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Attempt to Synthesize the (S) Enantiomer of a Glutathione Analog to be an Inhibitor of Glutathione Reductase

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Attempt to Synthesize the (S) Enantiomer of a Glutathione Analog to be an Inhibitor of Glutathione Reductase

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Abstract

Malaria is a disease of the blood that is caused by a parasite and affects over forty percent of the world population today. This small parasite can be treated in a very short period of time; however, there are many countries that do not have access to advanced medicine. This means that the people infected with this disease will usually die from complications arising from the illness. Malaria is prevalent in tropical and subtropical countries close to the equator where more than half of the world's population resides. Dominance of malaria in these areas is because of the low economic stability and the tropical areas have the perfect weather conditions for mosquitoes to blossom.

The unnatural peptide made from the pathways described in this paper is common in creating novel peptides. Manipulation of the unnatural peptide glutathione into the (*S*) configuration should inhibit glutathione reductase. The (*R*) configuration of glutathione is the natural state; therefore the (*S*) conformation creates an unnatural peptide. This enzyme inhibitor will allow for the cells to remain in increased oxidative stress state and kill the malaria parasite.

Acknowledgments

I would like to thank the University of Southern Mississippi's College of Science and Technology for providing me with the opportunity to complete an undergraduate thesis. Dr. Masterson and his graduate students have been instrumental in leading me down the correct path towards completing my thesis. Without them, I would not have been able to conduct this research. Lastly, I would like to thank my family and friends who have assisted me in every possible way throughout my time at The University of

Southern Mississippi. They have encouraged me to follow my dreams and have always been my biggest supporters.

Table of Contents

Table of Schemes

Introduction

Malaria is a parasitic disease that infects over forty percent of the world's population. This disease, caused by the parasite *Plasmodia* is passed from human to human by the bite of a female mosquito. After the initial infection, the parasite enters the blood stream and travels to the liver.[1] After the parasite enters the liver, it changes form and enters back into the blood stream where it infects red blood cells. The term Malaria comes from the Italian word meaning bad air. This disease was previously known as the marsh fever because it was so closely associated with swamps and marshlands. In the late 1800's, a French military hospital observed that there were parasites inside the red blood cells of the infected soldiers.[1] This was the first time a protist was identified as a disease causing agent. A protist is a diverse group of microorganisms in the kingdom Protista. After the disease causing agent was identified, there was work performed that could be used to identify the life cycle so that different preventative measures can be taken. The life cycle of Malaria can be identified as two different stages called a sporozoite and a merozoite. The initial motile infective form of the parasite is called the sporozoite. The sporozoite travels through the blood stream to the liver where it produces many merozoites. These can infect new red blood cells and the infective stage of the life cycle is now activated. Only female mosquitoes feed on blood; male mosquitoes feed on plant nectar, and thus do not transmit the disease.

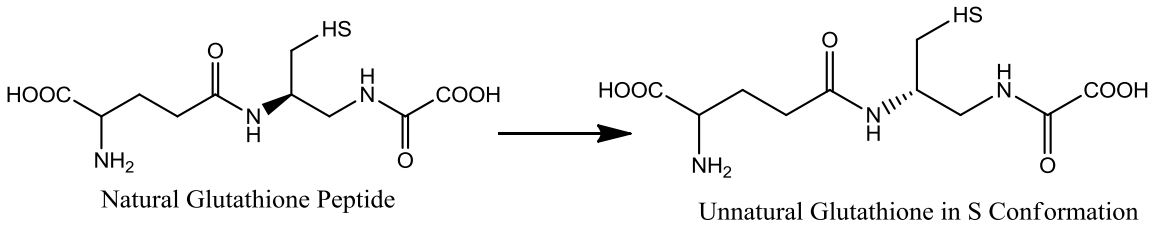
Once the malaria parasite enters and infects the host, many complications can arise from the disease. Malaria can cause many symptoms that can persist for many days and some can last several weeks. The illness can be stopped if treated within 48 hours of the onset of symptoms; however, most of the cases that are reported around the world are

in poverty stricken places where medicine is hard to acquire. Some of the symptoms include fever, nausea, vomiting, shakes, and kidney failure.[1] Diagnosis of malaria in the beginning stages of the disease is very important. This is so that treatment can be administered early to prevent those later stages of the illness that sometimes can cause irreversible damage to the host. Malaria is usually diagnosed by a microscopic examination of blood. However, in regions that cannot afford or do not have access to laboratory diagnostic testing, it has become routine to use only a history of subjective fever as the indication to treat for malaria.

