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**Synthesis and Quantitative Analysis of The Alkylation Products of
2-Deoxycytidine With 2-(Methylthio)ethyl-N-nitrosocarbamate
In Aqueous Solution at pH 8 and 9**

Thesis

Submitted to

The Chemistry Department of the

University Of Dayton

In Partial Fulfillment of the Requirements for

The Degree

Master of Science in Chemistry

By

Rhonda Ann Reid

Dayton, Ohio

May, 2008

APPROVED BY:



Dr. Kevin Church
Research Advisor
Committee Chairman



Dr. Gary Morrow
Committee Member



Dr. Howard Knachel
Committee Member

ABSTRACT

Synthesis And Quantitative Analysis Of The Alkylation Products Of 2-Deoxycytidine With 2-(Methylthio)ethyl-N-nitrosocarbamate In Aqueous Solution At pH 8 and 9.

Name: Rhonda Ann Reid
University of Dayton

Advisor: Dr. Kevin Church

Alkylating agents such as Lomustine and Carmustine have been used clinically for the treatment of primary and metastatic brain tumors and as a secondary therapy in Hodgkins's disease for many years. These alkylating agents decompose to a cyclic chloronium ion intermediate which eventually crosslinks DNA. Two new N-nitrosocarbamates were developed in Dr. Benin's lab bearing either a 2-(methylthio)ethyl (**I**) or 2-(dimethylamino)ethyl (**II**) group. These carbamates decompose under basic conditions to form a sulfonium ion or an aziridinium ion. We were able to follow the ability of (**I**) to alkylate a nucleoside in pH 8 and 9 aqueous buffer solution. Authentic alkylation standards were synthesized by chemical means and then used to quantitate the formation of alkylation products by HPLC. The rate constant, and half life could not be determined for alkylated cytidine (**1**) using nitrosocarbamate (**I**) at either pH. Nitrosocarbamate (**I**) alkylates thymidine following first order kinetics with rate constant of 0.00480 min^{-1} in pH 9 buffer solution. The total % yields (based on cytidine reactant) of alkylated product are 0.31% for (**I**) at

pH 8 and 0.32% at pH 9. The total % yield (based on thymidine reactant) of alkylated product for **(I)** is 2.91%.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS/ SYMBOLS

DNA- Deoxyribonucleic Acid

N- Nitrogen

O- Oxygen

S- Sulfur

HPLC- High Pressure Liquid Chromatography

DMSO- Dimethyl Sulfoxide

IR- Infra Red

^1H - Proton

^{13}C - Carbon 13

COSY- Correlation Spectroscopy

HETCOR- Heteronuclear Correlation

HMBC- Heteronuclear Multiple Bond Correlation

NMR- Nuclear Magnetic Resonance

TBAF- Tetrabutyl Ammonium Fluoride

THF- Tetrahydrofuran

TIPDS- 1,1,3,3-Tetraisopropylidisiloxanylidene

PTSA- p-Toluene sulfonamide

TMS-Tetramethylsilane

PTC- Phase Transfer Catalysis

MeOH- Methanol

TLC- Thin Layer Chromatography

DEPT NMR- Distortion Enhancement by Polarization Transfer

mM- Millimolar

min- Minutes

ln- Natural log

C- Concentration

mm- Millimeter

μ m- Micrometers

ml- Milliliter

Å- Angstrom

UV- Ultraviolet

Cm- Centimeters

mmol- Millimoles

g- Grams

μ l- Microliters

Chapter 1

Introduction

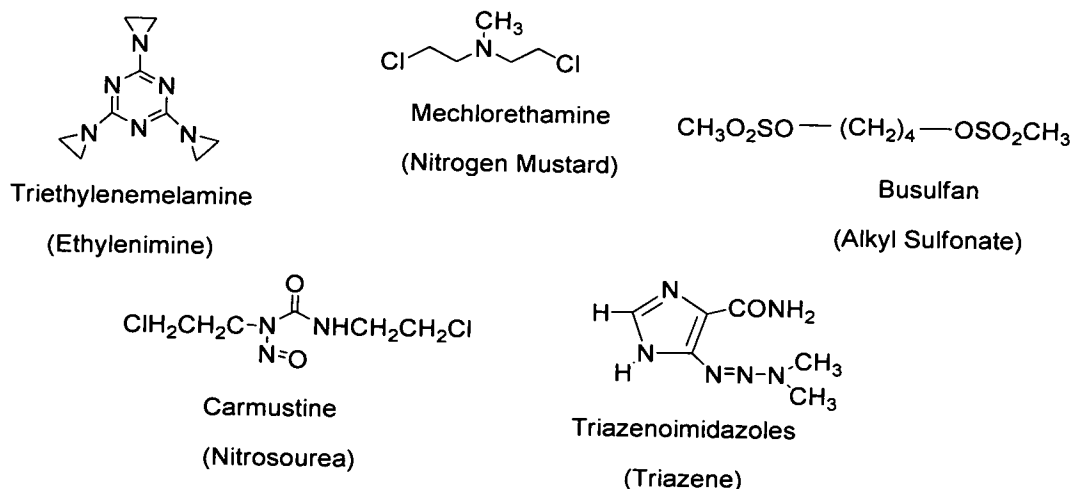
The chemotherapy of neoplastic disease has become increasingly important in recent years.¹ Cancer chemotherapy has undergone no spectacular breakthrough like the discovery of penicillin provided for antibacterial chemotherapy.¹ However, there has been substantial progress in many aspects of cancer research. In particular, an increased understanding of tumor biology has led to elucidation of mechanisms of action for antineoplastic agents.¹ What prompted the success of alkylating agents in cancer research were the studies done on nitrogen mustards to see if they showed any toxicity to cancer cells. A variety of nitrogen mustards were synthesized and found to show selectivity towards lymphoid tissue. This observation led to the crucial suggestion that nitrogen mustards be tested against tumors of the lymphoid systems in animals. Success in this area was followed by cautious human trials that showed mechlorethamine to be useful against Hodgkin's disease and certain lymphomas. This work was classified during World War II, but was finally published in a classic paper by Gilman and Phillips in 1946.² In this paper, the chemical transformation of nitrogen and sulfur mustard to cyclic "onium" cations was described and the locus of their interaction with cancer cells which, however

showed toxicity to rapidly proliferating cells in bone marrow and the gastrointestinal tract.¹

The major types of alkylating agents used in medicine today and in the past include: nitrogen mustards, ethylenimines, alkyl sulfonates, nitrosoureas, and triazenes,³ shown below in (Figure 1).

Figure 1

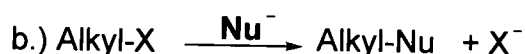
Examples of Five Of The More Important Classes of Alkylating Agents Utilized in Cancer Chemotherapy



These alkylating agents represent a class of cytotoxic molecules that have the ability to become strong electrophiles that form covalent linkages to electron-rich groups (nucleophiles) of DNA.³ These particular alkylating agents shown in (Figure 1) have the ability to replace a hydrogen atom with an alkyl group under physiological conditions. They are generally described as substitution reactions by N, O, and S heteroatomic nucleophiles reacting with electrophilic alkylating agents.¹ The two most common types of nucleophilic substitution reactions are

S_N1 (a), a step wise reaction via an intermediate carbenium ion, and S_N2 (b), a concerted reaction,¹ as shown in (Scheme 1) below:

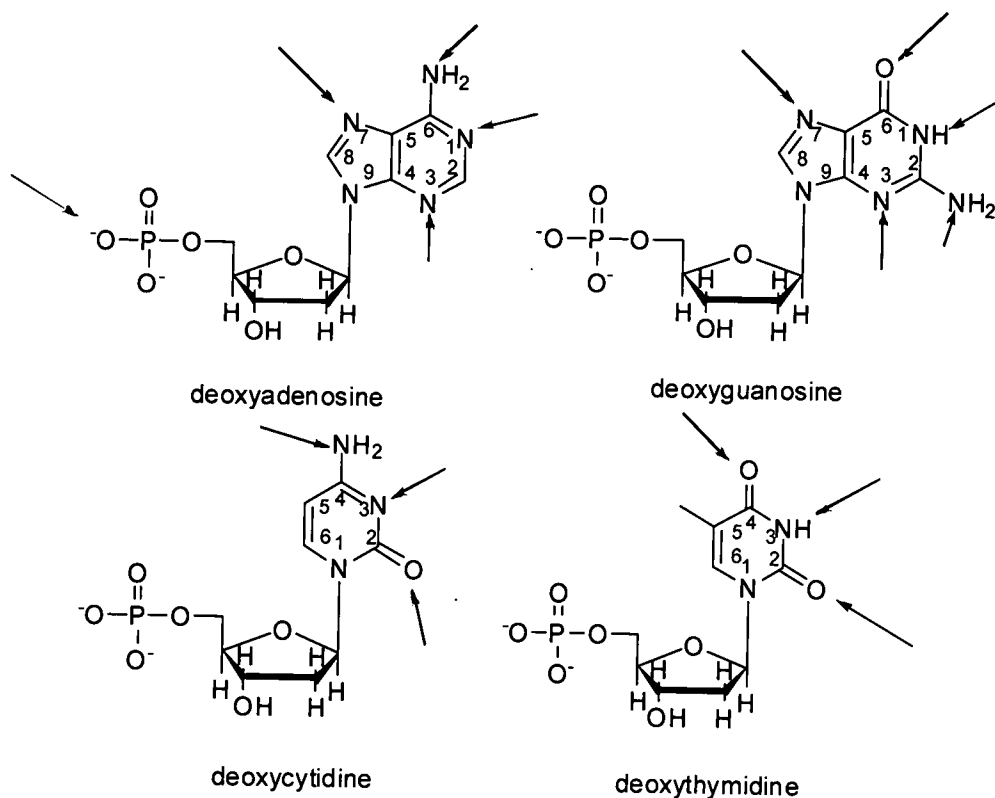
Scheme 1
The Most Common Types of Nucleophilic Substitution Reactions



Alkylating reagents can be divided into S_N1 and S_N2 types based on the mechanism of alkylation. The alkylation susceptibility of each site on the bases or backbone of DNA varies depending on the reagents used. The resulting lesions also have different mutagenic and cytotoxic effects.⁵ Simple alkylating agents such as ethylenimines and methanesulfonates undergo S_N2 reactions. Alkylating agents such as nitrogen mustards, which have a nucleophile capable of anchimeric assistance (neighboring group participation), can undergo S_N1 or S_N2 type reactions, depending on the relative rates of the aziridinium ion formation and the nucleophilic attack on the aziridinium ion.¹

It has been shown that the most reactive nucleophilic sites on DNA are N-7 guanine > N-3 adenine > N-1 adenine > N-1 cytidine, N-3 cytosine, O-6 guanine. The phosphate groups also can be alkylated,^{1,4} which is shown below in (Figure 2).

Figure 2
Possible Places of Alkylation On The Bases Of DNA



The regioselective modification of DNA by electrophiles has been attributed to steric factors, electronic factors, and hydrogen-bonding effects, but attempts to mechanistically model and predict alkylation patterns have had limited success.⁶ For example, some of the nucleophilic sites are in the interior of the DNA double helix and are sterically blocked. Only nucleophilic centers in the major and minor grooves or in the walls of the double helix are accessible to alkylating agents.¹ On the other hand the weakly carcinogenic alkylating agents (e.g., methyl methanesulfonate) primarily modify the pyridine-type ring nitrogen sites (e.g., the 7-position of guanine residues)⁷ while the more potent

carcinogenic alkylating agents (e.g., the N-alkyl-N-nitroso compounds) modify exocyclic oxygen centers (e.g., O⁶ of guanine residues) in addition to ring nitrogen sites.^{8,9} Many anticancer drugs display only a low degree of selectivity, often causing severe or even life threatening toxic side effects, thus preventing the application of doses high enough to kill all cancer cells. These limitations have led to the continued search for antineoplastic agents with improved selectivity to malignant cells.¹⁰

There has been limited research on the alkylation of 2-deoxycytidine, while there has been an enormous amount of research done on the identification of O⁶-alkylguanine from reaction of DNA with 1-alkyl-1-nitrosoureas using alkyl groups such as methyl^{11,12}, ethyl^{13,14}, and n-butyl¹⁵ derivatives, which are already reported in vitro. The nitrosoureas, including streptozotocin, BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea), and related compounds are useful agents for the treatment of lymphomas and certain other malignancies. These anticancer agents are highly active with a broad spectrum in experimental systems and have an established clinical role as antitumor agents.¹⁶ Their clinical roles, however, are limited because they show delayed and cumulative toxic side effects. Bone marrow suppression is prevalent and dose limiting. Many attempts to obtain new analogs with higher effectiveness and /or lower toxicity have so far not been convincingly successful.

