The Clean Cut: Design, Synthesis, Assay Optimization, and Biological Evaluation of Compounds That Can Produce Double Strand Breaks in Deoxyribonucleic Acid

the past four years. Dr. Patrick Ulrich and Dr. Linsey Moyer have been extremely supportive and willing to advise me on my engineering coursework and my thesis, and I have learned a great deal about the engineering approach to solving problems from them.

I would also like to thank my Thesis Committee members, Professor Christina M. Woo, Professor Emily Balskus, and Professor Neel Joshi, for taking the time to support my academic interests and evaluate my thesis.

I thank all my professors, teaching fellows, and peers for contributing to my academic journey at Harvard. I have learned so much about my passion for biomedicine, and my interactions with my peers and teaching staff in class and office hours have nurtured my love for science. Many days, I spent countless hours in the CCB Department Center working on my thesis or homework, and I would like to thank the CCB Custodial Staff and the Bauer Café staff for always checking in on me and having great conversations with me.

Lastly, I would like to thank my friends, Pforzheimer House staff, and family for their constant support and cheerleading. Whether I needed a hug, a second eye to read, or a Post-It reminder to cheer me up, my friends were always there to lend me a hand. Finally, my family has never given up on me and always pushed me to pursue my passion. Thank you to my mother for reading all of my drafts and being my science communication audience member. Thank you to my brother for his support and banter. My pursuit of a Chemistry-Biomedical Engineering joint degree has pushed me to understand the intersection of medicine, science, and engineering, and my future goals of practicing medicine and conducting research could have not been realized without all of the people mentioned above.

This thesis is dedicated to my mother Angeliki Brouard, my brother Dimitri O. Brouard, and my grandfather Dimitrios Koutlas for supporting my academic pursuits and reminding me education is the journey, not just the destination. I thank God for their love and guidance.

"Be passionate, be determined. Learn to love and love to learn."

-Maria Brouard

The Clean Cut: Design, Synthesis, Assay Optimization, and Biological Evaluation of Compounds that can Produce Double Strand Breaks in Deoxyribonucleic Acid

Abstract

Brouard, M., Kokkonda, P., and Woo, C.M. **2018**

Delivery of drug molecules to desired targets has been of utmost importance to maximize efficacy and minimize off-target toxicity. Currently, cancer patients receive chemotherapy which affects malicious and healthy cells in the body, leading to universal damage throughout the body. Antibody drug-conjugates (ADCs), which are composed of an antibody linked to a drug molecule with a chemical chain, have potential to deliver cytotoxic drugs to cancer cells that express unique receptors recognized by the antibody. This targeted drug therapy can minimize the undesired and harmful side effects of cytotoxic chemotherapy currently available. In the early stages of this project, juglone-derivatives were synthesized as potential double-strand break inducing agents and tested using bacterial DNA in a DNA Cleaving Assay. This process entailed optimizing visualization of DNA cleavage using gel electrophoresis. Though the juglone-derivatives did not cleave effectively, nitracrine-derivatives were found to create single-stranded breaks, showing potential for the use of nitracrine compounds as DNA cleaving agents on ADCs for cancer treatment.

Table of Contents

List of Figures

List of Schemes

List of Tables

Chapter 5

Chapter 1: Introduction

1.1 Specializing Cancer Treatment

The fight against cancer is one of the most pressing medical issues of the twenty-first century. In the United States, about 1.7 million cases are expected to be diagnosed in 2018.[1] The main methods of cancer treatment include radiation therapy, chemotherapy, and surgery which can have harsh side effects such as hair loss and weakness. Though these methods can be successful, there are instances of reoccurrence. Cancer is caused by one or more mutations in a human cell, causing the cell to proliferate and grow uncontrollably. These mutated cells cause damage to nearby healthy cells and tissues which can eventually lead to death. If the patient's cancer cells could be specifically targeted, the patient could be cured of their cancer with minimal side effects caused by current therapies available.

1.2 Gene Editing to Treat Cancer

The potential of gene editing to cure disease has led to the increased research and application of gene therapies for patient treatment. Modifications to Deoxyribonucleic Acid (DNA) would change an individual's genotype potentially allowing for the expression of a new phenotype; this concept could be used to change patients' genes for treatment of diseases that were thought of as incurable or untreatable. The discovery of defensive small RNAs in prokaryotes called Clustered Regularly Interspaced Short Palindrome Repeats (CRISPR) RNAs (crRNAs) has provided insight into the different approaches nature has evolved to build immunity.[2] Pharmaceutical companies such as CRISPR Therapeutics are using CRISPR technology to be able to modify a patient's cells and return the cells back to the patient's body for proliferation of the corrected cells; the company also delivers CRISPR machinery to target organs for correction of cells within the patient's body.[3] Modification of disease-causing single-gene mutations within an individual's cells could potentially cure the patients of their disease

completely. Therefore, CRISPR treatment has high potential in medical application; however, there is still a negative stigma for a large portion of the population on the ethics and potential issues around gene editing as a treatment. Moreover, there are delivery issues with the technology since many proteins and structures are involved in the process.

1.3 Antibody-Drug Conjugates as a New Form of Treatment

Antibody drug conjugates (ADCs) are an emerging mechanism of targeted drug therapy, with the potential advantage of limiting untargeted and undesired side effects. There are three parts to the antibody drug conjugate complex: the antibody, the drug molecule, and the linker (**Figure 1**).

