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Reaction of Halogenated Hydrocarbons with Cysteine and Nucleotides

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REACTION OF HALOGENATED HYDROCARBONS WITH CYSTEINE AND NUCLEOTIDES

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Honors College Distinction at Western Kentucky University

By

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Western Kentucky University
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ABSTRACT

Our objective is to develop a simple, inexpensive model to better understand the biologically relevant reactions of halogenated hydrocarbons and characterize them by NMR spectroscopy. We currently have a model that mimics the adduct created by the reaction of ethylene dibromide (a known toxin and carcinogen) with cysteine and guanosine 5'-monophosphate. Early attempts led to side products including ethylene oxide and ethylene glycol; however, our most promising method to date reacts cysteine with 2-bromoethanol in sodium methoxide/methanol followed by reaction of the 2-hydroxyethyl adduct with HCl and later with guanosine 5'-monophosphate. By reacting other halogenated hydrocarbons through the same method, we can directly compare their unknown reactivity to the known toxicity of ethylene dibromide. If the adducts are similar, additional research on these chemicals can be conducted and if determined toxic, classify them in a means that prevents their use. However through this method, reactions with 3-bromopropanol and 70% 1-bromo-2-propanol/ 30% 2-bromo-1-propanol have failed to convert from their hydroxypropyl adduct to their hydrochloropropyl adduct, indicating the model needs additional research and development.

Keywords: Halogenated Hydrocarbons, Ethylene Dibromide, Cysteine, NMR Spectroscopy, Toxicology

Dedicated to two of my biggest and proudest supporters, my Grandma and late Grandpa, whom I love and miss tremendously.

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FIELD OF STUDY

Major Field: Biochemistry

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CHAPTER 1

INTRODUCTION

Halogenated hydrocarbons are prevalent in our society; for many years they have been used as solvents, fumigants, propellants and have also served as intermediates in the production of many materials like plastics and textiles [1]. However, recent research is questioning the safety of these chemicals, in particular, ethylene dibromide (EDB). EDB was once used as a gasoline additive and was a popular pesticide and fumigant in the mid 1900's [2]. As its use became extensive, scientists identified EDB in the atmosphere, ground water, soil, and food supplies [3]. In 1983, the Environmental Protection Agency ordered an immediate emergency suspension of the use of EDB, citing significant contamination and carcinogenic and mutagenic studies in animals [4].

Ethylene dibromide's toxicity is not well known in humans due to limitations in study design, latency periods, and incomplete exposure data [5]. However extensive studies in animals have shown EDB to be carcinogenic; the International Agency for Research on Cancer found sufficient evidence of carcinogenic activity in animals, including tumors, adenomas, and carcinomas at numerous sites of the ingestion, absorption, and inhalation routes of exposure [5]. This scientific evidence led the

Environmental Protection Agency to list ethylene dibromide as a Group B2, probable human carcinogen [6].

In vivo, ethylene dibromide reacts with thiol containing molecules like cysteine and glutathione (GSH). Cysteine, one of the twenty α amino acids found commonly in proteins, is a critical component in the three-dimensional structure of proteins. The presence of the thiol group makes cysteine highly reactive, participating in enzymatic reactions and acting as a nucleophile. Glutathione, a tripeptide, contains a cysteine residue. It acts as an antioxidant, protecting the cell from reactive oxygen species.

Both cysteine and glutathione are activated in vivo. When cysteine is activated, it is incorporated into the enzyme alkylguanine transferase; it is present in the active site as amino acid number 145 [7]. In contrast, glutathione is not a part of the enzyme that activates it; it is activated by glutathione transferase. Both of these enzymes increase the reactivity of the cysteine residue by deshielding the sulfur atom. In addition, they increase the nucleophilicity of the sulfur atom, which is crucial in the bioactivation of EDB.

Ethylene dibromide has a known mutagenic biological mechanism [8]: the cross link of cysteine with ethylene dibromide and then with the nucleotide, guanine. First, EDB reacts with thiol containing molecules through a bimolecular substitution reaction (S_N2). A S_N2 reaction occurs when a nucleophile (electron rich species) attacks an electrophile (electron poor species) which releases a leaving group and forms a new bond. Cysteine, when activated by alkylguanine transferase in vivo, is a strong nucleophile. Similarly, EDB is an excellent electrophile with bromine as a sufficient

leaving group. Thus, a single bromine from EDB is displaced by the cysteine residue, as follows:

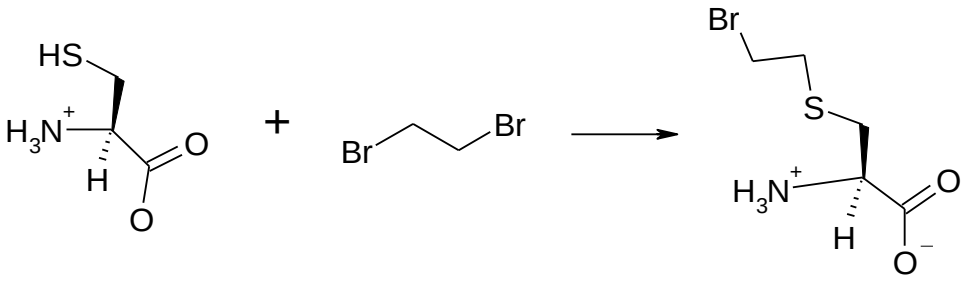

Figure 1.1 Bimolecular substitution reaction with cysteine and ethylene dibromide

Another bimolecular substitution reaction occurs, however this one is intramolecular. The remaining bromine is displaced by the sulfur atom, forming an episulfonium ion. This ion is an excellent electrophile and has shown increased reactivity toward DNA [9]. In particular, the ion has shown reactivity toward the N7 atom of guanine. Through another S_N2 reaction, the N7 atom of guanine attacks the episulfonium ion, as follows, forming the DNA adduct shown:

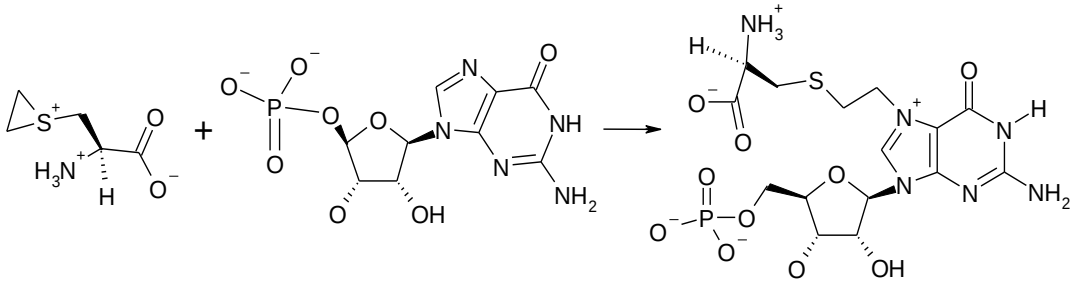

Figure 1.2 Bimolecular substitution reaction with cross-linked cysteine and ethylene dibromide and guanosine 5'-monophosphate

The alkylation of the guanine nucleotide weakens the glycocidic bond between the base and the sugar, resulting in an abasic site. During DNA replication, DNA polymerase places an adenine across from the guanine, instead of guanine's compliment, cytosine. When replication occurs again, adenine's compliment thymine will be in place of the original guanine, resulting in a guanine to thymine transversion. The cysteine is most likely a part of a larger polymer; even though DNA repair proteins are present, the bulk of the polymer is so large that DNA repair proteins are unable to access the base to repair the abasic site. Thus this guanine to thymine transversion has been shown to be mutagenic in bacterial cells and is most likely to be mutagenic in mammalian cells as well [10]. EDB has also been shown to react with other nucleotides at a reduced rate [10].

In Kentucky's 2001 Air Quality Report, several halogenated hydrocarbons were detected near seven metropolitan areas; some of these compounds are ethylene dichloride, dichloromethane, trichlorofluromethane, and bromomethane [11]. Each of these compounds has been reported as a toxic air pollutant. However in contrast to ethylene dibromide, the reactivity of most of these compounds has not been extensively studied. Thus, it is important to understand their reactivity with biological molecules.

Since these halogenated hydrocarbons have similar structure to ethylene dibromide, they most likely react similarly too. Thus, our objective is to develop a simple, inexpensive model to mimic the biological reaction of EDB with cysteine and guanine. Then, this model would be used with other halogenated hydrocarbons, like the ones found in Kentucky's air quality report, to better understand their biologically relevant reactions. If they react similar to EDB, they may have a similar toxicity. Thus,

this model would elicit more monitoring, development of biomarkers for exposure, and education to consumers on their presence.

In the first experiments, we attempted to mimic the biological mechanism of ethylene dibromide. Some of the first attempts resulted in biologically irrelevant molecules, including ethylene bridged cysteine residues, ethylene glycol and ethylene oxide. However, the final attempt successfully mimicked the crosslink of ethylene dibromide to cysteine and then to guanine. The model used 2-bromoethanol instead of 1,2-dibromoethane and a methanol/sodium methoxide mixture instead of deuterium oxide, both to prevent side product formation. The alkylated guanosine 5'-monophosphate adduct was characterized by NMR spectroscopy.

In the latter experiments, 3-bromo-1-propanol and a 70% 1-bromo-2-propanol/30% 2-bromo-1-propanol mixture were reacted with cysteine through the same mechanism. With the use of 2D NMR spectroscopy, their intermediates were characterized. However they did not show similar reactivity through the model. Previous research by James et al. had shown that a 1,3-dibromopropane crosslink with cysteine is a rather stable metabolite and is excreted in rats [12]. Thus the lack of reactivity of 3-bromo-1-propanol was expected. These failures facilitated an understanding of the structure-activity relationship of three carbon chained halogenated hydrocarbons and most importantly their episulfonium ion intermediate.

CHAPTER 2

METHODS

The NMR spectrum was collected on a JOEL 500 MHz instrument. The mass spectrometry data was collected on a Varian LC/MS 500 Ion Trap.

I. Reaction of Ethylene Dibromide in Deuterium Oxide

A. 1:1 *N*-acetyl-L-cysteine and ethylene dibromide: 8.15 mg of *N*-acetyl-L-cysteine was dissolved in 1 mL of deuterium oxide. 4.32 μL of ethylene dibromide was added. Sodium deuterium oxide was used to adjust the pH from 2.54 to 10.25. The reaction was monitored over time by NMR spectroscopy.

B. *N*-acetyl-L-cysteine and excess ethylene dibromide: 12.1 mg of *N*-acetyl-L-cysteine was dissolved in 1 mL of deuterium oxide. 0.1 mL of the mixture (1.21 mg of *N*-acetyl-L-cysteine) was placed in an NMR tube. 900 μL of deuterium oxide and 8.6 μL of ethylene dibromide were added. Sodium deuterium oxide was used to adjust the pH from 4.46 to 10.25. The reaction was monitored over time by NMR spectroscopy.

II. Reaction of 2-Bromoethanol in Deuterium Oxide

A. *N*-acetyl-L-cysteine and excess 2-bromoethanol with guanosine 5'-monophosphate: 12.1 mg of *N*-acetyl-L-cysteine was dissolved in 1 mL of deuterium oxide. 50 μL of the mixture (0.60 mg of *N*-acetyl-L-cysteine), 900 μL of deuterium oxide, and 20 μL of 2-bromoethanol were pipetted into an NMR

tube. Then, 30.5 mg of guanosine 5'-monophosphate was added. Using sodium deuterium oxide, the pH was adjusted to approximately 10. The reaction was monitored over time by NMR spectroscopy.

B. 2-bromoethanol and guanosine 5'-monophosphate: 900 μL of deuterium oxide and 20 μL of 2-bromoethanol were pippeted to an NMR tube. 20.5 mg of guanosine 5'-monophosphate was added. The initial pH was 7.22 and did not need adjusting. The reaction was monitored by NMR spectroscopy.

C. 5:1:1 2-bromethanol, guanosine 5'-monophosphate, and *N*-acetyl-L-cysteine: 12.1 mg of *N*-acetyl-L-cysteine was dissolved in 1 mL of deuterium oxide. 20 μL of the mixture (0.24 mg of *N*-acetyl-L-cysteine), 800 μL of deuterium oxide, and 5.25 μL of 2-bromoethanol were pippeted into an NMR tube. 6.03 mg of guanosine 5'-monophosphate was added. Sodium deuterium oxide was used to adjust the pH from 3.90 to 9.86. The reaction was monitored by NMR spectroscopy.

