine was dissolved in 70mL of 1M NaOH in a reaction flask, then 8.294g of phthalic anhydride was added. The reaction was stirred at room temperature for several days. Thin layer chromatography (TLC) was used to check the formation of the product and the completion of the reaction. The reaction solvent was extracted using ethyl acetate and water. Then the organic layer was extracted with saturated brine solution followed by drying with Na_2SO_4. 2.240g of product **7** was collected.

The next step would have been the addition of acetyl groups onto the OH groups on the sugar. However, the rotovap - vacuum pump was not effective in removing the pyridine solvent, the next step was put on hold to wait for a stronger vacuum pump.

IIC. Future work

The completion of the synthesis in scheme 2. After synthesizing the glycosyl halide **9** optimizations of the overall method would take place including testing on the effects of different solvents, different protecting groups, different temperatures, and different ratios of the reactants. The optimizations would allow us to determine the best conditions for our method with the best overall yield of the desired. Then the scope of our method would be expanded by utilizing different types of glycosyl halides, structures **10**-**15**, and alkene/alkynes, structures **16-24**. By doing this the reaction can be expanded to include many different types of starting materials and become a more universal method that would not be limited to a single type of reaction.

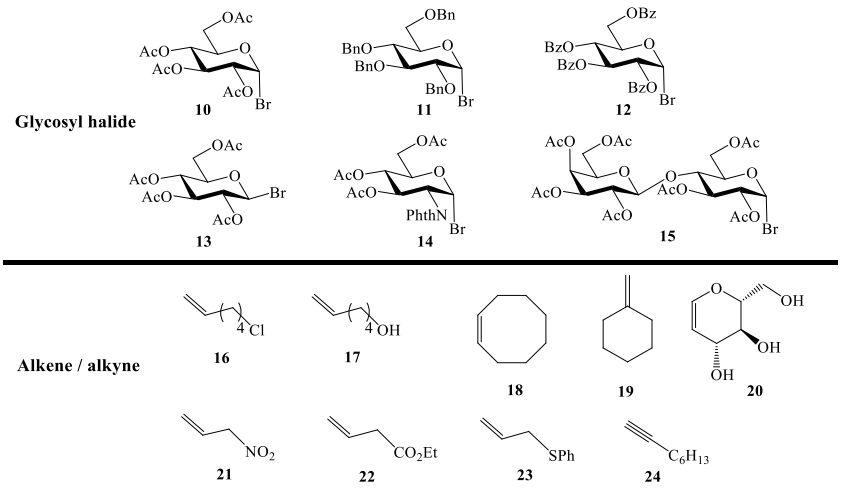


Figure 3: Different glycosyl halide and alkene/alkyne for expansion of the method

The mechanism of the reaction, figure 4, would be studied so that everything that happens in the reaction can be explored and investigated. In the proposed mechanism the glycosyl bromide will be radicalized by the photoirradiation by the low-pressure mercury lamp to form structures **28** and **29**. Structure **28** will then proceed to react with structure **26** to form structure **30**. Simultaneously the bromide, structure **29**, will also react with structure **26** to form structures **32** and **31**. Structure **32** will then loose a hydrogen to structure **30** in order to form structure **27**. Structure **27** is the desired product of the C-glycosidic bond. Structure **31** will react with itself to form byproduct structure **33**. Understanding the mechanism whould help us to understand more about our method of C-glycosylation method and allow us to make necessary modifications to achieve higher yields.