A cheap and effective anti-malarial drug that could be distributed to third world countries would mean saving the lives of many people each year. This could be possible by manipulation of the molecule Glutathione. Glutathione is a tri peptide that is created in the body from three amino acids; glutamate, cysteine, glycine. This molecule is a strong antioxidant, which means that it inhibits oxidation and removes oxidizing agents in the cell. It exists in two different states within the cell, the oxidized state and the reduced state. The reduced state of Glutathione is highly reactive due to the fact that it can lose an electron readily and react with itself to form the oxidized state. Still, the only way Glutathione can cycle from one state to the other is by the use of the enzyme Glutathione Reductase.[2] This enzyme also boosts liver functions in humans, helps the body produce natural killer cells, prevents the buildup of oxidized fats, and the lack of it will cause the cells to increase their oxidative stress. This oxidized state of cells is unfavorable to the malarial parasite and therefore in theory could kill it.[2] However, keeping cells in this state is the problem. There are inhibitors of the enzyme Glutathione Reductase that allow for this to occur. The natural configuration of cysteine in

Glutathione is in the *(R)* conformation. Creating an analog of Glutathione in which the cysteine is in the *(S)* configuration will act as an inhibitor of Glutathione Reductase. This inhibitor will allow for the cells to remain in an increased oxidative stress state and kill the malaria parasite. Figure 1 shows the natural state of the Glutathione molecule transformed into the unnatural peptide that is attempted to be made in this project.

Figure 1: Comparison of Glutathione and Analog being Synthesized



Natural Glutathione Peptide

Unnatural Glutathione in S Conformation

Literature Review

Glutathione is a molecule in the body that has been extensively researched.[2] Glutathione Reductase is an enzyme that maintains a proper ratio of GSH/GSSG in the human body. GSH is another name for Glutathione and GSSG is the oxidized form, Glutathione disulfide.[3] It enables the cell to get rid of free radicals and reactive oxygen species and is a target enzyme for anti malarial and anticancer drugs.

There have been many analogs of Glutathione prepared in hopes of finding cures for diseases or making advancements toward more powerful medications. An analog is a creation of something new from something that is already known. In a study performed in 2002, a Glutathione analog was formed by cyclization of the original molecule. This meant adding disulfide bridges to the linear molecule. According to "Synthesis and Biological evaluation of the disulfide form of the Glutathione Analog λ-(L-glutamyl)-Lcysteinyl-L-aspartyl-L-cysteine," adding disulfide bridges to a molecule, "stabilizes specific backbone conformations and limits the rotational space of the side chains." This modification in the original structure of Glutathione introduced new biological activity to the molecule.[2] This activity was studied to determine if creating the disulfide bridges would allow the molecule to function as a Glutathione reductase inhibitor. An inhibitor is what the scientists wanted to create because it would allow for the further advancement towards better drugs to fight against different pathogens. In order to test the effectiveness of the biological activity of the cyclic Glutathione for inhibition qualities, the enzyme is added to two different batches of the substrate. The activity of the enzyme in the presence of the Glutathione analog is tested. There was little inhibition activity taking place due to the fact of the restricted conformational freedom of the carbons in the ring structure. This

experiment proved that the synthesis of different analogs do provide different biological activity of the molecule and could potentially serve as significant pathogenic treatments against microorganisms. In this project, an analog of Glutathione will be made for the purpose of changing its biological activity to inhibit Glutathione Reductase.

Analogs of Glutathione not only aid in pathogenic microorganism treatments, but cancer treatments as well. In a paper called, "Glutathione and Mercapturic Acid Conjugates of Sulofenur and their Activity Against a Human Colon Cancer Cell Line," two analogs were created to serve as possible leads in anticancer agents.[3] These two analogs are made from Mercapturic Acid and Glutathione. Proteins bind to these conjugates in the presence of another cancer treatment agent, Sulofenur. It is a broad spectrum medication that is still in the trail stage. It is treating tumor cells; however, it is also causing anemia in the blood stream. In this study, Xiangming Guan found that both the glutathione conjugate and the mercapturic acid conjugate were present in the urine once the Sulofenur was introduced. They also discovered that these two conjugates "exhibited cell growth-inhibitory effects against the colon cancer cell line with potency compared to Sulofenur." However, even though this phenomenon is occurring in the cells, it is unknown whether or not the inhibitor of Glutathione Reductase is what is causing the cancer cells to decline in their growth. More studies will need to be performed to conclude that these conjugates do possess some anticancer activities and their development along with other analogs could lead to a novel class of anticancer medications. The analog in this project could be used in drugs to act as a Glutathione inhibitor and to cure other diseases as well.