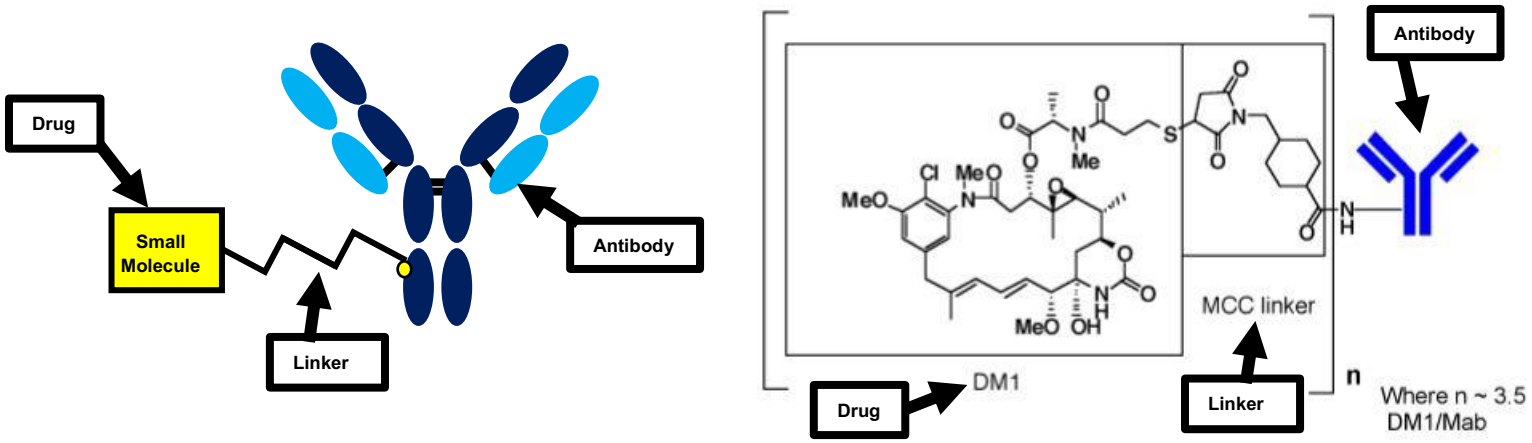


Figure 1: There are three major components to an ADC (left): the antibody which binds a specific surface protein, the cytotoxic drug molecule, and the linker attaching the antibody to the drug molecule. KADCYLA (right) by Genentech is an ADC in the market for breast cancer.[4] The antibody is trastuzumab, and DM1 is a small molecule mircrotubule inhibitor. The MCC linker is a thioether linker.

The antibody portion of the ADCs construct would be designed to bind to specific proteins or receptors on the cell membrane of the malignant cell. A drug molecule, attached by a linker to the antibody, would then be in proximity to the cell. There are two types of linkers: cleavable linkers are cut by the lysosome when the ADC is internalized and non-cleavable linkers remain attached to the drug molecule while the antibody is broken down by other processes occurring in

the cell.[5] Once the ADC is taken up by the cell, a cleavable linker can be dissembled to release the drug molecule and eventually kill the cell. The drug molecule or cytotoxic payload attached to the antibody can vary in function and interaction with the target pathway.[6] Part of the development of an effective ADC is to find a combination of an antibody which attaches to a desired target and is taken up by the cell while being able to attach a stable drug molecule or more to the antibody for cytotoxic treatment.

With the opportunity for effective targeted drug therapy, ADCs have been explored for use in treatment and eradication of tumors produced by various cancers. An effective ADC treatment would minimize off-target effects of the cytotoxic payload attached to the antibody, meaning the number and severity of the side effects caused by the payload can be decreased. While current ADC therapy is intravenous, this treatment can add to the many cancer treatments available today. There are currently two approved ADCs in the market. One of these ADCs targets HER2, a common target for breast cancer.[4] As previously mentioned, the potential benefits of ADCs are that they are a targeted therapy, allowing for potent drugs, guided by the specificity of the antibody binding, to be used for patient care.[7] Effective ADCs would only bind malignant cells, reducing the contact of the drug molecule with other healthy cells. In order to make sure the drug molecule is directly delivered to the cells, one would seek to target receptors expressed only on the malignant cells.

This type of treatment could potentially significantly reduce the number of side effects a cancer patient would have during treatment: rather than delivering a compound with the same potency as chemotherapy throughout the body, the antibody portion of the ADC would only attach to proteins expressed by cancer cells, making ADCs analogous to targeted chemotherapy. However, as with all drug treatments, issues of targeting and delivery remain. Many ADC

treatments are limited to locations were the entire complex can have access to the cancerous cells via the bloodstream. There is ongoing research to find ways to penetrate important immune blockades such as the blood-brain barrier.[8] Some ADCs, such as Mylotarg by Pfizer (**Figure 2**[9]), have been removed from the market or clinical trials due to non-specific effects of ADCs.[9] This emphasizes the needed development of these complex structures in order to provide alternative treatment to cancer patients.