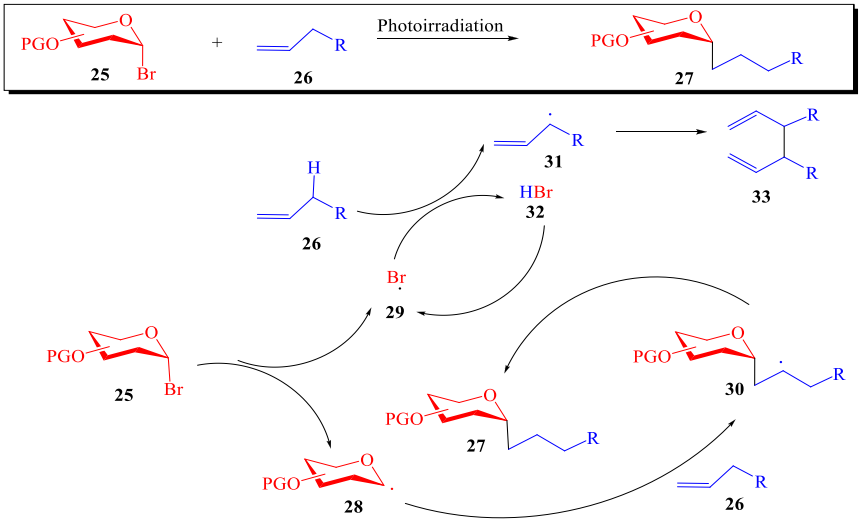


Figure 4: proposed mechanism for our method of c-glycosylation

III. Synthesis of rhodamine and biotin probes for the detection of cysteine containing proteins.

IIIA. Background

Cysteine is one of the many amino acids used in the creation of proteins. Cysteine is also one of the most reactive of the amino acids due to its nucleophilic tendencies due to the sulfur atom. The cysteine plays important roles for maintaining redox homeostasis protein structure and regulating cell signaling[6, 7]. Cysteine residues can undergo a variety of oxidative modifications. These posttranslational modifications can be reversible or irreversible and can cause changes in the surrounding environment which could potentially lead to deregulation of protein functions. Figure 5 shows examples of the different posttranslational modifications that can occur in cysteine. Changes in the concentration of the cysteine containing proteins

can have effects on biological processes. Elevations in the concentration of cysteine can contribute to diseases such as Alzheimer's, cardiovascular diseases, and osteoporosis just to name a few[8]. Deficiencies in the concentration of cysteine can cause hair depigmentation, lethargy, liver damage, and muscle and fat loss[9-10]. Due to the importance of the cysteine containing proteins on the body there is a desire for probes that identify the proteins containing cysteine and to quantify them. These probes can be used in a variety of ways such as diagnostics for early disease detection as well as understanding the functions of these cysteine containing proteins.