One of the main diseases that have been studied in conjunction with glutathione analogs and glutathione reductase is Malaria. If a cheap and easily made medication against malaria can be created, it could save many lives each year. An article written in 2009, entitled "In vitro Inhibition of Human Erythrocyte Glutathione Reductase by Some New Organic Nitrates," tells of twelve organic nitrate derivatives that were created and treated against human erythrocyte Glutathione reductase.[4] These molecules were shown to be strong glutathione reductase inhibitors and could possibly be used in anti malarial drugs. Another article written by R. Heiner Schirmer discusses the uses of Methylene Blue in the formation of anti malarial drugs. According to this article, Methylene Blue has been used in anti malarial agents since the late 1800's.[5] The reason this molecule is used is because it is used in the treatment of methemoglobinemia. This is a blood disorder in which hemoglobin is not able to release oxygen to the tissues effectively and is a complication resulting from malaria. The Methylene Blue interaction with the methemoglobin is a biochemical process. Schirmer found that through his and his partner's experiments that the elements of Methylene Blue should be high in concentration in a drug so that the Methylene Blue can act as a Glutathione reductase inhibitor.[5]

The reason that finding an inhibitor of glutathione reductase is a key point in anti malarial drugs is because the parasite is hypothesized to be more susceptible to oxidative stress than their hosts.[6] In a study performed by Ya Zhang, Ernst Hempleman and R. Heiner Schirmer, BCNU and HeCNU molecules were formulated to figure out if they could be used as possible anti malarial drugs. After their experiment, they determined that if the enzyme can be inhibited, the red blood cells that have been infected with the

parasite are exposed to oxidative stress.[5] The molecules they made inactivated Glutathione reductase and are being further analyzed by X-Ray crystallography to determine if they can be used in anti malarial drugs.