III. Reaction of Ethylene Dichloride in Deuterium Oxide

1:1 *N*-acetyl-L-cysteine and ethylene dibromide: 8.15 mg of *N*-acetyl-L-cysteine was dissolved in 1 mL of deuterium oxide. 3.95 μL of ethylene dibromide was added. Sodium deuterium oxide was used to adjust the pH from 2.36 to 10.04. The reaction was monitored over time by NMR spectroscopy.

IV. Reaction of 2-Chloroethanol in Deuterium Oxide

1:1 *N*-acetyl-L-cysteine and 2-chloroethanol: 8.15 mg of *N*-acetyl-L-cysteine was dissolved in 1 mL of deuterium oxide. 4.82 μL of ethylene dibromide was added.

Sodium deuterium oxide was used to adjust the pH from 3.60 to 10.02. The reaction was monitored over time by NMR spectroscopy.

V. Reaction of Bromomethyl Acetate in Methanol/Sodium Methoxide

1:3:1 cysteine, sodium methoxide, and bromomethyl acetate: 3 mixtures were prepared. Mixture #1 was 60.8 mg of cysteine in 10 mL of dry methanol. Mixture #2 was 81.4 mg of sodium methoxide in 10 mL of dry methanol. Mixture #3 was 49.3 μL of bromomethyl acetate in 10 mL of dry methanol. Mixture #2 was added dropwise to mixture #1 and stirred for 15 minutes. Mixture #3 was added dropwise to the combined mixture and stirred for 15 minutes. The methanol was evaporated off via rotovap. 1 mL of deuterium oxide was placed in the round bottom flask and swirled. The deuterium oxide was placed in an NMR tube and monitored by NMR spectroscopy.

VI. Reaction of Ethylene Dibromide in Methanol/Sodium Methoxide

1:3:1 cysteine, sodium methoxide, and ethylene dibromide: 3 mixtures were prepared. Mixture #1 was 60.8 mg of cysteine in 10 mL of dry methanol. Mixture #2 was 81.4 mg of sodium methoxide in 10 mL of dry methanol. Mixture #3 was 43.5 μL of ethylene dibromide in 10 mL of dry methanol. Mixture #2 was added dropwise to mixture #1 and stirred for 15 minutes. Mixture #3 was added dropwise to the combined mixture and stirred for 15 minutes. The methanol was evaporated off via rotovap. 1 mL of deuterium oxide was placed in the round bottom flask and swirled. The deuterium oxide was placed in an NMR tube and monitored by NMR spectroscopy. An LC/MS was also performed on the sample

VII. Reaction of 2-Bromoethanol in Methanol/Sodium Methoxide

A. 1:2:1 cysteine, sodium methoxide, and 2-bromoethanol: 3 mixtures were prepared. Mixture #1 was 60.8 mg of cysteine in 10 mL of dry methanol. Mixture #2 was 54.3 mg of sodium methoxide in 10 mL of dry methanol. Mixture #3 was 35.4 μL of 2-bromoethanol in 10 mL of dry methanol. Mixture #2 was added dropwise to mixture #1 and stirred for 15 minutes. Mixture #3 was then added dropwise to the combined mixture and stirred for an additional 15 minutes. The methanol was evaporated off via rotovap. Then 1mL of deuterium oxide was placed in the round bottom flask and swirled. The deuterium oxide was placed in an NMR tube and monitored by NMR spectroscopy.

B. 1:2:1 cysteine, sodium methoxide and 2-bromoethanol with hydrochloric acid and guanosine 5'-monophosphate: 3 mixtures were prepared. Mixture #1 was 182.4 mg of cysteine in 30 mL of dry methanol. Mixture #2 was 162.9 mg of sodium methoxide in 30 mL of dry methanol. Mixture #3 was 106.2 μL of 2-bromoethanol in 30 mL of dry methanol. Mixture #2 was added dropwise to mixture #1 and stirred for approximately 1 hour. Mixture #3 was added dropwise to the combined mixture and stirred for 15 minutes. The methanol was evaporated off via rotovap. 9 mL of hydrochloric acid was added to the round bottom flask and heated to 90^0C for 6 hours. The hydrochloric acid was evaporated off via rotovap. Then, the adduct was recrystalized by adding 9 mL of isopropanol followed by gravity filtration. The supernatant was transferred to a round bottom flask, and the isopropanol was evaporated via rotovap. 16.0 mg of the recrystalized adduct was dissolved in 1 mL of deuterium oxide. 44.8 mg of

guanosine 5'-monophosphate was added. The reaction was monitored over time by NMR spectroscopy.

VIII. Reaction of 3-Bromo-1-Propanol in Methanol/Sodium Methoxide

A. 1:3:1 cysteine, sodium methoxide, and 3-bromo-1-propanol: 3 mixtures were prepared. Mixture #1 was 12.2 mg of cysteine in 2 mL of dry methanol. Mixture #2 was 16.28 mg of sodium methoxide in 2 mL of dry methanol. Mixture #3 was 8.73 μL of 3-bromo-1-propanol in 2 mL of dry methanol. Mixture #2 was added dropwise to mixture #1 and stirred for 15 minutes. Mixture #3 was added dropwise to the combined mixture and stirred for 15 minutes. The methanol was evaporated off via rotovap. 1 mL of deuterium oxide was placed in the round bottom flask and swirled. The deuterium oxide was placed in an NMR tube and monitored by NMR spectroscopy.

B. 1:2:1 cysteine, sodium methoxide, 3-bromo-1-propanol with hydrochloric acid and guanosine 5'-monophosphate: 3 mixtures were prepared. Mixture #1 was 91.2 mg of cysteine in 20 mL of dry methanol. Mixture #2 was 81.5 mg of sodium methoxide in 20 mL of dry methanol. Mixture #3 was 66.4 μL of 3-bromo-1-propanol in 20 mL of dry methanol. Mixture #2 was added dropwise to mixture #1 and stirred for 30 minutes. Mixture #3 was added dropwise to the combined mixture and stirred for 15 minutes. The methanol was evaporated off via rotovap. 10 mL of hydrochloric acid was added to the round bottom flask and heated to 90^0C for approximately 6 hours. The hydrochloric acid was evaporated off via rotovap. Then, the adduct was recrystalized by adding 9 mL of isopropanol followed by gravity filtration. The supernatant was transferred to a round bottom

flask, and the isopropanol was evaporated via rotovap. 5.0 mg of guanosine 5'-monophosphate was added to 1 mL of deuterium oxide, which was added to round bottom flask and swirled. The deuterium oxide was placed in a NMR tube. The reaction was monitored over time by NMR spectroscopy.

IX. Reaction of 70% 1-Bromo-2-Propanol/30% 2-Bromo-1-Propanol in Methanol/Sodium Methoxide

A. 1:3:1 cysteine, sodium methoxide, 70% 1-bromo-2-propanol/30% 2-bromo-1-propanol: 3 mixtures were prepared. Mixture #1 was 12.2 mg of cysteine in 2 mL of dry methanol. Mixture #2 was 16.3 mg of sodium methoxide in 2 mL of dry methanol. Mixture #3 was 9.16 μL of 70% 1-bromo-2-propanol/30% 2-bromo-1-propanol in 2 mL of dry methanol. Mixture #2 was added dropwise to mixture #1 and stirred for 15 minutes. Mixture #3 was added dropwise to the combined mixture and stirred for 15 minutes. The methanol was evaporated off via rotovap. 1 mL of deuterium oxide was placed in the round bottom flask and swirled. The deuterium oxide was placed in an NMR tube and monitored by NMR spectroscopy.

B. 1:3:1 cysteine, sodium methoxide, 70% 1-bromo-2-propanol/30% 2-bromo-1-propanol with hydrochloric acid and guanosine 5'-monophosphate: 3 mixtures were prepared. Mixture #1 was 12.2 mg of cysteine in 2 mL of dry methanol. Mixture #2 was 16.3 mg of sodium methoxide in 2 mL of dry methanol. Mixture #3 was 7.92 μL of 70% 1-bromo-2-propanol/30% 2-bromo-1-propanol in 2 mL of dry methanol. Mixture #2 was added dropwise to mixture #1 and stirred for approximately 15 minutes. Mixture #3 was added dropwise to the combined

mixtures and stirred for 15 minutes. The methanol was evaporated off via rotovap. 5 mL of hydrochloric acid was added to the round bottom flask and heated to 90^0C for approximately 6 hours. The hydrochloric acid was evaporated off via rotovap. Then the adduct was recrystalized by adding 5 mL of isopropanol followed by gravity filtration. The supernatant was transferred to a round bottom flask, and the isopropanol was evaporated via rotovap. 3.0 mg of guanosine 5'-monophosphate was added to 1 mL of deuterium oxide, which was placed in the round bottom flask swirled. The deuterium oxide was placed in a NMR tube and monitored over time by NMR spectroscopy

CHAPTER 3

RESULTS

I. Reaction of Ethylene Dibromide in Deuterium Oxide

Reactions in deuterium oxide utilized *N*-acetyl-L-cysteine, instead of cysteine. With a net negative charge, *N*-acetyl-L-cysteine is more soluble in the polar solvent, than the zwitterion, cysteine. Also, previous research determined that reactions with cysteine, at pH 10, involved both the amine and sulfur atom of the thiol [13]. Reactions with the amine are biologically irrelevant; thus, *N*-acetyl-L-cysteine was utilized to prevent such side-reactions.

Also, in these reactions, the pH was adjusted to approximately 10. Previous research determined that halogenated hydrocarbons did not react with cysteine at reasonable rates at pH 7; however, at pH 10, the reactions occurred much more readily [13]. The basic conditions mimic the activation of cysteine or glutathione in vivo [14]. The sulfur atom becomes partially deprotonated, increasing its nucleophilicity and reactivity.

A. 1:1 *N*-acetyl-L-cysteine and Ethylene Dibromide

In the ^{1}H spectrum of 0.050 M *N*-acetyl-L-cysteine and 0.050 M ethylene dibromide (Figure 3.1), three singlets were present at 1.9 ppm, suggesting that three *N*-acetyl-L-cysteine adducts formed. (1.9 ppm is the chemical shift of the

methyl group on *N*-acetyl-L-cysteine.) Also, three doublet of doublets at 4.3, 4.2, and 4.1 ppm were present, corresponding to three alpha hydrogens.

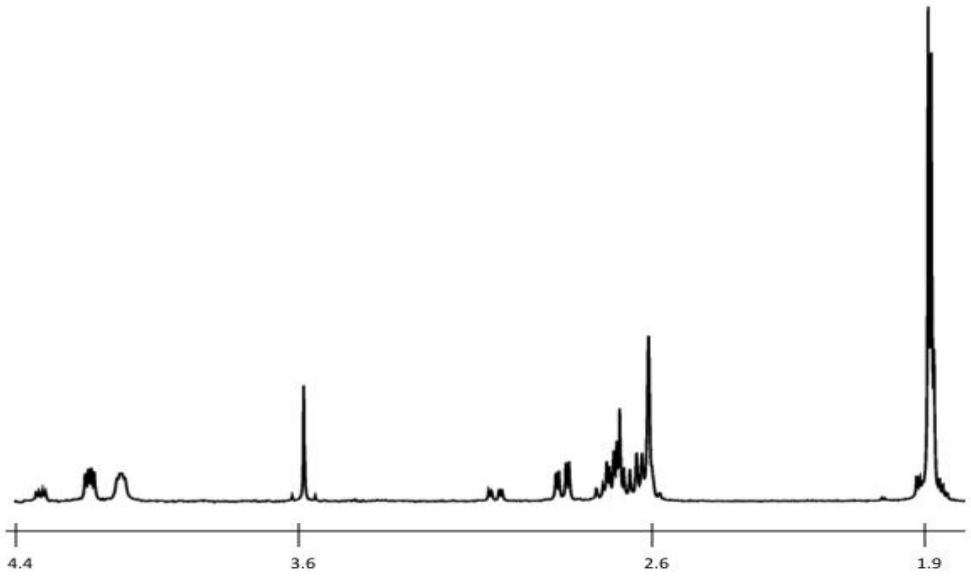

Figure 3.1 ^{1}H spectrum of 1:1 *N*-acetyl-L-cysteine and ethylene dibromide

The first adduct had a doublet of doublets at 4.1 ppm coupled to two doublet of doublets between 2.7 and 2.8 ppm. In a separate experiment, *N*-acetyl-L-cysteine was dissolved in deuterium oxide at pH 10. The unreacted *N*-acetyl-L-cysteine had the same chemical shifts as the observed adduct. Thus, at equal molarities, unreacted *N*-acetyl-L-cysteine was present.

The second adduct had a doublet of doublets at 4.2 ppm coupled to two doublet of doublets at 2.75 and 2.9 ppm. Also, a singlet at 2.7 ppm increased proportionally, as the product formed. The chemical shifts suggested the formation of an ethylene bridged *N*-acetyl-L-cysteine dimer.