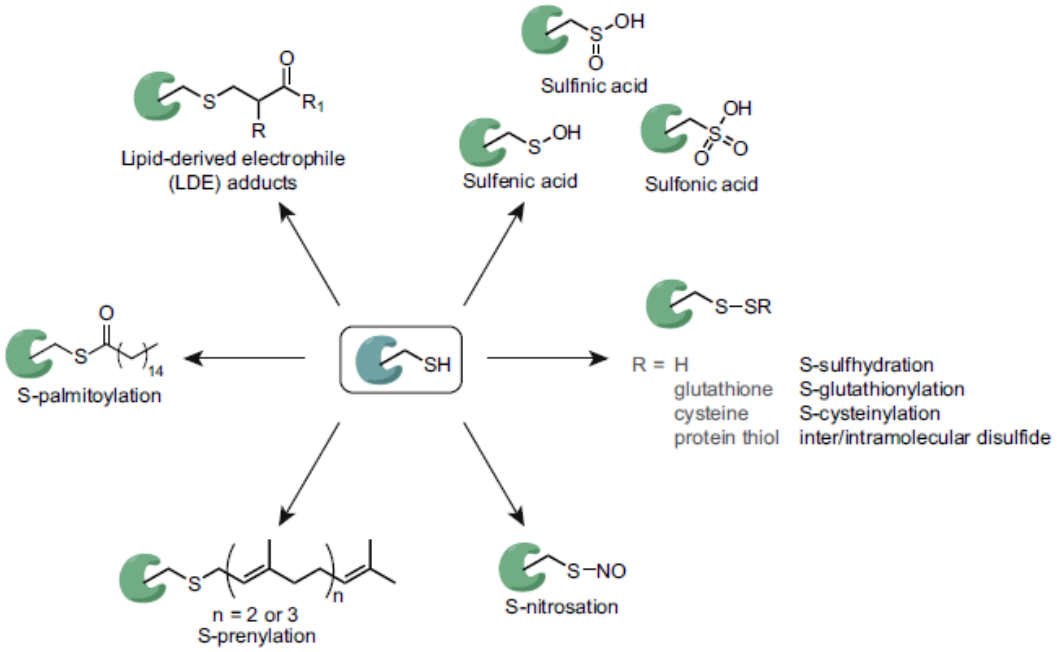


Figure 5: Different modifications of the amino acid cysteine

There are currently a variety of probes for cysteine containing proteins already available[11] as in Figure 6. There are probes that use fluorescence and ones that focused on analytical methods. These probes use a variety of different mechanisms such as Michael addition reaction[12], cyclization[13], substitution reactions[14], metal ion interactions[15], as well as cleavage of disulfide bonds[16]. Fluorescence can be provided from the attack of the thiol on the dye used or from the cleavage of the fluorescent moiety. Disadvantages of some of the current probes include the use of metal ions which would contain little spectra color changes[17] as well as the potential toxicity of the metal ions[18-20]. These medals may also potentially react with negatively charged residues which would decrease the sensitivity of the probe[21]. Some of the probes out there have poor detectability in Polyacrylamide gels[22], they could yield undesired background fluorescence[21], or could even be hydrolyzed[23].

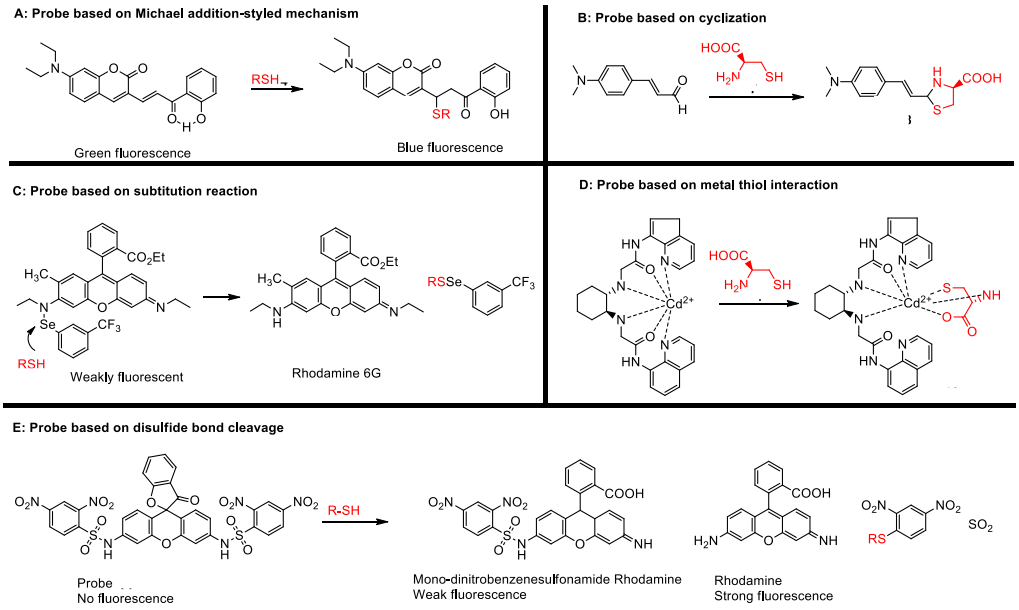
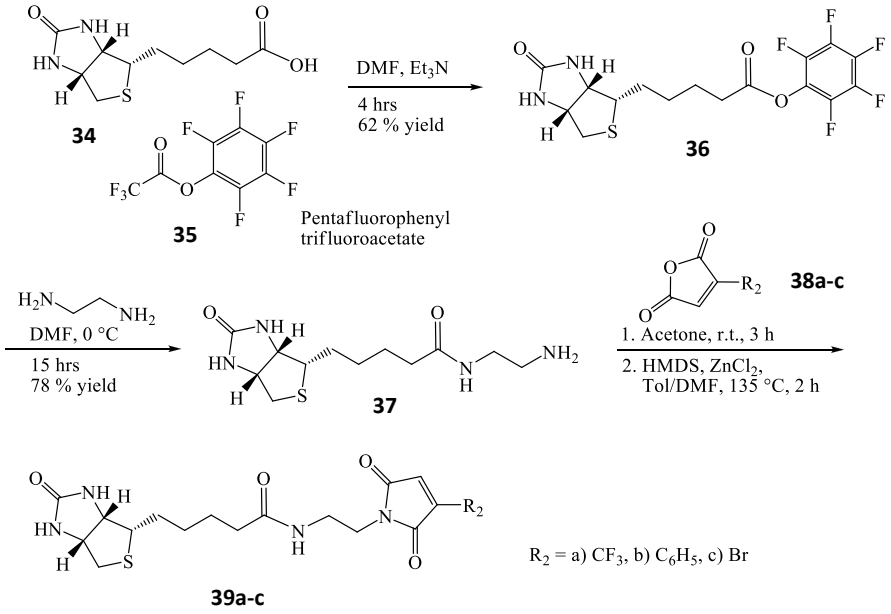