Our goal is to study the reactivity of nucleosides or DNA with new N-nitrosocarbamates synthesized by Dr. Benin's¹⁷ lab. These N-nitrosocarbamates are model compounds related to nitrosoureas that have been shown to have

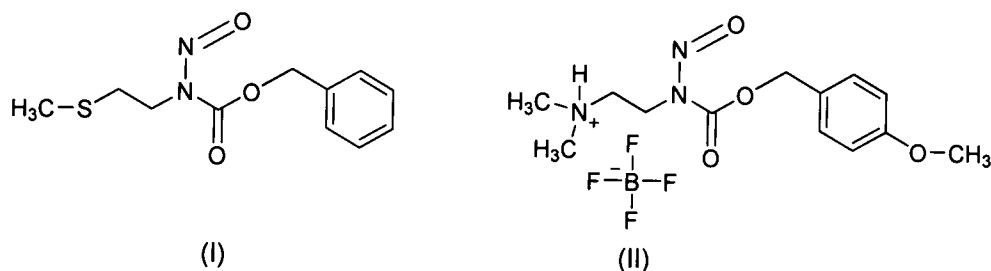
anticancer activity. Their toxicity should be reduced since isocyanate is not formed in their aqueous decomposition. The following questions will be answered:

- Can alkylated product standards be synthesized, and characterized?
- Can we follow the degree of alkylation by reverse phase HPLC methods?
- Can we determine the half life, rate constant, and the mechanism of alkylation?

We synthesized alkylated nucleosides to act as authentic standards to follow the formation of the compounds that we believe will be produced. Isolating these standards is very important because when we react cytidine, thymidine or adenosine with 2-(Methylthio)ethyl-N-nitrosocarbamate (I) or 2-(Dimethylamino)ethyl-N-nitrosocarbamate (II)¹⁷, (Figure 3) we are expecting low alkylation yields when decomposed in aqueous solution. We predict very low yields due to the fact that water is nucleophilic and most of the sulfonium ion or aziridinium ion will react with the water, giving alcohol products. Reaction with buffer is also possible which will further reduce yields.

Figure 3

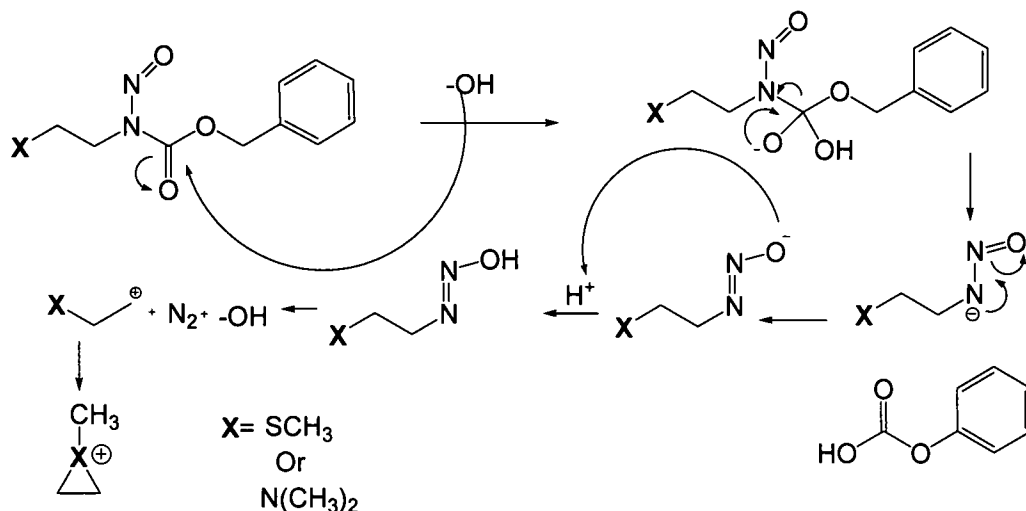
N-alkyl-N-nitrosocarbamates



These N-alkyl-N-nitrosocarbamates decompose under basic conditions to form a benzyl alcohol and a sulfonium ion (electrophile) or an aziridinium ion (electrophile) depending on if **(I)** or **(II)** is used, as shown in (scheme 2) below¹⁸.

Scheme 2

Decomposition of 2-(Methylthio)ethyl-N-nitrosocarbamate



Once this reactive specie is produced, its rates, extents, and sites of reaction on cellular macromolecules, such as DNA, would be governed by their intrinsic electrophilic reactivity. This reactivity could lead to substitution at a variety of sites on the multidentate heterocyclic nucleic acid base components of 2-deoxycytidine which is shown in (figure 4), 2-deoxyadenosine which is shown in (figure 5), and thymidine which is shown in (figure 6).¹⁹ Once the alkylation takes place at one of these positions an interstrand cross-link is formed, leading to both single and double-stranded DNA breaks and initiating both apoptotic and necrotic cell death by induction of p53 and / or p21.²⁰ Methylating agents result in monofunctional adducts on DNA and is known to be both mutagenic and

carcinogenic. O⁶-Methylguanine adducts in DNA disrupt guanine-cytosine base pairing, resulting in stalling of DNA polymerase during DNA synthesis, followed by insertion of incorrect base. Thymidine is incorporated instead of cytosine, resulting in GC-AT transitions.²¹ This initiates mismatch repair (MMR) at O⁶-MG:T sites, setting up a futile cycle which eventually activates ATM- and ATR-dependent signaling pathways. This process is dependent on functional MMR at low drug concentrations.²²

Figure 4
Possible Alkylated Products of 2-deoxycytidine

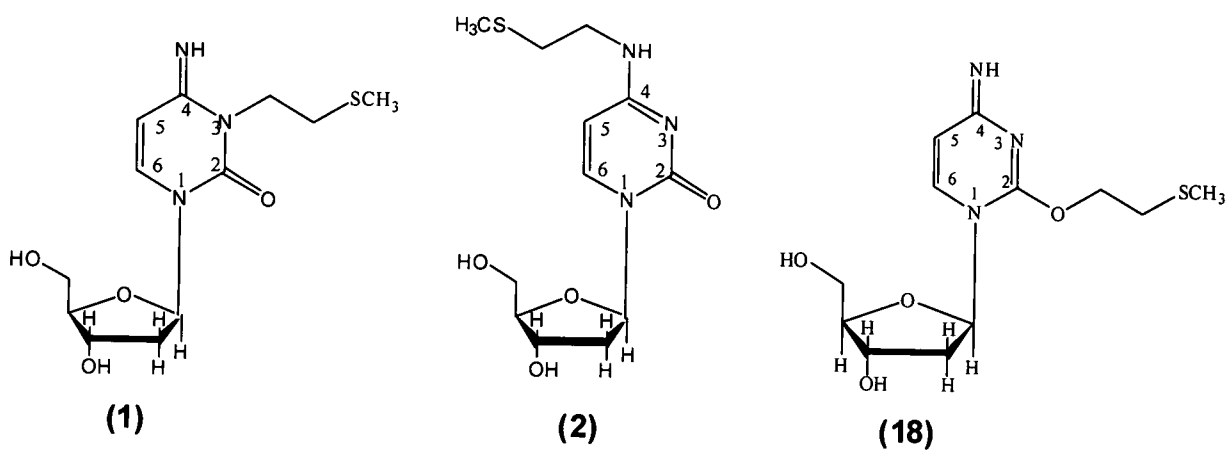


Figure 5

Possible Alkylated Products of 2-deoxyadenosine

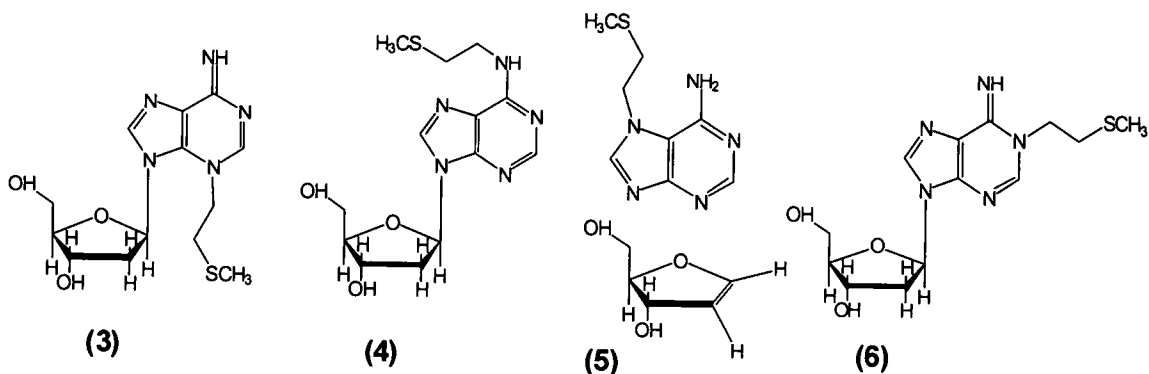
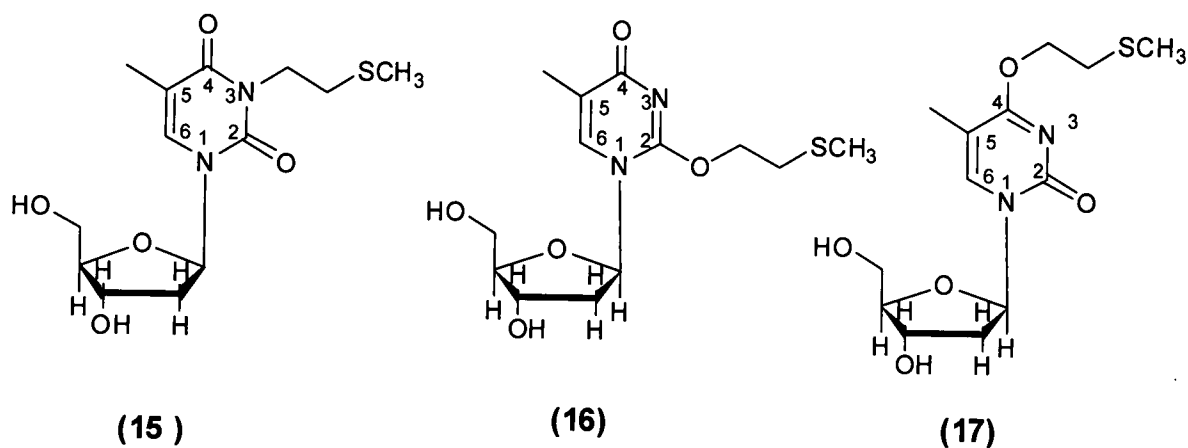


Figure 6

Possible Alkylated Products of Thymidine



Our primary goal has been to generate the onium ions derived from the new N-nitrosocabamates in aqueous solution and react it with a nucleoside. Doing this allows us to determine the mechanism of alkylation and to predict the reaction with DNA and possible therapeutic efficacy. All authentic standards will be purified by flash chromatography and structures will be determined by IR, MS,

H^1 NMR, C^{13} NMR, and 2D NMR. A reverse phase HPLC methodology will then be developed to follow the formation of alkylation products.

