STRUCTURAL STUDIES OF N^6-(DEOXY-D-*ERYTHRO*-PENTOFURANOSYL)-2,6-DIAMINO-3,4-DIHYDRO-4-OXO-5-N-METHYLFORMAMIDO-PYRIMIDINE AND 8-(DEOXYGUANOSIN-N^2-YL)-1-AMINOPYRENE

By

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Thesis
Submitted to the Faculty of the
Graduate School of Vanderbilt University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE
in

Chemistry
May, 2016
Nashville, Tennessee

Approved: Date:

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To my family,
Lori, Scott, and Lexi

ACKNOWLEDGEMENTS

To begin, I would like to thank Dr. Michael Stone for giving me the opportunity to work in his lab. The Stone lab members, Dustin Politica, Drew Kellum, Stephanie Bamberger, and Liang Li, have been amazing coworkers, peers, and friends. Major thanks are in order for Dr. Markus Voehler, director of the Biomolecular NMR Facility, for his willingness to assist me on my projects. Markus has always been a steady stream of guidance, and much of this work would not be possible without his input. I would like to thank Dr. Carmelo Rizzo and his lab members at Vanderbilt, Tracy Johnson-Salyard and Dr. Chanchal Malik, for their work on synthesizing the MeFapy samples. Furthermore, I would like to thank Dr. Ashis Basu at the University of Connecticut and Dr. Chanchal Malik, once again, for their work on synthesizing the aminopyrene samples.

Of course, graduate school is not possible without a strong support system. I would like to thank my best friends, Jessie, Brandon, Jessica, Greg, and Bobby, for their incessant support. Also, I would like to thank Dr. Susan Verberne-Sutton for her support and advice about surviving graduate school. I would not be on the path I am without her help. This would not be a proper acknowledgement section without thanking my parents, Lori and Scott, and my sister, Lexi. They instilled the motivation and courage to embark on this journey. They were always available during this entire process, and I could not thank them enough.

Last, but certainly not least, I want to thank God for providing me with a plethora of opportunities to get where I want to be in my life. The work presented here is a step in the right direction.

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CHAPTER 1

Introduction

Deoxyribonucleic Acid Structure and Damage

Deoxyribonucleic acid (DNA) is an exceedingly important biomacromolecule responsible for storing the genetic information of cells.[1] DNA has a distinct structure that allows it to be recognized by proteins such as the transcription machinery.[2] Whenever the structure of DNA is perturbed via carcinogens or modifications, mutations can arise which have the potential to be detrimental to the cell. An example is UV-induced thymine dimers which have the ability to inhibit DNA synthesis.[3] The mutagenic profiles of damaged DNA vary with the type of damage or lesions involved.

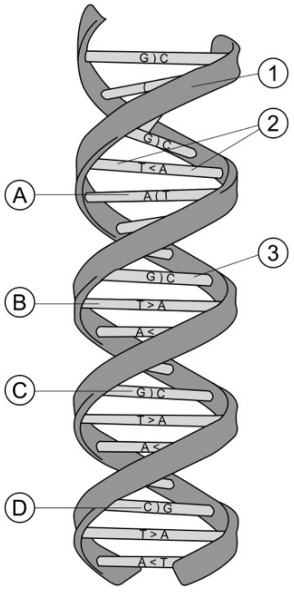

Figure 1. General Structure of DNA[4]:

A picture illustrating the DNA double helix as well as the base pairs.

(A) Adenine, (B) Thymine, (C) Guanine, (D) Cytosine, (1) Phosphate backbone, (2) Base pairs, (3) Nitrogenous base

The most common structure of DNA is B-type double helix. While other structural forms of DNA exist, such as A-DNA and Z-DNA, B-DNA is the predominant form within the biological context.[5] The DNA macromolecule has three main components: the phosphate backbone, the deoxyribose sugar moiety, and the nucleobase. The phosphate backbone orients the nucleosides correctly, and it is responsible for the net negative charge that DNA is known to have. The sugar moiety is one of the structural differences between DNA and RNA. In DNA, the sugar moiety is a deoxyribose sugar unit indicating that it does not have a hydroxyl group at the 2' position. Finally, connected to the deoxyribose sugar is the nucleobase responsible for the genetic information. There are four nucleobases in DNA: adenine (A), thymine (T), guanine (G), and cytosine (C) (**Figure 2**).

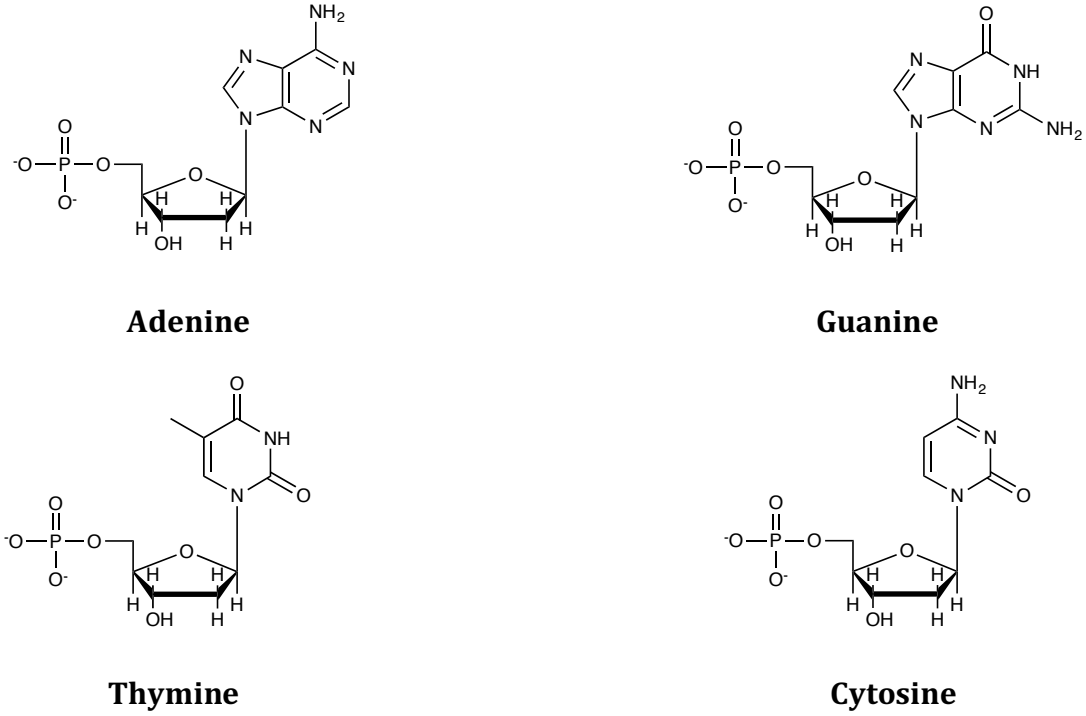

Figure 2. The four nitrogeneous bases connected to the deoxyribose sugar moiety which is connected to the phosphate backbone.

Adenine and guanine are the purines of the nucleobases while thymine and cytosine are the pyrimidines. These structures are highly important because they ultimately dictate how the double helix will form. Since the two strands of DNA are required to form the double helix, the nucleobases must form base pairs to each other on the opposite strands. Adenine and thymine form a Watson-Crick base pair with two hydrogen bonds, while guanine and cytosine form a similar base pair with three hydrogen bonds. Therefore, guanine and cytosine base pairs are more stable. The bonding network in DNA does provide stability to the overall macromolecule. However, substantial stability is achieved by the π-stacking (base-stacking) interactions between neighboring bases in the strands. Since the bases are aromatic, their π-electrons can conjugate and stabilize the DNA duplex structure.

DNA damage has been a significant area of research. Genetic approaches to parse disease characteristics often revealed mutations in the DNA that caused downstream effects. Various types of mutations have been characterized; however, the shear complexity of the human genome has proven difficult to detail all of the mutations for every protein. As research has shifted to a proteomic perspective, research has demonstrated that the structures of interacting components in a biological system is significant for maintaining the function of those components. Therefore, by studying the structural perturbations that suspected carcinogens cause the global DNA structure, insight can be generated about how proteins will interact with these damaged oligonucleotides. By understanding the biological processing behind some of these lesions, there is the potential for more research to lead to better drugs or treatments for certain disorders.

Methyl Formamidopyrimidine

Methylation of deoxyribonucleic acid (DNA) has been a growing area of interest in genomic studies, particularly with cytosine and guanine.[6,9] With its relevance to epigenetic regulation and varying levels of damage it causes to DNA, more focus is being placed on understanding the mechanisms involved with methylation and the biological processing of its products. Methylation of DNA can be induced from various endogeneous, exogeneous, and occupational sources.[7] Furthermore, a few chemotherapeutic agents are known methylating agents.[8] Methyl-formamidopyrimidine (MeFapy; **Figure 3**) is a product of methylation that occurs in DNA.[18] While it is hypothesized that tobacco carcinogens and food-associated nitrosamines can ultimately induce MeFapy-dG formation, chemotherapy drugs like temazolamide have been suggested to form MeFapy-dG lesions during methylation events.[9]

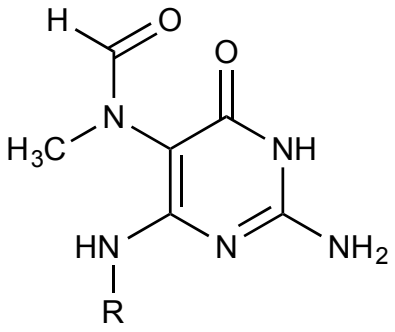

Figure 3. Structure of MeFapy-dG.

MeFapy occurs in the context of guanine residues (dG) due to the nucleophilic nature of guanine's N7 site.[10] While Fapy-dG remains the simplest Fapy system to study, MeFapy-dG is an important step toward studying more complex alkylated Fapy species which is the fundamental significance of this work. As mentioned previously, it has been hypothesized that

MeFapy-dG is generated during the use of chemotherapy that uses methylating agents.[11] The formation of MeFapy-dG differs from the generation of Fapy-dG lesions. Formation of MeFapy begins with methylation of guanine at the N7 position. This position has been classified as the most nucleophilic site in DNA which is crucial in the formation of MeFapy-dG.[12] Once the guanine residue has been methylated to form 7-methylguanine (7-meG), the next step involves the addition of hydroxide to C8 follow by breaking the C8-N9 bond of the imidazole ring. Opening of the imidazole ring is slow at neutral pH, therefore depurination is favored at this pH range.[13] However, under alkaline conditions, the ring opening reaction proceeds readily (**Figure 4**).

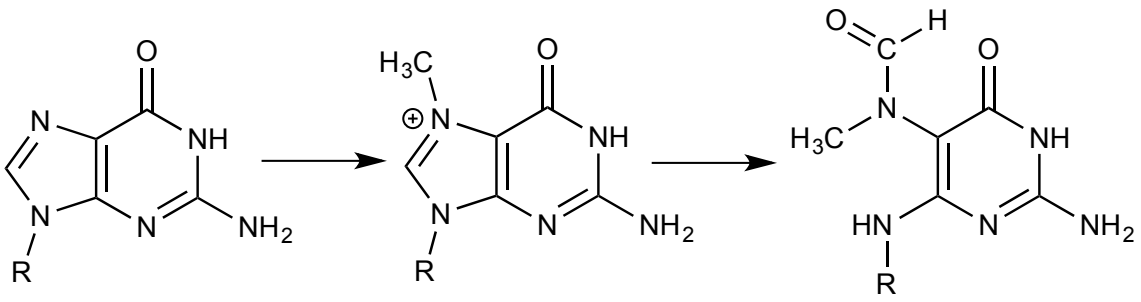

Figure 4. Proposed formation of MeFapy-dG. The N7 atom becomes methylated which leads to N7 being positively charged. Under alkaline conditions, this positive charge is readily neutralized by a hydroxyl group. Then the imidazole ring ruptures to form MeFapy-dG.