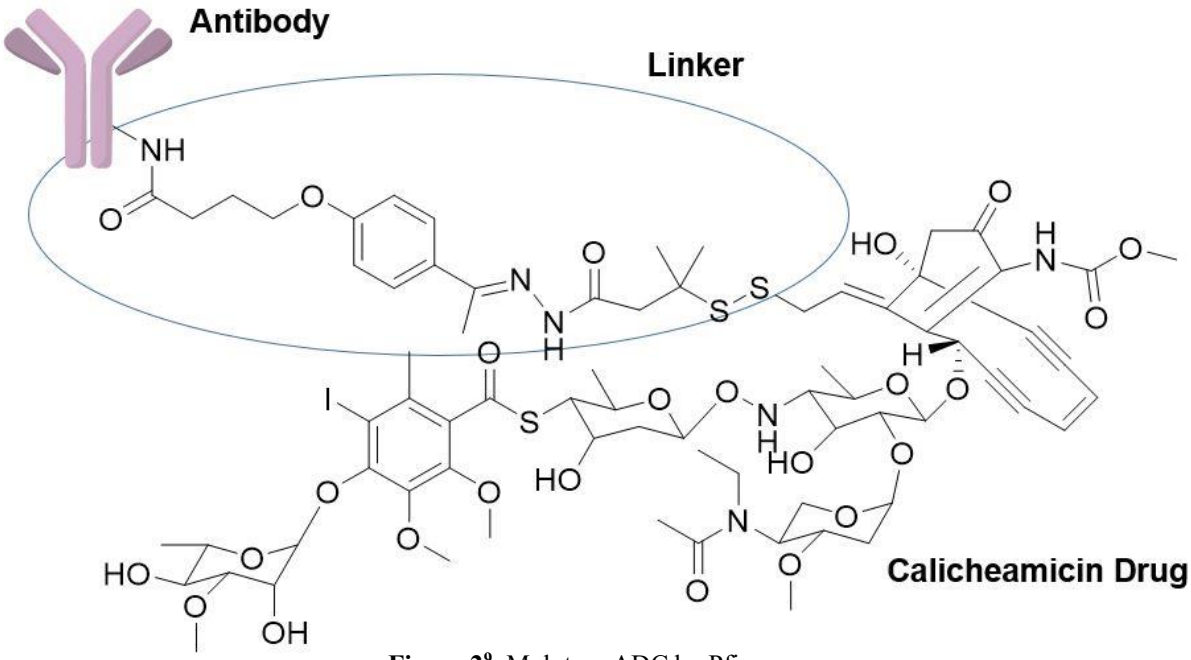


Figure 2[9]: Mylotarg ADC by Pfizer

1.4 Breaking Down the ADC

Since there are many different approaches to optimizing ADCs, I chose to take a chemical approach to thinking about the ADC. With regard to synthetic chemistry, one can think to either develop a new linker for the ADC complex or design potential drug candidates to attach to the antibody. I chose to pursue my interest in medicinal chemistry and decided to think about drug design and synthesis.

DNA cleavage could provide an interesting way to treat cancer because one could kill cancer cells by directly interacting with DNA rather than with proteins that interact with DNA. As discussed above, CRISPR is used by bacteria as a defense mechanism, and bacteria are able to create double strand breaks in DNA for protection. Being able to synthesize a compound that could double-strandedly cleave DNA could create a potent drug that can be used in ADCs. **My design project is focused on the drug component of an antibody-drug conjugate through the design, synthesis, and testing of potential DNA cleaving agents.**

Double stranded cleaving agents have been explored in the past, and some compounds have been developed that have the ability to cleave two DNA strands. However, many of these compounds have metal complexes, and it has been found that metal-based compounds were not deliverable to the human body tissue.[10] Therefore, development of a completely organic compound that could create double-strand DNA breaks could potentially reduce delivery problems in the body. The design and approach to creating this compound will be described in the next chapters. ADC optimization could give more options to patients and cytotoxicity of the drug component is extremely important to allow for quick and permanent elimination of cancerous cells.

Chapter 1 References

(1) American Cancer Society. *Cancer Facts and Figures 2018*. Atlanta: American Cancer Society, 2018.

(2) Pierce, B.A. *Genetics: A Conceptual Approach*; W.H. Freeman and Company: New York, 2014; pp 360, 404-407.

(3) CRISPR Therapeutics. http://www.crisprtx.com/crispr-platform/crispr-cas9-gene editing.php?section=therapeutic-approach (accessed February 25, 2018). CRISPR Platform Page.

(4) Genentech, Inc. KADCYLA® [ado-trastuzumab emtansine]- Prescribing Information, (2016).

(5) Junutula, R. et al. Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index. *Nat.Biotech*. **2008,** *26*, 925-932.

(6) Diamantis, N. and Banerji, U. Antibody-drug conjugates -an emerging class of cancer treatment. *Brit. J. Can.* **2016,** *114*, 362-367.

(7) Verma, S. et al. Trastuzumab Emtansine for HER2-Positive Advanced Breast Cancer. *New Engl. J. Med*. **2012,** *367*, 1783-1791.

(8) Yasunaga, M., Manabe, S., Tsuji, A., Furuta, M., Ogata, K., Koga, Y., Saga, T., and Matsumura, Y. Development of Antibody-Drug Conjugates Using DDS and Molecular Imaging. *Bioengineering*. **2017**, *4*, 1-13.

(9) Pfizer. Mylotarg® (gemtuzumab ozogamicin)- Prescribing Information, (2010). http://labeling.pfizer.com/showlabeling.aspx?id=119.

(10) Cannan, W.J. and Pederson, D.S. Mechanisms and Consequences of Double strand DNA Break Formation in Chromatin. *J. Cell Physiol.* **2016,** *231*, 3-14.