The ^{1}H spectrum of the dimer is simplified, because a mirror image exists that is chemically and magnetically equivalent (Figure 3.2). The three sets of doublets of doublets correspond to the alpha hydrogen and beta hydrogens of the *N*-acetyl-L-cysteine adduct, respectfully. Also, the two homotopic protons of the ethylene bridge are equivalent, signaling the singlet.

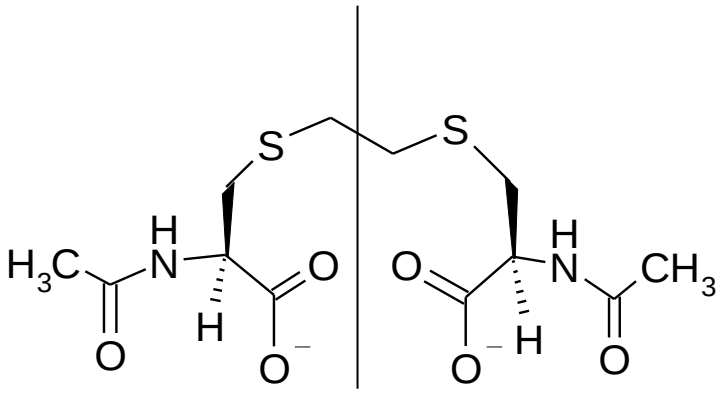

Figure 3.2 Symmetry of ethylene bridged *N*-acetyl-L-cysteine dimer

The formation of the *N*-acetyl-L-cysteine dimer has two possible mechanisms. In the first possible mechanism, an episulfonium ion forms, following a similar mechanism to the mutagenic cross-link of cysteine and guanine via ethylene dibromide. In the second mechanism, two S_N2 reactions occur (Figure 3.3):

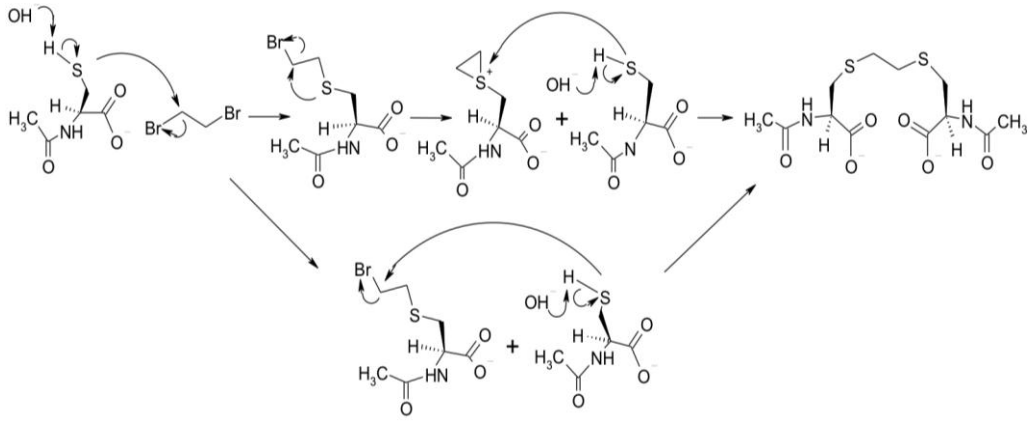

Figure 3.3 Proposed mechanisms to form ethylene bridged *N*-acetyl-L-cysteine dimer

In a separate experiment, guanosine 5'-monophosphate was added to the mixture and a cross-link with the nucleotide was not observed.

The third adduct had doublet of doublets at 4.3 ppm coupled to two doublet of doublets at 2.8 and 3.1 ppm. In a separate experiment, *N*-acetyl-L-cysteine was dissolved in deuterium oxide at pH 10. Over the span of several hours, an adduct with similar chemical shifts formed. According to the Merck Index, aqueous solutions of *N*-acetyl-L-cysteine at alkaline pH is likely to oxidize upon contact with the air to form *N*-acetyl-L-cystine—two disulfide bridged *N*-acetyl-L-cystienes [15] (Figure 3.4).

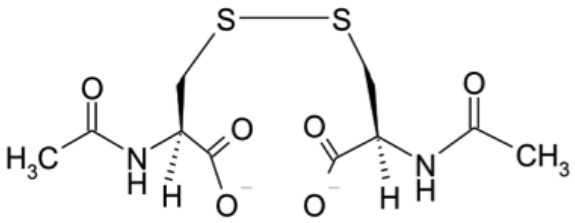

Figure 3.4 Structure of *N*-acetyl-L-cystine

B. *N*-acetyl-L-cysteine and Excess Ethylene Dibromide

In this experiment, a 13:1 ratio of ethylene dibromide to *N*-acetyl-L-cystine was utilized. By increasing the amount of ethylene dibromide, the probability of episulfonium ion formation increases. With more episulfonium ions available, the likelihood of a cross-link with guanosine 5'-monophosphate is greater, mimicking the biological mechanism of EDB.

The ^{1}H spectrum of excess ethylene dibromide had two singlets at 1.9 ppm and two doublet of doublets at 4.3 and 4.2 ppm, suggesting the formation of two *N*-acetyl-L-cystine adducts (Figure 3.5). The chemical shifts of both adducts were similar to the ^{1}H spectrum of 0.050 M *N*-acetyl-L-cysteine and 0.050 M ethylene dibromide (Figure 3.1). After comparing the 2D spectra, it was concluded that the ethylene-bridged *N*-acetyl-L-cysteine dimer and *N*-acetyl-L-cystine formed. Also, due to the excess ethylene dibromide, all of the *N*-acetyl-L-cysteine reacted.

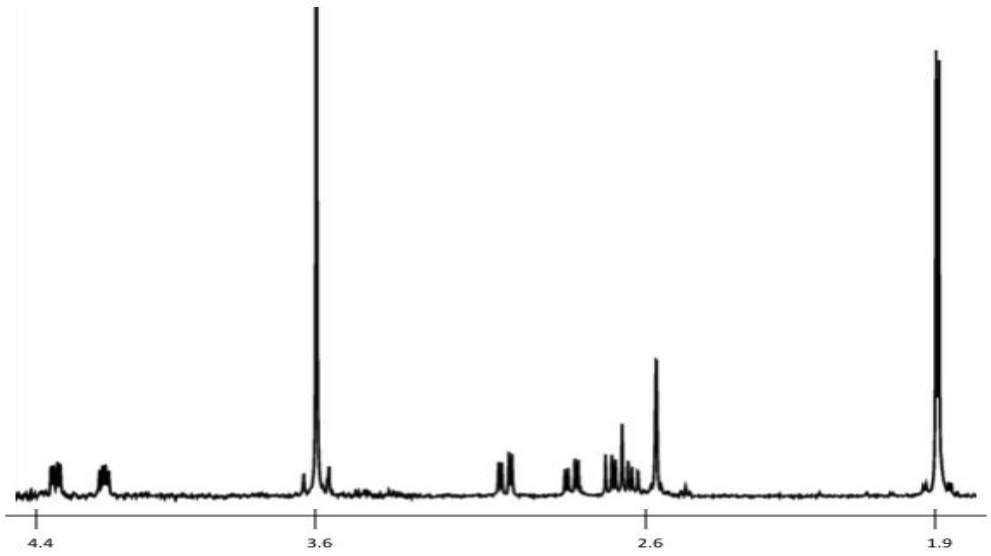

Figure 3.5 ^{1}H spectrum of *N*-acetyl-L-cysteine and excess ethylene dibromide

In a separate experiment, guanosine 5'-monophosphate was added to the 13:1 ratio of ethylene dibromide to *N*-acetyl-L-cysteine. The possible cross-link of *N*-acetyl-L-cysteine to the nucleotide via ethylene dibromide was not observed.

II. Reaction of 2-Bromoethanol in Deuterium Oxide

In these experiments, 2-bromoethanol was utilized instead of 1,2-dibromoethane. By replacing the second bromide with a hydroxyl, the likelihood of episulfoium ion formation in the presence of *N*-acetyl-L-cysteine is decreased, preventing the formation of the ethylene bridged *N*-acetyl-L-cysteine dimer.

A. *N*-acetyl-L-cysteine and excess 2-bromoethanol with guanosine 5'-monophosphate

A 75:13.5:1 ratio of 2-bromoethanol, guanosine 5'-monophosphate (5'-GMP), and *N*-acetyl-L-cysteine, respectfully, was utilized. After 10 days and a pH of approximately 10, the ^{1}H spectrum (Figure 3.6) revealed a probable

reaction with 5’-GMP.

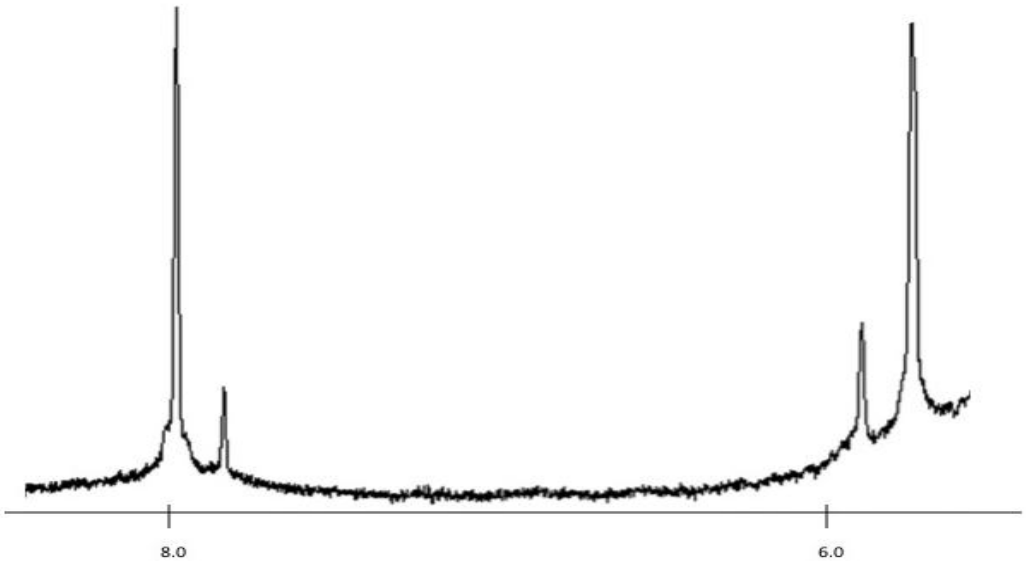

Figure 3.6 ^{1}H spectrum of *N*-acetyl-L-cysteine and excess 2-bromoethanol with guanosine 5’-monophosphate

Reactions with 5’-GMP and dibromoethane typically occur at the N7 atom of the base [16]. After alkylation, two protons, one bonded to the C8 atom of the base and one bonded to the C1’ atom of the sugar (Figure 3.7), experience changes in their electronic environments, affecting their chemical shifts. The N7 alkalyation diminishes the deshielding effect, altering the environment of the C8 proton. Also, the alkylation weakens the base to sugar bond, altering the environment of the C1’ proton.

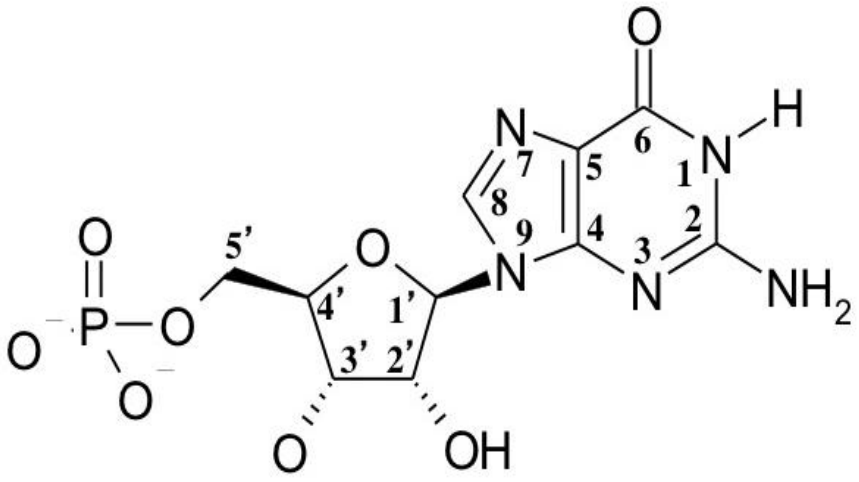

Figure 3.7 Unreacted guanosine 5'-monophosphate

However, an alkylation at the N7 atom usually shifts the protons bonded to C8 and C1' downfield. In Figure 3.6, the shifts observed in the 5'GMP reaction for C8 is upfield, which the shift for C' is downfield. The site of alkylation of ethylene oxide to 5'GMP was not determined.