Figure 6: Different probes currently available

IIIB. Experimental section

The overall outcome of this project is to synthesize different maleimide probes, with the difference in the two probes being the fluorescent moiety, that are selective for the detection of cysteine. Benefits of producing a maleimide probe is that it can identify intact proteins without any upfront processing it can isolate cysteine containing proteins from complex mixtures as well as it can be used qualitatively and quantitatively for analyzing cysteine containing proteins. Rhodamine was used as one of the fluorescent moieties due to it being able to be detected within a wide range of emission and it can be observed directly. Biotin is used in the probes due to its rapid crossing of the plasma membrane and can be used to detect quantify purify and identify proteins throughout the cell. The biotin can be detected using Western blotting.

The synthesis of the biotin probe, derivatives **39a-c**, which is shown in scheme 3. The first reaction is the installation of the leaving group pentaflyorophenyl into the biotin-carboxylic acid to form product **36**. Addition of ethyl diamine to **36** leads to the formation of compound **37**. Lastly the maleimide **38a-c** is installed to form the Biotin probes **39a-c**. The different R2 groups on the maleimide would lead to different analogues of the probes



Scheme 3: Synthesis of Biotin probe

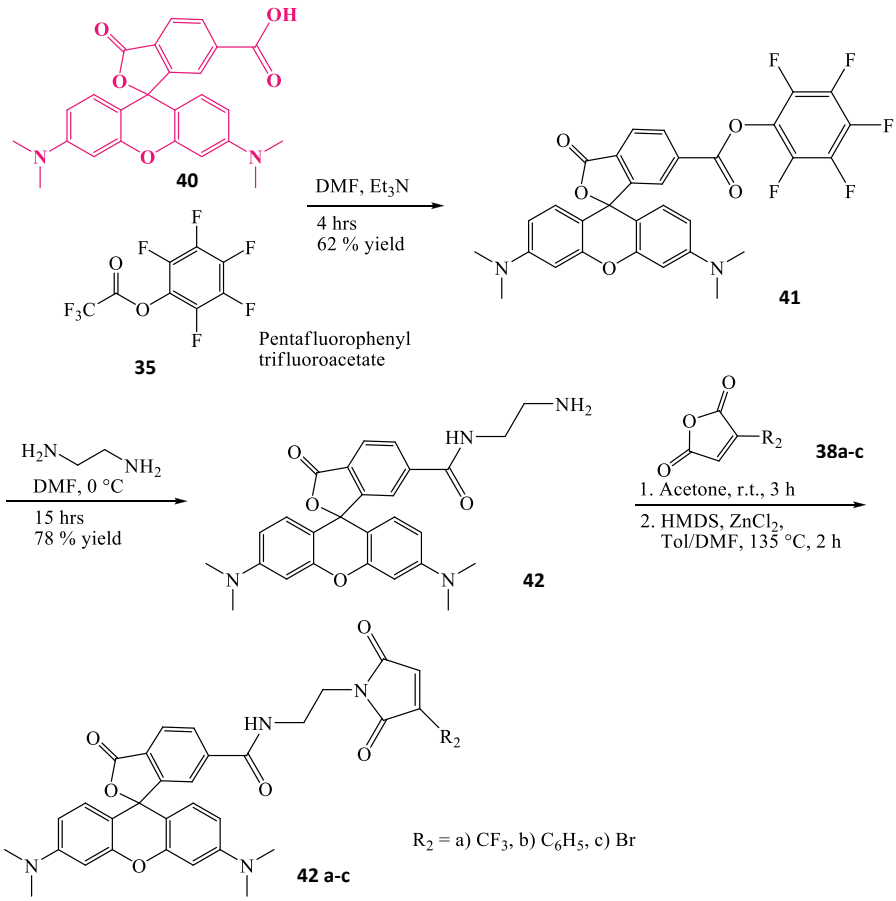
The synthesis of the biotin probe was done in a few attempts with the last attempt getting the farthest along. To start this synthesis 0.5g of biotin was added to a flame dried reaction flask and dissolved in 5.0mL of DMF. 0.55mL of triethylamine and 0.014mL of pentaflurophenyl was also added to the reaction flask. The reaction was stirred at room temperature overnight. TLC was used to check the formation of the product and the completion of the reaction. The reaction solvent was extracted using ethyl acetate. The organic layer was then extracted using saturated sodium bicarbonate. Product **36** was then purified using column chromatography.