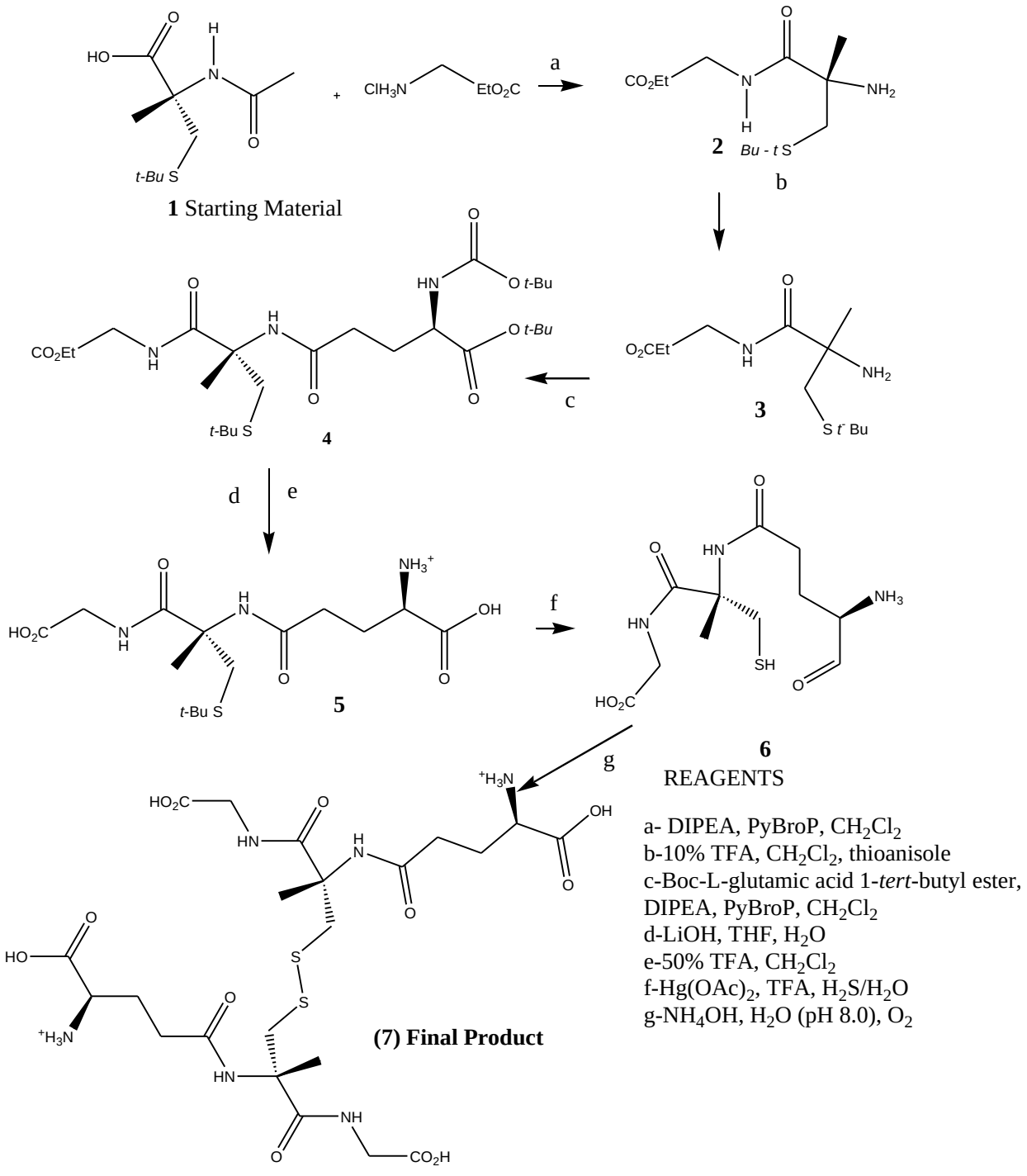
Hypothesis

There have been many molecules synthesized to act as Glutathione reductase inhibitors. The hypothesis for my project is to create an analog of Glutathione in which the cysteine residue is in the *(S)* configuration instead of the *(R)* configuration. This is hypothesized to create an inhibitor of the enzyme because the natural state of Glutathione is in the *(R)* configuration. This could be an inhibitor because changing any part of the Glutathione backbone changes its biological activity. The other analogs that have been tried have seemed to have some type of inhibition qualities; therefore, the *(S)* enantiomer could be the key in discovering a more effective anti malarial treatment.[6]

This research is important because Malaria is a major pathogen that affects over 40 percent of the world's population. More than two million people die every year of this disease. Finding a cheap, cure or treatment for this pathogen could save many lives every year. [7]

Proposed Project

There have been many unnatural amino acids synthesized and many have been found to have interesting applications in the medical community. For this project, the unnatural peptide Glutathione in the *(S)* enantiomeric form will be synthesized. In nature, this peptide is found in the *(R)* form. In the lab, many different Organic Chemistry techniques will be utilized to synthesize this molecule. One of the techniques is solution phase synthesis. This forms peptides through the use of selecting specific protecting groups that are easily removed and added to the growing polypeptide. To start out, a molecule in the *(R)* configuration is given. This molecule is used to prepare Glutathione analogs and to illustrate its versatility in the solution phase synthesis. The scheme that will be followed in this synthesis is taken from a paper entitled, "An Improved Method for the Preparation of Protected *(R)*-2-Methylcysteine: Solution-Phase Synthesis of Glutathione Analog," written by Douglas S. Masterson, Brant L. Kedrowski, and Amanda Blair. This scheme has been modified a little to fit my particular project.[8] The scheme below is the original scheme that was used previously to create the same intermediates. However, once the synthesis reactions were performed in this particular project, revisions to this scheme needed to be made to accommodate different obstacles.



Scheme 1: Project Summary (Initial Scheme)

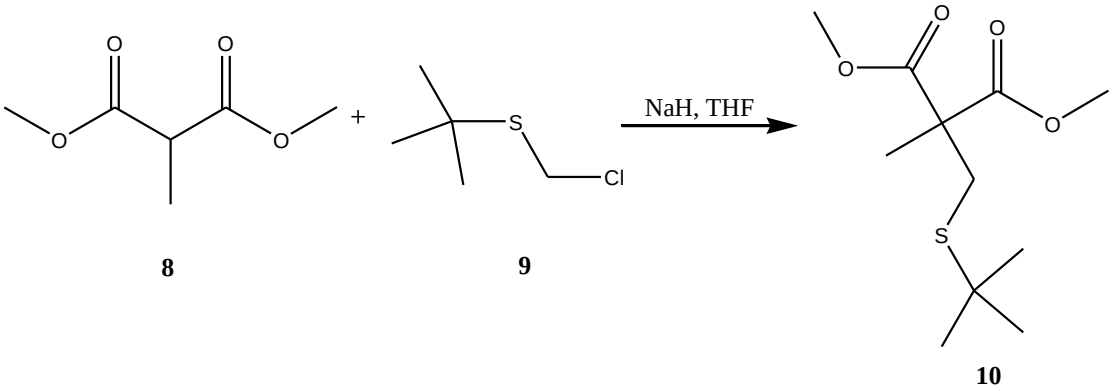
The molecule that will be provided as the starting material in the lab is **1** in the above scheme. The first thing done with this molecule is to add bromotripyrrolidinophosphonium hexafluorophosphate (PyBroP) as the coupling agent to create molecule **2**. In molecule **2** there is a protecting group on the amino side and it is "removed by 10% trifluoroacetic acid (TFA) and thianisole as a cation scavenger."[8] This forms molecule **3** in good yield and is coupled with Boc-L-Glutamic acid 1- *tert* butyl ester forming molecule **4**. The ester and the other protecting groups need to be removed. These will be removed by standard removal procedures forming the molecule **5** seen in the scheme. One last step in creating the reduced glutathione analog wanted is to remove the *tert*-butyl protecting group by using mercuric acetate. The peptide that is left is the molecule **6** seen in the above scheme. The last thing that needs to be done is to pass the peptide through a basic solution with oxygen. This will form the disulfide bond seen in molecule **7**. This molecule is in the *(S)* configuration and is the molecule that my entire project centers around. The different reagents that are used in the sequential steps are shown in the box below the scheme above. They are recognized in the scheme by the letters a-g. The first molecule in this scheme showed that it was a suitable reagent for the creation of the Glutathione analog that is needed for the rest of my project. Once this molecule is created, it will be tested on Glutathione to see if it acts as an inhibitor of Glutathione reductase using a Glutathione reductase assay kit that will be provided in the lab. The instructions for the assay are given in the assay kit. The assay kit will show a graph of what the glutathione reductase activity is before molecule **7** is added into the mixture. The slope of that line will be found. After this is done, molecule **7** will be added and the slope of the line for the activity of Glutathione reductase should decrease,