In a separate experiment, 5'GMP was placed in deuterium oxide at pH 10. The ^{1}H spectrum had signals at 8.0 and 5.8, corresponding to the protons of C8 and C1' atoms, respectfully. Similar peaks were present in the ^{1}H spectrum of the initial experiment. However, there were also signals at 7.9 and 5.9, corresponding to the protons of C8 and C1' atoms, respectfully, after the alkylation of 5'-GMP. But, after analyzing 2D spectra, we could not characterize the 5'GMP adduct or site of alkylation.

B. 2-Bromoethanol and guanosine 5'-monophosphate

In an attempt to characterize the 5'GMP adduct, a 5:1 ratio of 2-bromoethanol to 5'GMP was prepared. The initial pH of the solution was 7.22

and not adjusted. An ^{1}H spectrum four days later revealed no reaction with 5'GMP (Figure 3.8).

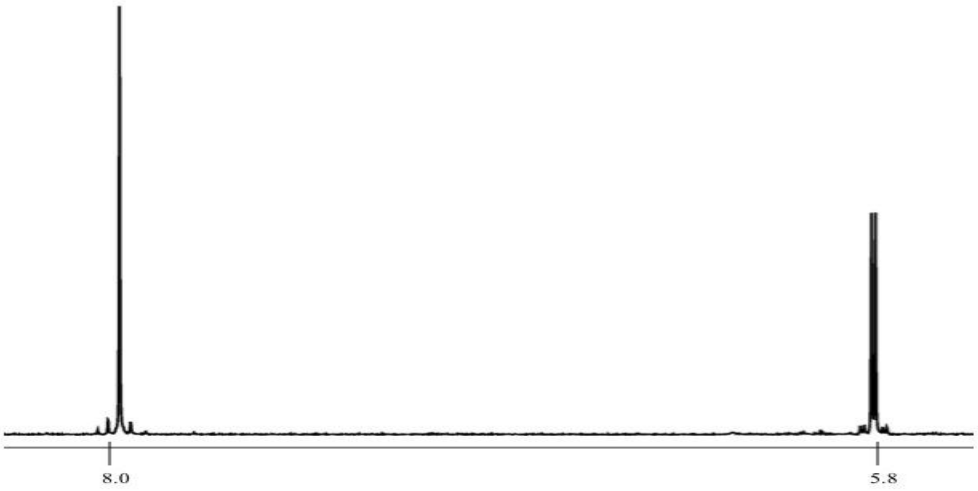

Figure 3.8 ^{1}H spectrum of 2-bromoethanol and guanosine 5'-monophosphate at pH 7 (1)

Then, the pH of the solution was adjusted to 9.95. The following day, an ^{1}H spectrum revealed a reaction with 5'GMP.

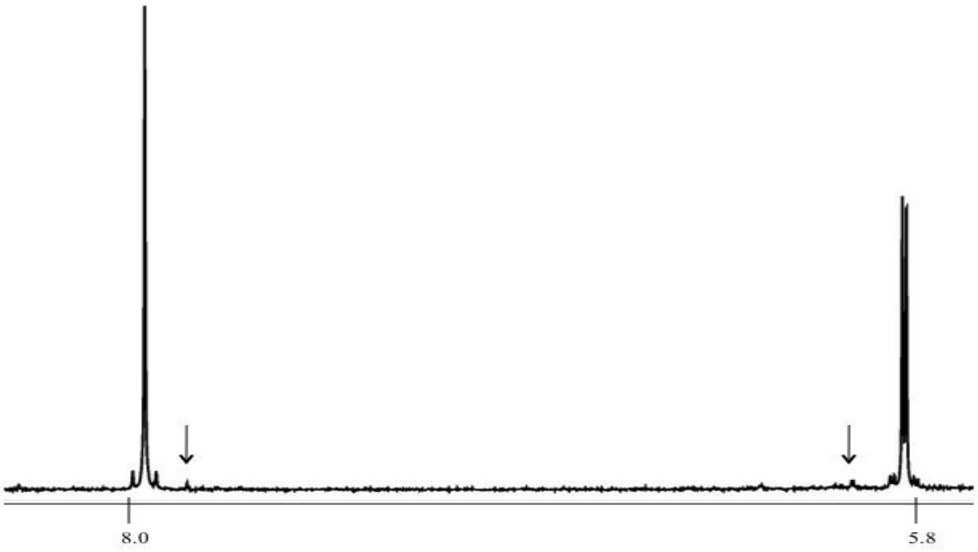

Figure 3.9 ^{1}H spectrum of 2-bromoethanol and guanosine 5'-monophosphate at pH 10 (1)

To characterize the adduct, the ^{1}H spectra before and after the pH adjustment were compared. The spectra were almost identical, except for a singlet at 2.72 ppm (Figure 3.10 and 3.11). The singlet was present at a very low absorbance before the pH adjustment. However, after the adjustment, the absorbance increased dramatically, suggesting the formation of the molecule was pH dependent. Also, the reaction with 5'GMP occurred after the pH adjustment, correlating the reaction with the formation of the molecule. In addition, the singlet at 2.72 ppm was observed in Figure 3.6—the reaction of 2-bromoethanol, 5'-GMP, and *N*-acetyl-L-cysteine at pH 10.

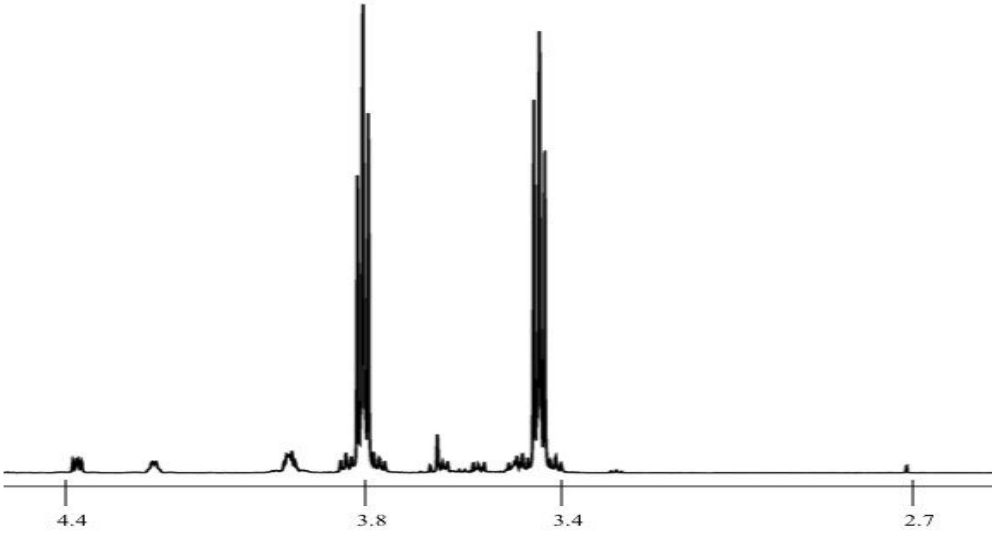

Figure 3.10 ^{1}H spectrum of 2-bromoethanol and guanosine 5'-monophosphate at pH 7 (2)

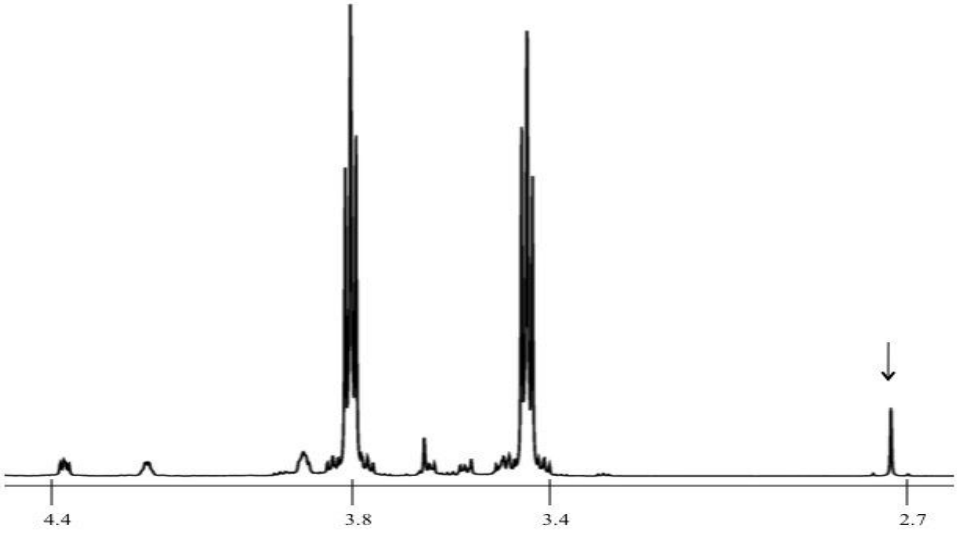

Figure 3.11 ^{1}H spectrum of 2-bromoethanol and guanosine 5'-monophosphate at pH 10 (2)

To further understand the origin of the singlet at 2.72 ppm, two solutions of 2-bromoethanol in deuterium oxide were prepared. The first solution had an

initial pH of 1.43 and was not adjusted. The second solution had a similar initial pH but was adjusted to 10.10. The singlet at 2.72 ppm was not observed in the ^{1}H spectrum of the first solution. However, the singlet was present in the ^{1}H spectrum second solution. Therefore, the singlet was a result of 2-bromoethanol at high pH.

It is well known that halohydrins in basic solution undergo intramolecular S_N2 reactions to form epoxides. When 2-bromoethanol undergoes the intermolecular S_N2 reaction, ethylene oxide forms (Figure 3.12). Ethylene oxide contains a mirror image that is magnetically and chemically equivalent. Therefore, one set of the homotopic protons will appear in an ^{1}H spectrum as a singlet, which we observed in the ^{1}H spectrum of 2-bromoethanol and guanosine 5'-monophosphate at pH 10.

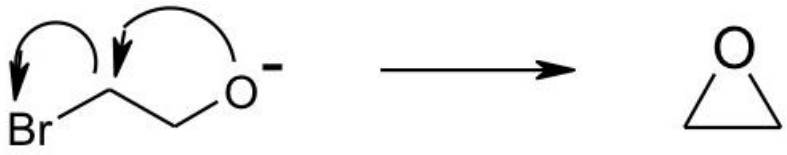

Figure 3.12 Proposed mechanism of ethylene oxide formation

The three membered ring of ethylene oxide is highly strained and reactive. And, through an S_N2 mechanism, 5'GMP can attack the three membered ring, alkylating the base. The product is consistent with the reaction observed in the ^{1}H spectrum of 2-bromoethanol and guanosine 5'-monophosphate at pH 10 (Figure 3.9). Also, it is, mostly likely, the 5'GMP observed in the ^{1}H spectrum of *N*-

acetyl-L-cysteine and excess 2-bromoethanol with guanosine 5'-monophosphate (Figure 3.6).

C. 5:1:1 2-Bromoethanol, guanosine 5'-monophosphate, and *N*-acetyl-L-cysteine

In order to determine the next step in our experiment, it was important to understand why the cross link between *N*-acetyl-L-cysteine and 5'GMP via 2-bromoethanol did not occur. A solution with a 5:1:1 ratio of 2-bromoethanol, 5'GMP, and *N*-acetyl-L-cysteine, respectfully, was prepared. The lower ratio allows the *N*-acetyl-L-cysteine and any of its reactions to be easily detected.

In the initial ^{1}H spectrum, 3 singlets were present at 1.9 ppm, suggesting the formation of 3-*N*-acetyl-L-cysteine adducts (Figure 3.13). However, the adducts were hard to identify in the ^{1}H spectrum, so a COSY and HMQC were preformed. The 2D spectra revealed signals at 4.33, 4.23, and 4.19 ppm, corresponding to possible alpha hydrogens. In addition, the COSY revealed coupling consistent with 3 sets of alpha and beta hydrogens. However, during the 2D spectra, the initial pH of 9.86 dropped to 8.01, making the adducts hard to identify.