CHAPTER II

RESULTS AND DISCUSSION

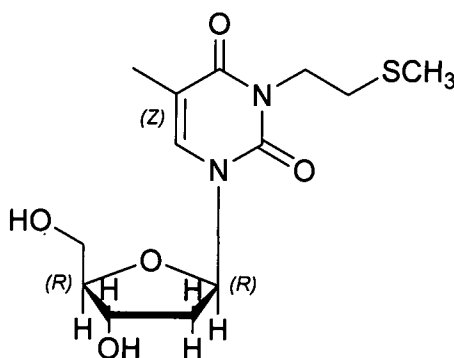
Overview

We have isolated the protected nucleosides **(7)** and **(8)** which are shown in scheme 4 and 9, by reacting each nucleoside with 1,3 Dichloro-1,1,3,3-tetraisopropyldisiloxane in pyridine to generate 5', 3' bis protected cytidine, and adenosine as shown in (Schemes 4 and 9 respectively). In our efforts to isolate authentic standards for alkylated cytosine and adenosine, we were successful in reacting **(9)** with **(8)** as illustrated in (Scheme 7). This was done using N,N-Diisopropylethylamine as the base and benzene as the solvent. Using these reactants we were able to isolate **(10)** (which is shown in scheme 7) in an overall yield of 75.3%. We were also successful in removing the disiloxane protecting group by reacting **(10)** with TBAF in tetrahydrofuran (THF). Preparatory TLC was performed to obtain an overall yield of 70%. These structures were confirmed using C^{13} NMR, H^1 NMR, 2-Dimensional HETCOR, COSY, and techniques such as HMBC. After isolating the authentic standard by chemical means, we were able to use the standard to quantitate the formation of alkylated product by High Pressure Liquid Chromatography (HPLC). A calibration curve was generated to calculate the total yield of each simulated biological reaction of alkylated

nucleoside (**1**). As stated in the introduction we were initially going to study two N-nitrosocarbamates (I), (II), which upon hydrolysis should form cyclic sulfonium ion or aziridinium ion intermediates. We studied N-nitrosocarbamate (I) and its ability to alkylate a nucleoside in pH 8 and 9 aqueous buffer. We were able to follow the rate of alkylation for these reactions at pH 8 and 9 but we were not able to determine rate constants or half life due to the fact that the data obtained from the HPLC was on the edge of detection limits, but we were able to determine how much was actually formed. The total % yields (base on cytidine reactant) of alkylated product are 0.31% for (I) at pH 8 and 0.32% at pH 9. These percent yields seem low, but the major nucleophiles present in the reaction are the water and buffer solutions.

Figure 7

3-[2-(Methylthioethyl)]thymidine



(15)

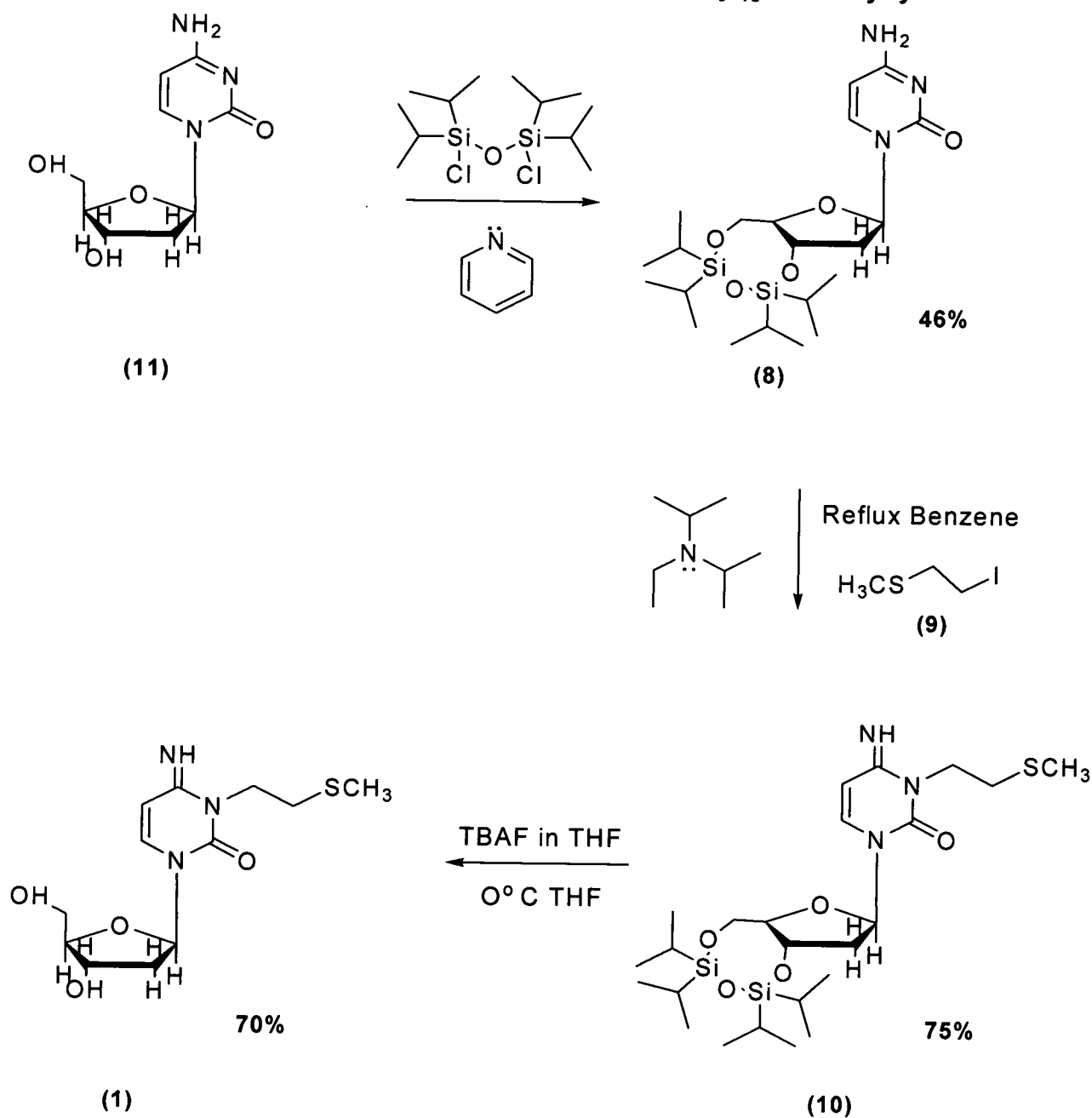
Heidi Wheatcraft²³ had the opportunity to work on thymidine using the same N-nitrosocarbamates. Her authentic standard (**15**), which is shown in (Figure 7), was used to determine how much alkylated product is produced at pH

9. Her experiments with **(15)** at pH 8 followed first order kinetics with a rate constant of $.00393 \text{ min}^{-1}$ for nitrosocarbamate (I). Total % yield of 1.34%.

Shown below in (scheme 3) is the overall multiple step synthesis of the authentic standard **(1)**.

Scheme 3

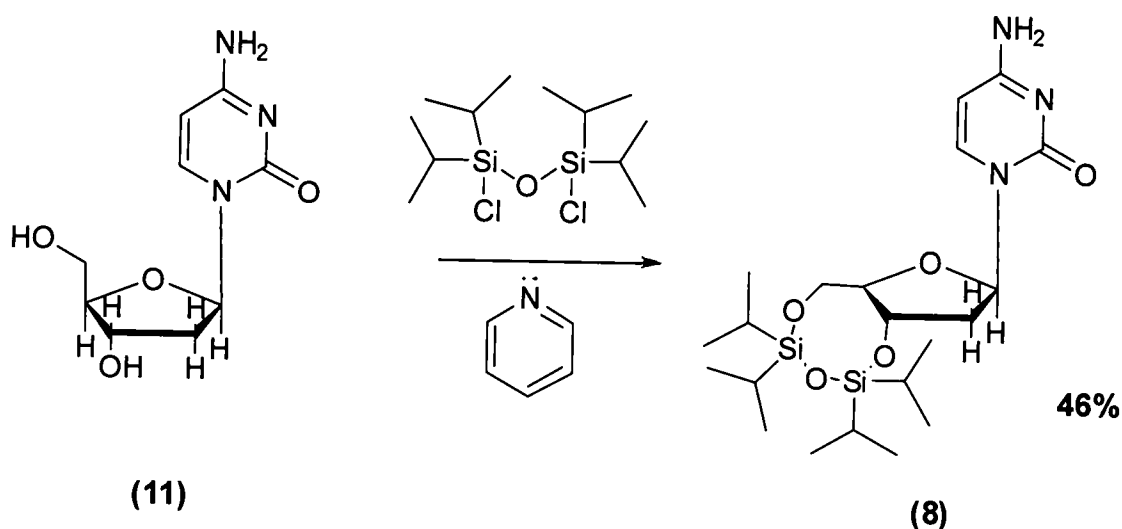
Multiple Step Synthesis of 3-[2-(methylthioethyl)]-2-deoxycytidine



2.1 Generation of 3',5'-[1,1,3,3-tetraisopropylidisiloxy] -2-deoxycytidine

Scheme 4

The protection of 3' and 5' Hydroxyl Groups on 2-deoxycytidine



The simultaneous protection of the 3' and 5' hydroxyl groups of nucleosides is a common problem in organic synthesis.²⁴ An alternative silicon-based group offers a wider repertoire of conditions for mild deprotection and such a group was devised by Markiewicz: the 1,1,3,3-tetraisopropylidisiloxanylidene group (abbreviated TIPDS).²⁵⁻²⁶ TIPDS groups are stable to water, 0.3M PTSA in dioxane, 10% CF₃COOH in CHCl₃, 5M NH₄OH in dioxane-H₂O (4:1), and tertiary amines in pyridine. This particular protecting group is used specifically for 1,3- and 1,4-diols, and can be deprotected with fluoride or TMS-I. Knowing that this protecting group is useful for this type of chemistry and that it is stable under a wide variety of conditions, it was used to protect the 1,3-diol in (11). These positions on the sugar were protected so there would be no alkylation taking

place on the deoxyribose sugar and leaving only the positions on the pyrimidine ring accessible to alkylation.

The isolation of **(1)** was performed in a step wise synthesis, with the first step outlined in (Scheme 4). Commercially available 2-deoxycytidine·H₂O was reacted with 1.1 equivalents of 1,3 Dichloro-1, 1, 3,3-tetraisopropyldisiloxane in pyridine. The lone pair on the 3', 5' oxygens forms a strong bond to silicon displacing the chlorides on both ends of the disiloxane. This 8-membered ring, which is formed by rapid reaction first at the least hindered hydroxyl group, (primary hydroxyls react approximately 1000 times faster than secondary hydroxyls) is followed by a second intramolecular silylation with the next proximate hydroxyl at C-4.²⁷⁻²⁸ Pyridine is needed to deprotonate the resultant oxonium ions in the mechanism. Acid extraction was done followed by flash chromatography for complete purification. We were able to obtain a 45% yield of **(8)**, whose structure was verified by H¹ NMR, C¹³ NMR, and 2D NMR.

2.2 Attempted preparation of 3',5'-[1,1,3,3-tetraisopropyldisiloxy]-3-[2-(methylthioethyl)]-2-deoxycytidine

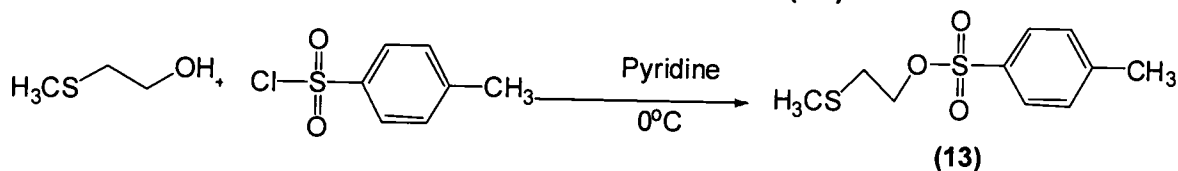
One of our earlier approaches in alkylating the N-3, O-4, O-6 position of **(11)** was to try phase transfer catalysis using 2-chloroethylmethylsulfide, according to a literature procedure. This study reported a detailed study of N⁶- or N¹-alkylation of adenosines via 6-N-acylated species by the use of phase transfer catalysis (PTC). It was found that, when the benzoyl group was chosen as the N-

acyl group, N1-alkylated products were obtained along with 6-N-alkylated products.²⁹

Since phase transfer catalysis has been proven to work by other researchers,²⁹ we decided to use this route using chloride instead of bromide. We reacted **(8)** with 2-chloroethyl methyl sulfide in benzene in a vigorously mixed solution of H₂O, tetrabutylammonium bromide, and sodium hydroxide. We used a larger amount of the Bu₄NBr because the literature²⁹ stated that if a small amount of the catalyst is used the reaction rate is extremely slow, and a 12hr reaction time was necessary to ensure completion. The reaction also proceeds very slow when a weaker base is used in the aqueous phase.²⁹ We were not able to generate any compound with increasing the catalysis or using a stronger base; this could have been due to the weaker leaving group (Cl) relative to bromide. We attempted to synthesize tosylate **(13)** by reacting 2-(methylthio) ethanol with p-toluenesulfonyl chloride in pyridine, which is shown below in (scheme 5). This reaction was performed to see if we could isolate a compound with a better leaving group that is cable of reacting faster with the protected cytidine to produce **(1)**.