The precursor species to MeFapy-dG, 7-meG, has generally been assumed to be harmless and observed to occur often in DNA,[17] yet evidence suggests that MeFapy-dG can be more detrimental to the cell in terms of cytoxicity. Work on MeFapy-dG and its interaction with various polymerases and enzymes is ongoing; however, previous work has detailed that MeFapy-dG is a substrate for the human 8-oxo-G glycosylase enzyme (hOGG1) and the bacterial Fapy glycosylase enzyme (Fpg).[14] When the lesion is present in a duplex, it becomes a very persistent

lesion unlike its precursor 7-meG which was effectively repaired over time.[15] The mutagenicity of MeFapy-dG is still being parsed; however, one of the more dominant mutations observed was the G→T transversion. Furthermore, the cytotoxic activity of MeFapy-dG has been shown multiple times *in vitro*.[11] Interestingly, the mutagenicity of the lesion seems to be highly sequence dependent with differing mutagenic profiles based on the neighboring bases around the lesion.[16] In the work by Christov et al. it was shown that MeFapy-dG was a strong block to replication *in vitro*.[18] It was further hypothesized that the various anomers associated with MeFapy-dG were significantly related to its cytotoxic activity. The MeFapy-dG:C pair caused a strong thermodynamic destabilization of a DNA duplex by lowering the melting temperature relative to a normal G:C base pair. When examined in a polymerase bypass assay, MeFapy-dG was shown to be bypassed less efficiently than 8-oxoG by *E. coli* DNA polymerase I.[19] As mentioned, MeFapy-dG can adopt various anomers (**Figure 5**) and rotamers (**Figure 6**) due to the increased degrees of conformational freedom permitted by the ruptured imidazole ring. The two anomers, β- and α- have interesting biological activities as the α-anomer was hypothesized to be the anomer responsible for blocking replication.[20,21]

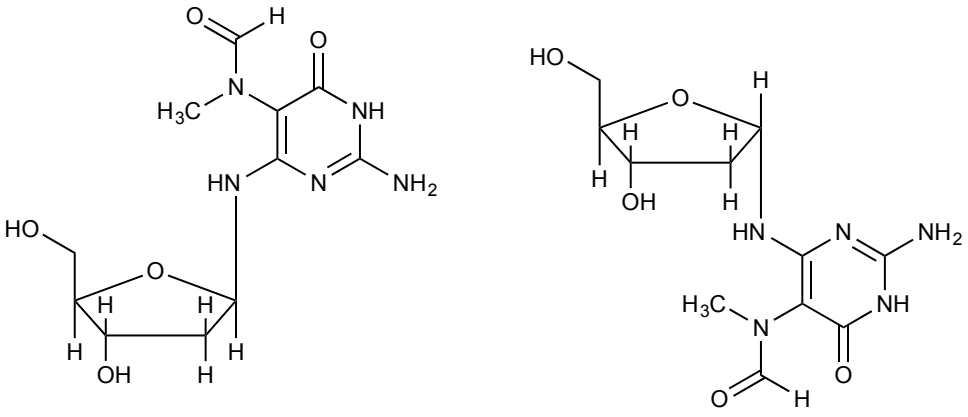

Figure 5. A depiction of β the (left) and α (right) anomers of MeFapy-dG.

Rotamers exist around the formyl group as well as various rotamers involving rotations of the N^5 (previous N^7 in dG) bond in MeFapy-dG.[22]

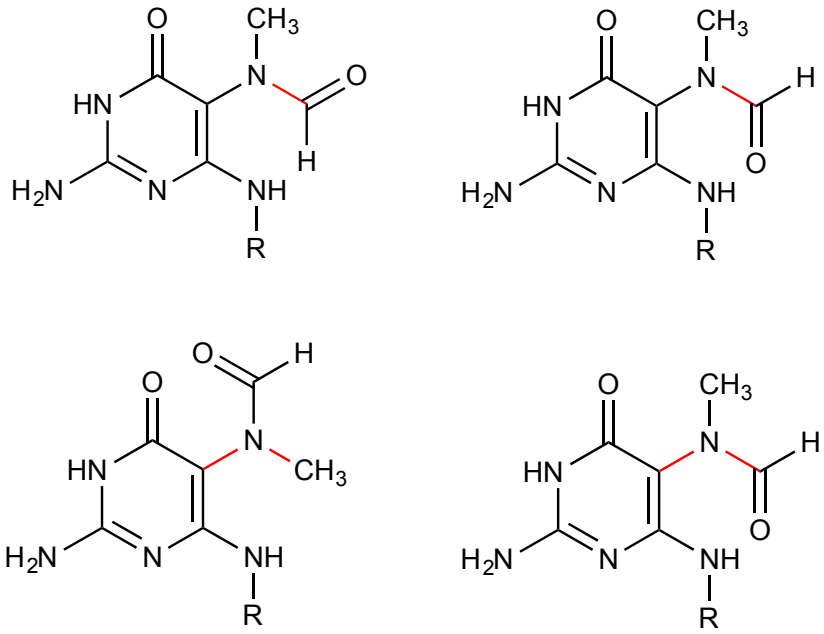

Figure 6. The various rotamers MeFapy-dG can form. It can form *Z* and *E* isomers around the formyl bond (top pictures). Furthermore, it potentially can exhibit rotations around the N^5 bond.

Therefore, the work presented here sought to parse those structural characteristics of the MeFapy-dG lesion via NMR spectroscopy.

Aminopyrene Introduction

The polyaromatic hydrocarbon (PAH) 1-nitropyrene (1-NP) is an abundant chemical pollutant found in urban air particulates and diesel exhaust.[23] Upon inhalation of 1-NP, it is metabolized via the nitroreduction pathway which leads to the formation of aminpyrene (**Figure 7**).[24]

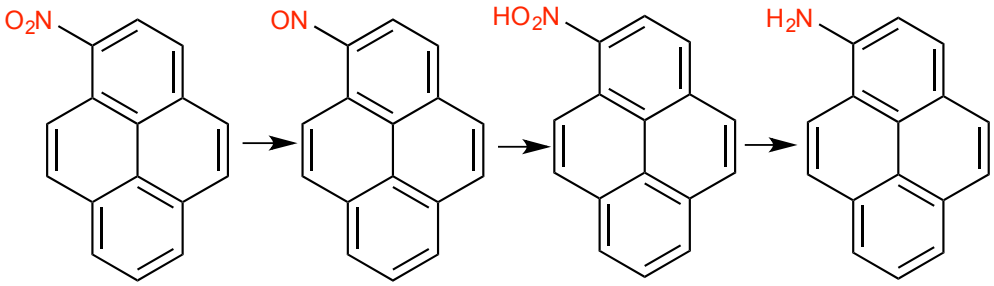

Figure 7. The proposed path of formation for aminopyrene from 1-nitropyrene.

The adduction sites of 1-NP has been characterized at the C^8 and N^2 positions on guanine residues. Most of the previous work on 1-NP and its metabolized product, aminopyrene (AP) has been at the C^8 attachment point. 1-NP is reduced to N-hydroxy-1-aminopyrene which is responsible for adduction to DNA.[25] Structural studies done on C^8-AP-dG have found that the global B-DNA conformation is sustained and bypass of the lesion in the M13mp2 bacteriophage was efficient and almost error-free.[26] However, the C^8-AP-dG adduct ultimately displaced the guanine it was attached to into the major groove with the adduct intercalating into the duplex. Interestingly enough, due to the strength of the base stacking interactions induced by the AP adduct, the intercalation of the adduct actually stabilized the duplex.[27]

The cellular mutagenicity of the adduct suggested that the mutations spectrum was organism-sequence dependent with frameshifts dominating in bacteria and G→T transversions in mammals.[28,29] While bypass was efficient by low-fidelity polymerases, it was suggested that the adduct completely stalled high-fidelity replicative polymerases.[30]

Little work has been done on N^2-AP-dG; however, other adducts at the N^2 position have been analyzed to use as a comparison. The structural analysis of N^2-benzo[a]pyrene diol epoxide

(**Figure 6**) in an 11-mer duplex was shown to be positioned in the minor groove, pointing in the 5' direction of the primary strand.[31] Furthermore, the N^2-B[a]P was shown to minimally perturb the B-DNA helix.[32] While the the B[a]P structure is not immediately similar to AP, it does provide some insight into the context of bulky N^2 adducts.

Other bulky N^2 adducts, such as N^2-acetylaminofluorine (N^2-AAF and N^2-benzo[*a*]pyrene diol expoxide; **Figure 8**), had similar degrees of perturbation and were suggested to be repaired by nucleotide excision repair (NER) like other bulky adducts.[33]

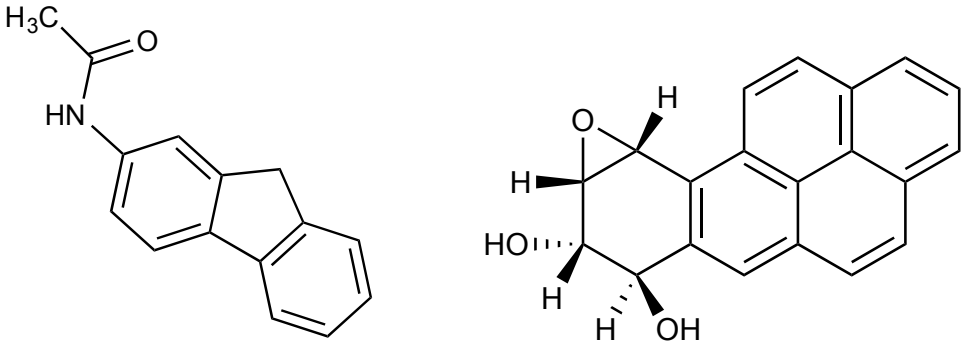

Figure 8. N^2-AAF (left) and N^2-benzo[*a*]pyrene diol epoxide (right).

Likewise, N^2-IQ-dG (**Figure 9**) was studied structurally, and it was found to displace the complementary base into the major groove. Ultimately, this allowed the IQ adduct to intercalate into the duplex with the modified base remaining in the *anti* configuration.[34]

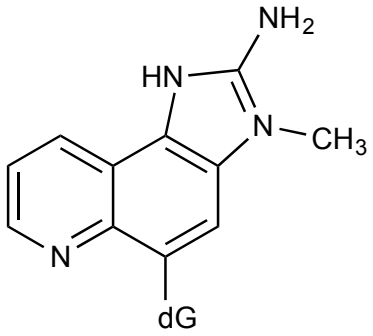

Figure 9. The N^2-IQ-dG adduct intercalated into the duplex unlike the benzo[*a*]pyrene diol expoxide.

As previously mentioned with the adduct, large bulky adducts often require the TLS polymerases (often in the Y-family) to bypass them in the DNA. Polymerase bypass studies found that hPolκ was the most efficient enzyme at bypassing various bulky N^2 adducts with hPolη showing signs of stalling and misincorporation.[35] The work here seeks to explain the structural characteristics of the N^2-AP-dG adduct (**Figure 10**). Since the adduct is attached near the hydrogen-bonding interface, it is hypothesized there will be some perturbance of the DNA structure.

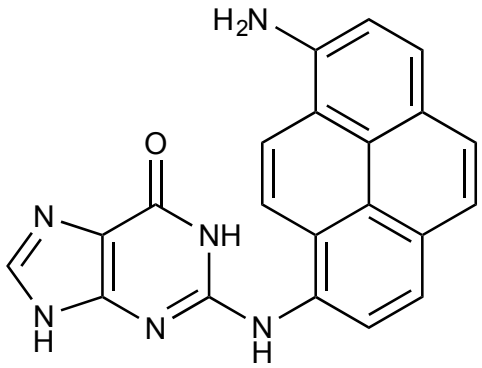

Figure 10. N^2-Aminopyrene-dG.

Structural Information by Nuclear Resonance Spectroscopy

Structural biology and biophysical chemistry have developed powerful tools and methods to parse the structural characteristics of nucleic acids, proteins, and biological processes. There are various modalities used such as nuclear magnetic resonance spectroscopy (NMR), X-ray crystallography, computational methods, cryo electron microscopy (cryo-EM), small angle X-ray scattering (SAXS), and others. While all of these approaches are powerful, they are also limited in different ways. Therefore, it is common to use multiple modalities to complement one another. NMR has proven to be useful because it is able to generate solution structures of nucleic acid and proteins. This is a significant advantage because it is more closely related to biological conditions, unlike X-ray crystallography which can only generate static structures with no conclusive insight into dynamics. Multiple NMR experiments and pulse sequences can be used to generate structural information for nucleic acids.

Correlation Spectroscopy (COSY): The COSY experiment is a 2D homonuclear experiment that measures through-bond interactions.[36] Protons that are within three bonds of each other will be observable through the transfer of magnetization that occurs. Unlike TOCSY which can observe long distance through-bond interactions in a spin system, COSY is a short distance through-bond approach.[37] For this project, the primary purpose of the COSY was to observe the H5-H6 scalar couplings of cytosines in the duplex. Ultimately, the information in COSY ensures that the correct number of cytosine cross-peaks are present, and it helps to simplify NOESY spectrum assignment since the chemical shifts of the cytosine cross-peaks will be the same in both experiments.

Nuclear Overhauser Effect Spectroscopy (NOESY): The NOESY pulse sequence revolves around the NOE (nuclear Overhauser effect) which is a transfer of magnetization through-space.[38,40] It is a 2D homonuclear approach like the COSY. However, unlike the COSY, the NOESY experiment observes through-space interactions which provide most of the structural information. Protons that are within five angstroms of each other can transfer magnetization between each other which generates a cross peak for that interaction in the spectrum. Therefore, the NOE is very sensitive to the distance between protons and quickly falls off at a rate of $1/r^6$.[39,30] The NOESY spectra are more complicated than the COSY spectra, however, assignment strategies previously detailed and explained are in place to parse the structural information from the data.[40]

CHAPTER II

Materials and Methods

Synthesis of Samples

Unmodified and Complementary Stands: All unmodified oligonucleotides were synthesized by Midland Reagent Company (Midland, Texas).