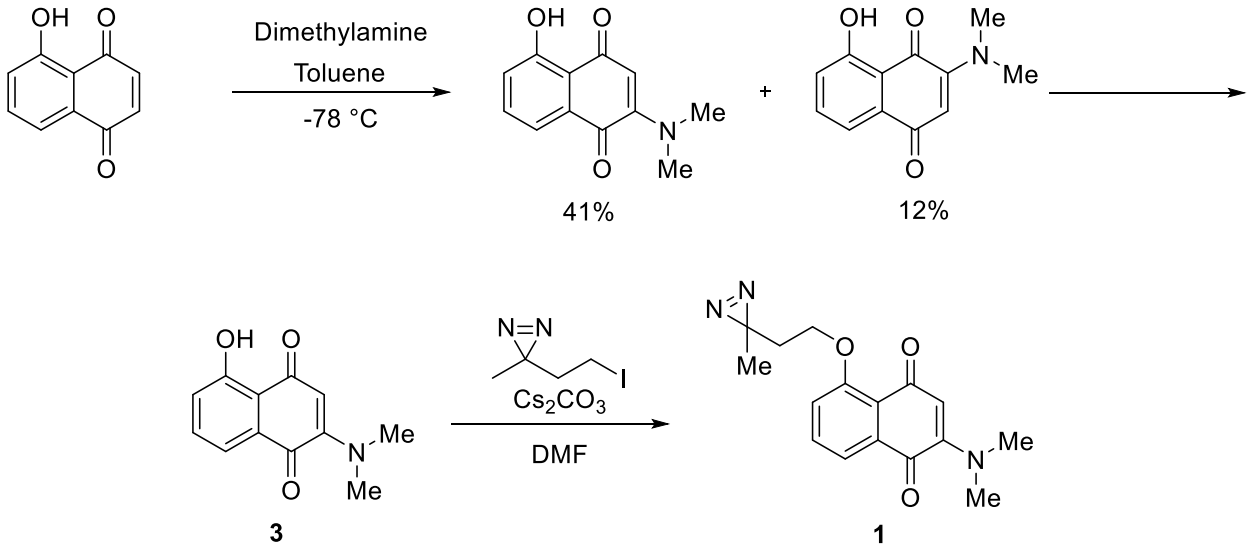
Chapter 2: Initial Investigation of DNA Cleaving Agents for Targeted Design Approach

2.1 Motivation

The preliminary portion of my thesis project consisted of testing potential double-strand breaking compounds and optimizing the DNA cleaving assay and visualization of cleavage results. The main two types of DNA cleavage by small molecules are single-stranded and double-stranded breaks. There are many other mechanisms used in medicine and research for cleaving DNA including enzymes and metal-containing compounds, the later which have existed for decades.[1] However, there are significantly fewer double-stranded cleaving agents available. In order find the most optimal design approach for a double-stranded cleaving compound, we decided to explore compounds that have either been shown to achieve double-strand breaks or compounds which had functional groups that could be modified to create two nucleophilic sites for two strand breaks.

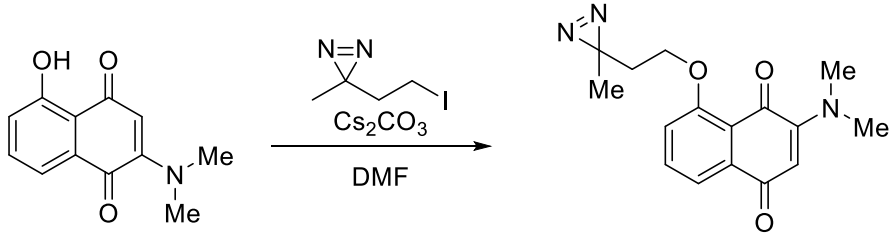
2.2 Juglone Compounds

My initial synthesis was of a juglone with a diazirine moiety as shown in Scheme (1).[2,3,4,5] Juglone compounds are known to at least nick DNA; the planar nature of the juglone allows for intercalation between DNA base pairs, allowing for nucleophilic attack on the DNA backbone through a radical mechanism.[6,7]



Scheme 1[2,3,4,5]**:** Alkylation of juglone compound to create diazo-juglone Compound (**1**).

By adding a diazirine moiety to the alcohol functional group of the juglone, we hypothesized that through UV radiation, the diazirine would abstract a proton from or covalently link to the DNA causing a one strand break while the quinone radical mechanism would be able to cleave the other strand of DNA. To probe this hypothesis, we synthesized Compound **1** and its regioisomer Compound (**2**) as seen in Scheme (2)[2,3,4,5].



Scheme 2[2,3,4,5]**:** Alkylation of juglone compound to create diazo-juglone Compound (**2**).

2.3 Development and Design of DNA Cleaving Assay

A common technique used for visualizing DNA is gel electrophoresis.[8] In order to test the efficacy of **1**, **2**, and **3**, the compounds were incubated with pcDNA 3.1 (+) for 30 minutes in the dark and 30 minutes under UV radiation (365 nm). The samples were then loaded into an agarose gel, and electrophoresis at 120 V was performed for 30 minutes. 0.8% agarose gels

(instead of 1% agarose gels) were used to produce clearer results. The generalized workflow pictured in **Figure 3.**