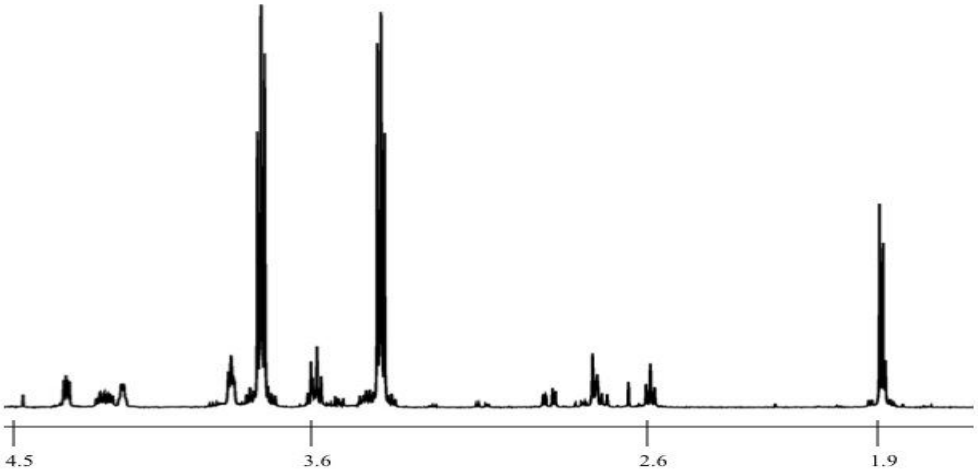

Figure 3.13 ^{1}H spectrum of ethylene oxide and guanosine 5'-monophosphate adduct

The first adduct had a coupling pattern of 4.19 ppm to 2.76 and 2.89 ppm. In a separate experiment, a solution of *N*-acetyl-L-cysteine in deuterium oxide at pH 8 was prepared. The chemical shifts were consistent with the coupling pattern of the first adduct. Therefore, unreacted *N*-acetyl-L-cysteine was present and identified in the ^{1}H spectrum.

The second adduct had a coupling pattern of 4.33 ppm to 3.09 and 2.80 ppm. The chemical shifts were consistent with the coupling pattern of oxidized *N*-acetyl-L-cysteine found in the ^{1}H spectrum of 0.050 M *N*-acetyl-L-cysteine and 0.050 M ethylene dibromide. With the initial alkaline solution, it is likely that *N*-acetyl-L-cystiene air oxidized to form *N*-acetyl-L-cystine [14].

The third adduct had a coupling pattern of 4.23 ppm to 3.07 and 2.87 ppm. However, there were also two triplets coupled to each other at 3.58 and 2.58

ppm. The signals were not identified in the 2-bromoethanol and 5'GMP ^{1}H spectrum, and, most likely, were attributed to the presence of *N*-acetyl-L-cysteine. The third adduct was not characterized until further experiments were performed (listed in section III). Yet, in hindsight, the coupling patterns corresponded to a low yield of an hydroxyethyl *N*-acetyl-L-cysteine adduct formed by the following mechanism:

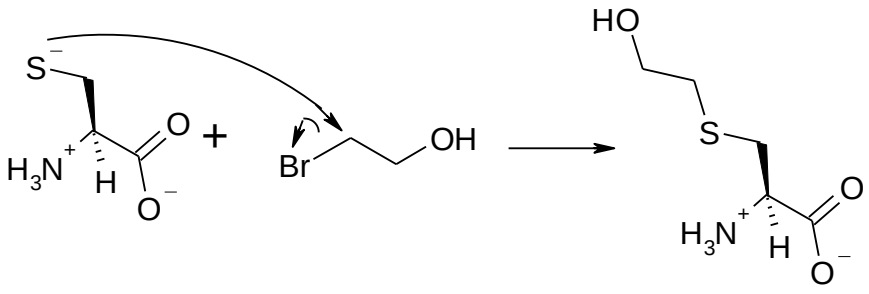

Figure 3.14 Proposed mechanism of hydroxyethyl *N*-acetyl-L-cysteine adduct

III. Reaction of Ethylene Dichloride in Deuterium Oxide

Ethylene dichloride is similar in structure to ethylene dibromide. However, the halogen is different. Chloride, in comparison to bromide, is a poor leaving group. Thus, the reactions with ethylene dichloride should proceed slower, possibly preventing dimer formation and/or oxidation of *N*-acetyl-L-cysteine.

In the ^{1}H spectrum of 0.050 M *N*-acetyl-L-cysteine and 0.050 M ethylene dichloride (Figure 3.15), three singlets were present at 1.9 ppm, suggesting that

three *N*-acetyl-L-cysteine adducts formed. Also, three doublet of doublets at 4.35, 4.20, and 4.05 ppm were present, corresponding to three alpha hydrogens.

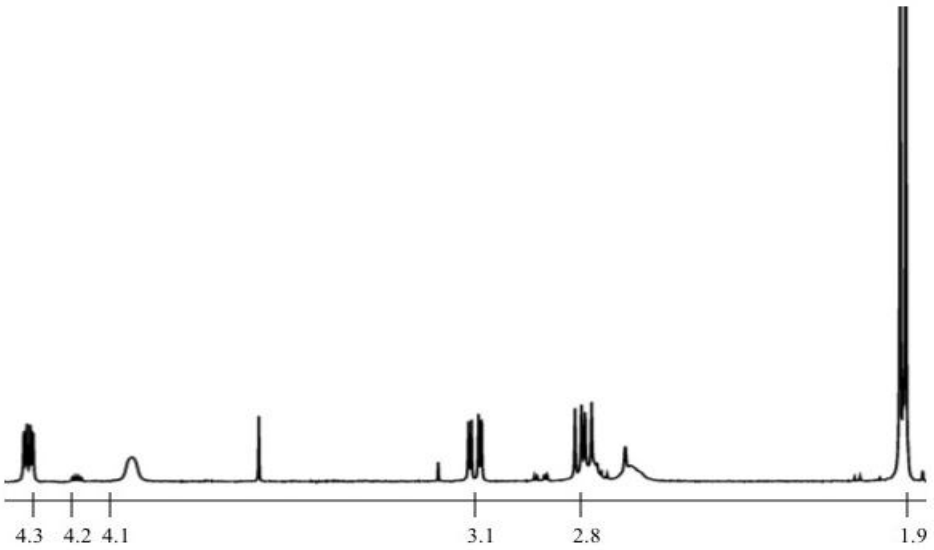

Figure 3.15 ^{1}H spectrum of 1:1 *N*-acetyl-L-cysteine and ethylene dichloride

The adducts observed in Chapter 2, Section I, Reaction A (1:1 *N*-acetyl-L-cysteine and ethylene dibromide) were identical to the ones observed in the ethylene dichloride reaction. Unreacted *N*-acetyl-L-cysteine, ethylene bridged *N*-acetyl-L-cysteine dimer, and *N*-acetyl-L-cystine were characterized. However, the *N*-acetyl-L-cysteine dimer formed at a much slower rate. The dimer was not observed until two weeks into the reaction. Four weeks later, the product was still minute in comparison to *N*-acetyl-L-cystine and unreacted cysteine.

(The mechanism for the formation of the ethylene bridged *N*-acetyl-L-cysteine dimer is the same as shown in Figure 3.3.)

IV. Reaction of 2-Chloroethanol in Deuterium Oxide

Similar to ethylene dibromide and ethylene dichloride, 2-bromoethanol and 2-chloroethanol are very similar in structure. Although the chloride containing halogenated hydrocarbon should react slower, the same adducts and reactions should be observed.

In the ^{1}H spectrum of 0.050 M *N*-acetyl-L-cysteine and 0.050 M ethylene dichloride (Figure 3.16), two singlets were present at 1.9 ppm, suggesting that two *N*-acetyl-L-cysteine adducts formed. Also, two doublet of doublets at 4.32 and 4.22 ppm were present, corresponding to two alpha hydrogens.

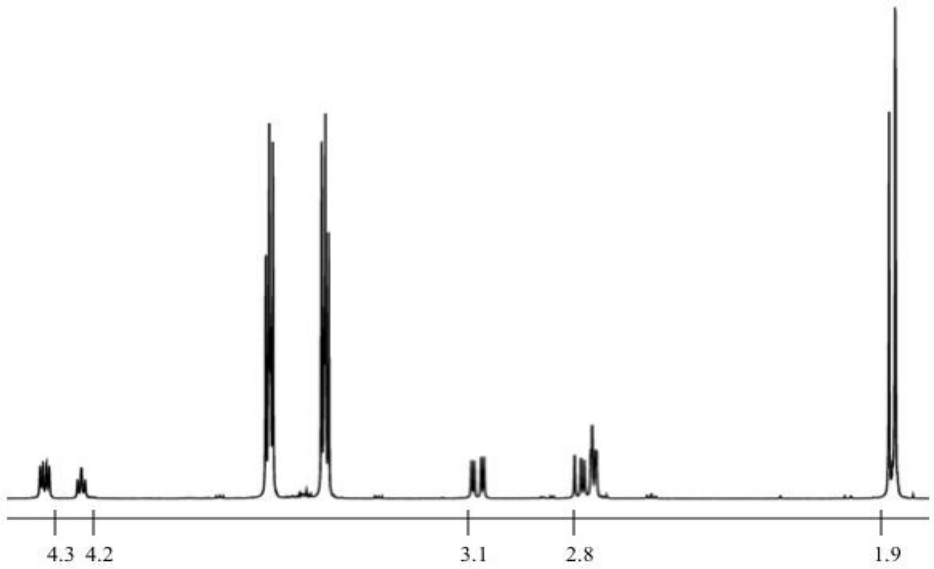

Figure 3.16 ^{1}H spectrum of 1:1 *N*-acetyl-L-cysteine and 2-chloroethanol

The adducts observed in Chapter 2, Section II, Reaction C (*N*-acetyl-L-cysteine and excess 2-bromoethanol) were identical to the ones observed in the 2-

chloroethanol reaction. The two main products, unreacted *N*-acetyl-L-cysteine and -acetyl-L-cystine were characterized. At first, there were no signs of the hydroxethyl adduct. When the sample was checked four weeks later, very small signals were present at 3.6 and 2.6 ppm corresponding to the hydrogens in the alcohol chain. However, the amount is so minute that the adduct should not be considered as a significant product of the reaction.

V. Reaction of Bromomethyl Acetate in Methanol/Sodium Methoxide

In an article by Marsh et. al, an experimental method to form DNA adducts by crosslinking nucleotides and glutathione via halogenated hydrocarbons was described. The method utilized a 0.80 mM solution of glutathione dissolved in methanol, a 1.25 mM solution of sodium dissolved in methanol, and a 0.85 mM solution of bromomethyl acetate. The solutions were added dropwise as described. Then, the crosslink of glutathione and bromomethyl acetate was precipitated out via centrifugation [17].

We adopted a similar method described in Chapter 2 Section III. After the method was completed, a ^{1}H spectrum was performed (Figure 3.17). It appeared that the signals at 4.30, 3.90, and 3.80 ppm were possible alpha hydrogens. A COSY, HMQC, and DEPT-135 were collected to aid in characterization. However, the products were unable to be characterized.

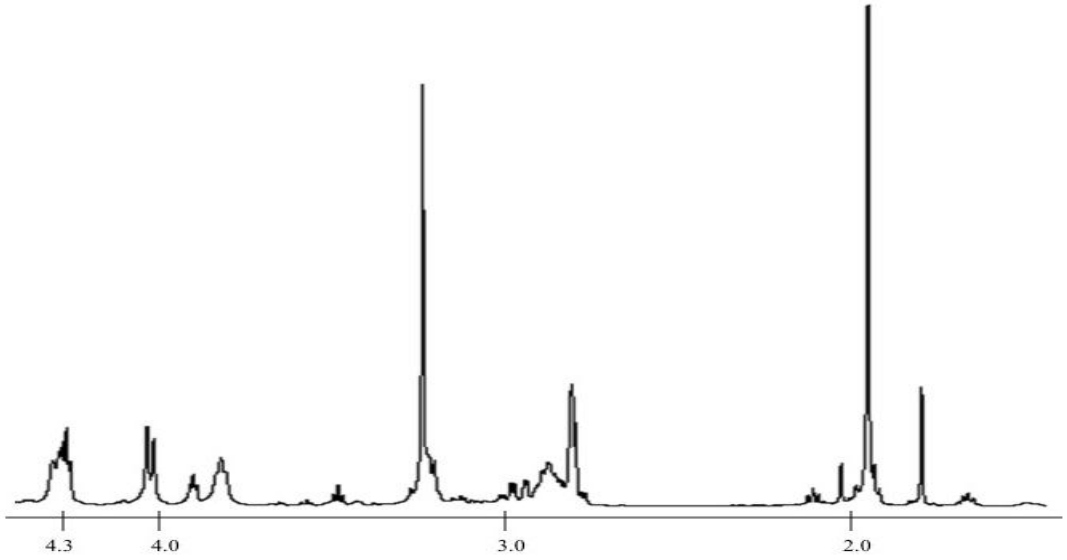

Figure 3.17 ^{1}H spectrum of cysteine and bromomethyl acetate in methanol/sodium methoxide

To aid in characterization, LC/MS was utilized. A 0.1% formic acid solution in acetonitrile and 0.1% formic acid in water were used. The solvent was an 50/50 isocratic solution, without a column, and in positive ion mode. The results were inconclusive and the products were unable to be characterized.