MeFapy: The MeFapyG modified oligonucleotide was initially synthesized and purified by Tracy Salyard-Johnson and Dr. Chanchal Malik, collaborators in the Rizzo laboratory at Vanderbilt University. The sequence was 5'-($G^1C^2T^3A^4G^5T^6\underline{X}^7G^8G^9T^{10}C^{11}C^{12}$)-3' where $\underline{X}^7$ is MeFapyG. Dr. Plamen Christov of the Rizzo lab previously developed the synthetic scheme for this sample.[21] Briefly, the N7-position of 5'-DMTr-dG is methylated using methyl iodide. To achieve the ring opening to MeFapy-dG, the reaction is treated with sodium hydroxide, then the base is neutralized with hydrochloric acid. Once the MeFapy-dG nucleoside is synthesized, it is then converted into a phosphoramidite so that it can be incorporated into an oligonucleotide via solid phase synthesis.

Aminopyrene: Synthesis and initial purification of the oligonucleotide containing N^2-AP-dG was accomplished by the research group of Dr. Ashis Basu and Dr. Chanchal Malik at the University of Connecticut. The sequence was 5'-($G^1T^2G^3C^4\underline{X}^5T^6G^7T^8T^9T^{10}G^{11}T^{12}$)-3' where $\underline{X}^5$ is the N^2-AP-dG adduct. This sequence was selected due to its relevance to codon 273 in the p53 gene.[41] The original synthetic protocol was characterized by Dr. Debasis Chakraborti in Dr. Basu's lab at the University of Connecticut. The synthesis utilized a Buchwald-Hartwig palladium-catalyzed amination reaction. Nitropyrene with a leaving group (such as a halogen) was allowed to react

with a nucleoside. The optimized cross-coupling reaction was affected by a Pd catalyst, the presence of a base, and an electron rich ligand.[42]

Ultraviolet-Visible Spectroscopy

UV/Vis was used to obtain an optical density (OD) measurement. Ultimately, this would provide a concentration of each individual strand. The unmodified and modified strands were dried with a Labconco centrivap. For MeFapy, 1.00 mL of pH 7.4 phosphate buffer (10 mM phosphate buffer with 0.01 mM EDTA and 100 mM NaCl) was added to the sample. For aminopyrene and the unmodified strands, 1.00 mL of milli-pure deionized water was added. The samples were subsequently vortexed then centrifuged. A blank cuvette was filled with 1.00 mL of the phosphate buffer or water depending on the sample and mixed in the cuvette. Then the Varian Cary 100 Bio spectrometer was blanked. Next, 1.00 μL of the DNA sample was added to the blank cuvette and an OD reading was obtained. Using the IDT OligoAnalyzer tool, the extinction coefficient could be obtained along with the OD to nanomole conversion factors.

MALDI Mass Spectrometry

All single strand oligomers, including modified and unmodified, were characterized using a Voyager MALDI-TOF instrument. The matrix was composed of 3-hydroxypicolinic acid and ammonium hydrogen citrate. All readings were collected in the negative-ion mode. DNA was dissolved in 500 μL of milli-pure water. Then 1.00 μL of the DNA was mixed with 10 μL of 3-hydroxypicolinic acid and 5 μL of ammonium hydrogen citrate. Upon vortexing and centrifuging, 1.5 μL of the sample/matrix mixture was plated on a MALDI plate and allowed to dry. Upon drying, another 1.00 μL of the sample/matrix mixture was added.

Preparation of DNA Duplex

Once the concentration of the single-stranded oligonucleotides had been obtained, a duplex was made by adding the same concentration (nanomoles) of each strand to a centrifuge tube. Upon adding the correct amount of material of both strands to a tube, the DNA sample was placed in a heat bath to denature the strands for 10 min. After ten min., the heat bath was turned off, and the sample was allowed to sit overnight to ensure that the strands would anneal to form the duplex. The optical density of the duplex was checked on UV-Vis.

High Performance Liquid Chromatography

Both samples (MeFapy-dG and Aminpyrene) were purified using reverse-phase high performance liquid chromatography (RP-HPLC). Initially, each single strand was purified separately before being annealing into a duplex. The dried oligomers were suspended in 500 µL of deionized water. Aliquots of the sample were then injected onto the HPLC depending on the concentration. Upon purification of the single strands, the duplex was formed, and the purity of the duplex was checked using HPLC again. 0.1 M, pH 7.0 ammonium formate buffer with acetonitrile was used as the mobile phase using gradient elution. The method started at 5% acetonitrile and was maintained for 5 minutes. Then from 5 minutes to 55 minutes, the acetonitrile ratio was gradually increased to 16% and held for 5 minutes. From 60 minutes to 65 minutes, the acetonitrile ratio was gradually increased further to 35%. Finally, from 65 to 70 minutes, the acetonitrile concentration was dropped down to 5% gradually. The column was purchased from Phenomenex, and it was a preparative Gemini C-18 250x10 mm column. In some runs, an analytical Luna C-18 250x4.6 mm column was used for enhanced purification. Once the single strand oligomers were purified, they were annealed to their respective

complementary strands. After the purification, the samples were freeze dried using a lyophilizer to remove the acetonitrile.

Hydroxyapatite Anion Exchange Chromatography

Once the duplexes were formed, a hydroxyapaptite column was employed to separate excess single strand oligomers from the duplex. The HAP column acts as an anion exchange column which allows further purification of the duplex oligonucleotides. The buffers used in this purification were 10 mM and 100 mM sodium phosphate, each with 100 mM NaCl and 0.05 mM EDTA. After elution, the samples were dried down in the Labconco centrivap.

Size Exclusion Chromatography

The HAP buffers introduced a large concentration of salt to the samples. Therefore, a G25 Sephadex desalting column was used to remove the excess salt. This type of column utilizes size-exclusion chromatography to separate the duplex (or single strand oligomers) from the salt. The sample is dissolved in 1.00 mL of milli-pure water and placed onto the column. Milli-pure water is used as the mobile phase with an isocratic method. Ultimately, the sample and salt eluted at separates times. After the sample was desalted, the sample was dried down in the centrivap.

Nuclear Magnetic Resonance Spectroscopy

General: Once the duplexes had been dried down and desalted, the sample was dissolved in 180 µL of a 10 mM phosphate buffer with 0.01 mM EDTA and 100 mM NaCl (pH 7.4 for MeFapy-dG, pH 7.0 for N^2-AP-dG). The samples were then dried down in the centrivap. In order to examine the non-labile protons, a D_2O exchange was carried out three times. The sample was

then dissolved in 99.996% D_2O. All spectra were analyzed using Topspin and Sparky software. Furthermore, all unmodified samples correlating to MeFapy-dG and N^2-AP-dG were treated in the exact same manner.

Unmodified duplex: NOESY and magnitude COSY experiments were performed on Bruker 800 (MeFapy-dG) and 900 (N^2-AP-dG) MHz instruments at 298 K with 2048 real data points in the t_2 dimension and 512 real data points in the t_1 dimension. The NOESY spectra were obtained using a mixing time of 250 ms. The NOESY and COSY spectra were zero-filled during processing to obtain final matrices of 2048·1024 data points.

MeFapy-dG: Prior to collecting the 2D data, 1D scans were carried out which consisted of 32768 data points on a 800 MHz Bruker NMR spectrometer at 298 K. Using the same approach mentioned for the unmodified duplex, 2D NMR data was collected for the MeFapy-dG sample.

N^2-AP-dG: Prior to collected of 2D data, 1D scans were carried out which consisted of 32768 data points on a 900 MHz Bruker NMR spectrometer at 298 K. The same approach used for the unmodified and MeFapy-dG duplexes was used for the N^2-AP-dG duplex.

NOESY Sequential Walk: The NOESY spectra have a distinct area of the spectrum denoted as the "walking region", and it is representative of interactions between H1' protons on the sugar between H8 protons on purines and H6 protons on pyrimidines. Starting at the 5'-end of each individual strand, the interactions between the H1' and H8/H6 protons can be sequentially labeled, or "walked", all the way to the 3'-end. The figure below illustrates the sequential walk.

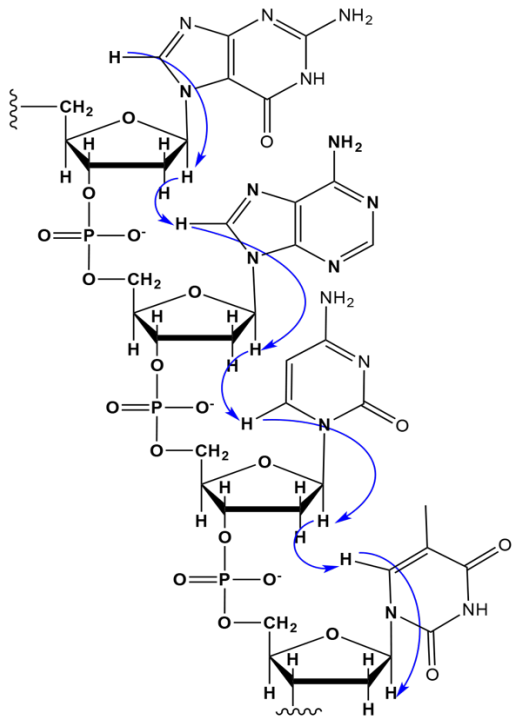

Figure 11. A depiction of the interactions that are analyzed in the NOESY walking region between H1' of the sugar and H8/H6 protons of purines and pyrimidines, respectively.

For unmodified samples, the walk should be continuous since unmodified duplexes exhibit typical B-DNA structures. However, it is common for modified duplexes to have breaks or discontinuities due to structural perturbations. Once the sequential walk is established, it can be used to help assign other cross-peaks throughout the spectrum.

CHAPTER III

Methyl Formamidopyrimidine Results and Discussion

Introduction

Ken Dempster in the Stone laboratory previously analyzed the unmodified version of the MeFapy-dG sequence. The NMR assignments were checked further and confirmed. However, complete analysis of the modified MeFapy-dG sample had not been completed. The MeFapy-dG duplex sequence was 5'-d($G^1C^2T^3A^4G^5T^6X^7G^8G^9T^{10}C^{11}C^{12}$)-3'-5'-d($G^{13}C^{14}A^{15}C^{16}C^{17}C^{18}A^{19}C^{20}T^{21}A^{22}G^{23}C^{24}$)-3'. Initial assignments on the modified sample and NMR parameters were originally completed and set by Ken Dempster; however, some corrections and further assignments were made to the existing data.

Analysis of the Modified D_2O COSY NMR Spectra

The unmodified duplex yielded typical B-type DNA behavior, and it acted as a reference for the modified duplex. The COSY spectra are used to locate cytosine H5-H6 scalar spin couplings. Ultimately, this information is used as a reference point in the more complicated NOESY spectra. It is important to note that the COSY spectra contains more structural information, but the H5-H6 scalar couplings for cytosine are the primary purpose of using the experiment. The data was collected at 298 K on an 800 MHz Bruker spectrometer.

The MeFapy-dG sequence contains eight cytosines. Therefore, it would be expected to see eight cross-peaks in the COSY spectrum corresponding to the H5-H6 scalar couplings (F1, 5.10 to 6.15 ppm ;F2, 7.1ppm to 7.85 ppm). This can be seen in **Figure 12** below. Interestingly,

the C20 cross-peak appears to be broadening, potentially indicating that the base is experiencing multiple environments. This could be evidence of the MeFapy lesion's various rotameric species.

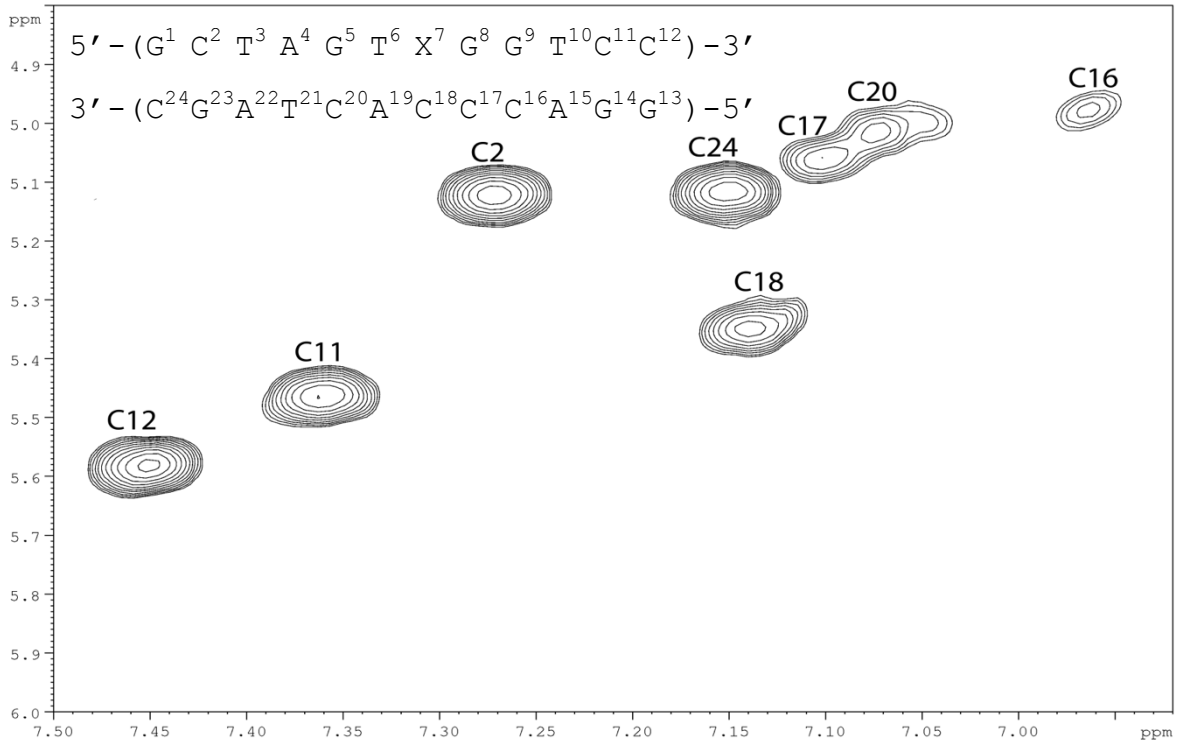

Figure 12. H5-H6 scalar coupling region of the MeFapy COSY spectrum taken at 298 K on an 800 MHz instrument.