Scheme 5

Attempted Preparation of **(13)**



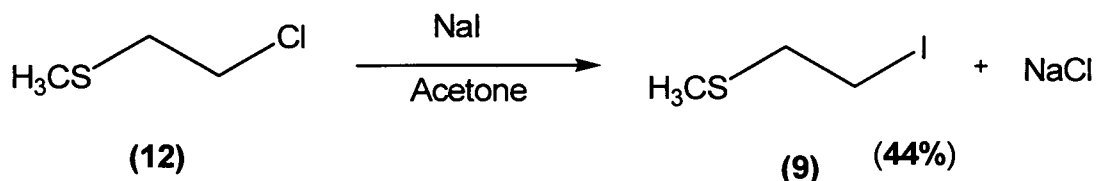
Scheme 5 was not successful. No compounds were isolated using the phase transfer route. Since the first two routes were unsuccessful we decided to pursue a different route.

2.3 Preparation of 2-Iodoethylmethylsulfide

As mentioned previously in the results some of our early attempts to generate **(1)** were unsuccessful. This is likely due to the leaving group, which is known to serve two purposes in a S_N2 reaction, it must polarize the C-X bond making the carbon atom electrophilic, and it must leave with the pair of electrons that once bonded it to the electrophilic carbon atom.³⁰

Scheme 6

Preparation of S_N2 alkyl halide from 2-chloroethylmethylsulfide



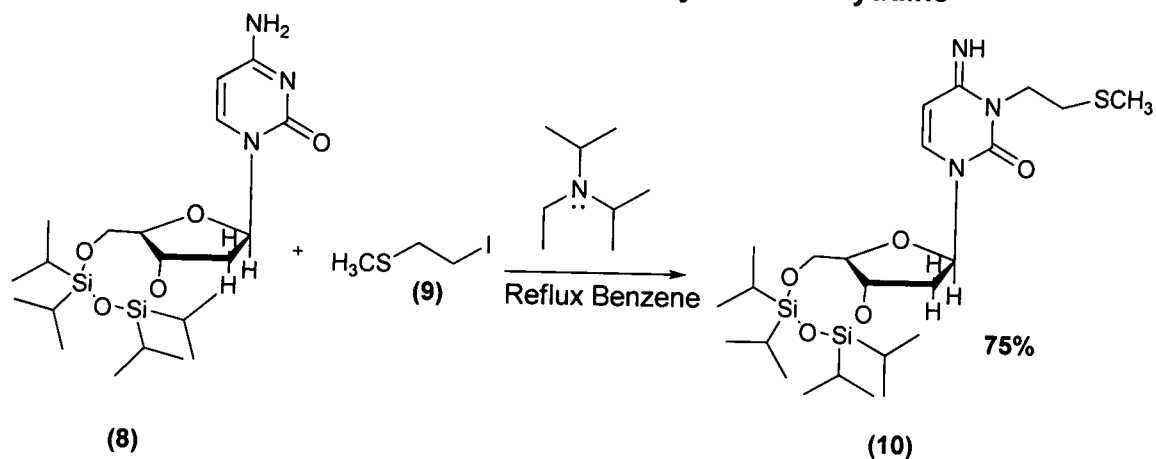
Examining these characteristics, we decided to generate a leaving group that possesses a better chance to fulfill the requirements. The chloride **(12)** that was once used in the earlier experiments was converted into a better substrate by replacing chloride with iodide using NaI in acetone to generate **(9)**. This particular reaction is known as a halogen exchange reaction, and is shown in (Scheme 6).³⁰ This exchange is fast because iodide is a stronger nucleophile and chloride

is considered a moderate nucleophile. This was a straightforward reaction and we were able to generate 44% of this alkyl halide, which was verified by ^1H NMR.

2.4 Isolation of 3',5'-[1,1,3,3-tetraisopropylidisiloxy]-3-[2 (methylthioethyl)]-2-deoxycytidine

Scheme 7

Generation of Protected Alkylated N-3 Cytidine



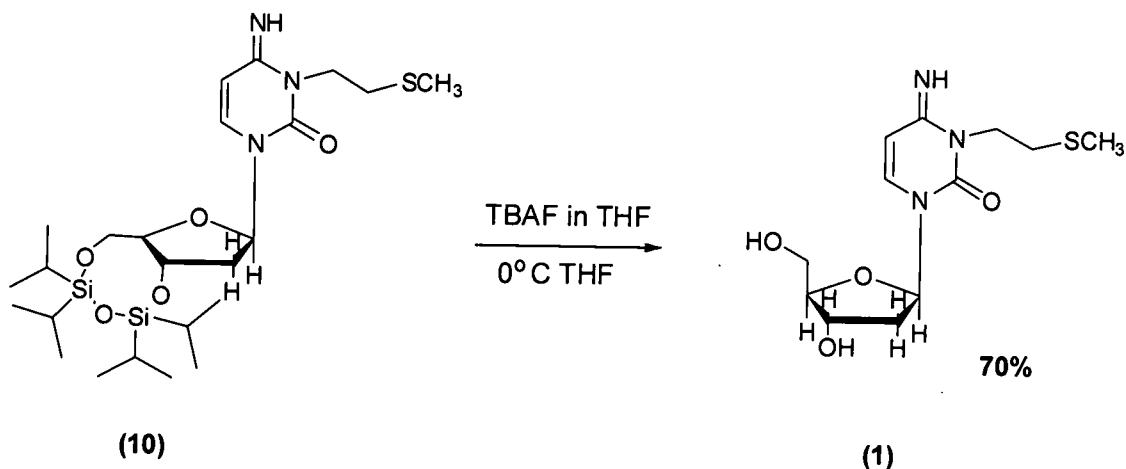
The next step in the multiple step synthesis was to react (8) with (9) in benzene using N,N-Diisopropylethylamine as the base to generate (10). We presumed that the lone pair electrons on either the N-3 or N-4 position of the pyrimidine base were nucleophilic enough to attack the carbon bearing the iodide, displacing the iodide producing a positively charged nitrogen at the heterocyclic nitrogen. The base was utilized to remove one of the hydrogen's at the N-4 position. This reagent was chosen because it is a good base but weak nucleophile. Our goal was to choose a base that was good for deprotonating but not nucleophilic enough to attach to the alkyl halide. This reaction was

successful. We were able to generate **(10)** in 75% isolated yield. This yield was impressive because we did not know if the N-3 position was nucleophilic enough to produce any of the two possible alkylation products of cytidine. This product was characterized by ^1H , ^{13}C , 2D COSY, and HETCOR. HMBC spectroscopy was utilized to determine the position of alkylation for N-3 or N-4. The CH_2 protons next to N-3 showed 3 bond coupling to the C-2, C-4 position. N-4 alkylation should only correlate to C-4.

2.5 The generation of 3-[2-(methylthioethyl)]- 2-deoxycytidine (**1**)

Scheme 8

Deprotection of Alkylated Cytidine To Generate 3-[2-(methylthioethyl)]-2-deoxycytidine



The last step in the generation of **(1)** was to remove the disiloxane at the 3' and 5' positions of the deoxyribose sugar. Many conditions can be used to rapidly cleave TIPDS groups including TBAF, 0.2 M HCl in aqueous dioxane, or 0.2 M NaOH in aqueous dioxane.³¹ There are other alternatives, a more cost

effective route is using an excess amount of ammonium fluoride in refluxing MeOH, which is also effective in deprotecting TIPDS derivatives of nucleosides.³¹ In our research we used TBAF to desilylate (**10**) in THF. The Si-F bond is much stronger than the Si-O bond causing the silicon to displace from the oxygen's, forming alkoxides. Water was used to protonate the alkoxides formed, thus generating (**1**). We had to use preparatory TLC in order to separate the bands; it could not be purified by flash chromatography. We were able to isolate (**1**) in 70% yield and its' structure was determined by ^1H NMR, C^{13} NMR, and 2D NMR, in which COSY, and HETCOR is shown in figure 8, 9 below:

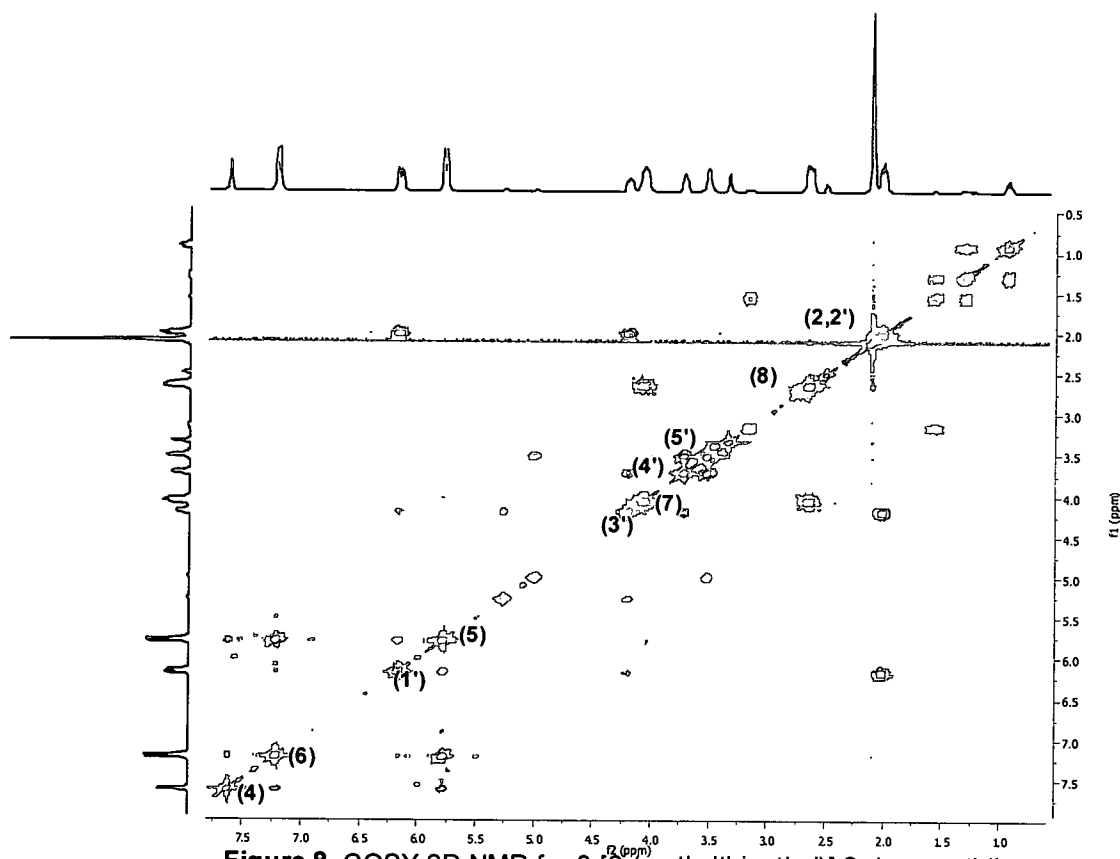


Figure 8. COSY 2D NMR for 3-[2-(methylthioethyl)]-2-deoxycytidine

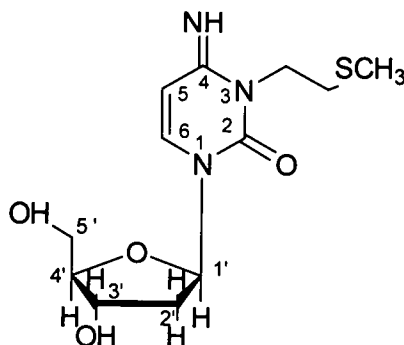


Figure 8a. Structure representation of 3-[2-(methylthioethyl)]-2-deoxycytidine

Shown in (Figure 8) is a correlation technique (Correlation Spectroscopy) that was used to determine 3 bond proton connectivities. Looking at the spectrum you can see that the absorbance that is labeled (4) is not coupled to any protons. Knowing this helped us to determine that the most deshielded peak is the imine because it is electronegative and it's attached to the heterocyclic ring making it accessible to anisotropy thus deshielding it to about 7.6 ppm. Protons (5) (5.8ppm) and (6) (7.2ppm) are directly coupled and adjacent to one another on the heterocyclic ring and occurring in the vinylic region which is shown in (Figure 8a). Since they are adjacent to one another and they have one proton attached, it is assumed that it will show up as a doublet based on the $n+1$ rule, which is clearly shown in (Figure 8). Proton (6) is more deshielded because it is next to electronegative nitrogen. Proton (1') (6.2ppm) is on a chiral carbon that correlates with position (2') (2.02ppm) which has hydrogen's that are non-equivalent. Since they are different (H 1') appears as a doublet of doublets, as is shown in the spectrum. Proton (1') is more deshielded then (H 2') because it is next to nitrogen and oxygen. Proton (2') is also correlated to (H 3') (4.2ppm)

which is coupled to 3 protons. Proton (3') is more deshielded than (H 4') because (H 3') has an alcohol group attached directly. The (H 4') (3.7 ppm) is also a chiral carbon and it is correlated with (H 5') (5.0 ppm) which also has non-equivalent hydrogen's. The (H 4') carbon is more deshielded than (5') because it is adjacent to oxygen that is electronegative. The alkyl side chain on N3 has two triplet peaks (H 7) and (H 8) each coupled to each other, but not to any other proton, which is represented in (Figure 8). Proton (7) is more deshielded than (H 8) because it is next to electronegative nitrogen.