VI. Reaction of Ethylene Dibromide in Methanol/Sodium Methoxide

Ethylene Dibromide was studied through the methanol/sodium methoxide method. After the methanol was evaporated off, an NMR was acquired. The ^{1}H spectrum showed five signals (4.10, 4.00, 3.85, 3.70 and 3.50 ppm) possibly corresponding to alpha hydrogens (Figure 3.18). 2-dimensional NMR, including, HMQC, COSY, and DEPT-135, were performed. However, the products were unable to be characterized.

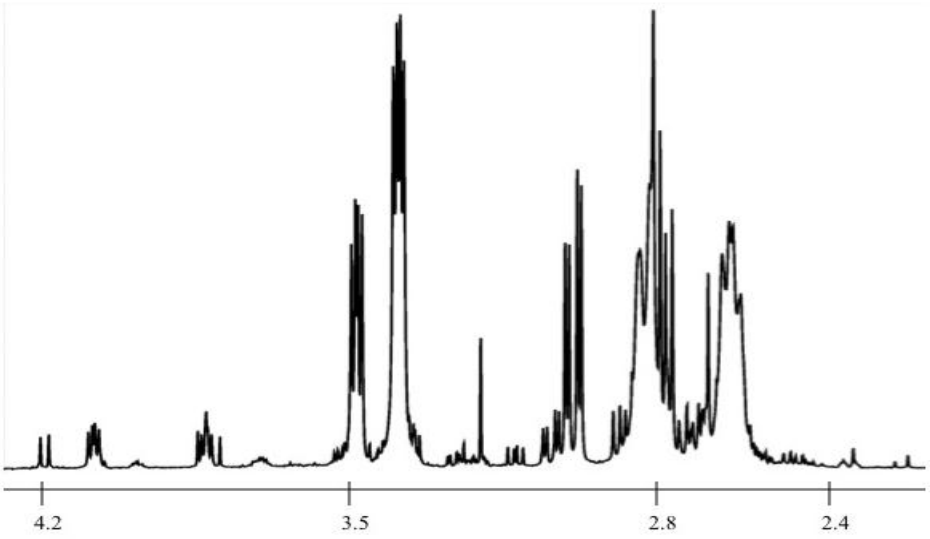

Figure 3.18 ^{1}H spectrum of ethylene dibromide and cysteine in methanol/sodium methoxide method

To aid in characterization, LC/MS was utilized. A 0.1% formic acid solution in acetonitrile and 0.1% formic acid in water were used. The solvent was an 50/50 isocratic solution, without a column, and in positive ion mode. Again, the results were inconclusive and the products were unable to be characterized.

VII. Reaction of 2-Bromoethanol in Methanol/Sodium Methoxide

A. 1:2:1 cysteine, sodium methoxide, and 2-bromoethanol

The reaction of 2-bromoethanol with cysteine was also studied through the methanol/sodium methoxide method. After the methanol was evaporated off via rotovap, a proton NMR was performed. In the ^{1}H spectrum (Figure 3.19), three sets of doublet of doublets and two triplets were observed—signals similar to the reaction of 2-bromoethanol with *N*-acetyl-L-cysteine in deuterium oxide. (The large singlet at 3.2 ppm corresponds to residual methanol.)

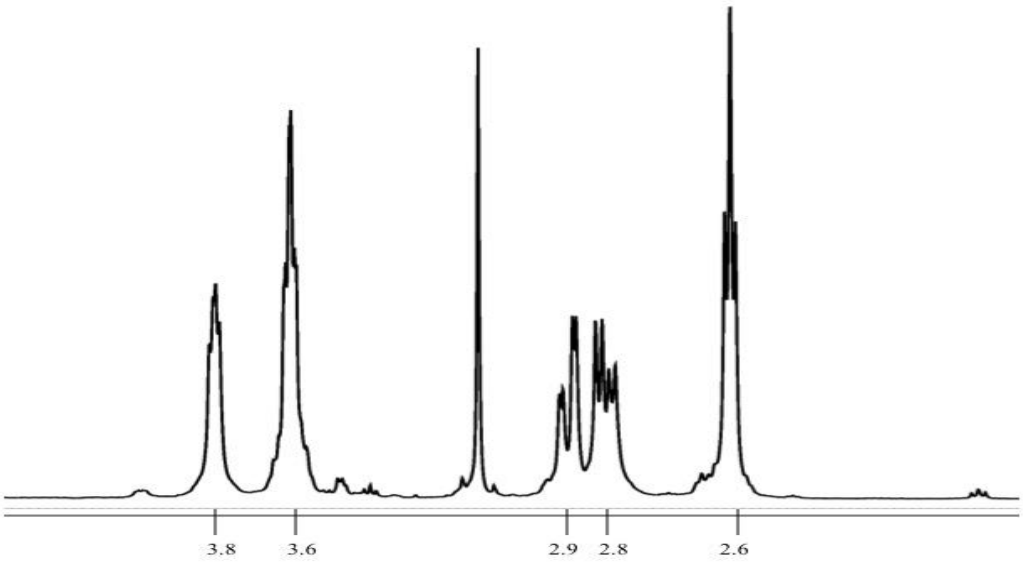

Figure 3.19 ^{1}H spectrum of hydroxyethyl cysteine adduct

The doublet of doublets at 3.8 ppm and the doublet of doublets at 2.9 and 2.8 ppm corresponded to a typical alpha and beta hydrogens pair. However, the triplets at 3.6 and 2.6 ppm were not, and, after a 2-deminsional COSY was performed, it was determined that the triplets couple to each other. The signals correspond to the two sets of homotopic protons in the alcohol, confirming the formation on the hydroxyethyl adduct. The adduct forms through the same mechanism in Figure 3.14.

In a separate experiment, guanosine 5'-monophosphate was added to the hydoxyethyl adduct in 1 mL of deuterium oxide. An NMR was performed. The ^{1}H spectrum revealed no reaction with 5'GMP.

B. 1:2:1 cysteine, sodium methoxide and 2-bromoethanol with hydrochloric acid and guanosine 5'-monophosphate

The hydoxyethyl adduct was unreactive in the presence of 5'GMP, due to the poor leaving ability of the hydroxyl group. Through an additional reaction, the hydroxyl was replaced with chloride, which is a fair leaving group. After replacement, the chloroethyl adduct was reacted with 5-guanosine-monophosphate. The ^{1}H spectrum revealed a reaction with 5'GMP. And, after integration, it was determined that 15% of the 5'GMP was alkylated (Figure 3.20).

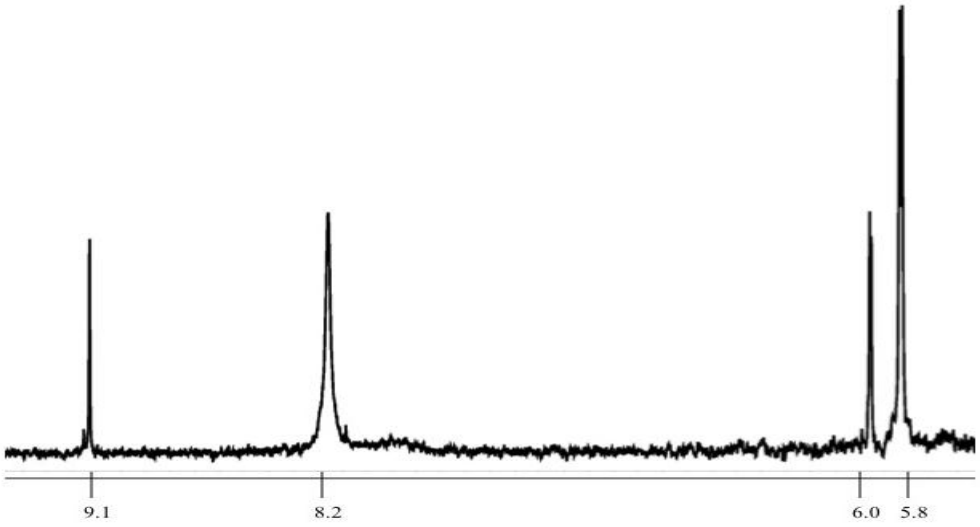

Figure 3.20 ^{1}H spectrum of chloroethyl cysteine adduct and guanosine 5'-monophosphate reaction

The set of signals at 8.2 and 5.8 ppm correspond to the C8 and C1' of unreacted 5-GMP, respectfully. The downfield set of signals at 9.1 and 6.0 ppm correspond to the C8 and C1' of alkylated 5-GMP, respectfully. Upon alkylation, the glycocidic bond between the sugar and base is weakened, causing the bond to

break. The C1' is no longer being shielded by the nitrogenous base, causing a slight shift downfield. Similarly, the C8 is no longer being shielded by the deoxyribose sugar, causing a significant shift downfield. The reaction mimics the biological mechanism of ethylene dibromide and occurs through the mechanism in Figure 3.21 as followed:

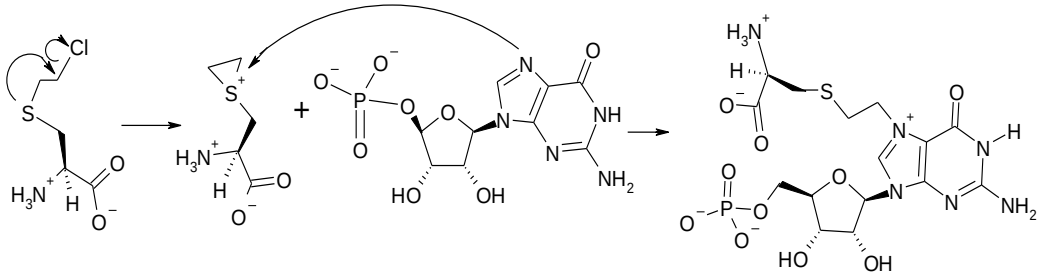

Figure 3.21 Proposed mechanism of hydroxychloro cysteine adduct and guanosine 5'-monophosphate reaction

VIII. Reaction of 3-Bromo-1-Propanol in Methanol/Sodium Methoxide

A. 1:3:1 cysteine, sodium methoxide, and 3-bromo-1-propanol

After successfully mimicking the reaction of ethylene dibromide with 5'GMP via cysteine, the reaction of similar halogenated hydrocarbons began. The first haloalkane was 1,3-dibromopropane. The corresponding alcohol, 3-bromo-1-propanol was utilized.

Through the methanol/sodium methoxide method, 3-bromo-1-propanol was reacted with cysteine. After the methanol was evaporated, a proton NMR was preformed. In the ^{1}H spectrum (Figure 3.22), three doublet of doublets, triplet, pentet, and doublet of triplets were observed. (The singlet at 3.2 is

residual methanol).

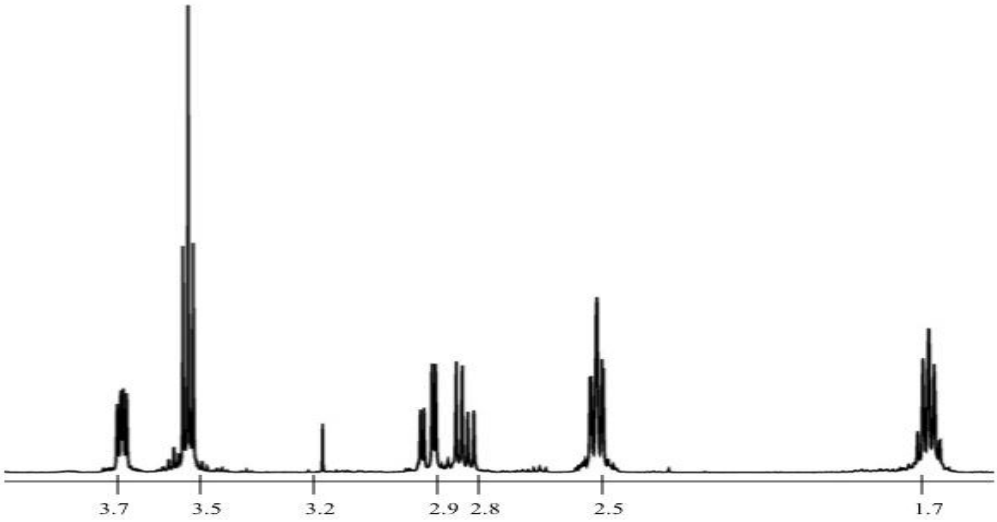

Figure 3.22 ^{1}H spectrum of hydroxypropyl cysteine adduct

The doublet of doublets at 3.72 ppm and doublet of doublets at 2.85 and 2.95 ppm correspond to an alpha hydrogen and beta hydrogens pair. The doublet of triplets at 2.5 ppm corresponds to carbon 1 in the propyl chain, the pentet at 1.65 ppm corresponds to carbon 2 in the propyl chain, and the triplet at 3.5 ppm corresponds to carbon 3 in the propyl chain (Figure 3.23) The characterization was further confirmed by a 2-dimensional COSY that confirms the predicted coupling patterns.