Analysis of the Modified D_2O NOESY NMR Spectra

The modified sample offered some interesting insights. On the modified strand of the modified MeFapy sample there were a couple of breaks in the walk indicating structural perturbations. For instance, the sequential walk stops after T6, the 5'-neighbor base to the MeFapy lesion, and it does not resume until T10. An explanation of this could be that the neighboring base cross-peaks have broadened due to various conformations MeFapy-dG can adopt, potentially indicating that MeFapy-dG does not adopt a single conformation in solution.

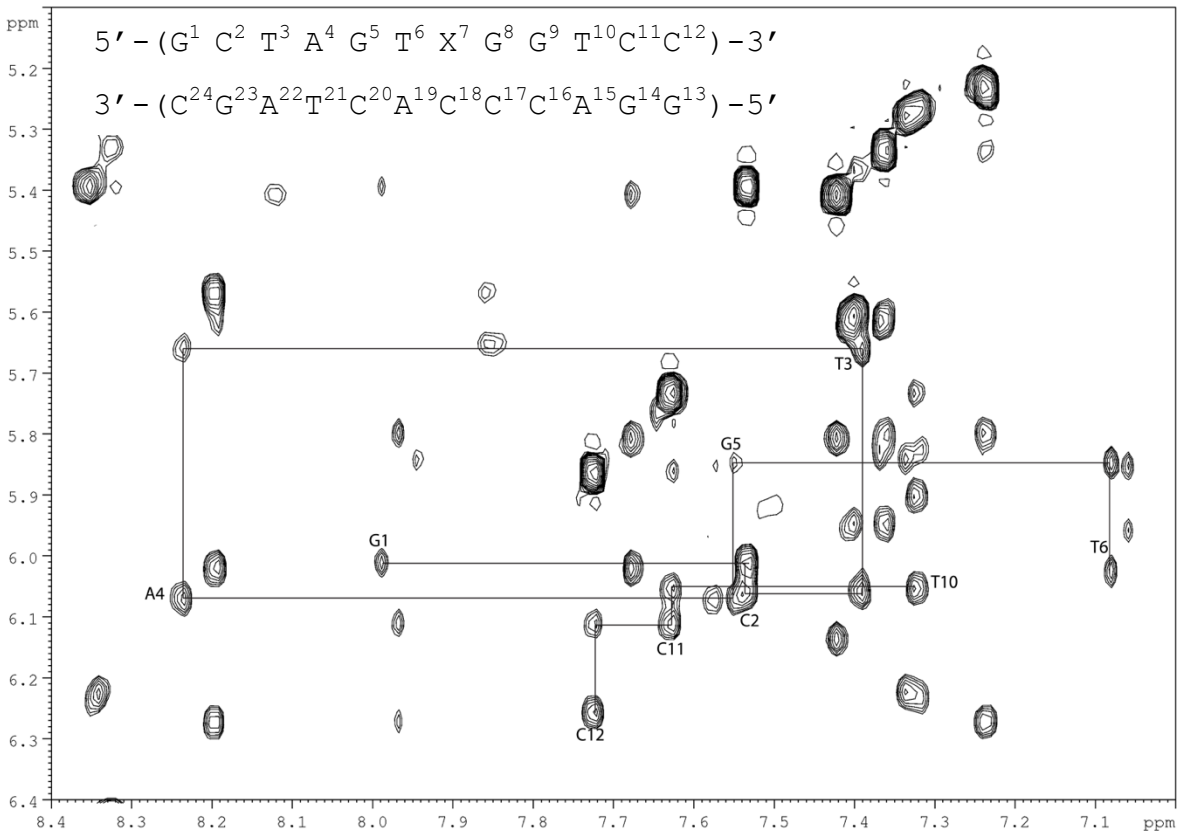

Figure 13. The NOESY walking region of the modified strand.

Furthermore, the complementary strand exhibits a couple of breaks in the walking region, as well. The walk is mostly continuous up until C18 where the cross-peak from C18 H1' to A19 H8 has broadened and/or is missing. The walk breaks again at C20 where it does not pick up until A22. Therefore, it is obvious that the MeFapy-dG lesion is causing some structural perturbations around the lesion site. However, work is still ongoing to determine predominant MeFapy-dG structural conformations in solution.

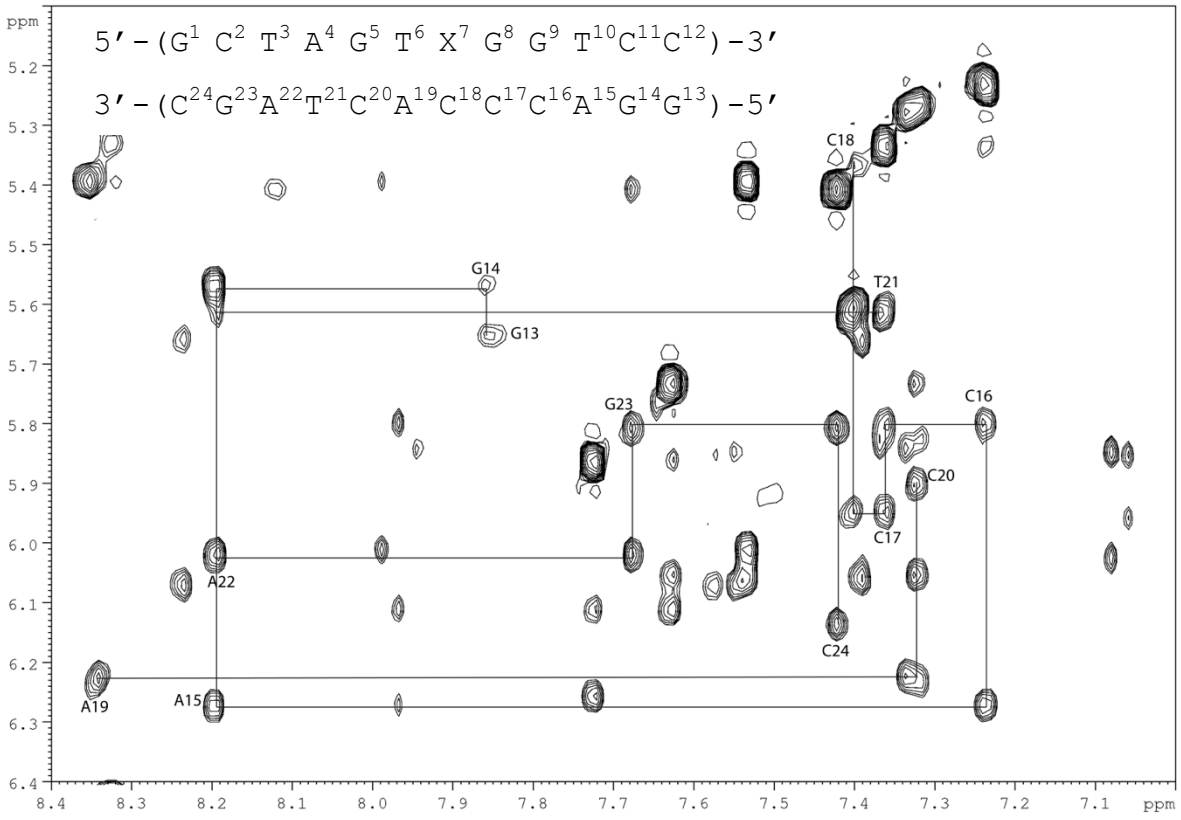

Figure 14. The NOESY walking region of the complementary strand.

Parsing out the MeFapy-dG methyl cross-peak amongst the thymine methyl cross-peaks was an important step in locating other lesion peaks throughout the spectra. Therefore, work was done to conclusively determine which methyl peak belonged to the MeFapy-dG lesion. **Figure 15** below demonstrates the several of the methyl peaks in the MeFapy-dG D_2O spectrum. In the figure, only one of the MeFapy-dG peaks was conclusively located, and it is labeled as X7Me-H8. Therefore, this indicates that this a cross-peak of the MeFapy-dG methyl group with the formyl (denoted as H8) proton of the lesion. Other methyl peaks belonging to the lesion were expected, but they have not been conclusively identified at this time.

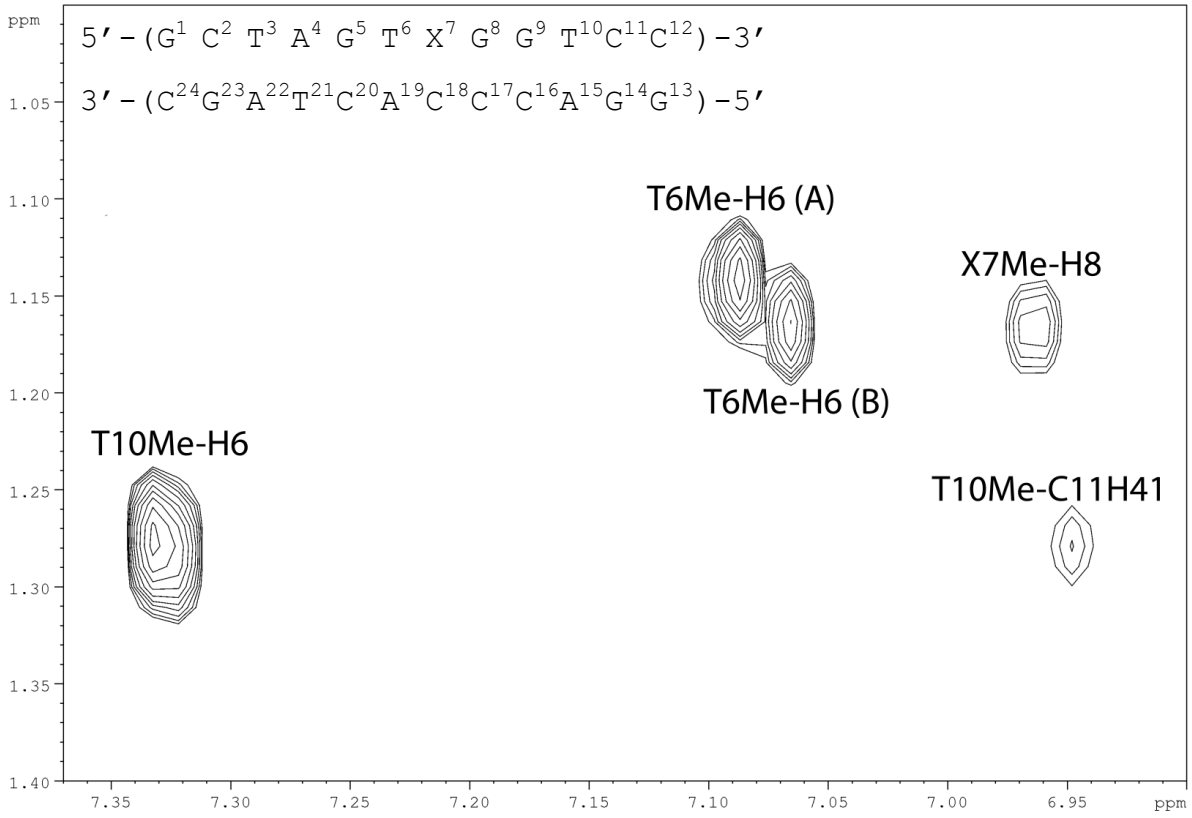

Figure 15. The methyl region of the MeFapy-dG spectrum.

Figure 16 illustrates how the MeFapy-dG methyl peak discussed in Figure 15 interacts with other known lesion cross-peaks in the spectrum. The top panel of Figure 16 is the methyl region. The middle panel is the H2'1/H2'2 region of the spectrum. Finally, the bottom panel is adjacent to the diagonal in the 2D NOESY spectrum. The assigned MeFapy-dG methyl cross-peak aligns with the lesion peaks in the H2'1/H2'2. Interactions can be traced down to the diagonal where the formyl proton of the MeFapy-dG is located. The bottom panel shows that the signal on the diagonal also lines up with a cross-peak which is an interaction between the formyl proton of the lesion and the H6 proton of the 5'-neighboring base, T6.