To synthesize compound **37**, 5mL of DMF was added to the flask containing product **36** followed by 0.1417mL of ethyl diamine. The reaction was stirred overnight in an ice bath. The organic layer was extracted using ethyl acetate and saturated NaHCO3 solution as the aqueous layer. The organic layer was then transferred to a pre-weighed round bottomed flask. All of the solvent was removed under vacuum to yield product 37.

Compound **37** was then carried to the next step. 5mL of chloroform was added to the flask containing **37** followed by 0.360g of phenylmaleic anhydride. The reaction was stirred at room temperature overnight. All solvents were evaporated off and then weighed. The product formed by this reaction was then suspended in diethyl ether and then vacuum filtered. The solid was then dissolved in 10mL of a toluene DMF mixture (9:1) and 0.268g of zinc chloride. Once dissolved 1.37mL of a 50/50 mixture of toluene and bis(trimethylsilyl)amine

over 20 minutes. The contents were then heated to reflux for 3 hours at 160°C. During this time the solution turned blue. After three hours, the reaction was stopped, and all solvents were completely evaporated off to finish forming the final products **39a-c**. No analysis was able to be completed on the product due to the pandemic not allowing anyone else back on campus after fall break. Analysis was not ran after returning for spring semester due to the potential of degradation of the product.

I have also worked on the synthesis of the Rhodamine probes. Scheme 4 provides the method for the synthesis of the rhodamine probe. It was challenging to work with the rhodamine compounds because it was light sensitive. While working with these compounds, all lights need to be turned off or avoided as much as possible.



Scheme 4: Synthesis of the rhodamine probe

This reaction was set up by flame drying a round bottomed flask and the air was replaced with nitrogen gas. Once that was complete, 5g of commercially available Rhodamine B, structure **40**, was added to the reaction flask followed by 5mL of DMF, 2.8mL of triethylamine, and 0.1mL of pentafluorophenyl trifluoroacetate (structure **35**). The reaction flask was wrapped in aluminum foil since the rhodamine is light sensitive. The reaction was stirred at room temperature overnight. The organic layer was extracted using ethyl acetate, water, and saturated $NaHCO_3$ solution for the aqueous layer. The organic layer was then dried with anhydrousNa_2SO_4 and then transferred to a round bottomed flask. The next steps will be continued by other students in the future.

IIIC. Future work

The next step in this project would be to test the products formed (structures 39 a-c and 42 a-c) to confirm product formation. After that, the probes would be tested to see if they are selective for cysteine over other aminos acids. This will be done by adding the probes to a pool of amino acids and seeing if the probes are only binding to the cysteine. Next the probe will be tested by adding it to a pool of proteins, some of them containing cysteine and others not. This is done to ensure that the probe will be able to distinguish between the proteins containing cysteine and those that do not. This is demonstrated by figure 7. The probe will need to be tested in cell cultures to assess toxicity and to see if it will even cross the cell membrane. The probes being designed here are relatively large molecules and may not be able to pass through the cell membrane.