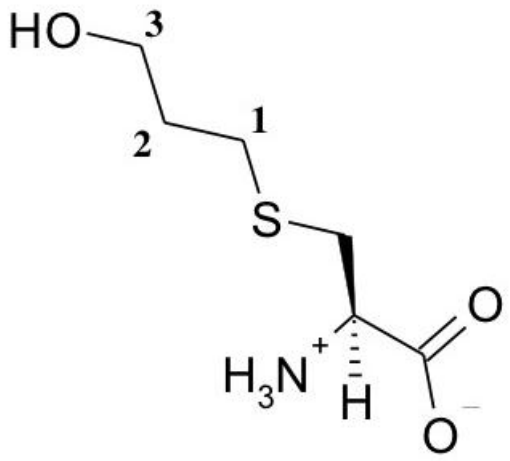

Figure 3.23 Hydroxypropyl cysteine adduct

The hydroxypropyl adduct forms through a similar mechanism as the hydroxyethyl. The mechanism is shown below (Figure 3.24):

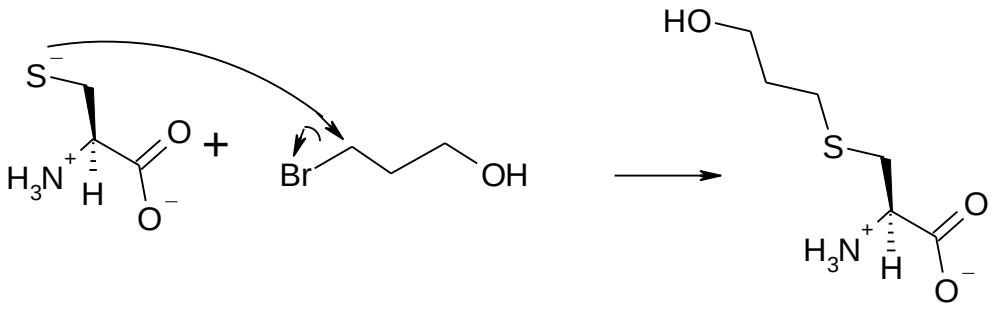

Figure 3.24 Proposed mechanism of hydroxypropyl cysteine adduct

B. 1:2:1 cysteine, sodium methoxide, 3-bromo-1-propanol with hydrochloric acid and guanosine 5'-monophosphate

Through the same method, the 3-bromo-1-propanol adduct was heated in

hydrochloric acid to replace the hydroxyl group with a chloride atom. Then, in another reaction, guanosine 5'-monophosphate was added to the mixture. A proton NMR was performed. The ^{1}H spectrum revealed no reaction with 5'GMP (Figure 3.25). It also revealed that the hydroxyl to chloride conversion was unsuccessful.

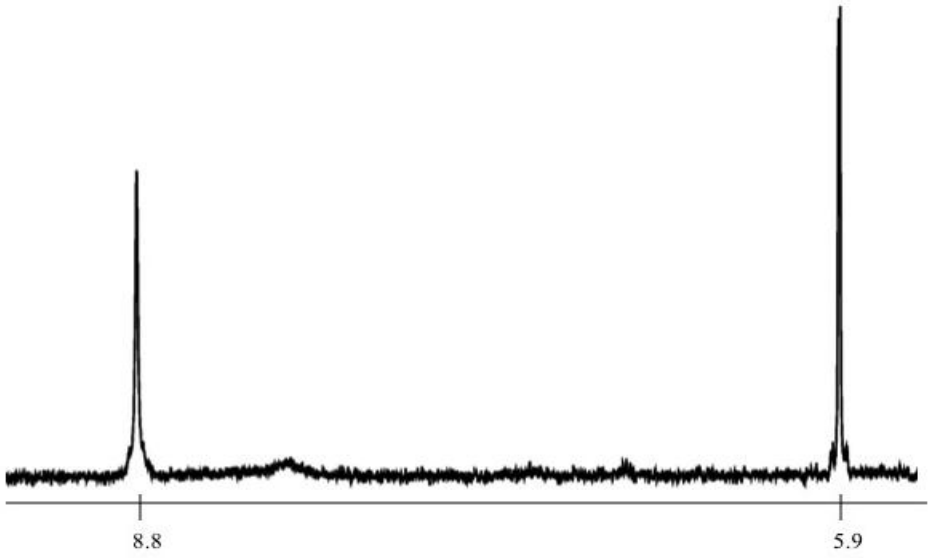

Figure 3.25 ^{1}H spectrum of unsuccessful chloropropyl cysteine adduct and guanosine 5'-monophosphate reaction

The method was repeated to ensure there was not human error. Again, a proton NMR revealed that the hydroxyl group was not replace with a chloride atom. The method was repeated again heated to above $100^{0}C$. The same results were observed. In a last attempt, the incubation time was extended to 10 hours, yet the same results were observed. Without a successful hydroxyl to chloride conversion, a reaction with 5'GMP will not occur.

IX. Reaction of 70% 1-Bromo-2-Propanol/30% 2-Bromo-1-Propanol in Methanol/ Sodium Methoxide

After the unsuccessful conversion on hydroxypropyl cysteine adduct to chloropropyl cysteine adduct, a new halogenated hydrocarbon was tested—a 70% 1-bromo-2-propanol/30% 2-bromo-1-propanol mixture. The haloalkane mixture was a combination of the two compounds—it has a three carbon chain like 3-bromo-1-propanol, however the alcohol is located at the second carbon, similar to 2-bromoethanol.

A. 1:3:1 cysteine, sodium methoxide, 70% 1-bromo-2-propanol/30% 2-bromo-1-propanol

The 70% 1-bromo-2-propanol/30% 2-bromo-1-propanol mixture was reacted with cysteine through the methanol/sodium methoxide method. After the methanol was evaporated, a proton NMR was performed. The ^{1}H spectrum revealed a successful reaction between the 70%/30% mixture and cysteine. (The reaction scheme is shown in Figure 3.26) However the spectrum wasn't as clean as the other adducts, making characterization difficult (Figure 3.27)

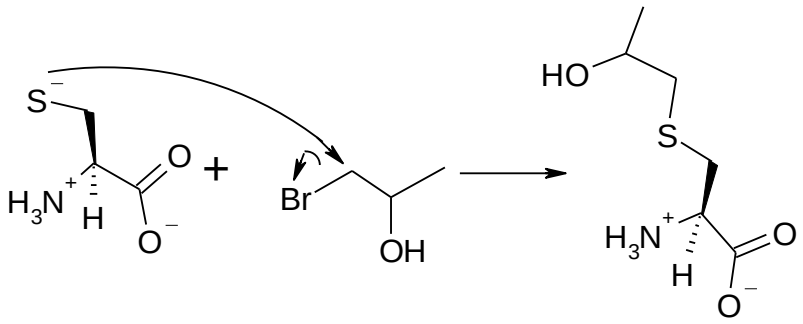

Figure 3.26 Proposed mechanism of 70% 1-bromo-2-propanol/30% 2-bromo-1-propanol crosslink with cysteine

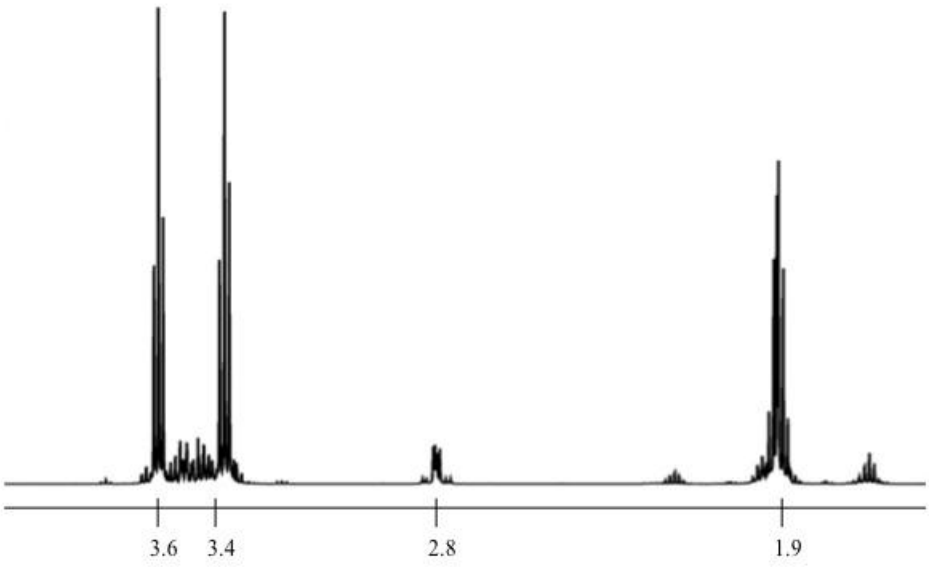

Figure 3.27 ^{1}H spectrum of 70% 1-bromo-2-propanol/30% 2-bromo-1-propanol crosslink with cysteine

A COSY, HMQC, and DEPT-135 were performed to characterize the adduct. The DEPT-135 revealed a set of 9 carbons that contained 4 pairs less than 0.1 ppm apart. The pairs correspond to the slightly different shifts of the 1-bromo-2-propanol cross-linked to cysteine diastereomers (Figure 3.29)

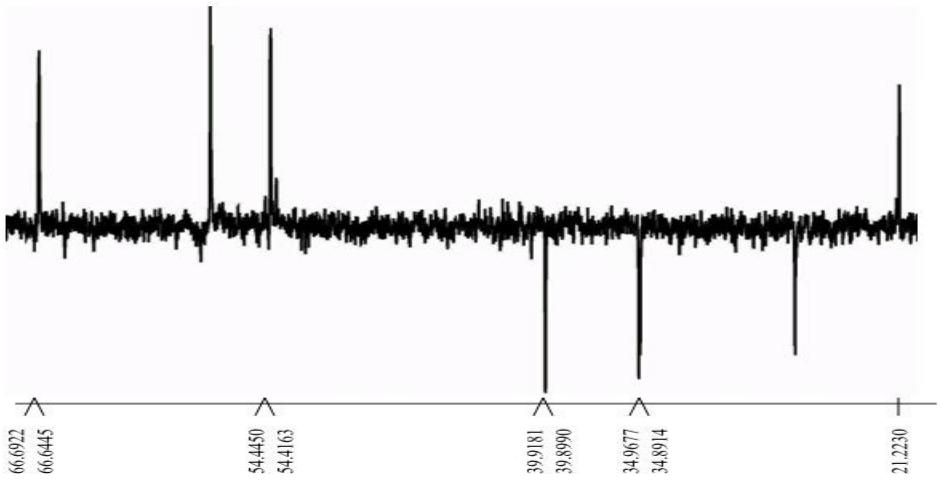

Figure 3.28 DEPT-135 spectrum of 70% 1-bromo-2-propanol/30% 2-bromo-1-propanol crosslink with cysteine

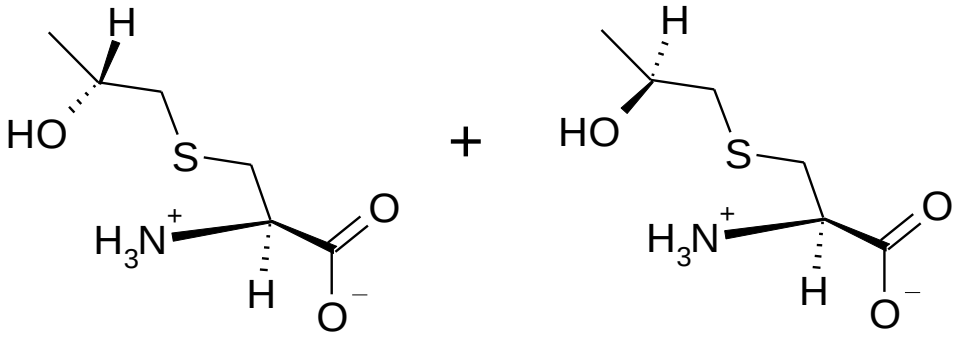

Figure 3.29 70% 1-bromo-2-propanol/30% 2-bromo-1-propanol crosslink with cysteine diastereomers

The characterization is as followed: The set of signals at 66.6922 and 66.6445 ppm corresponds to carbon 1 in the alcohol chain. The set of signals at 54.4450 and 54.4163 ppm corresponds to the beta hydrogens. The set of signals at 39.9181 and 39.8990 ppm corresponds to carbon 2 in the alcohol chain. The set of

signals at 34.9677 and 34.8914 ppm corresponds to carbon 3 in the alcohol chain. Lastly, the signal at 21.2230 corresponds to the alpha carbon. The characterization was aided by the coupling patterns observed in the 2-dimensional COSY.