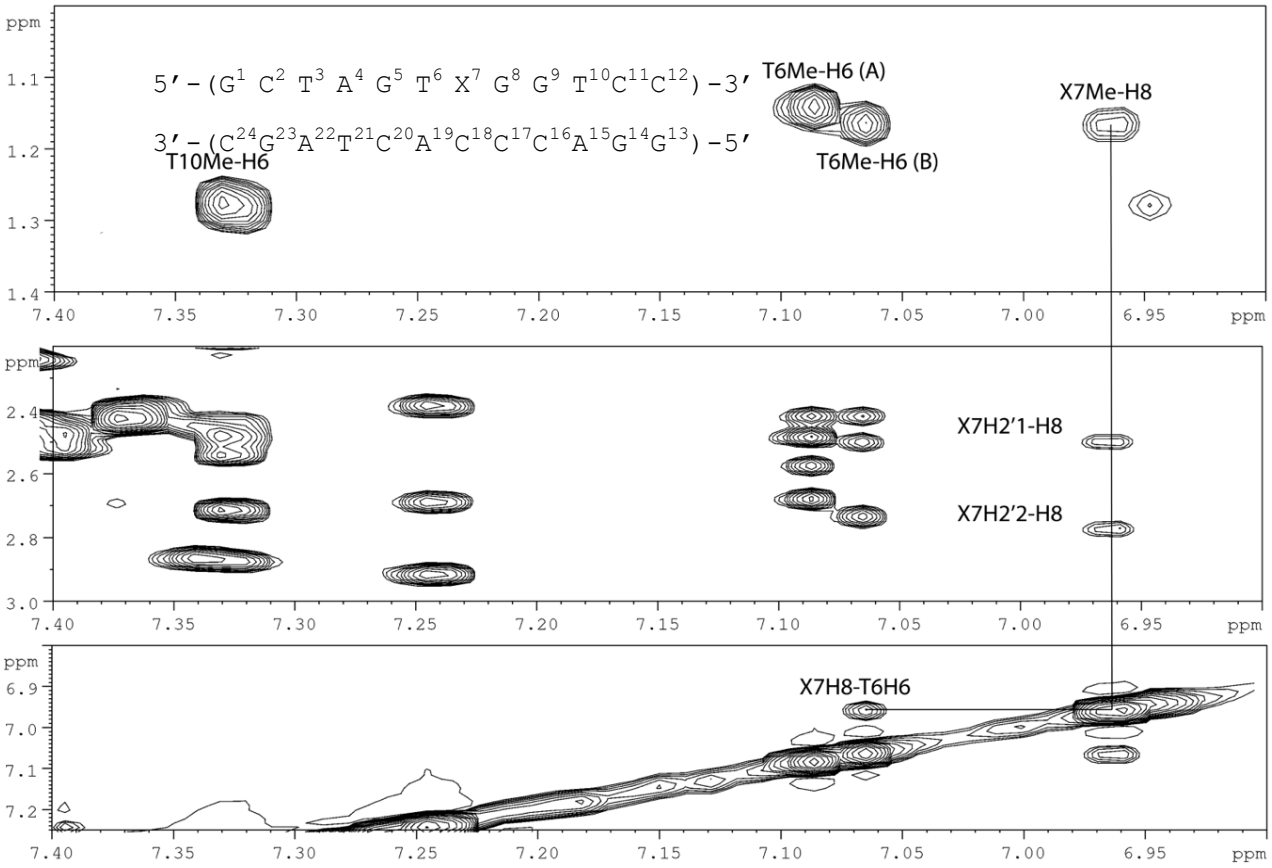

Figure 16. Tile plot linking interactions of the formyl (H8) proton of the MeFapy-dG lesion.

Assignments in the modified sample were subsequently compared to assignments in the unmodified sample. As previously mentioned, MeFapy-dG can adopt various conformations. Therefore, work was done to determine if the two anomers (α and β) were visible in the spectrum. Interestingly, the H2'1/H2'2 region offered some valuable insight. **Figure 17** shows a comparison of the modified (left panel) and the unmodified (right panel) at 298 K. It appears that in the modified sample, there is a "doubling" of neighboring base cross-peaks. This provides potential evidence of the various anomeric populations of MeFapy-dG in the solution. If this is

the case, the three peaks in the modified sample corresponding to the same chemical shift as the three peaks in the unmodified panel would indicate the β-anomer.

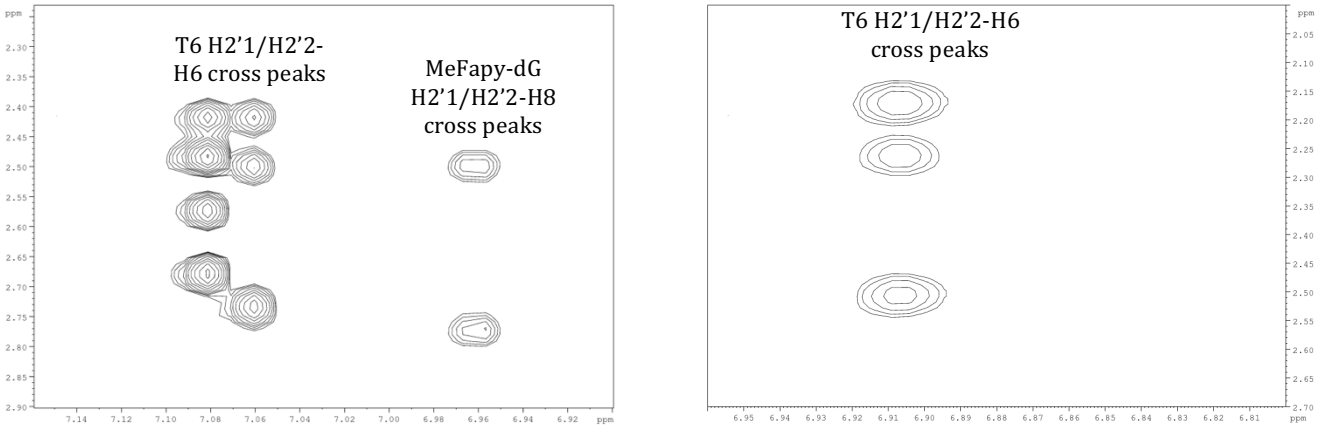

Figure 17. Comparison of modified (left) and unmodified (right) H2'1/H2'2 region. The modified panel illustrates doubling of peaks of the neighboring T6 base. The peaks furthest to the right in the modified sample are the X7 H2'1-H8 and H2'2-H8 cross-peaks. The doubled peaks are the T6 H2'1-H6 and H2'2-H6 peaks.

CHAPTER IV

N^2-Aminopyrene-dG Results and Discussion

Introduction

The N^2-AP-dG adduct was placed in the 5'-d($G^1T^2G^3C^4\underline{X}^5T^6G^7T^8T^9T^{10}G^{11}T^{12}$)-3'-5'-d($A^{13}C^{14}A^{15}A^{16}A^{17}C^{18}A^{19}C^{20}G^{21}C^{22}A^{23}C^{24}$)-3' sequence where the adduct is attached to a guanine represented as $\underline{X}^5$. The sequence is biologically significant because it represents codon 273 in the p53 tumor suppressor gene which is a known mutational hotspot for various cancers.[32] Initially, an unmodified sequence was annealed and analyzed. The unmodified sample would act as a control or a basis of comparison for the modified duplex.

Analysis of the Unmodified D_2O NOESY Spectrum

Figure 18 shows the unmodified strand of the unmodified sample sequential walk. As expected, the walk was continuous since the sample was unmodified. The walk can be easily traced down the unmodified strand, from 5'→3' (G1 to T12). This is evidence that the unmodified sequence exhibits B-type DNA structure.

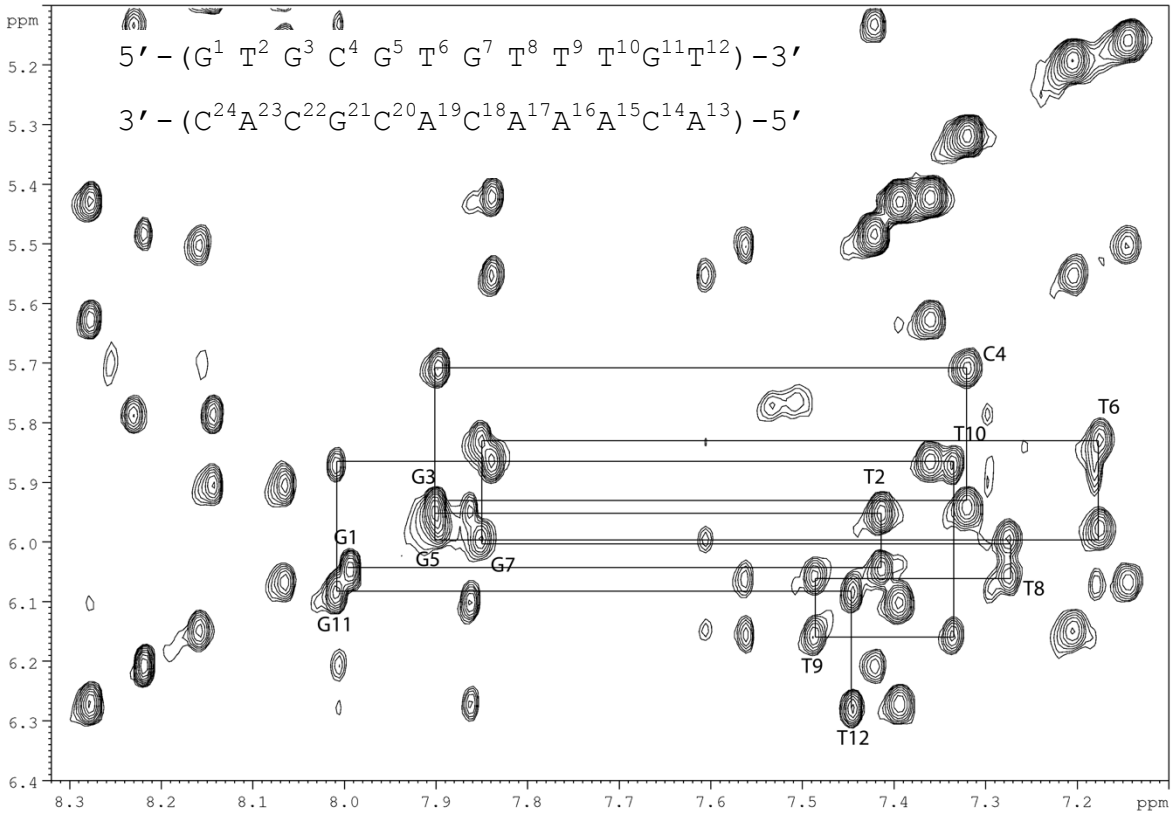

Figure 18. Walking region of the unmodified strand of the unmodified sample at 308 K on a 900 MHz instrument.

Similarly to the unmodified strand of the unmodified sample, the complementary strand (**Figure 19**) exhibited an unbroken continuous walk from A13 to C24, indicating B-DNA structure. Other regions throughout the spectrum were also assigned, but they are not detailed here.

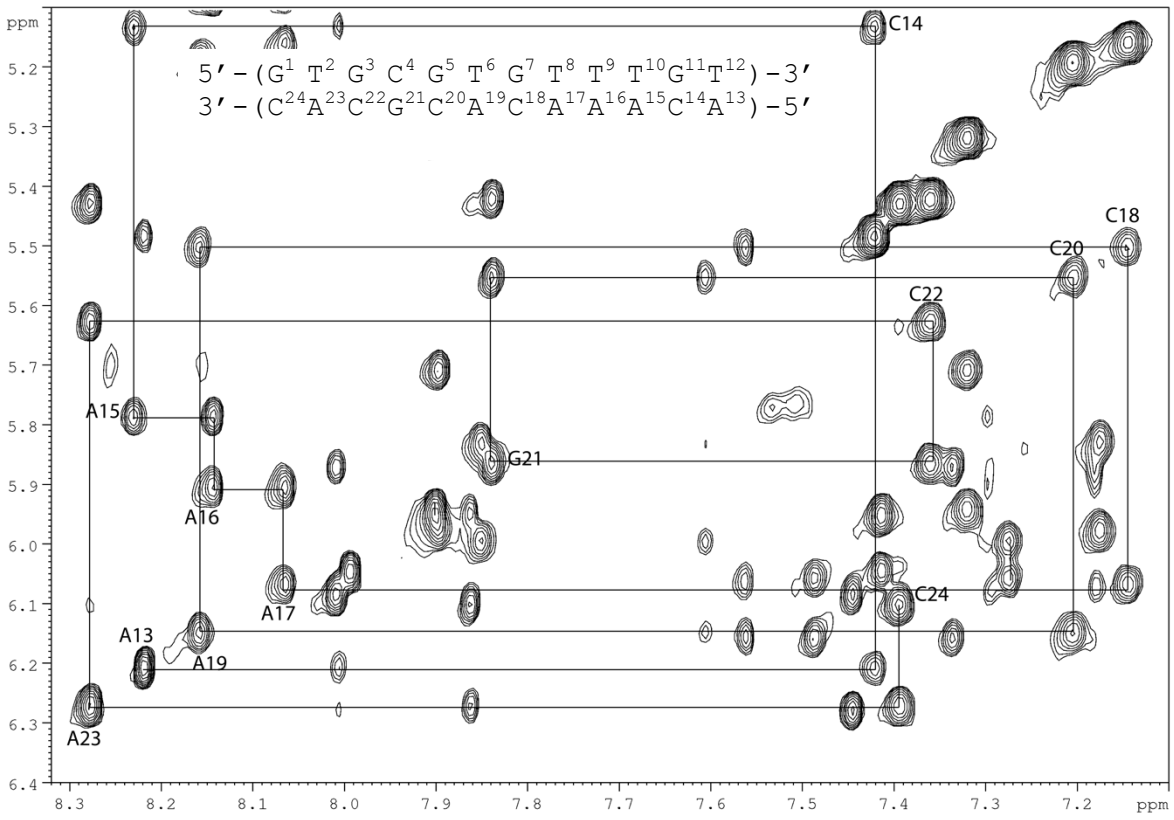

Figure 19. Walking region of the complementary strand of the unmodified sample at 308 K on a 900 MHz instrument.