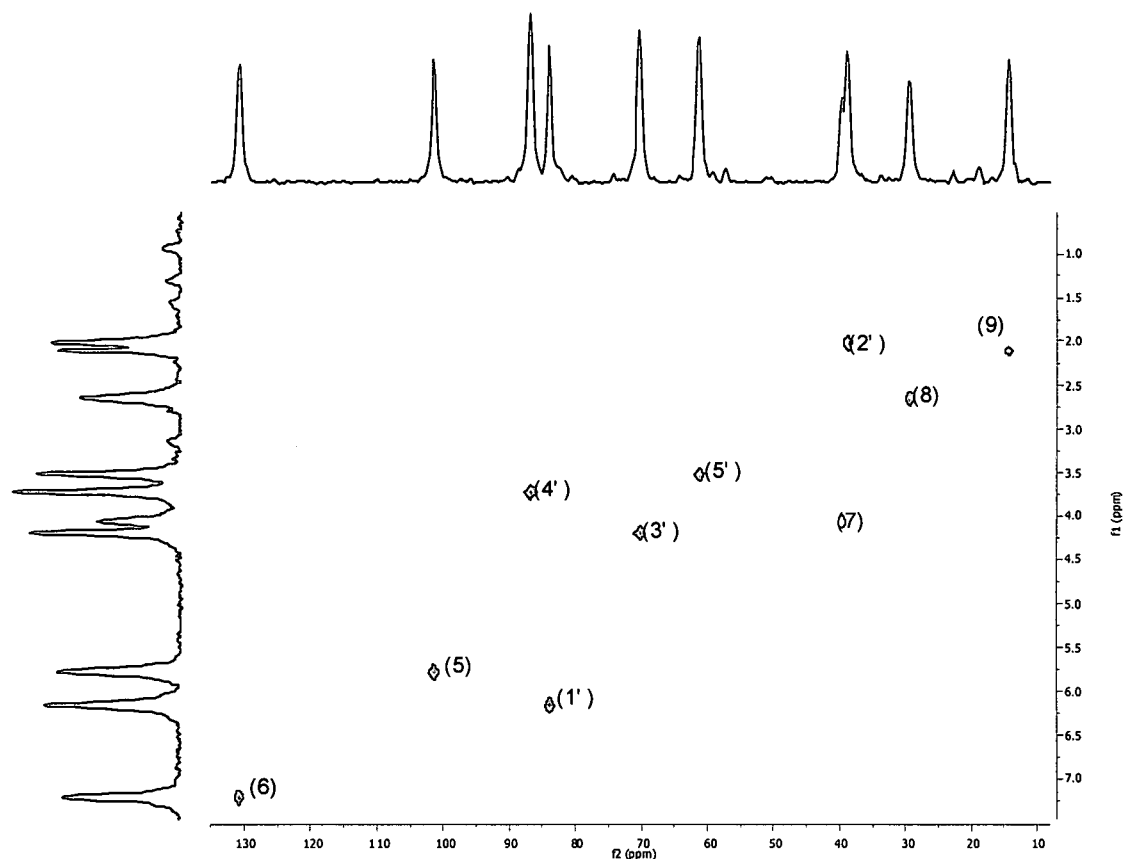


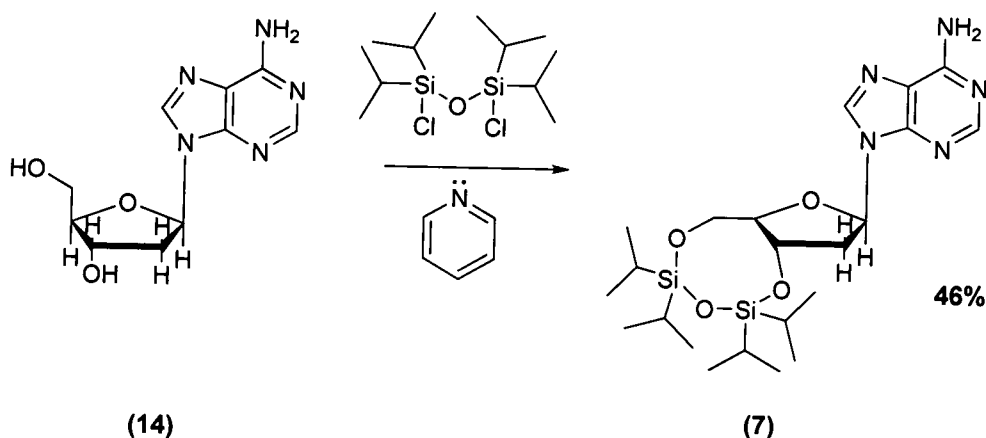
Figure 9. HETCOR 2D NMR for 3-[2-(methylthioethyl)] - 2- deoxycytidine (1)

The ^{13}C - ^1H COSY (HETCOR) experiment shown in (Figure 9) is the correlation of ^{13}C nuclei with directly attached protons, only the carbons that are directly attached to a ^1H are detected.³¹ The X-axis shows the ^1H ppm range and the Y-axis shows the carbon ppm range. The spectrum shows that (C 6) peak at (130.9 ppm) correlates with (H 6) (7.2 ppm), (C 5) at (102 ppm) is directly attached to (H 5) (5.8 ppm), (C 4') at (87 ppm) correlates with (H 4') (3.7 ppm), (C 1') at (84 ppm) is attached to (H 1') (6.2 ppm), (C 3') at (70 ppm) is attached to (H 3) (4.2 ppm), (C 5') at (61 ppm) correlates with (H 5') (3.5 ppm), (C 7) at (40 ppm) correlates with (H 7) at (4.1 ppm), (C 2') at (39 ppm) is directly attached to (H 2') at (2.0 ppm), (C 8) at (30 ppm) correlates with (H 8) at (2.7 ppm), and (C 9) at (15 ppm) correlates with (H 9) at (2.1 ppm). All the remaining carbons (C 2, C 4) which absorb in the aromatic region cannot be interpreted from the HETCOR as they don't have any hydrogen atoms attached to them. However a peak at 156 ppm can be assigned to (C 2) and a large peak at 150 ppm can be assigned to (C 4).

2.6 The generation of 3',5'-[1,1,3,3-tetraisopropylidisiloxy] – 2 deoxyadenosine (7)

Scheme 9

The protection of 3' and 5' Hydroxyl Groups on 2-deoxyadenosine

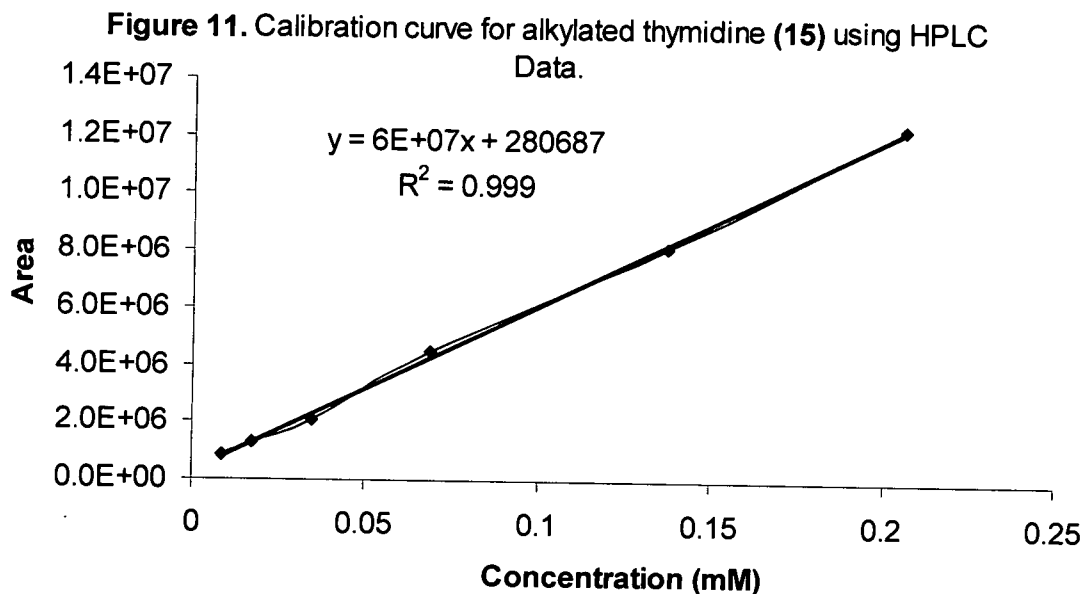
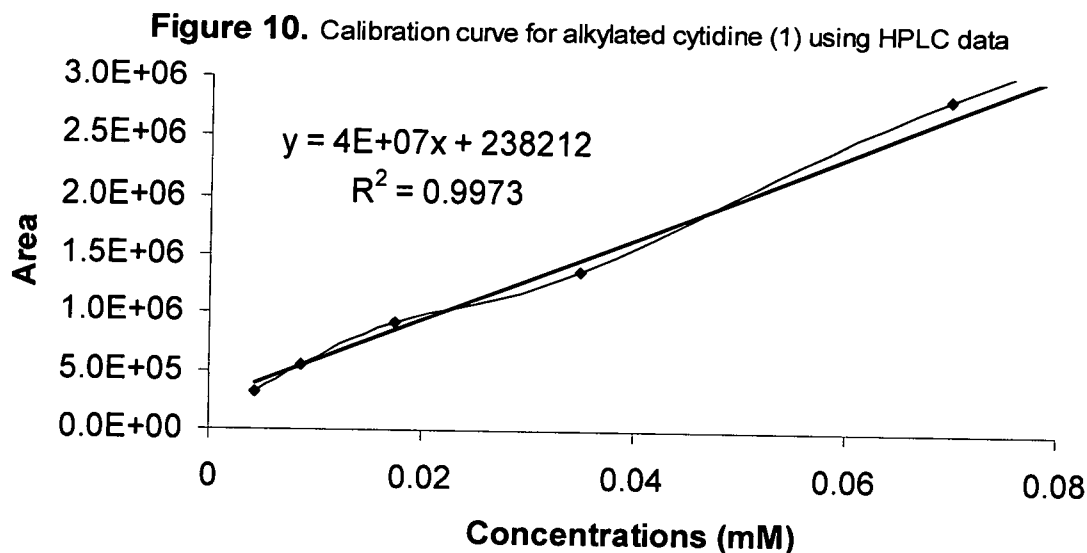


Commercially available 2-deoxyadenosine was reacted with 1.1 equivalents of 1,3 Dichloro-1,1,3,3-tetraisopropylidisiloxane in pyridine. This reaction was performed the same way as cytidine. We were able to obtain a 28% yield of (7). We were not successful with generating any of the authentic standards that were shown in (Figure 5) by means of phase transfer, and reacting (7) directly with (I). Reacting (7) with (9) was also attempted. This was unsuccessful.

HPLC Quantitation of Alkylation Products

Calibration curves were prepared using the data generated from the HPLC runs with the authentic standard solutions for both alkylated cytidine (1) and alkylated thymidine (15). These calibration curves, which are shown in (Figures 10 and 11), were used for the determination of the amount of alkylated

nucleoside produced in each of the simulated biological reactions. Looking at the calibration curves in figure 10 and 11 one can see that the R^2 values are great and shows almost perfect linearity.