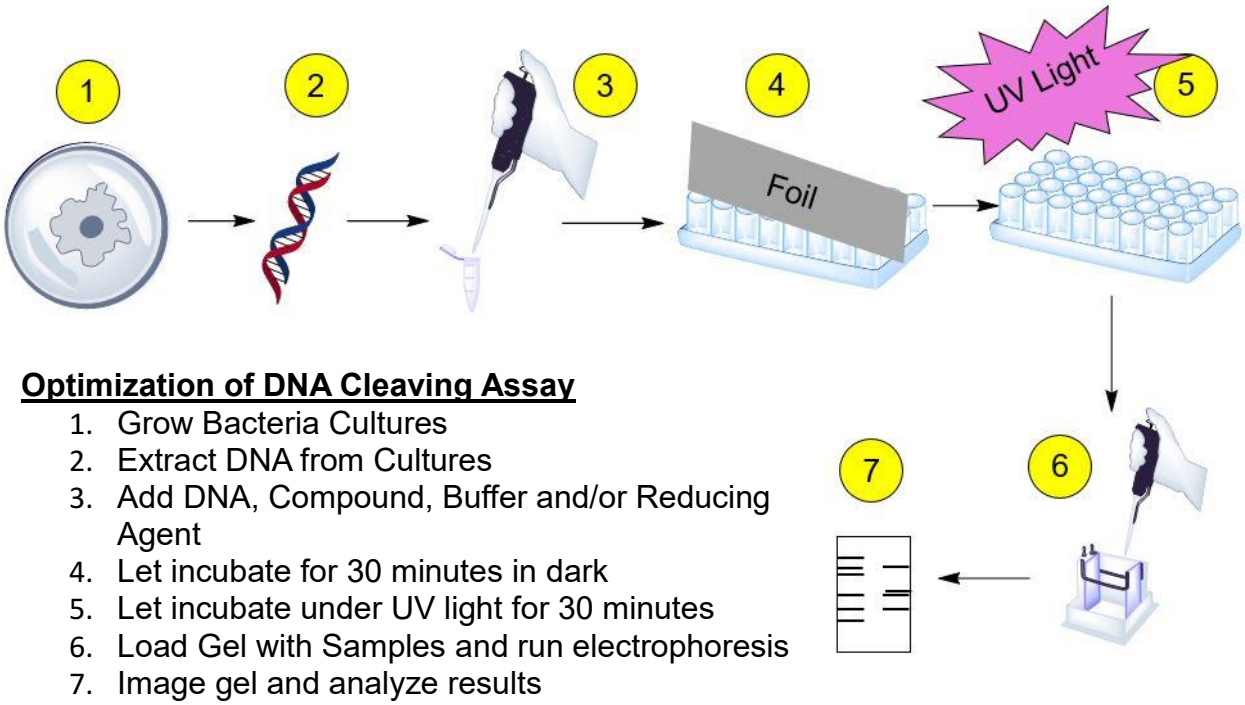


Figure 3: All compounds were tested using the process specified in this workflow which is described in detail in the Materials and Methods section. (1) Bacteria were cultured and inoculated for two days. (2) DNA was extracted from the samples, and yielded concentration was determined. (3) Compound was added to a solution of DNA with or without a reducing agent. (4) The mixture was incubated for 30 minutes in darkness. (5) Samples were radiated under UV light (365nm) for 30 minutes. (6) 0.8% agarose gel was loaded with samples and ran at 120 V for 30 minutes. (7) Gel was imaged, and cleavage was observed.

2.4 Linear and Nicked DNA References

In order to determine whether a single-strand (nicked DNA) or double-strand (linear DNA) break was achieved by the compound, a restriction enzyme was used as a reference. Restriction enzyme digests produce double-strand breaks in DNA and appear as linear DNA in gel electrophoresis. If activity was seen, the gel was run again with a nicked DNA reference (another enzyme digest) to confirm that linear and not nicked DNA was present in the samples.

Initially, the restriction enzyme digest showed two bands, suggesting the protocol provided by the producer was not sufficient to cleave the pcDNA entirely. The protocol was optimized and scaled to produce full digestion of the DNA (**Figure 4**).

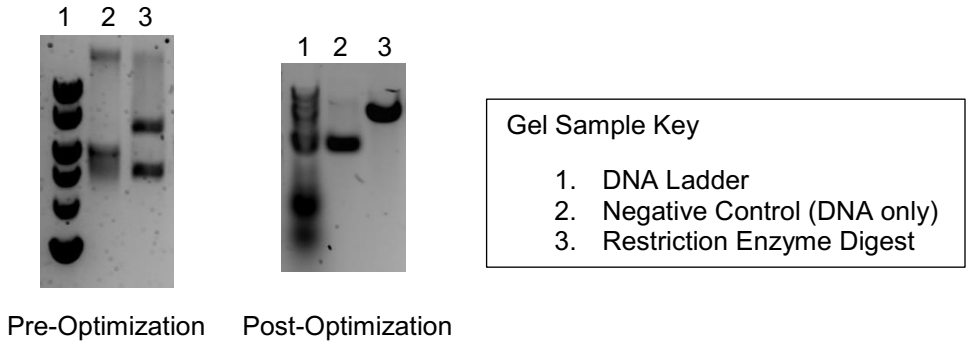


Figure 4: Optimization of the restriction enzyme digest and loading of the DNA produces clearer results in the 0.8% agarose gel.