Characterization of the Modified N^2-AP-dG Duplex

Once the unmodified duplex was analyzed, data was collected on the modified duplex. The first task was to locate the aminopyrene protons in the COSY spectra. The cross-peaks located there could then be used in the NOESY to trace interactions between the adduct and the DNA. Magnitude COSY spectra were collected at various temperatures. **Figure 20** shows the aminopyrene cross-peaks at 283 K. Interestingly enough, there only appears to be two adduct cross-peaks at this temperature while four were expected. It could be that at certain temperatures the scalar couplings between the protons of the adduct are isochronous.

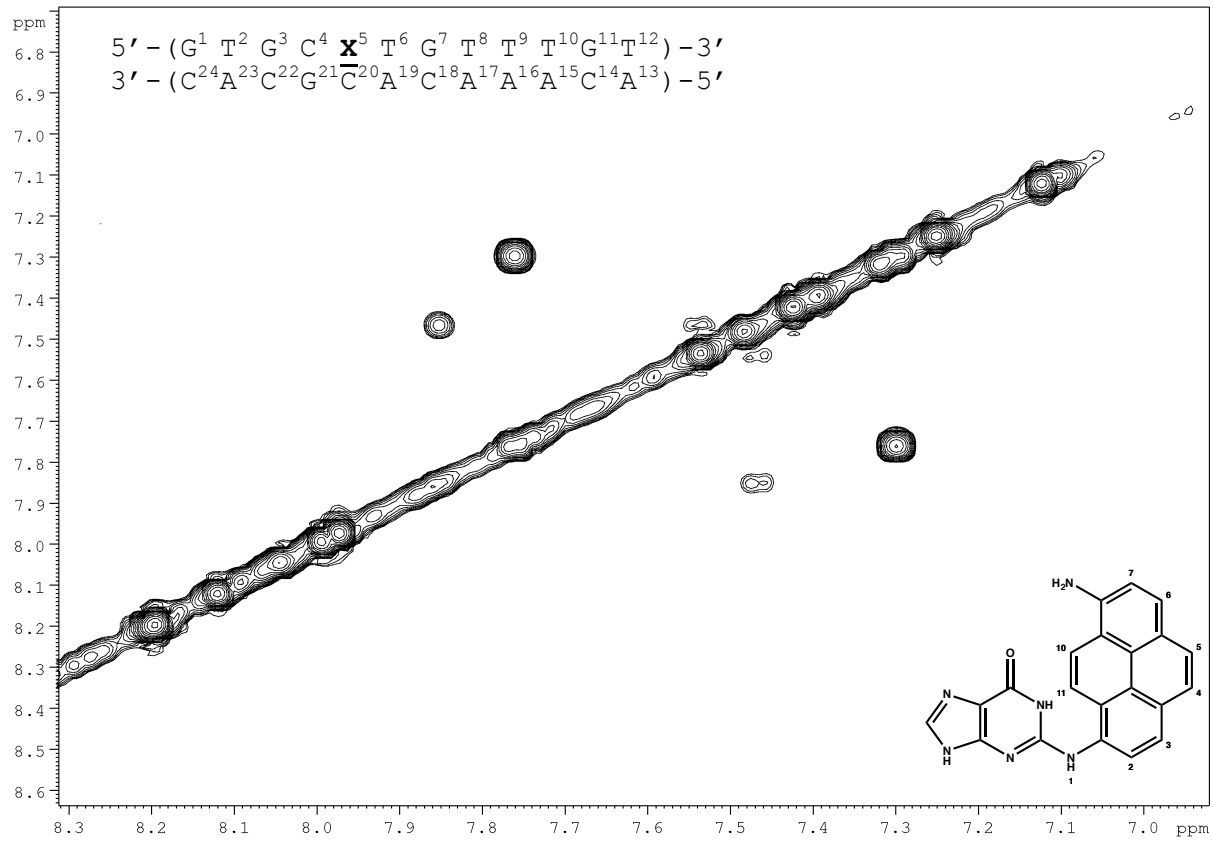

Figure 20. The aminopyrene proton cross-peaks in the magnitude COSY spectrum at 283 K on a 900 MHz instrument.

Furthermore, if the temperature is increased to 308 K, more dispersion is noted in the same region. **Figure 21** illustrates this and shows that more cross-peaks are starting to arise. Thus, this indicates there is some temperature dependence to the lesion. This dependence could be attributed to motion of the adduct, or perhaps the scalar couplings of the adduct protons are too low to be fully visualized in a magnitude COSY experiment. Therefore, a separate experiment, such as a DQF-COSY would be more beneficial. A magnitude COSY is a great tool when the coupling constants are large (such as is the case for the cytosine H5-H6 scalar couplings). However, it is not the ideal experiment if the *J*-constant is lower than 4 Hz.

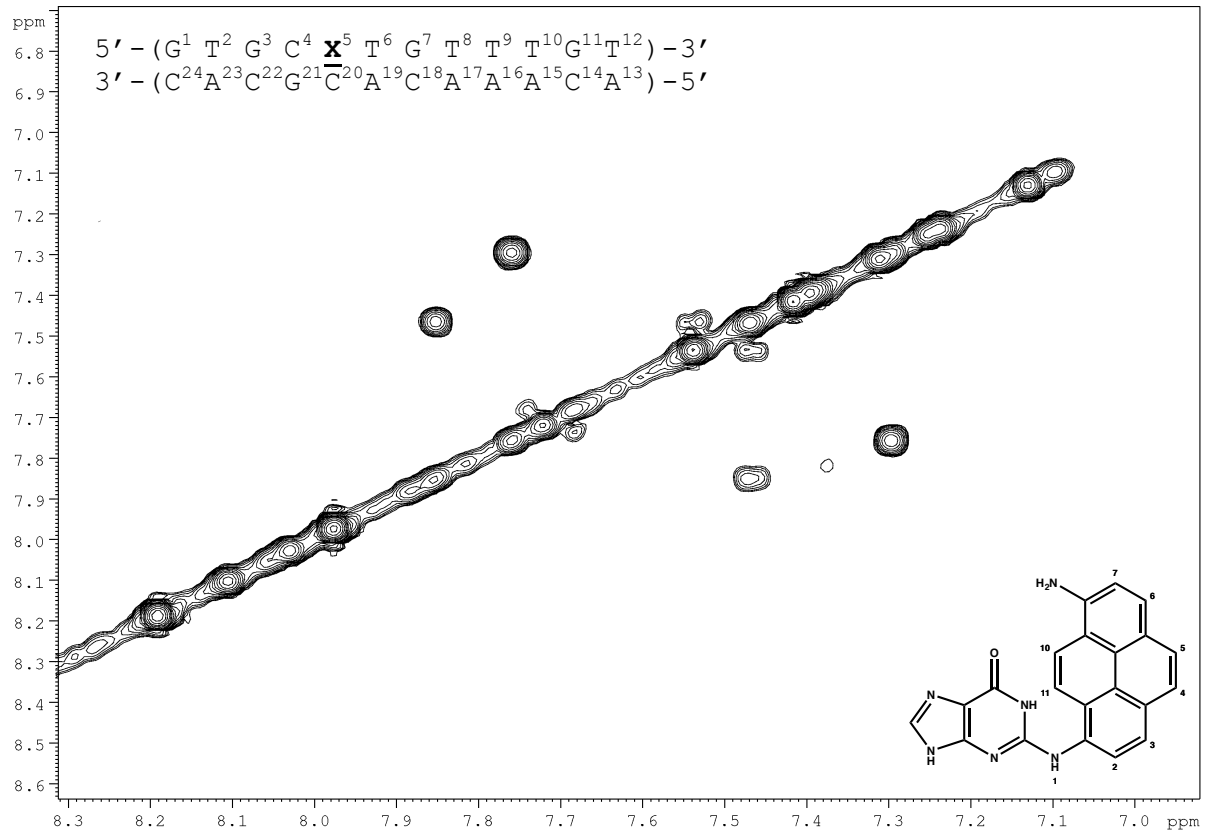

Figure 21. The aminopyrene proton cross-peaks in the magnitude COSY spectrum at 288 K on a 900 MHz instrument.

To determine if the adduct proton cross-peaks became more intense as the temperature was increased, spectra were acquired at other temperatures. The peaks were most dispersed at 308 K. This can be seen in **Figure 22**. Therefore, this temperature should be chosen for future experiments.

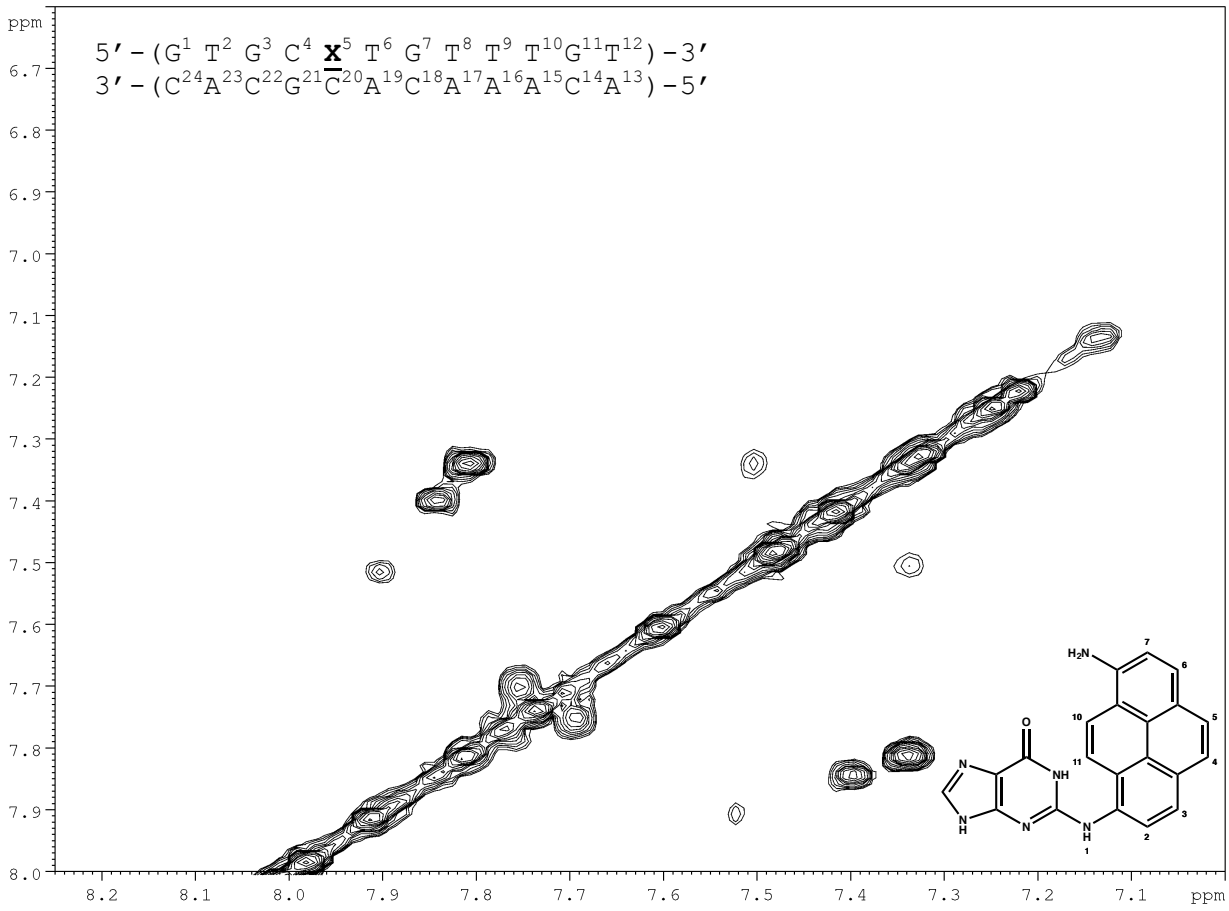

Figure 22. The aminopyrene proton cross-peaks in the magnitude COSY spectrum at 308 K on a 900 MHz instrument.