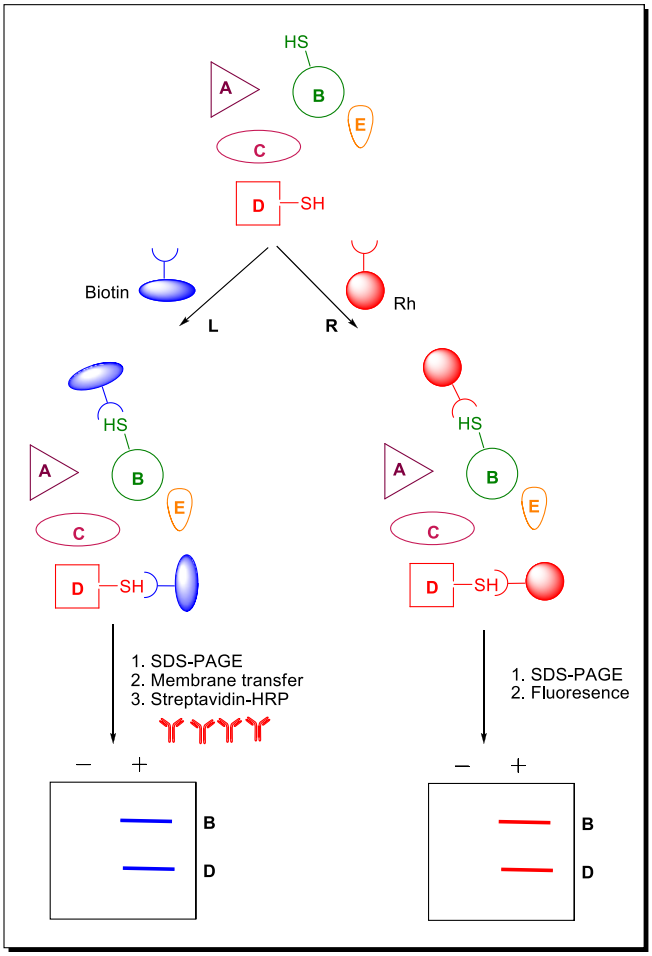
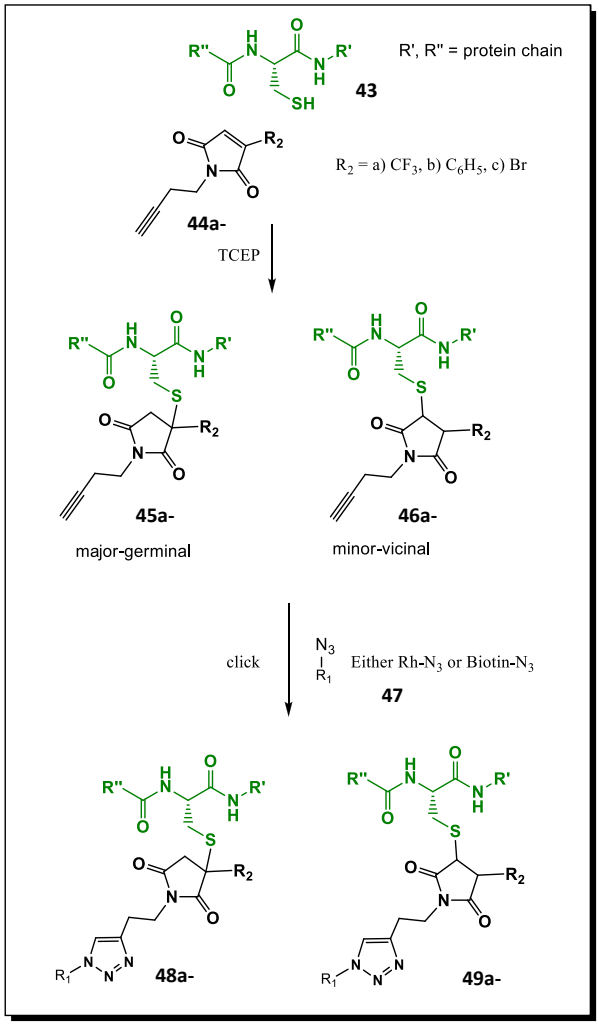


Figure 7: Testing the selectivity of the probes for cysteine

If the probe is not able to pass through the cell membrane, there is a backup plan that would then need to be explored. The backup plan still ends up forming similar probes to the initial methods, however this probe is formed using click chemistry where the fluorescent moiety clicks on to the maleimide portion after it has already bound to the cysteine. The synthesis of this probe is detailed in scheme 5. In this scheme the pieces of the probe are added individually so that the smaller pieces of the overall probe will have an easier time passing through the cell membrane. This would overcome the issue of the probe being too large to pass through the membrane. Figure 8 shows how the probe will work in its selectivity for cysteine as well how the two parts come together to form the probe.