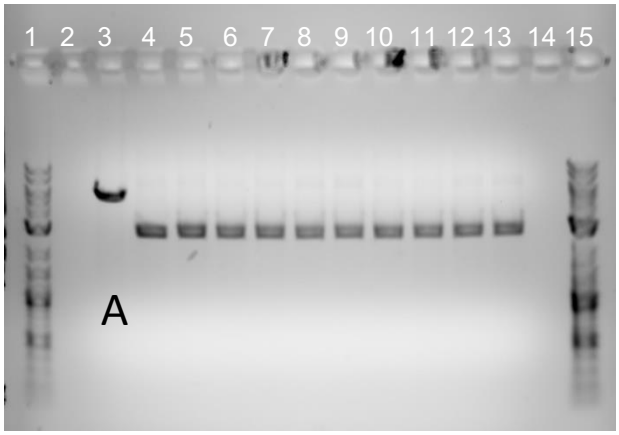
2.5 Results of Juglone Compounds

Compounds **1**, **2**, and **3** were all tested using the workflow described above (**Figure 3**). Three conditions were tested for each compound:

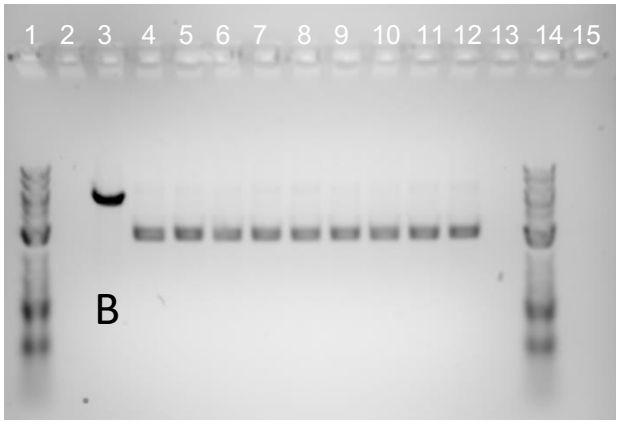
1. Compound and DNA in phosphate-buffered saline (PBS) with no UV radiation
2. Compound and DNA in PBS with UV irradiation for 30 minutes
3. Compound, reducing agent, and DNA in PBS with UV radiation for 30 minutes

In previous studies with juglones, reducing agents have been used to help activate the radical mechanism, and we initially tested with reducing agents in order to enhance the juglones' performance.[9] The reducing agents used in these studies (if present) were 1,4-Dithiothreitol (DTT) and Tris(2-carboxyethyl)phosphine hydrochloride (TCEP). Based on previous studies conducted in the past with juglone compounds, it was speculated that **1** and **2** would at least be able to cleave once, and the diazirine moiety would cleave the second DNA strand, resulting in a double strand break (DSB).[10]

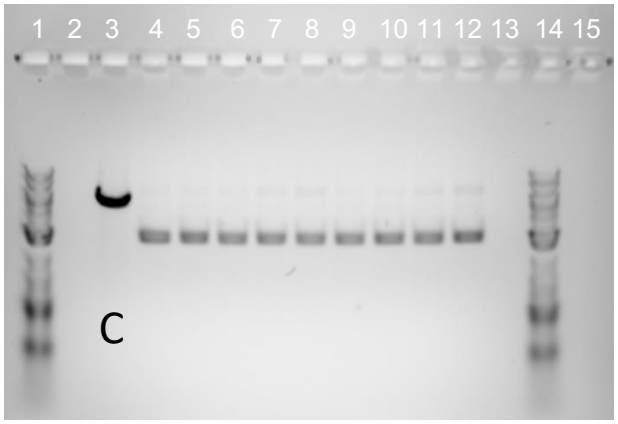
Unfortunately, **1-3** did not display DNA cleavage activity. **Figure 5** shows the results of the DNA cleaving assay for all three compounds.



Lane Number	Condition
1	DNA Ladder
2	SKIP
3	Restriction Enzyme
4	DNA Only (Negative Control)
5	16 µM Compound 4 (UV+)(DTT-)-Compound Degraded
6	5 mM Compound 1 (UV-)(DTT-)
7	10 mM Compound 1 (UV-)(DTT-)
8	5 mM Compound 1 (UV+)(DTT-)
9	10 mM Compound 1 (UV+)(DTT-)
10	5 mM Compound 1 (UV-)(DTT+)
11	10 mM Compound 1 (UV-)(DTT+)
12	5 mM Compound 1 (UV+)(DTT+)
13	10 mM Compound 1 (UV+)(DTT+)
14	SKIP
15	DNA Ladder



Lane Number	Condition
1	DNA Ladder
2	SKIP
3	Restriction Enzyme
4	DNA Only (Negative Control)
5	5 mM Compound 2 (UV-)(DTT-)
6	10 mM Compound 2 (UV-)(DTT-)
7	5 mM Compound 2 (UV+)(DTT-)
8	10 mM Compound 2 (UV+)(DTT-)
9	5 mM Compound 2 (UV-)(DTT+)
10	10 mM Compound 2 (UV-)(DTT+)
11	5 mM Compound 2 (UV+)(DTT+)
12	10 mM Compound 2 (UV+)(DTT+)
13	SKIP
14	DNA Ladder
15	SKIP



Lane Number	Condition
1	DNA Ladder
2	SKIP
3	Restriction Enzyme
4	DNA Only (Negative Control)
5	5 mM Compound 3 (UV-)(DTT-)
6	10 mM Compound 3 (UV-)(DTT-)
7	5 mM Compound 3 (UV+)(DTT-)
8	10 mM Compound 3 (UV+)(DTT-)
9	5 mM Compound 3 (UV-)(DTT+)
10	10 mM Compound 3 (UV-)(DTT+)
11	5 mM Compound 3 (UV+)(DTT+)
12	10 mM Compound 3 (UV+)(DTT+)
13	SKIP
14	DNA Ladder
15	SKIP

Figure 5: Compounds **1**, **2**, and **3** were tested in 0.8% agarose gel at 5 mM and 10 mM to determine the effects of UV radiation and a reducing agent (DTT). A) Compound **1**: There is a lane for Compound 4, a new compound that was being synthesized at the time, but the compound degraded. Compound **4** will be discussed further in the next section. No linear or nicked DNA was observed, suggesting Compound **1** did not cleave. B) Compound **2**: No linear or nicked DNA was observed, suggesting Compound **2** did not cleave. C) Compound **3**: No linear or nicked DNA was observed, suggesting Compound **3** did not cleave. The results above suggest that these juglone derivatives were not efficient cleaving agents.