To be thorough, an extra experiment was ran at 338 K to see what the adduct protons were doing once the strands were melted. It can be seen that the dispersion of peaks gained at 308 K was lost, and only two cross-peaks are observed (similar to the 283 K experiment). It was concluded that at very low and very high temperatures, the cross-peaks belonging to the adduct protons are isochronous, meaning that they are overlapping at the same chemical shift. The 338 K COSY is shown in **Figure 23**.

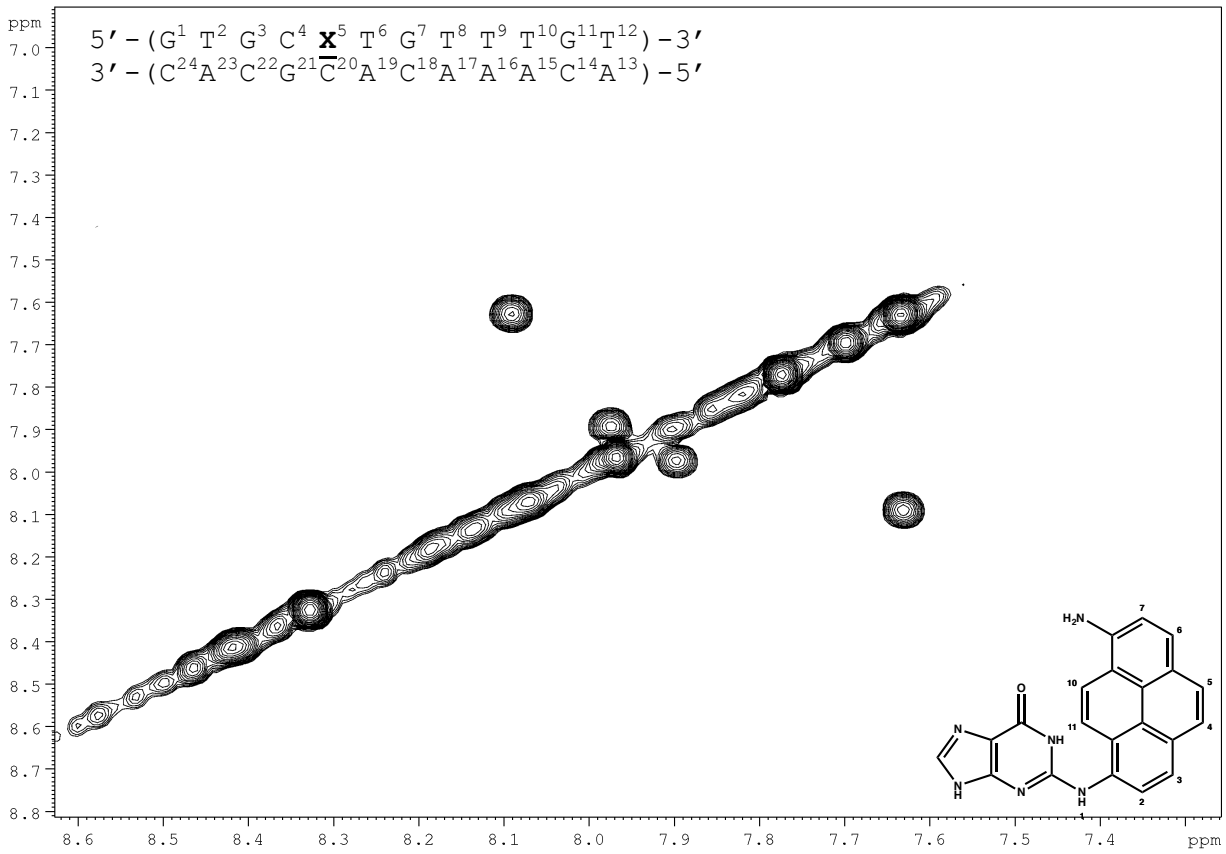

Figure 23. The aminopyrene proton cross-peaks in the magnitude COSY spectrum at 338 K on a 600 MHz instrument.

Once the COSY spectra were obtained, NOESY spectra were collected at 35°C on the modified duplex. Since the data demonstrated some unusual characteristics, it is important to note that the conclusions derived from the data are preliminary and only speculation. Further purification of a new N^2-AP-dG duplex is required for further analysis. **Figure 24** shows the walking region of the modified strand. The walk is continuous up until T6 where it breaks. It can be seen that T6 has shifted off of the panel further upfield, meaning that the T6 has become more shielded. This could be potential evidence for aminopyrene sitting in the minor groove and pointing in the 3' direction of the modified strand. Therefore, the two peaks that are missing are

the T6 H1'-H6 and T6 H1'-G7 H8 cross-peaks. The walk picks back up at G7 H1'-H8 and continues unbroken to the end of the strand.

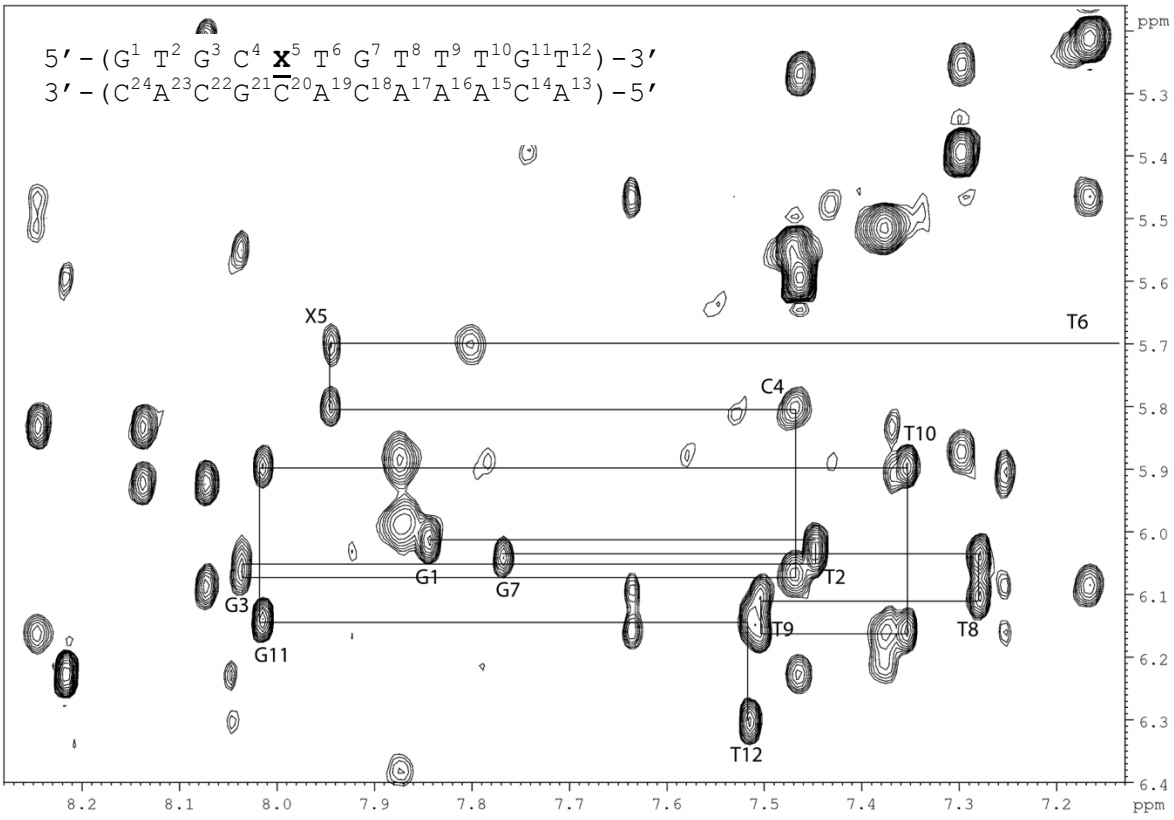

Figure 24. Walking region of the modified duplex, modified strand at 308 K on a 900 MHz instrument.

A closer inspection reveals that the X5 H1'-T6 H6 cross peak lines up with the other T6 cross-peaks including: T6 Me-H6, T6 H2'1-H6, T6 H2'2-H6, X5 H2'1-T6 H6, and X5 H2'2-T6 H6. This can be visualized in the NOESY spectrum shown in **Figure 25**. The large upfield chemical shift of the T6 base is indicative of increased shielding. Therefore, this is evidence that the AP adduct is sitting in the minor groove, pointing toward the 3' end of the modified strand.

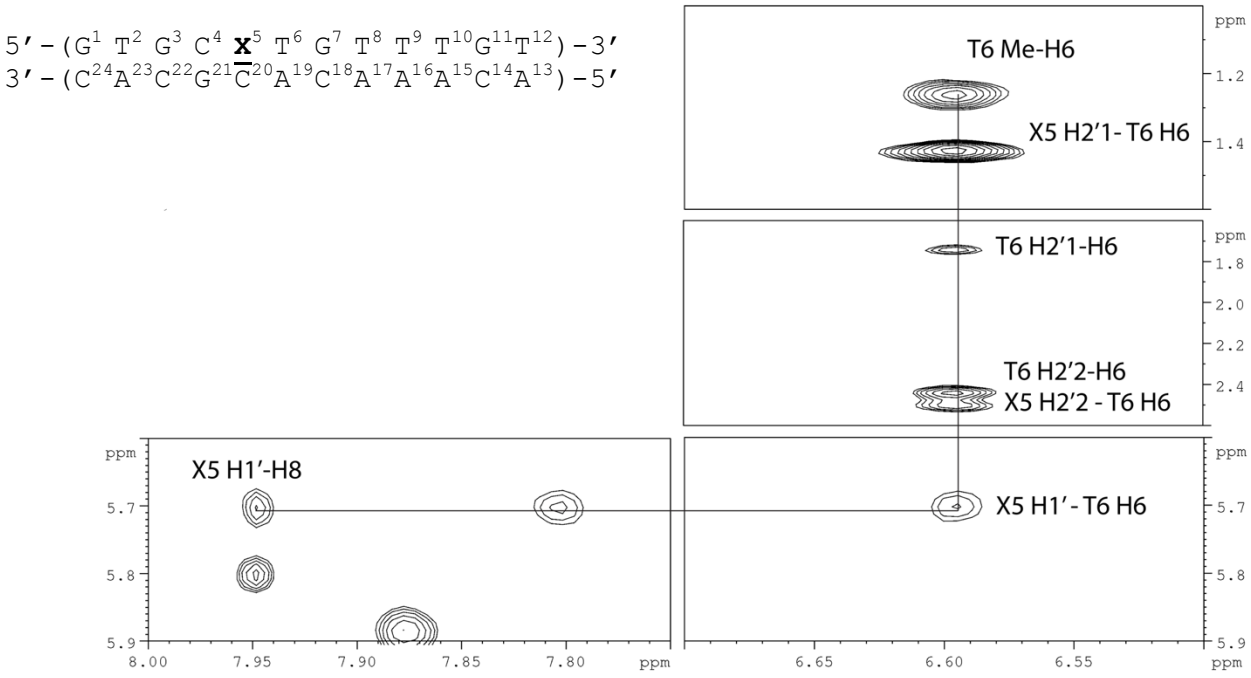

Figure 25. T6 connectivities, modified strand at 308 K on a 900 MHz instrument.

More problems are encountered on the complementary strand of the modified duplex. **Figure 26** shows the walking region of the complementary strand. The walk is continuous from A13 to A19, and then it breaks right around C20, which is the base opposite of the adducted base. The C20 H5-H6 cross-peak is seen, but the C20 H1'-H6 cross-peak was never located. If the C20 base was flipped out like it may be in the case of an intercalated structure, the cross-peak would still be present, but it would likely shift further downfield indicating more deshielding of the base. However, this is not observed for the aminopyrene sample. It could be that the C20 H1'-H6 base is overlapped with another cross-peak in the spectrum.

The walk picks up again at G21. Interestingly, the G21 cross-peaks have broadened out significantly throughout the spectrum. The walk breaks again at A23, and the peaks assigned for

A23 were highly speculative. Another unusual characteristic is that the C24 peaks have not been located. The C24 H5-H6 cross-peak could not be conclusively found in the COSY spectra similar to the NOESY spectra. This is unusual, and it is the main reason the conclusions from the NOESY data is speculative and not conclusive.