Scheme 5: Synthesis of the Probe using click chemistry

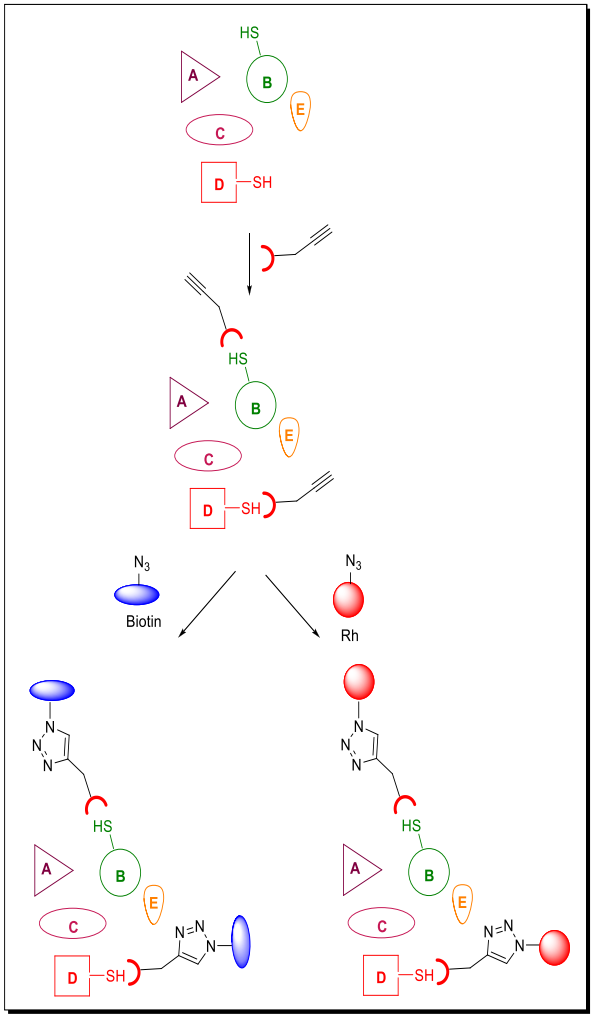


Figure 8: Testing the click chemistry probe for selectivity

IV. Literature review: Fluorescent probes for cysteine containing proteins

The topic of this review is fluorescent probes for cysteine containing proteins that have been developed over the last 10 years. There are different kinds of probes containing fluorescent moieties including chemical, metal ion, and quantum dot probes. The chemical probe is a compound that goes into the cell and binds to the cysteine, where then either the fluorescence is quenched or released depending on the mechanism. The most common form of the fluorescent probe is the chemical probe, with each probe operating under a different mechanism. For chemical probes the most popular mechanism is a Michaels addition reaction mechanism. Figure 6A details the reaction of the Michael addition reaction[24]. Where the fluorescence is triggered by the release of the fluorescent portion upon the binding to the thiol as seen in figure 9. When the fluorescence is released upon the binding of the thiol it can either be because the attachment of the thiol caused the probe to have fluorescence, as in figure 9, or it can be because the reaction with the thiol releases a molecule from the probe that then carried the fluorescence as in figure 10[25].

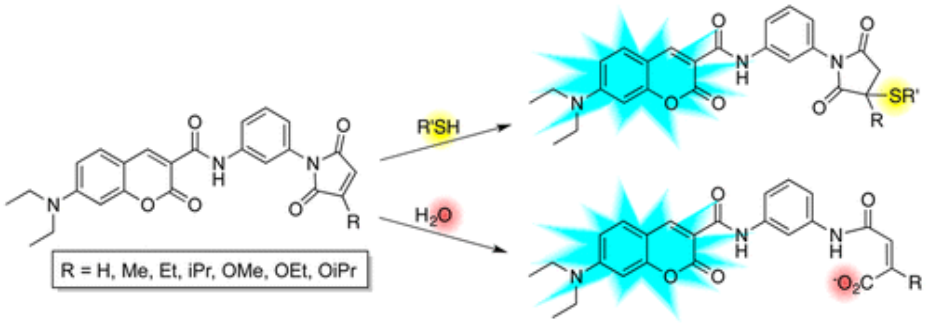


Figure 9: An example of a chemical probe where the fluorescence is turned on by the addition of the thiol