2.6 Juglone Derivatives Discussion

Juglones have been shown to be anti-cancerous, both through plasmid DNA cleaving and cell and tumor studies.[10,11] The juglone derivatives **1**, **2**, and **3** were not able to create DSBs in the DNA as there was no DNA present at the linear reference provided by the restriction enzyme digest. Moreover, there was a lack of even nicked DNA in the samples; there was a faint band in each of the samples, including the negative control, at a height around the linear reference, but since this was uniform across all the DNA, it is most likely due to the DNA used in the experiments. Given that juglones were at least expected to cleave once in previous studies, the results of these experiments were not particularly expected. One contribution to the lack of activity from the juglone derivatives could the UV radiation component of the procedure. UV radiation is required for the covalent bonding of the diazirine moiety to the DNA backbone for cleavage. However, the compounds could have potentially bonded to each other during UV radiation. The juglone class of molecules does not necessarily use UV light for activation. Moreover, radiation was done after the introduction of juglone compound rather than radiating the DNA before, which has shown to be more effective in similar research.[12] These results directed our attention away from juglone derivatives, and we decided to try a new line of compounds that had been developed for potential in cancer treatment.

2.7 Exploring Nitracrine Compounds as Cleaving Agents

The development of small molecules directly targeting DNA is growing as an additional alternative for cancer treatment including in ADC design. Research has included synthesizing compounds that can intercalate between DNA base pairs, bind to the major or minor groove, or connect to DNA in a staple-like orientation as seen in **Figure 6C**.[13]

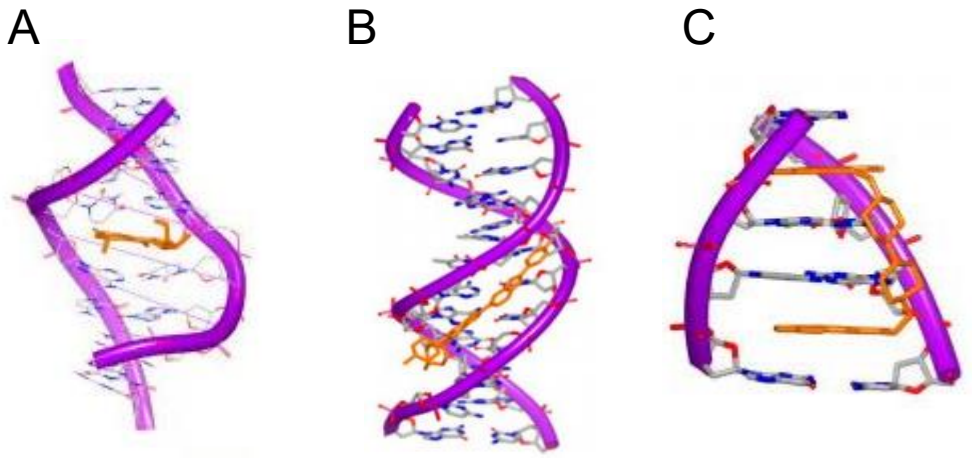


Figure 6[13]: Small molecules of various shapes and sizes have been developed to directly interact with DNA. The DNA is colored purple while the small molecule is colored orange. (A) This image shows a small molecule intercalated between base pairs in a DNA strand. (B) Small molecules have also been developed to bind the major and minor grooves of the DNA double helix. In this diagram, the small molecule is binding the minor groove. (C) This small molecule partially has properties of both of the molecules described in A and B. Two portions of the compound intercalate between base pairs while the third portion of the compound spans the groove of the DNA.

Given the previous work described with the juglone compounds, we decided to continue to work with the motif presented in **Figure 6A**.[13] Nitracrine compounds provided a promising option due to their planar nature and previous application in anti-cancer drug development.[14] Research has been conducted in modifying the functional groups on the base acridine structure **(Figure 7).**[15] As with the juglone compounds, the proposed mechanism of action for the nitracrine is radicalization at the amine group. Some nitracrine compounds have been shown to bind DNA well, and its photoactivation properties allow for the radical chemistry.[16] Nitracrines have also been tested directly in mammalian and bacterial cells and have shown cytotoxic activity.[15] The nitracrine compound would be expected to make at least one single strand break as predicted with the juglone derivatives due to the radical mechanism activated by UV radiation.

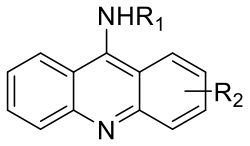
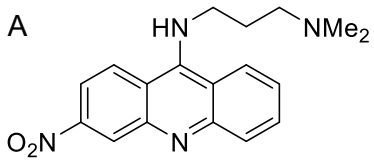


Figure 7: The general structure of nitracrine compounds where R_1 and R_2 are functional groups that are varied for the construction of a functional, anti-cancer drug.