causing the slope to decrease. A decrease means that the molecule created did the job we are hypothesizing. No decrease in slope or an increase in slope would disprove the hypothesis previously made.

Results (Revised Schemes)

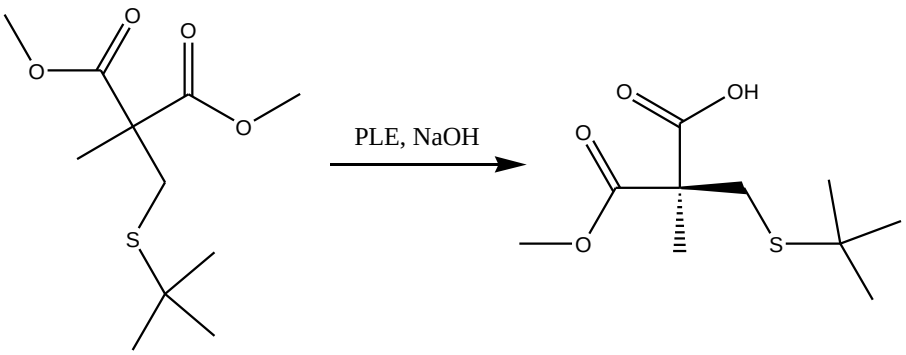
Scheme 1 was the original plan for this project; however, after deliberating and discussing options, changes to the original needed to be made. Due to having to repeat different steps and taking into consideration the cost of different reagents, changes needed to be made. Schemes 2-6 show the revised reagents and intermediates that were made in this project.

Scheme 2: Synthesis of Dimethyl 2(butylsulfamethyl)-2-methylmalonate



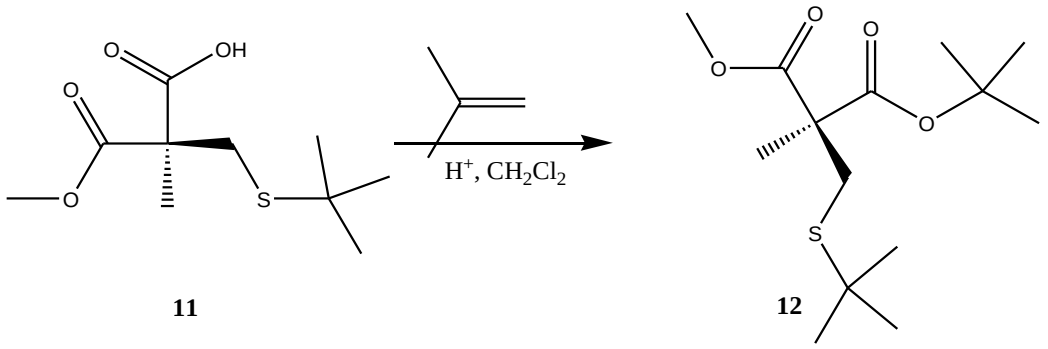
8 **9** **10**

Scheme 3: Synthesis of *(S)*-2-*tert*Butylsulfanylmethyl-2-methylmalonic Acid Monomethyl Ester