After generating the calibration curve and the HPLC data from the simulated biological reactions, the total yield for each simulated biological

reaction was calculated. From, this, the percent yield values could then be determined. After calculations were analyzed the percent yield values seemed very low for alkylated cytidine, 0.31% for alkylated cytidine (**1**) at pH 8, $0.32 \pm 0.051\%$ for alkylated cytidine (**1**) at pH 9, and thymidine 2.91 ± 0.299 at pH 9. At pH 9 the thymidine yield data showed an increase as the pH increased, which is shown in table 1.

Table 1. Alkylated thymidine (**15**) percent yields.

pH	Comparing % yields
7*	0.212%
8*	1.339%
9	2.91%

*Wheatcraft²³ yields after reacting N-nitrosocarbamate (I) with Thymidine

The next step was to use the HPLC data to determine the kinetics of the alkylation reactions under biological conditions. The time data versus the concentration was plotted for alkylated cytidine (**1**) and thymidine (**15**) at pH 9 to see if the concentration increases over time, which is shown below in (figures 12, and 13). The blue, yellow and pink were used to represent each trial that was run on (1) and (15) using HPLC.

Figure 12. The Increase in concentration over time for alkylated cytidine (1) using HPLC data

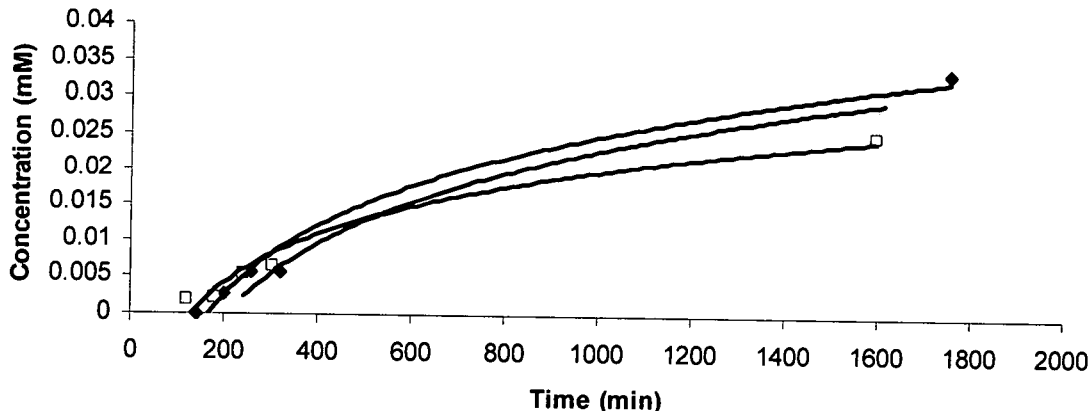
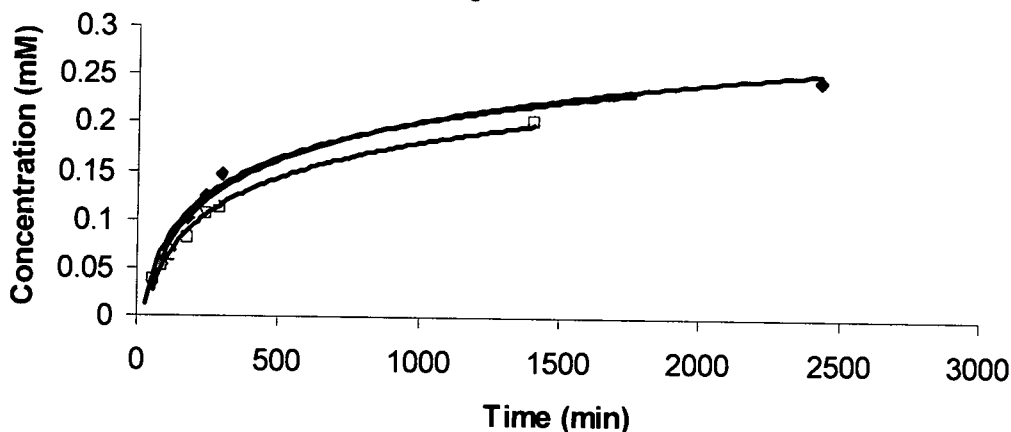


Figure 13. Increase in concentration over time for alkylated thymidine (15) using HPLC data



The data for alkylated cytidine (1) was on the edge of detection, so after a couple of hours we were then able to detect the concentration. This was only done for alkylated cytidine (1) at pH 9, because it was assumed that if it was on the edge of detection at pH 9, the results will be very unclear at pH 8.

Shown in (Figures 14-15) is the separation of 3-[2-(methylthioethyl)]-2-deoxycytidine (6.13 ± 0.61 min) from 2-deoxycytidine (which is represented by the tall peak at 1.82 ± 0.02 min), and the nitrosocarbamate (I) (which is not shown) at pH 8 and 9. Represented in (Figure 16) is the separation of 3-[2-

(methylthioethyl)]- thymidine (5.26 min) from thymidine (tallest peak at 2.08 min), and the nitrosocarbamate (I) (which is not shown) at pH 9. The determination of the identity of the peaks in (Figure 14, 15, and 16) was done by co-eluting the known standards with the reaction mixture.

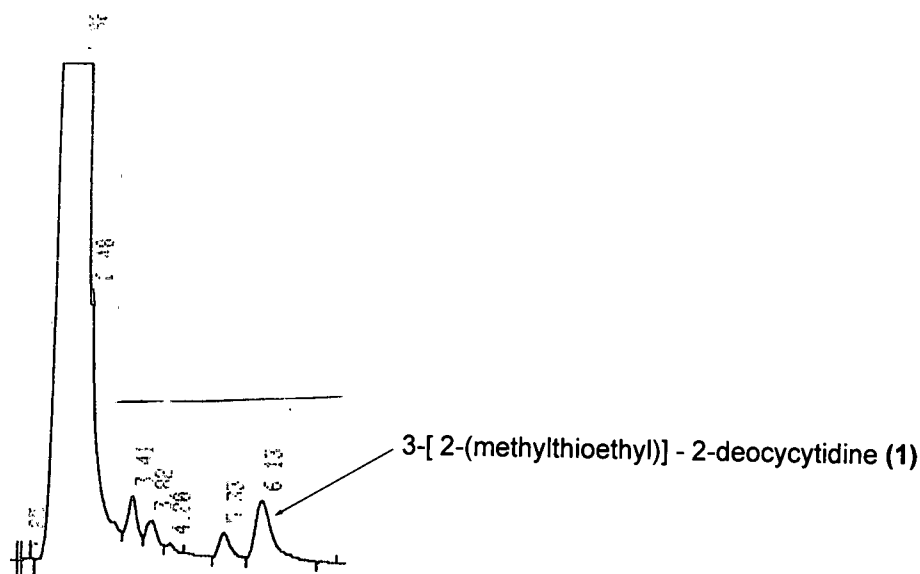


Figure 14. Representation of the separation of 3-[2-(methylthioethyl)]-2-deoxycytidine (1) in pH 8 buffer solution.

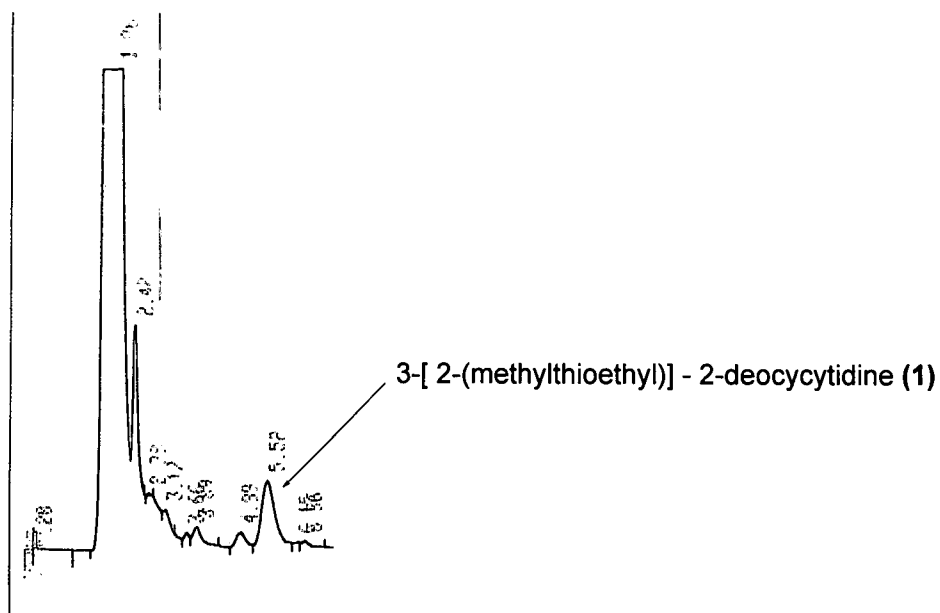


Figure 15. Representation of the separation of 3-[2-(methylthioethyl)]-2-deoxycytidine (1) in pH 9 buffer solution.

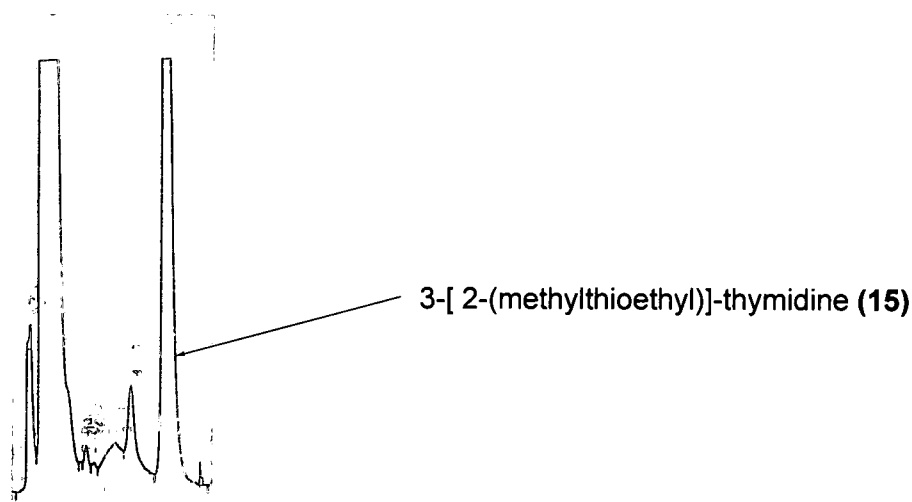
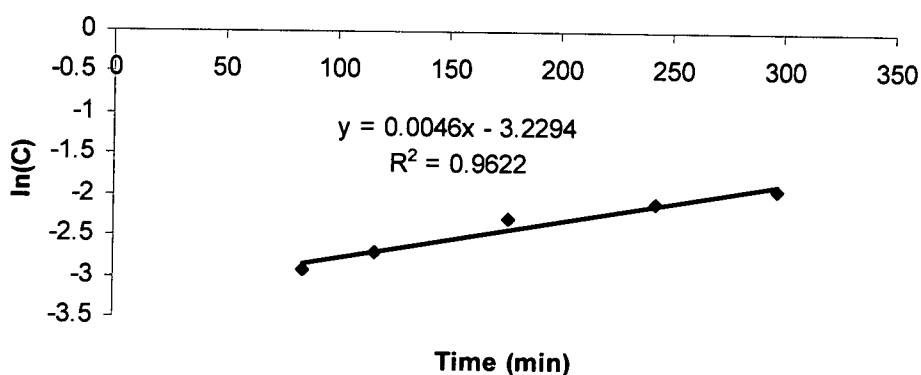


Figure 16. Representation of the separation of 3-[2-(methylthioethyl)]-thymidine (15) in pH 9 buffer solution.

The concentration of product versus time data was then plotted as the natural log of concentration of product versus time, and 1/concentration versus time to determine if alkylated cytidine or alkylated thymidine formation followed first or second order kinetics. From this you can also determined the rate

constant and half life. The kinetic data for alkylated cytidine (**1**) did not conform to either 1st or 2nd order rate law. The formation of alkylation product in this case may also follow more complicated kinetics than 1st or 2nd order reaction. We believe the data was on the edge of detection and somewhat inconsistent. Thymidine (**15**) conformed to a first order rate plot which is shown in (Figure 17) below.