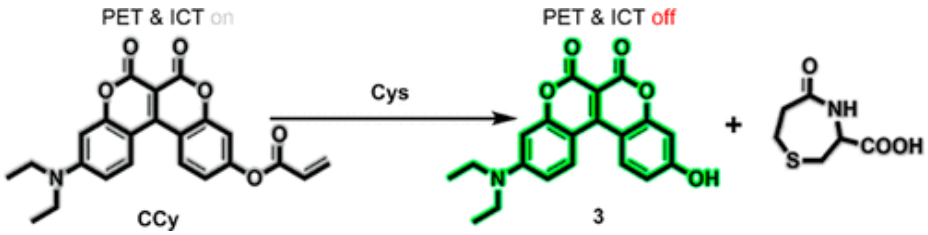


Figure 10: A coumarin based fluorescent probe where fluorescence is released upon the release of the fluorescent moiety

Probes containing the metal ions also work by emitting fluorescence. An example of a probe containing a metal ion is a guanine rich oligonucleotide probe that's conformation had been adjusted by the interactions between the guanine and a silver ion[26]. The oligonucleotide with the silver atom is the probe for cysteine. Since the silver ion changes the conformation

of the probe the florescence of the oligonucleotide shifts. However, once the probe comes into contact with cysteine, the structure of the probe returns to its unfolded structure thus causing the fluorescence to shift back to what is was before the silver ion was bound[26]. Figure 11 shows how this probe reacts first with the silver ion then in the presence of cysteine the reaction is reversed and releases the silver ion[26].

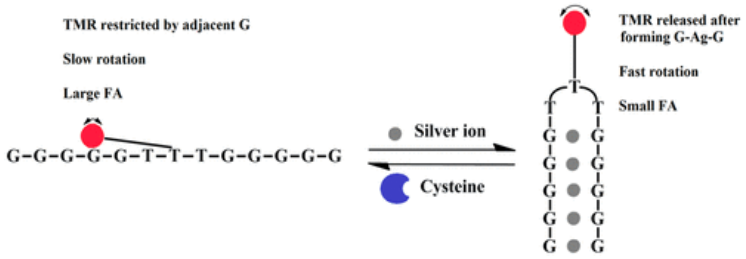


Figure 11: Oligonucleotide probe

Another type of probe is the quantum dot probes as in figure 12. This probe uses amino nitrogen quantum dots that are quenched by gold nano particles[27]. The fluorescence is recovered upon the binding of the cysteine to the gold nanoparticles.

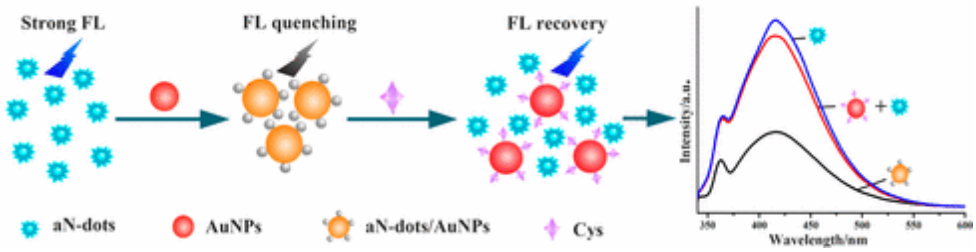


Figure 12: Probe using amino nitrogen quantum dots

Downsides of some of these probes is that they cannot distinguish between cysteine, homocysteine, and glutathione since these compounds are similar in structure. The review paper is still in the works, with future work on it being finishing the writing and editing for future publication which I shall continue working on after graduation.

V. Conclusions

Working on different projects has helped me expand my knowledge, techniques, and skill sets in biochemistry and organic chemistry. It has also allowed me to apply the knowledge I had gained in the classroom in a research setting. Besides doing bench work, I learned more about lab management, lab inventory and chemical ordering to begin a project. The literary review has allowed me to expand my knowledge of probes and the differing mechanisms that they may operate under. All of the things I have learned from working in

this research lab has provided invaluable experience for me and sparked an interest in doing more research.

VI. Acknowledgement

I would like to thank Dr. Son Nguyen, the faculty and staff of the Hollins University Chemistry Department, Hollins University, as well as the New Faculty Research Fund and the Faculty Development and Student Research Fund for supporting us and our projects.

VII. References

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