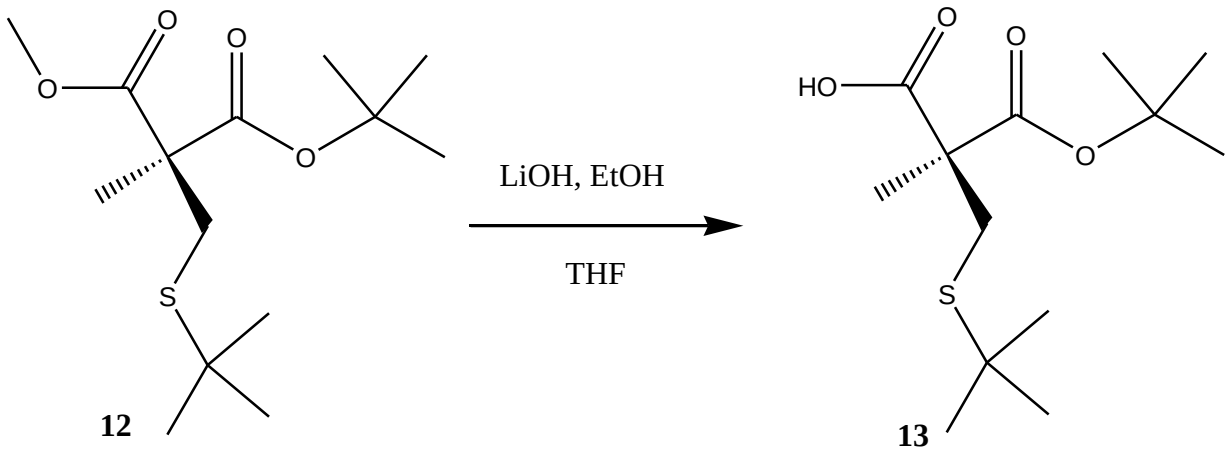
10 **11**

Scheme 4: Synthesis of *(S)*-4-*tert*-Butyl-1-methyl-2-(benzyloxymethyl)-2-methyl-succinate



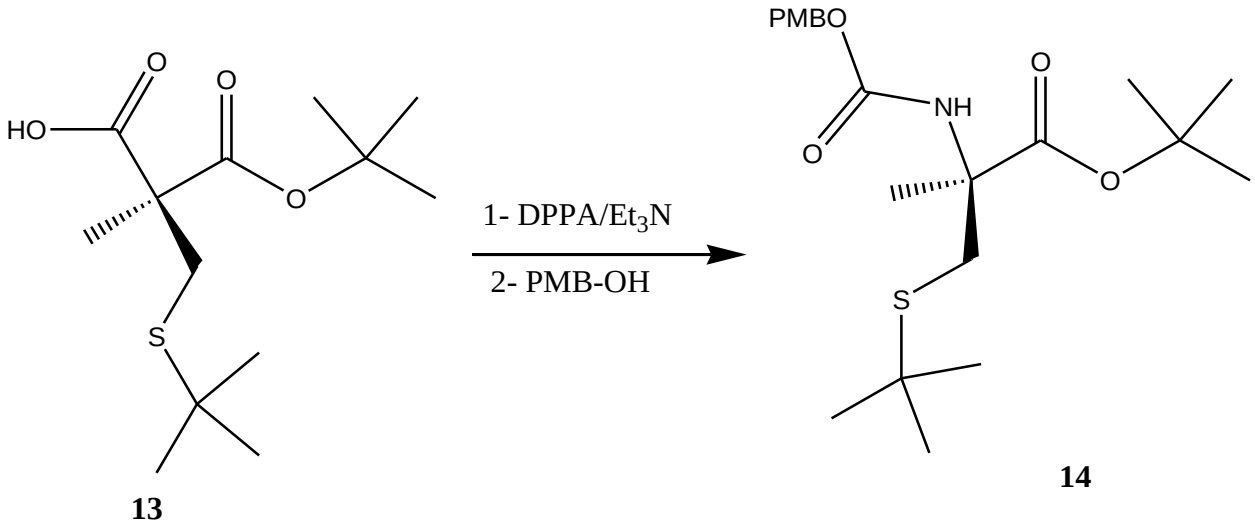
11 **12**

Scheme 5: Synthesis of *(S)*-2-*tert*-Butylsulfanmethyl-2-methylmalonic Acid Mono-*tert*-butyl Ester



12 **13**

Scheme 6: Synthesis of *(S)*-N-4-Methoxybenzyloxycarbonyl-S-*tert*-butyl-2-methylcysteine *tert*-butyl Ester



13 **14**

Discussion and Conclusion

The initial goal of this project was to create an unnatural peptide, *(S)*-d-methyl-Glutathione. However, as the project progressed, many corrections needed to be made to experimental procedures and with lab equipment. The final goal of this project became to correct the mistakes that led to improper synthesis of different intermediates along the pathway to creating *(S)*-α-methyl-Glutathione. We have demonstrated that it is possible to create different intermediates through the use of many different organic chemistry concepts such as, PLE hydrolysis and the Curtius rearrangement. We successfully synthesized the following intermediates on the pathway to forming *(S)*-Glutathione; diester, the acid ester, i-butyl Chloro Methylsulfide, mixed diester, and the half ester.