Figure 17. First order kinetics for alkylated thymidine (**15**) using HPLC data



The nitrosocarbamate (**I**) alkylates thymidine following first order kinetics with a rate constant of 0.00480 min^{-1} and a half life of 144 min at pH 9, which is shown in (Figure 17). It has been shown that at pH 8 the rate constant is slower by about 0.00087 min^{-1} . This could be due to the fact that nitrosocarbamates decompose faster at higher pH since it is a base catalyzed decomposition. We then determined if the electrophile formed from decomposition was selective for different nucleophiles. The selectivity factor (α) gives an indication of the selectivity of the electrophile for various nucleophiles in solution. In order to obtain the results, the total # of moles of nucleophile present was determined for

each reaction, which includes H₂O, buffer, methanol, and nucleoside. Then the total number of moles of cytidine or thymidine was divided into the total number of moles of nucleophiles present in the biological simulated reaction. This resulted in a statistical yield of 0.018% for alkylated cytidine at pH 8 and pH 9, and for thymidine 0.0148% pH 9. The average yield for the alkylated cytidine (**1**) and thymidine (**15**) was divided by the statistical yield to produce the selectivity value for both, as shown in (Figure 18).

Figure 18
Selectivity Determination

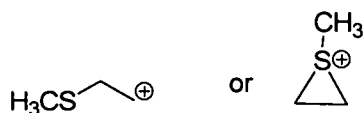
$$\alpha = \frac{\% \text{yield}}{\% \text{statistical Yield}}$$

For alkylated cytidine (**1**), the selectivity for cytidine is 18 times what you would expect to see statistically. Alkylated thymidine (**15**) gave a yield 196 times higher than the expected yield. This higher alkylation selection may be due to the fact that the pK_a of thymidine N-H is 8.1, at pH 8 about 50% is ionized, and pH 9 it is a 100% ionized. The conjugate base is a good nucleophile which gives higher alkylated yields. The second reason is that the nitrosocarbamate decomposes faster at a higher pH. Cytidine selectivity is much lower at both pH 8 and 9 presumably because the heterocyclic nitrogen's are less nucleophilic, so a higher pH does not matter as much. All of this indicates that the reactive intermediate shown in (Figure 19) is sensitive to nucleophilicity. Which form would be more sensitive? Probably the cyclic ion, because it is a soft acid and the N-3 position

on the 2-deoxycytidine is a soft base (relative to H_2O), thus conforming to the Acids and Bases principle which states that a hard acid prefers to bind to a hard base and a soft acid prefer to bind to a soft base.³¹

Figure 19

Reactive intermediates from Nitrosocarbamate (I)



Knowing this one can say that these yields are good compared to the expected yields, as shown by the selectivity values. This could result in high yields in DNA alkylations making these N-nitrosocarbamates of potential therapeutic use.

CHAPTER III

Experimental

General Procedures

HPLC experiments were run using two different columns, one being a Waters Radial Pak HPLC cartridge (8mm, C18, 10 μ m particles) with a flow rate of 2ml/min on a Waters 810 Baseline HPLC system and hp 3390A Integrator. The second column was a Waters Atlantis T3 column (5 μ m, 4 6X150mm, C18). All NMR experiments were run on a Bruker AVANCE 300 MHz system with tetramethylsilane (TMS) as the internal reference. All NMR chemical shifts are given in ppm. All Flash chromatography was performed on a silica gel (Merck, grade 9385, 230-400 mesh, 60Å) column and all TLC analysis was performed on glass backed silica plates (250 μ m analytical plates). All IR experiments were run on a Perkin Elmer IR Paragon with a KBr disk. All UV-vis experiments were run on a Hewlett-Packard 8453 UV-spectrometer using 1cm quartz cuvettes. The following chemicals such as methanol, chloroform, acetone, methylene chloride, petroleum ether, magnesium sulfate, and HCl were all purchased from Fisher Scientific. Reagents such as 2-deoxyadenosine, 2-deoxycytidine, pyridine, 2-chloroethylmethylsulfide, sodium iodide, thiosulfate pentahydrate, sodium sulfate, N,N-Diisopropylethylamine, tetrahydrofuran, and TBAF were all purchased from

Sigma Aldrich. Benzene was purchased from Acros organics. The reagent 1,3 Dichloro-1,1,3,3-tetraisopropylidisiloxane was purchased from Alfa Aesar.

Synthesis of Alkylated Standards

3',5'- [1,1,3,3-tetraisopropylidisiloxy] – 2 deoxyadenosine (7)

In the preparation of 3', 5'- [1,1,3,3,-tetraisopropylidisiloxy] – 2 deoxyadenosine, 30ml of pyridine was used to dissolve 1.7877g (6.6 mmol) of 2-deoxyadenosine. Then 2.4 ml (7.3 mmol) of 1,3 Dichloro-1,1,3,3-teraiso propylidisiloxane was added dropwise to the dissolved 2-deoxyadenosine via a syringe. The reaction was allowed to stir under nitrogen gas atmosphere over night. The solution was rotary evaporated to remove most of the excess pyridine. The residue was dissolved in chloroform and an extraction was done with dilute acidic water 3 times to remove any excess pyridine. The organic solution was dried with magnesium sulfate and filtered into a flask to be rotary evaporated down to a syrup. TLC analysis showed there were two products, the compound of interest and pyridine. Flash chromatography on silica gel was done to separate 3',5'-[1,1,3,3-teraisopropylidisiloxy] – 2 deoxyadenosine from any pyridine that was left using 30% acetone in methylene chloride as eluent. The appropriate fractions were collected and transferred to a flask to be rotary evaporated. The compound was put under high vacuum over night, and a white foamy compound formed with an overall yield of 29%. Structure determination was done using proton, carbon, DEPT, IR and 2D NMR. ¹H-NMR (CDCl₃); δ 1.06

(16H's (CH₃)₂-CH-Si , m, H 10, H 11), 2.67 (2 H, ddd, CH₂, HH' 2'), 3.90 (1H, ddd, H 4'), 4.05 (2 H , dd, ,H 5'), 4.95 (1 H, dd, H 3'), 5.90 (2 H, s, 6-NH₂), 6.30 (1 H, dd, H 1'), 8.04 (1H, s, H 8), 8.32 (1 H, s, H 2); ¹³C NMR (CDCl₃); δ 12.50,12.85,13.11,13.37 (4CH-Si, dd, 10), 16.90, 16.99, 17.04, 17.17, 17.35, 17.39, 17.40, 17.52 (8 CH₃-Si, 11), 39.92 (CH₂, C 2'), 61.69 (CH₂, C 5'), 69.72 (CH, C 3'), 83.14 (CH, C 4'), 85.16 (CH, C 1'), 120.3 (C, C 5), 138.9 (CH, C 8), 149.1 (C, C 4), 152.9 (CH, C 2), 155.5 (C, C 6). IR(KBr); 3166, 2946, 2868, 1654, 1599, 1466, 1331, 1300, 1249, 1035, 886, 777, 695 cm⁻¹.

3',5'- [1,1,3,3-tetraisopropylidisiloxy] – 2 deoxycytidine (8)

In order to isolate 3',5'- [1,1,3,3-teraisopropylidisiloxy] – 2 deoxycytidine, 90ml of pyridine was used to dissolve 2.5g (5.3 mmol) of 2-Deoxycytidine hydrate. Then 3.85 ml (7.9 mmol) of 1,3 Dichloro-1,1,3,3-tetraisopropylidisiloxane was added drop wise to the dissolved solution via syringe. The solution was allowed to stir over night under nitrogen gas atmosphere. The solution was then transferred into another flask and rotary evaporated down to remove most of the pyridine. Then 175 ml of chloroform was added to the solution and an extraction was done using dilute acidic water 3 times to remove any excess pyridine. The chloroform layer was saved then dried with magnesium sulfate, and filtered into a flask to be rotary evaporated. TLC analysis indicated two different compounds, pyridine and protected cytidine. Flash chromatography on silica was performed to separate the pyridine from the compound of interest using 30% acetone in chloroform as eluent. The appropriate fractions were collected and rotary evaporated and

placed on high vacuum, which formed a flakey white solid compound with an overall yield of 46%. The structure was determined using ^1H , ^{13}C , ^{13}C DEPT, COSY, and HETCOR. ^1H -NMR (CDCl_3); δ 1.04 (2 16H's(CH_3)₂-Si, m, H 7, H 8), 1.92 (2 H, s, 4-NH₂), 2.32 (2 H, CH₂, ddd, H 2'), 3.77 (1 H, dd, H 4'), 4.01 (2 H, CH₂, dd, H 5'), 4.16 (1 H, dd, H 3'), 5.68 (1 H, d, H 5), 6.05 (1 H, dd, H 1'), 7.88 (1 H, d, H 6); ^{13}C NMR (CDCl_3); δ 12.38, 12.96, 13.40 (3CH-Si), 16.84, 16.95, 17.04, 17.33, 17.35, 17.47, 17.52 (7CH₃-Si), 30.96 (CH, C 3'), 39.92 (CH₂, C 2'), 59.96 (CH₂, C 5'), 66.69 (CH, C 4'), 84.90 (CH, C 1'), 93.39 (CH, C 5), 141.1 (CH, C 6), 155.7 (1C, C 4), 165.8 (1C, C 2).

2-Iodoethylmethylsulfide (9)

In order to isolate 2-Iodoethylmethylsulfide, 0.89 ml (9 mmol) of 2-chloroethylmethylsulfide was dissolved in 20 ml of acetone, (which had been dried prior to using with magnesium sulfate), and 1.7594 g (11.7 mmol) of sodium iodide was added last. This solution was allowed to heat overnight at reflux under nitrogen gas using a heating mantle and reflux condenser. The solution was taken off the heating mantle and was allowed to cool for 5 min. At this time the solution was a dark brown color. Eight crystals of sodium thiosulfate pentahydrate were added to the solution and allowed to stir for about 45 min. The solution became a light caramel brown color. The solution was then poured into another flask and rotary evaporated down. Then 100ml of chloroform was added to the solid compound. The solution was then dried and filtered into another flask; 20ml of dried acetone was added to the flask. An extraction was performed

using 100ml of petroleum ether and 100ml of cold water. The pet ether layer was then extracted 3 times with a solution of sodium thiosulfate pentahydrate in 500ml of water. The ether layer was filtered into a beaker and dried with sodium sulfate. The solution was then rotary evaporated at low temp. We were able to isolate 2-iodoethylmethylsulfide as a light yellow liquid with an overall yield of 44%. Structure determination of 2-Iodoethylmethylsulfide was done by H^1 NMR. H^1 - NMR ($CDCl_3$); δ 2.16 (3 H, s, S-CH₃), 2.94 (2 H, t, S-CH₂CH₂), 3.31 (2 H, t, S-CH₂).

3',5'-[1,1,3,3-tetraisopropylidisiloxy]-3-[2-(methylthioethyl)]-2-deoxycytidine(10)

3',5'-[1,1,3,3-tetraisopropylidisiloxy]-3-[2-(methylthioethyl)]-2-deoxycytidine was prepared by adding 1.000 g, (2.1 mmol) of 3',5'- [1,1,3,3-tetraisopropylidisiloxy] – 2 deoxycytidine and 0.194 ml of N, N-Diisopropylethylamine to 30 ml of benzene. 1.8148 g, (8.6 mmol) of 2-Iodoethylmethylsulfide was added to the 100 ml flask slowly. The reaction was refluxed at 80⁰ C over night under nitrogen gas atmosphere. Flash chromatography on silica gel was performed to purify (10) using 20% acetone and chloroform. Fractions were collected in large test tubes and the appropriate fractions were rotary evaporated down and placed on the high vacuum over night. The compound was a light yellow solid with an over all yield of 75%. This structure was determined by H^1 , C^{13} , 2D COSY, DEPT, and HMBC NMR. H^1 -NMR ($CDCl_3$); δ 1.05 (2 16H's (CH₃)₂-CH-Si, m, H 10,H 11) 2.18 (3 H, s, H 9), 2.42 (2H,CH₂, ddd, H 2'), 2.80 (2H, t, H 8), 3.73 (1 H, dd, H 4'),

4.02 (2 H, dd, CH₂, H 5'), 4.25 (2 H, t, H 7), 4.45 (1H, dd, H 3'), 5.54 (1 H, d, H 5), 6.05 (1 H, dd, H 1'), 7.12 (1 H, d, H 6). C¹³ NMR (CDCl₃); δ 12.4, 13.0, 13.0, 13.4 (4CH-Si) 15.5 (CH₃, C 7, C 9), 16.8, 17.0, 17.0, 17.1, 17.3, 17.4, 17.5, 30.44 (CH₂, C 8), 39.83 (CH₂, C 2'), 40.62 (CH₂, C 7), 60.34 (CH₂, C 5'), 67.69 (CH, C 3'), 84.10 (CH, C 1'), 84.80 (CH, C 4'), 101.6 (CH, C 5), 130.9 (CH, C 6), 150.3 (C, C 4), 157.7 (C, C 2).