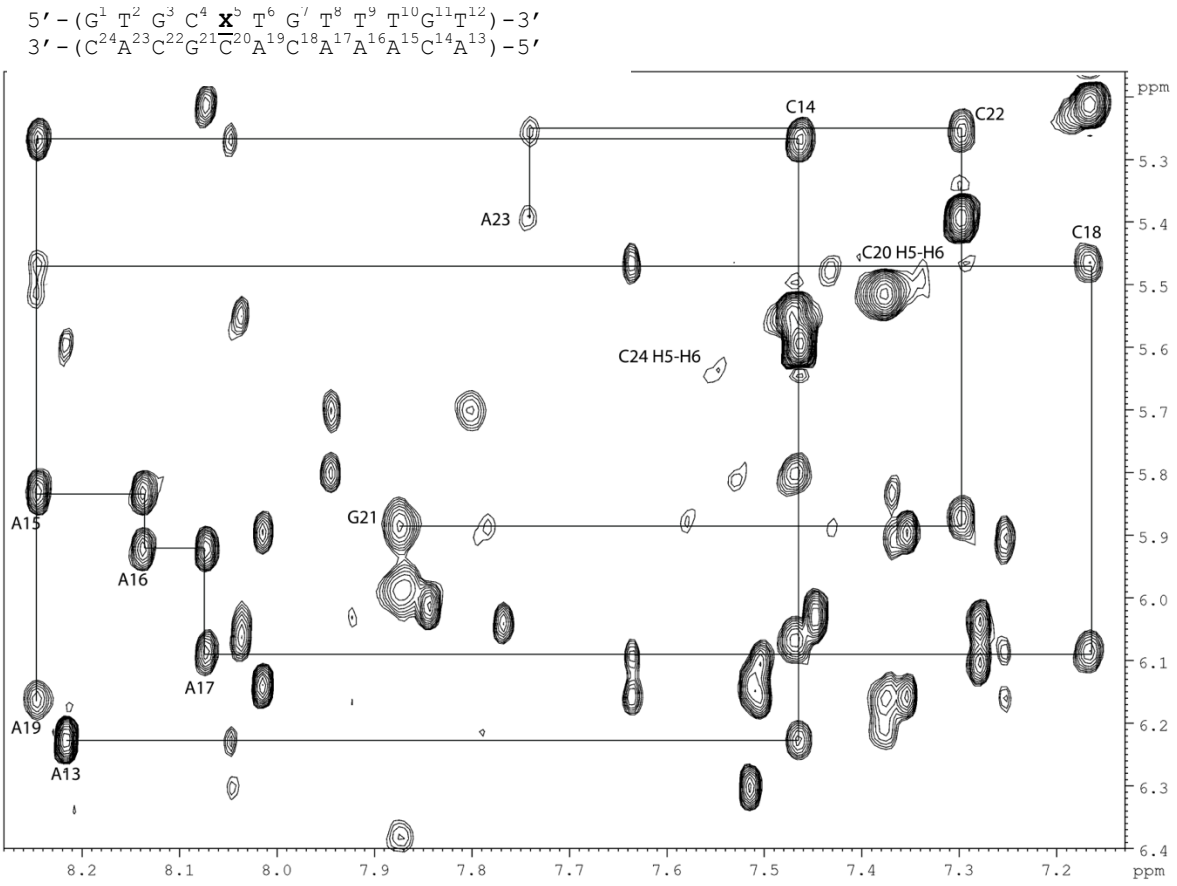

Figure 26. Walking region of the modified duplex, complementary strand at 308 K on a 900 MHz instrument.

Additional adduct interactions were located throughout the spectrum. For instance, interactions between the X5 and the G21 sugars and the adduct provide more evidence to the hypothesis that the aminopyrene is oriented in the minor groove. These interactions are shown in **Figure 27** and **Figure 28**.

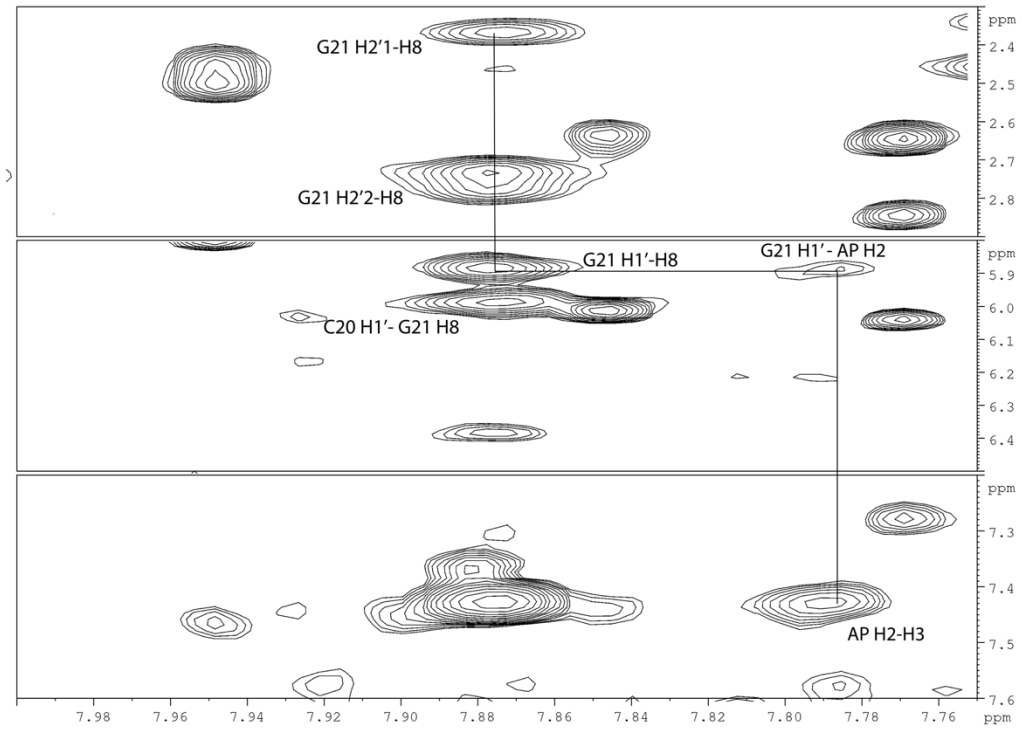

Figure 27. Interactions between G21H1' and AP H2 at 308 K on a 900 MHz instrument.

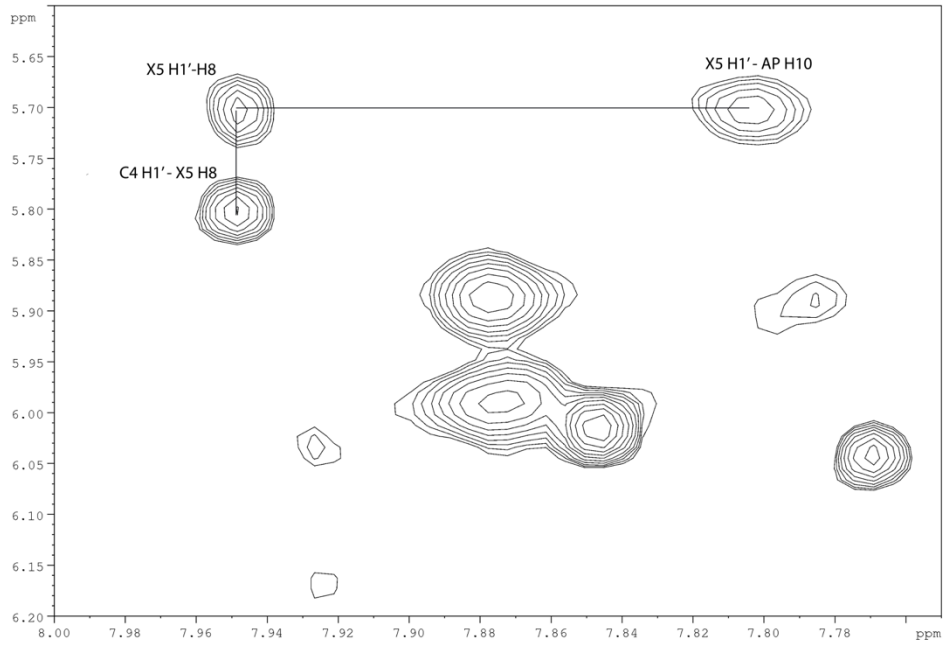

Figure 28. interactions between X5 H1' and AP H10 at 308 K on a 900 MHz instrument.

Both of the interactions of the adduct with the G21 and X5 sugars provide evidence of the aminopyrene sitting in the minor groove, pointing in the 3'-direction of the modified strand. For more conclusive results, attention was given to A19 H2. The H2 protons are great markers for adducts that are situated in the minor groove. The only way AP could have any cross-peaks with A19 would be if it is oriented in the minor groove, pointing in the 3' direction. Interestingly enough, there was a cross peak observed for such an interaction as illustrated in **Figure 29**.

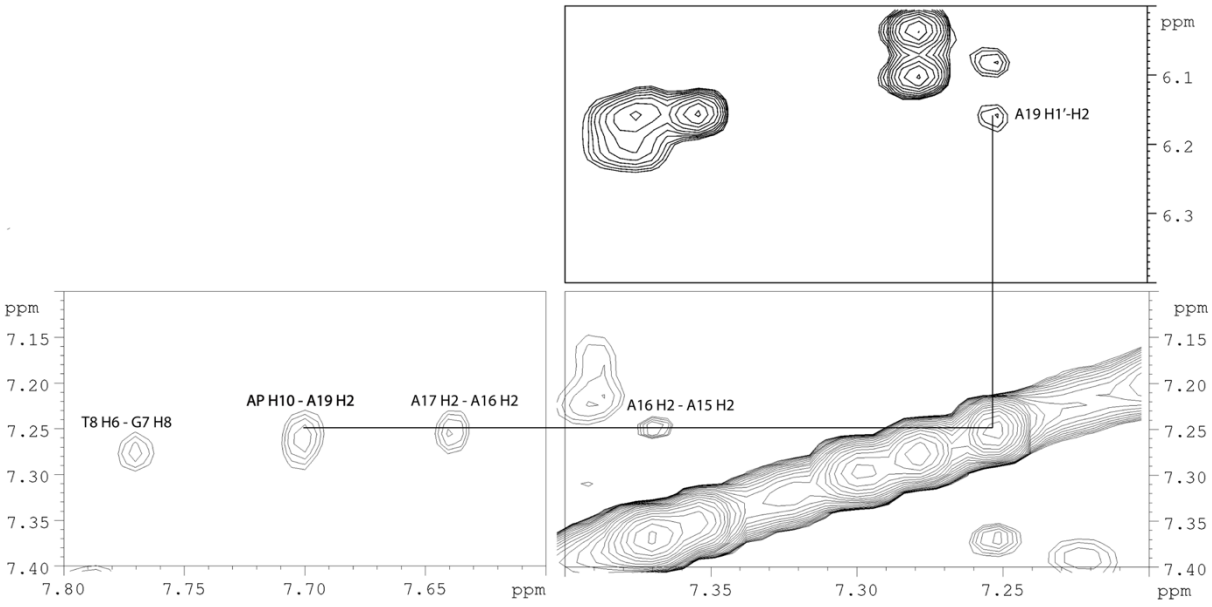

Figure 29. Interaction of AP H10 with A19 H2 at 308 K on a 900 MHz instrument.

CHAPTER V

Summary

Methyl Formamidopyrimidine-dG

There are significant differences between the unmodified and modified NMR spectra. The H1' walking region indiciates substantial structural perturbation around the lesion site. Throughout the spectra, the neighboring bases have their cross-peaks doubled, indicating that these bases are experiencing multiple chemical environments. The multiple chemical environments could be attributed to the various anomers and rotamers that MeFapy-dG can potentially adopt. There is evidence that the doubling of the neighboring base cross-peaks can be attributed to the α and β-anomers. Cross-peaks are present that indicate the formyl proton of MeFapy-dG interacting with the H6 proton of the neighboring T6 base. This provides evidence of at least one rotamer in solution where the methyl group is positioned toward the hydrogen bonding interface, and the formyl group is situated closer to the deoxyribose sugar.

N^2-Aminopyrene-dG

The unmodified sample demonstrates normal B-type DNA structure. Therefore, it will act as a control for future experiments. Various magnitude COSY experiments have shown that 35°C is an ideal temperature for collecting spectra. At this temperature there is dispersion of the adduct protons. Furthermore, this temperature is good for COSY experiments, and it is far from the melting temperature of the strand. At very high and very low temperatures, the adduct protons appear to be isochronous or severely overlapped. Furthermore, the modified NOESY spectra show that T6 has shifted further upfield and has become more shielded, indicating that aminopyrene may be oriented in the minor groove, pointing in the 3'-direction of the modified

strand. On the complementary strand, cross-peaks associated with C20, A23, and C24 are missing or cannot be conclusively assigned which is somewhat unusual in the case of A23 and C24. C20 cross-peaks could be overlapped with other cross-peaks in the region.

CHAPTER VI

Future Directions

Methyl Formamidopyrimidine-dG

There is still a plethora of information that should be mined from the NMR spectra. An important step in parsing the structural characteristics of MeFapy-dG in solution will be determining the predominant conformations. Therefore, an important experiment would be to perform an HSQC on the methyl, C^{13}-labeled sample. This sample has already been synthesized. By using an HSQC, interactions between the labeled methyl group and nearby protons can be assigned and analyzed. It is likely that the lesion may be too complicated for structural calculations due to the many rotameric configurations.

N^2-Aminopyrene-dG

Purification of a new modified duplex is necessary to parse out conclusive findings. It would be exceedingly beneficial to collect DQF-COSY spectra at varying temperatures to visualize the adduct protons. As previously mentioned, all of the COSY data presented were magnitude COSY spectra. If the adduct proton scalar couplings are below 4 Hz, then it would be difficult to see these cross-peaks in the spectrum. To get a full picture, it would be best to collect DQF-COSY on the duplex instead of magnitude COSY. Furthermore, more NOESY spectra will be needed on the new modified duplex to determine the adduct-DNA interactions conclusively.

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