The first attempt at the complete synthesis came to a halt at the Curtius Rearrangement step, **14**, because the solvent became charred and the product looked as though it was slightly decomposed. This could have been due to there not being a good reflux of solvent occurring causing the solvent to evaporate and the product to char when left overnight. After this halt in the synthesis process, we had to go back and create more intermediates starting with the diester because the entire product previously made was consumed in the Curtius Rearrangement. The goal of the project then became to fix the problems that were occurring during the reactions that were needed to scale up the intermediates leading up the Curtius Rearrangement. This is so that once that step was reached again, more products would be available to use.

Once the scale up process began, each synthesis step was carefully monitored so that each step could be carefully perfected and problems could be fixed as they occurred.

The synthesis of the diester, **10** scheme 2, was completed with no problems. The next step was the PLE hydrolysis, scheme 3. Most of the diester that was created was made pure after chromatography or pure without further purification steps; however, the MPT titrino needed to undergo a few changes so that the reaction would give a higher percent yield and not contain as many impurities. The MPT titrino needed to be recalibrated and the entire machine needed to be cleaned to remove any organic residues. This process did not take long and allowed for pure diester in higher quantitative amounts to be synthesized.

The biggest problem that occurred during this experiment was the third synthesis step which was *tert* butylation step, scheme4. This was the step that we had the most trouble on and caused the project to be changed from a synthesis of a new compound to an attempt to synthesize a new compound. After many failed attempts to make the pure product of the *tert* butylation reaction, we realized there must be something wrong with the reagents because the technique in this synthesis reaction is not difficult to master. There was always an impurity that needed column chromatography to purify it. Sometimes even after the column was run, the *tert* butylation product was still not found. After many failed attempts, we finally discovered the contamination issue. This contamination issue was in the isobutylene tank. The probe that the isobutylene gas was coming out of before it condensed was plastic and the plastic was eroding and melting into the condensed mixture. This plastic tip needed to be replaced by a glass one. This new tip allowed for the unwanted plastic particles in the reaction to be eliminated. Another alteration to the protocol was to add an excess of isobutylene and to make sure the sulfuric acid was pure. Once these few things were changed, the pure product was

made many times in high yield. Changing out the tip on the isobutylene tank not only advanced my project, but fixed a contamination concern for the entire lab and all of the projects that use isobutylene will now run more smoothly.

This project was completed with the successful synthesis of many intermediates that will lead to the creation of the analog of Glutathione containing (*S*)-α-methyl-Cysteine. However, there are a few more steps that need to be completed before the synthesis of a new compound. This is where more research needs to be conducted, such as moving forward in the progression of steps toward creating the unnatural cysteine analog. The complications that occurred in the steps that I completed are fixed; therefore, another researcher can now successfully make a large quantity of each intermediate step. They should then be able to continue the synthesis with more products so if anything goes wrong, there is more intermediate to work with. This hopefully will allow for no other researcher to have to start over at the Dimethyl methyl malonate step. Once the unnatural cysteine analog is successfully created, it can be tested to determine if it has inhibition qualities against glutathione reductase. Inhibition against this molecule could mean advancement towards a cheaper malaria drug, which could allow for countries in poverty the access to a drug that could slow or even kill the malaria parasite.

Materials and Methods

Dimethyl 2(tert-butylsulfamethyl)-2-methylmalonate (3)

A three necked round bottomed flask was charged with a 60% dispersion of NaH in mineral oil (2.703 g, 0.0675 mol), a stir bar, and 321.75 mL of THF. The flask was fitted with a rubber septum, a glass stopper, and a reflux condenser to which a nitrogen inlet was attached so the flask could be flushed. The mineral oil was washed with pentane and the flask was placed in an ice bath for 15 minutes to cool to 0˚C. A solution of di-methyl-methylmalonate (9.0 g) in 128 mL of THF dropwise to the reaction flask over 30 minutes with stirring. The reaction was removed from the ice bath and allowed to stir for an addition 60 minutes. Then, 10.71 g of i-butyl chloromethyl sulfide was added dropwise over 5 minutes with stirring. The resulting solution was then heated to reflux solvent overnight. The solution became cloudy with NaCl. The solution was allowed to cool and then diluted with 300 mL of ether and washed with cold HCl followed by brine. The solution was then dried with $MgSO_4$ filtered, and concentrated *in vacuo*. The weight of product, **10,** was 11.42 grams. The liquid was clear and colorless and was purified by chromatography (4:1 hexane/ether); TLC R_f=0.31 (4:1 hexane/ether); ^{1}H-NMR (300 MHz, $CDCl_3$): 1.37 (6H t), 1.41 (9H s), 1.5 (3H s), 3.1 (2H s), 3.7 (4H).