3-[2-(methylthioethyl)]-2-deoxycytidine (1)

In order to synthesize 3-[2-(methylthioethyl)]-2-deoxycytidine, 10ml of tetrahydrofuran (THF) was added to 0.9531 g (1.8 mmol) of 3',5'-[1,1,3,3-tetraisopropylidisiloxy]-3-[2-(methylthioethyl)]-2-deoxycytidine. The solution was allowed to stir in an ice bath for five minutes under nitrogen gas atmosphere. Then after five minutes 5.3 ml (5.3 mmol) of TBAF (1 M solution in THF) was added slowly to the flask and allowed to stir for 30 min. After 30 min 1 ml of H₂O was added and allowed to stir for another 30 min. After completion of the 30 min the solution was rotary evaporated under high vacuum to remove any excess water. TLC analysis was performed on a glass backed silica plate to visualize the number of compounds formed. There were a total of three compounds including the compound of interest. Preparatory TLC was performed using 40% methanol in chloroform as eluent. The large TLC plate was removed from the reservoir after 1hr and 30min. The plate showed three wide bands separated based on polarity. The bottom band was scraped off the silica plate and poured into a flask containing 40% methanol in chloroform and allowed to stir for 35 min to allow the

product to go into solution. The solution was then filtered from the silica gel and rotatory evaporated. This compound was then put on high vacuum over night. The compound was isolated as a yellow orange colored solid with an over all yield of 70%. The structure was determined by ^1H , ^{13}C , 2D COSY, DEPT and HETCOR NMR. ^1H -NMR (DMSO-d_6 ; δ 2.11 (3H, s, H₉), 2.65 (2H, ddd, H_{2'}), 3.52 (2H, dd, H_{5'}), 3.73 (1H, dd, H_{4'}), 4.10 (2H, t, H₇), 4.20 (1H, dd, 3'), 5.00 (1H, s, 5'(OH)), 5.27 (1H, s, 3'(OH)), 5.79 (1H, d, H₅), 6.17 (1H, dd, 1'(H)), 7.22 (1H, d, 6), 7.62 (1H, s, 4-NH). ^{13}C NMR (DMSO-d_6 ; δ 14.64 (CH_3 , C₉), 29.65 (CH_2 , C₈), 39.00 (CH_2 , C_{2'}), 39.77 (CH_2 , C₇), 61.46 (CH_2 , C_{5'}), 70.48 (CH, C_{3'}), 84.16 (CH, C_{1'}), 87.03 (CH, C_{4'}), 101.6 (CH, C₅), 130.9 (CH, C₆), 150.3 (C, C₄), 156.3 (C, 2).

HPLC Quantitation of 3-[2-(methylthioethyl)]- 2-deoxycytidine and 3-[2-(Methylthio)ethyl]thymidine

After generating the authentic standard by chemical means, the standard was used to quantitate the formation of alkylated product (**1**) under biological conditions by using High Pressure Liquid Chromatography (HPLC). UV-vis spectroscopy was employed to determine the λ max of alkylated cytidine (**1**). This was found to be 272 nm. In previous work done by Heidi Wheatcraft,²³ alkylated thymidine (**15**) absorbs light maximally at 268nm. Before running the biological simulation reactions, a calibration curve had to be generated with the alkylated cytidine authentic standard. This will be used to determine the amount of product formed from each simulated biological reaction. In order to find conditions for

best separation (**1**) was dissolved in methanol and pure commercially available 2-deoxycytidine was also dissolved in methanol. HPLC conditions were determined for the optimal separation of the two when co-injected (15 μ l of each were injected on to the column). A solvent ratio of 80% 0.071M HCO₂H H₂O/ 20% MeOH was used. Ion exchange chromatography was utilized in order to ensure one ionization state of the nucleoside and allow the compounds to separate. This system eluted (**1**) from the column at 4.27 \pm 0.04 minutes and easily separated from deoxycytidine. The calibration curve was prepared by injecting 20 μ l of (**1**) onto the column; from this an appropriate peak area was obtained. This injection was performed three times for each of the concentrations (0.1395 mM, 0.06975 mM, 0.0349 mM, 0.01744 mM, 0.008719 mM, 0.004436 mM) and the average peak area was calculated and plotted against concentration.

Biological Simulation

A biological simulation was set up to determine the amount and rate of formation of (**1**) upon decomposition of (**I**) in the presence of cytidine at 37°C \pm 1°C. The pH buffer solutions were made in order to keep the reactions at constant pH. 0.6073g of Tris [hydroxymethylaminomethane] or Trizma base was dissolved in 0.5L of water to generate a concentration of 0.01mol/L buffer solution to generate a pH 8 buffer. A small amount of concentrated hydrochloric acid was added to the pH buffer solution to bring the pH down to 8 using a pippet. The pH 9 buffer was prepared by weighing out 0.73 g of 1,4

Diethylpiperazine (liquid) into a beaker and then transferred with water into a 500ml volumetric flask. A small amount of concentrated HCl was dropped into the beaker to obtain a pH 9 buffer solution.

HPLC conditions for the decomposition of nitrosocarbamate (I) with 2-deoxycytidine was accomplished by using ion exchange chromatography. The amount of formic acid used was 0.071 M dissolved in 1.5L of H₂O. The solvent ratio of (84% H₂O/HCO₂H : 16% MeOH) was found to be the appropriate conditions for elution of (1) at 6.13 ± 0.61 min.

The decomposition reactions were performed by dissolving 10mg of 2-deoxycytidine in 5ml of pH 8.00 or 9.00 buffer and placed in a flask in a $37 \pm 1^\circ\text{C}$ water bath. Then 11.7 mg of (I) was dissolved in 0.5 ml of DMSO and added to the flask with a disposable pippet. The reaction was monitored using HPLC every 30 min for the first hour then 1 hour intervals after that for up to 5 hours. A final data point was injected at roughly 24 ± 4 hour time mark. To ensure that the predicted compound was formed for decomposition of (1), co-elutions were performed using a reaction sample and (1) dissolved in methanol. Two reactions were run for pH 8 and three for pH 9

In order to make sure that (1) and (15) were stable at pH 9 a stability test was run for both at pH 9. The decomposition was set up with a water bath at 37°C to follow the decomposition of the compound over time. In preparation, 5ml of pH 9 buffer was pipetted into a small round bottom flask, which was located in the water bath, and then 0.5 ml solution of alkylated cytidine or thymidine in MeOH was added to the solution and was allowed to stir. HPLC aliquots were

injected at the beginning and 30 min after and then by 1 hour up to 5 hour time. The reactions were then allowed to sit in the bath over night with out stirring at 37° C, to provide a final yield at 24 hrs. The alkylated cytidine and alkylated thymidine showed no signs of instability at pH 9.

As stated in the results, follow up work was performed on alkylated thymidine (**15**) to study the decomposition of nitrosocarbamate (**I**) in the presence of deoxythymidine at pH 9. Decomposition reactions had to be done for pH 9 using (**I**) only. A calibration curve was generated with Heidi Wheatcraft's²³ alkylated thymidine standard (**15**). In order to generate the curve for (**15**), the authentic standard was dissolved in 50 ml of methanol and diluted 5 times to obtain the following concentrations: 0.206 mM, 0.1375 mM, 0.06875 mM, .03438 mM, 0.01719 mM, .008594 mM, and then HPLC conditions were determined for the separation of (**15**) and thymidine. This was accomplished by co-injection. A solvent ratio of 50% 0.00121M HCO₂H/H₂O : 50% MeOH was used for the calibration curve. Ion exchange chromatography again was utilized in order to allow separation of compounds. These conditions allowed (**15**) to elute off at 5.35 ± 0.05 minutes. The calibration curve was then obtained by injecting 20µl of alkylated thymidine (**15**) onto the column and recording the area. Each determination was performed in triplicate. The average area was then calculated and plotted against concentration.

After the calibration curve was generated, the next step was to do the biological simulation for thymidine. This was accomplished the same way as cytidine, using the previously made pH 9 buffer. The only difference is that

methanol was used instead of DMSO to dissolve the carbamate reactant. We also had to change the solvent conditions to 51% 0.0012M HCO_2H : 49% H_2O in order to get a better separation. These conditions eluted **(15)** off the column at 5.26 ± 0.09 minutes. Three decomposition reactions were run using nitrosocarbamate **(I)** at pH 9.

CHAPTER IV

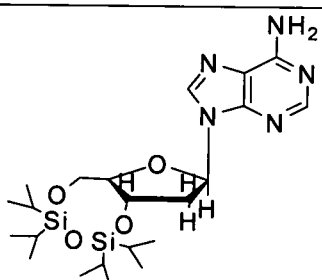
CONCLUSION

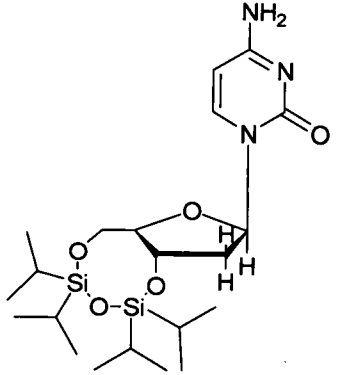
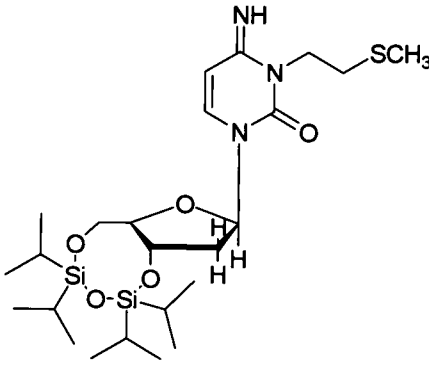
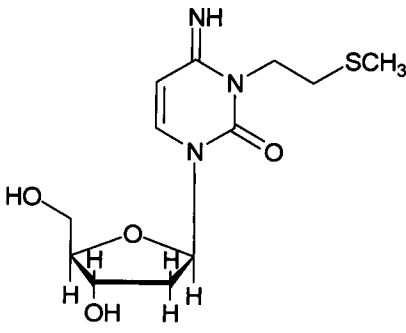
Our research has led to the following accomplishments as illustrated in Table 1:

- One authentic standard was prepared and characterized for cytosine (**1**).
- The protecting group on adenosine (**7**) was prepared and characterized.
- Alkylation of protected adenosine was unsuccessful
- We were able to determine how much of alkylated deoxycytidine (**1**) was formed at pH 8 and 9. We were unsuccessful at determining the rate constant and half life for alkylated deoxycytidine (**1**) at pH 8 and 9 because the data was on the edge of detection and inconsistent.
- The rate of formation, rate constant, and half life was found for alkylated thymidine (**15**) in pH 9.
- The following synthetic intermediates and targets were generated.

Table 2

List of compounds synthesized and their yields

Structure	Yield (%)
 3',5'- [1,1,3,3-tetraisopropylidisiloxy] - 2 deoxyadenosine (7)	28%

 <p>3',5'-[1,1,3,3-tetraisopropylidisiloxy]-2 deoxycytidine (8)</p>	46%
<p><chem>CSCH2CH2I</chem></p> <p>2-Iodoethylmethylsulfide (9)</p>	44%
 <p>3',5'-[1,1,3,3-tetraisopropylidisiloxy]-3-[2-(methylthioethyl)]-2-deoxycytidine (10)</p>	75%
 <p>3-[2-(methylthioethyl)]-2-deoxycytidine (1)</p>	70%

FUTURE PLANS V

Authentic standards will be made and characterize for adenosine, and guanosine. Once these standards are generated a biological simulation will be setup to react the nucleosides with the N-alkyl-N-nitrosocarbamates in aqueous solutions at a pH of 8 or 9. HPLC will be used to determine how much of the compound was actually formed and to follow the degree of alkylation. The rate constant will also be studied as well as the half life. Conducting this research can then make better generalizations about mechanism and selectivity for various nucleophilic positions.

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