The 2-bromopropanol adduct was not detected by NMR spectroscopy. Additional carbons were not observed in the DEPT-135 or HMQC, nor were additional coupling patterns observed in the COSY. Most likely, the adduct formed. However the signals were observed as noise, due to the minute concentrations.

B. 1:3:1 cysteine, sodium methoxide, 70% 1-bromo-2-propanol/30% 2-bromo-1-propanol with hydrochloric acid and guanosine 5'-monophosphate

The 70% 1-bromo-2-propanol/30% 2-bromo-1-propanol was boiled in hydrochloric acid to replace the hydroxyl group with a chloride atom. Then, in another reaction, guanosine 5'-monophosphate was added to the mixture. A proton NMR was performed. The ^{1}H spectrum revealed no reaction with 5'GMP (Figure 3.30). It also revealed that the hydroxyl to chloride conversion was unsuccessful. The experiment was repeated to ensure no human error, and the same results were observed.

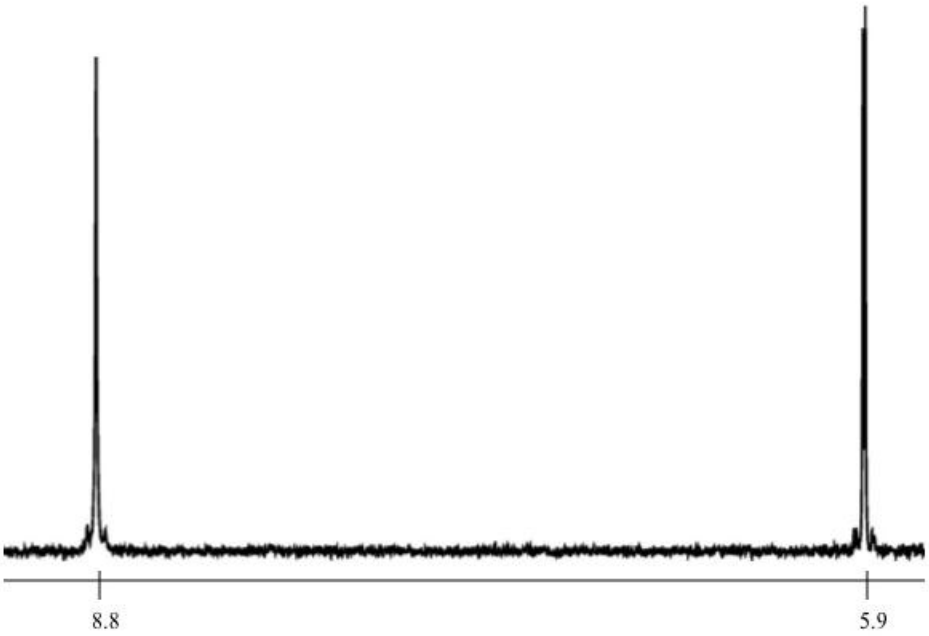

Figure 3.30 ^{1}H spectrum of unsuccessful 70% 1-bromo-2-propanol/30% 2-bromo-1-propanol cysteine adduct and guanosine 5'-monophosphate reaction

CHAPTER 4

DISCUSSION

In reaction IA, the formation of the ethylene bridged *N*-acetyl-L-cysteine dimer is not surprising. At pH 10, the thiol is partially deprotonated, forming an excellent nucleophile. And, the ethylene dibromide contains two bromines, which are excellent leaving groups. The first step, S_N2 reaction (alkylation of cysteine), is highly favored and understood. However, the second step is yet to be characterized.

In the first possible mechanism, the second step is an intramolecular S_N2 reaction that forms the episulfonium ion. The episulfonium ion that is formed is electron deficient, unstable, and highly reactive. With the additional deprotonated *N*-acetyl-L-cysteines available, another S_N2 reaction proceeds, forming the ethylene bridged *N*-acetyl-L-cysteine dimer.

In the second possible mechanism, the second step is a repetition of the first step. An S_N2 reaction occurs between a partially deprotonated *N*-acetyl-L-cysteine and the alkylated *N*-acetyl-L-cysteine. Determining the second step would be rather difficult because the intermediate concentration would be minute and unstable in the environment, making it unlikely to be detected.

However, it would be expected that, if the episulfonium ion formed, an alkylation with guanosine 5'-monophosphate would be favored. However, the partially

deprotonated thiol is a stronger nucleophile than the N7 atom of 5'GMP. Most likely, the *N*-acetyl-L-cysteine is out competing the 5'GMP in solution.

In reaction 1B, we attempted to increase the likelihood of alkylating guanosine 5'-monophosphate. The solution was made with a large excess of ethylene dibromide and 5'GMP. However, the ethylene bridged *N*-acetyl-L-cysteine dimer was still present and an alkylation with 5'GMP was not observed. The reaction confirmed that partially deprotonated thiol is a stronger nucleophile than the N7 atom of 5'GMP.

Also in reaction I A and B, the formation of oxidized *N*-acetyl-L-cysteine is expected. The sample is exposed to air, so an abundance of oxygen is available. The oxidation of cysteine and *N*-acetyl-L-cysteine is expected and was observed in all deuterium oxide reactions at pH 10.

In reaction II A and C, we attempted to stop the quick episulfonium ion formation by replacing the second bromine in ethylene dibromide with a hydroxyl group. The leaving ability of hydroxyl is poor. However, the leaving ability of water is fair and a possibility in the deuterium oxide solution. As seen in the results, 5'GMP was not alkylated and the ethylene bridged *N*-acetyl-L-cysteine dimer did not form. It can be concluded that the episulfonium ion did not form, due to the poor leaving ability of the hydroxyl group.

In additional to *N*-acetyl-L-cysteine side reactions, 2-bromoethanol formed ethylene oxide through an intramolecular S_N2 reaction. Although biologically irrelevant, the product reacted with 5'GMP. Therefore, 2-bromoethanol was not an ideal substrate in deuterium oxide reactions.

Although not known at the time, the hydroxyethyl adduct did form in reaction II A and C in a small concentration. However with the oxidized cysteine, ethylene oxide, and 5'GMP and ethylene oxide reaction, too many side reactions were present for the reaction to be used as a model.

To further understand the effects of leaving groups, reactions III (*N*-acetyl-L-cysteine and ethylene dichloride) and IV (*N*-acetyl-L-cysteine and 2-chloroethanol) were performed. Chlorine, in comparison to bromine, is a fair leaving group. And, the reactions were expected to proceed similarly to ethylene dibromide and 2-bromoethanol, however at a much slower rate.

The products of both reactions were similar. *N*-acetyl-L-cysteine oxidized in both reactions. However, in reaction III, the presence of the sulfur bridged *N*-acetyl-L-cysteine dimer was smaller. And, in reaction IV, the presence of hydoxyethyl adduct was significantly smaller. The lack of both products suggests that the episulfonium ion did not form as readily and the effects of replacing bromine with chlorine is significant.

Due to all the side reactions, we proceeded to the methanol/sodium methoxide method described in Marsh et. al [16]. Our first attempt was to mimic their cross link of bromomethyl acetate with S-(1-Acetoxymethyl)glutathione. Instead of using a tripeptide, we proceeded with cysteine (reaction V). However, we were not successful. We were unable to characterize the adducts formed in the reaction from the NMR spectrums. And, the predicted products were not observed in the LC/MS data. Further LC/MS experiments will be conducted, including experiments in negative mode.

Similarly, our second attempt with ethylene dibromide (reaction VI) was also unsuccessful. We were unable to characterize the adducts formed in the reactions from the NMR data. And, the predicted products were not observed in the LC/MS spectra.

However, our third attempt was successful (reaction VII). We cross-linked 2-bromoethanol with cysteine to form the hydroxyethyl adduct. In contrast to the deuterium oxide reaction, the cysteine did not oxide and the reaction was very clean. However, as expected, the hydroxyethyl adduct did not react with 5'GMP, because of the poor leaving group.

To proceed in the reaction, the hydroxyl group needed to be replaced with a better leaving group. Although replacing the hydroxyl group with a bromine atom is ideal and an exact mimic of ethylene dibromide, we decided to proceed with chlorine. Hydrobromic acid is rather dangerous and deemed unsafe to use in our application. So, the hydroxyethyl adduct was heated in hydrochloric acid instead.

By looking at the NMR data, approximately 50% of the hydroxyethyl adducts reacted to form the chloroethyl adduct. The yield is a concern that will be addressed in future experiments. The hydroxyethyl adduct will be boiled in hydrochloric acid with zinc chloride as a catalyst. Regardless of the yield, the chloroethyl adduct did react with 5'GMP, mimicking the ethylene dibromide mutagenic reaction.

Two additional halogenated hydrocarbons were utilized in the methanol/sodium methoxide method (3-bromo-1-propoanol in reaction VIII and 70% 1-bromo-2-propanol/30% 2-bromo-1-propanol in reaction IX). Both adducts formed a clean hydroxypropyl and 2-hydroxypropyl adduct, respectfully. However, neither reaction successfully replaced the hydroxyl group with a chloride atom.

The unsuccessful replacement may be due to the extra distance between the nucleophillic sulfur and leaving group. The extra carbon makes the intramolecular reaction less favorable. Also, if the leaving group replacement proceeds through the episulfonium ion, as we predict, water is a poor leaving group. (The hydroxyl picks up a proton from the hydrochloric acid). However, the 2-hydroxypropyl adduct should not be effected by the additional carbon, since the replacement is occurring at carbon 2. Addition experiments, including the zinc chloride catalyzed reaction, will be done to understand the chemistry.

However, the unsuccessful hydroxyl to chloride replacement in the 70% 1-bromo-2-propanol/30% 2-bromo-1-propanol may be due to steric hindrance. The bromine is located on a 2° carbon, in comparison to a 1° carbon in ethylene dibromide. The unsuccessful conversion reinforces the S_N2 character of ethylene dibromide and suggests that EDB is unique amongst halogenated hydrocarbons in episulfonium ion formation. Other halogenated hydrocarbons like 3-bromopropane and 2-bromopropane may proceed through a different mutagenic mechanism in comparison to ethylene dibromide.

CHAPTER 5

CONCLUSION

Through many unsuccessful experiments, the reactivity of *N*-acetyl-L-cysteine and cysteine under different conditions was explored. In deuterium oxide at pH 10, *N*-acetyl-L-cysteine formed many irrelevant side products, including an ethylene bridged *N*-acetyl-L-cysteine dimer and *N*-acetyl-L-cystine. Similarly, cysteine formed an ethylene bridges cysteine dimer and cystine. In addition, we found that 2-bromoethanol at pH 10 formed ethylene oxide, which reacted with 5'GMP. And, it was found that dichloroethane and 2-chloroethanol reacted similarly to dibromoethane and 2-bromoethanol, however, at a much slower rate.

Both *N*-acetyl-L-cysteine and cysteine formed the hydroxyethyl adduct with 2-bromoethanol in deuterium oxide at pH 10. However, there were to many irrelevant side products for the reaction to be a model. New conditions were explored in a sodium methoxide and methanol medium. A successful alkylation occurred by reacting cysteine with 2-bromoethanol, replacing the hydroxyl leaving group with chloride, and cross-linking with 5'GMP. However, the reaction could not be mimicked with similar halogenated hydrocarbons, including 3-bromo-1-propanol, and a 70% 1-bromo-2-propanol/30% 2-bromo-1-propanol mixture.

Although the biological mechanism was mimicked, a simple, inexpensive model was not successfully developed. However, the unsuccessful experiments lead to understandings and new ideas for other possible models. One possibility is the use of cysteine containing peptides or proteins to reduce the number of side reactions. In a few experiments, I attempted to use glutathione, a tripeptide that includes cysteine. However, the peptide was not soluble in deuterium oxide or methanol. Another possibility is the use of alkylguanine transferase or cysteine proteases, cysteine containing proteins. Also, the use of peptides and proteins are more biologically relevant.

Other future reactions will form hydroxyl adducts with other halogenated hydrocarbons through the methanol/sodium methoxide method. Currently, a 3-bromo-1,2-propanediol experiment is awaiting characterization. Also, other leaving groups will be explored to find a safe, yet reactive, alternative to chloride. Lastly, an LC/MS technique will be developed to aid in characterization of adducts, and will be used to characterize the adducts of ethylene dibromide and bromomethyl acetate in methanol/sodium methoxide.

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