(*S*)-2-*tert*-Butylsulfanylmethyl-2-methylmalonic Acid Monomethyl Ester (4)

The purified diester, **10**, (2.4 g, 9.6 mmol) was suspended in 0.1 N phosphate buffer (250 mL, 7.4 pH). PLE was added (90 units/mol; 17 mg = 1 unit; 50 mg PLE) to the mixture and it was stirred rapidly. The pH was maintained at 7.4 by addition of 1.21 N NaOH, using a 798 MPT titrino in the pH Stat mode. The reaction was complete after the

addition of 17 mL of titrant. The reaction was worked up by addition of 2 N NaOH to reach pH 9, and the mixture was washed with ether once. The pH was then adjusted to pH 2 by addition of 1.2 N HCl and extracted with ether three times. The combined extracts were washed with brine, dried over $MgSO_4$ filtered, and concentrated *in vacuo*. The product, **11,** yielded was a clear, colorless oil (1.37 g, 3.4 mmol). ^{1}H-NMR (300 MHz, $CDCl_3$): 1.31 (3H t), 1.34 (9H s), 1.55 (3H s), 3.1 (2H q), 4.4 (2H s).

***(S)*-4-*tert*-Butyl-1-methyl-2-(benzyloxymethyl)-2-methyl-succinate (5)**

An amount of 2.2 grams of **11** (11.3 moles) was dissolved in 12 mL of CH_2Cl_2 and placed in a 20 mL sealable pressure tube at -10˚C. A quantity of 0.72 mL of concentrated sulfuric acid and 7.2 mL of condensed Isobutylene was added to the pressure tube. The tube was capped, and the reaction was allowed to stir overnight at room temperature. The tube was then placed on an ice bath for 15 minutes; it was then opened and allowed to stir at room temperature for 2 hours to allow for evaporation of any remaining Isobutylene. The solution was diluted with 60 mL CH_2Cl_2 and washed three times with 2.2 N NaOH, dried over $MgSO_4$ and concentrated *in vacuo*. The product, **12**, was a clear, vicous oil; 0.96 grams (need to purify). Add methanol to the product to get the polymer precipitate out (34.28% yield). TLC (30% ether/hexane) R_f = 0.52. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.44 (9H, s), 1.47 (3H, s), 3.52 (2H, s), 3.79 (1H), 3.9 (1H), 4.63 (2H, s), 7.53 (5H).

***(S)*-2-*tert*-Butylsulfanmethyl-2-methylmalonic Acid Mono-*tert*-butyl Ester (6)**

An amount of 0.45 grams (1.72 mmol) of **12** was dissolved in 13.6 mL of EtOH. An amount of 0.136 g (1.3 mmol) of LiOH was dissolved in 1.5 mL of water and added to

the reaction flask. The reaction mixture was allowed to stir at room temperature for 48 hours after which time TLC (30% ether/hexane) showed that the reaction was no longer progressing. THF was evaporated and the reaction was diluted with water and acidified to pH 3.0 with cold 2N HCl. The mixture was extracted three times with ether and the combined extracts were washed with brine. The solution was dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The product, **13**, was a clear, colorless oil that crystallized upon standing. (0.13g product, 0.527 mmol, 28.7% yield). . ^{1}H-NMR (300 MHz, $CDCl_3$): 1.47 (9H, s), 1.49 (3H, s), 3.80 (1H), 4.58 (2H, s), 7.43 (5H).

_(S)_-N-4-Methoxybenzyloxycarbonyl-S-_tert_-butyl-2-methylcysteine _tert_-Butyl Ester (7)

An amount of 0.13 g (0.527 mmol) of **13** was dissolved in 1.0 mL of dichloroethane in a 50-mL round-bottomed flask with a magnetic stirbar. A volume of 93.2 mL of Et_3N, and 136.5 mL of DPPA was added to the solution and brought to reflux solvent for 1.5 hours, at which time 131.43 mL of PMB-OH was added to the solution and brought to reflux for 12 hours. The solvent was evaporated giving yellow oil, and concentrated *in vacuo* and the protected ester was purified by flash chromatography (6:1 hexane/EtOAc). R_f=0.54. The product we got may have run too long because it looked like it was charred and slightly decomposed.

*Repeat experiments of these five steps were performed during this process to gain a higher yield of some products to take to the next steps.

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