2.8 Testing Nitracrine-Derivative

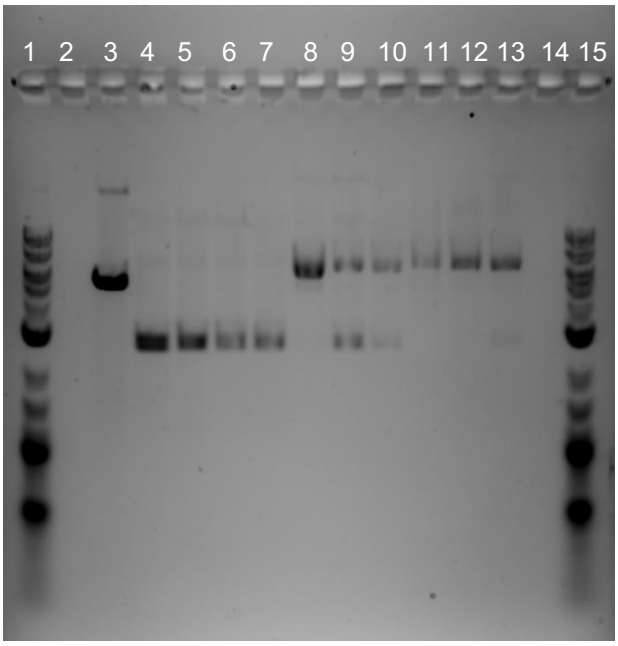
We prepared **4** (PK-52), and **4** was tested in the same preliminary millimolar range as **1**, **2**, and **3**, and the same cleavage assay protocol was used. The initial experiment gel was run for one hour, and results are shown in **Figure 8**.

A



4

B



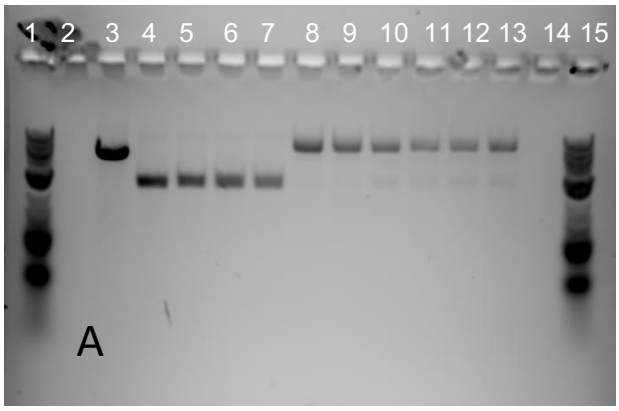
Lane Number	Condition
1	DNA Ladder
2	SKIP
3	Restriction Enzyme
4	DNA Only (Negative Control)
5	1 mM Compound 4 (UV-)(DTT-)
6	5 mM Compound 4 (UV-)(DTT-)
7	10 mM Compound 4 (UV-)(DTT-)
8	1 mM Compound 4 (UV+)(DTT-)
9	5 mM Compound 4 (UV+)(DTT-)
10	10 mM Compound 4 (UV+)(DTT-)
11	1 mM Compound 4 (UV+)(DTT+)
12	5 mM Compound 4 (UV+)(DTT+)
13	10 mM Compound 4 (UV+)(DTT+)
14	SKIP
15	DNA Ladder

Figure 8: (A) Structure of **4** (B) This 0.8% agarose gel run for 1 hour shows nicked DNA, suggesting **4** is active both in the with and without the presence of a reducing agent (DTT). It is clear that UV radiation is needed for **4** to be active, which also supports the radical mechanism discussed previously and in other literature.

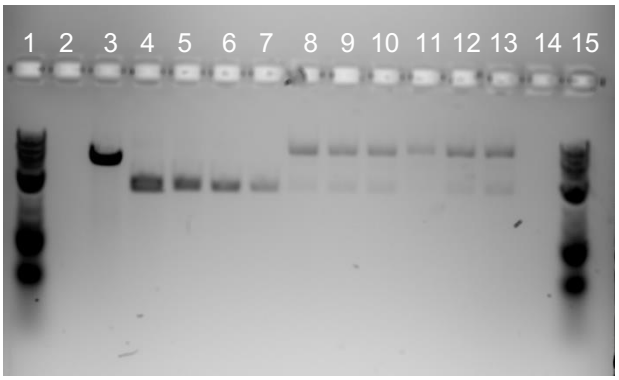
Nicked DNA was present after incubation with **4**, leading to further examination of **4** to test for the functional concentration range, the necessity for a reducing agent, and required UV exposure time.

2.9 Functional Concentration Range of Compound 4

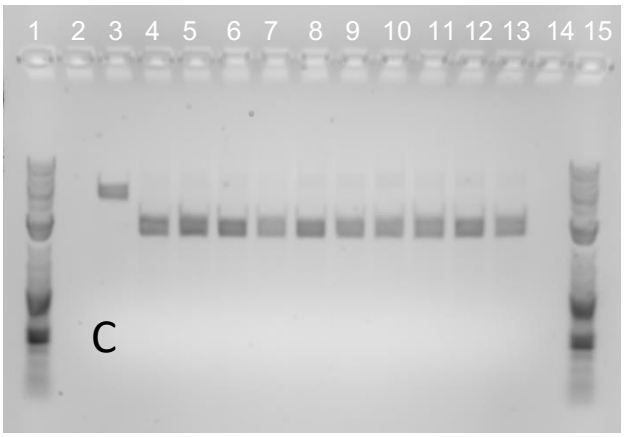
First, it was important to understand the operating range at which **4** was actively cleaving DNA; most drugs are functional in the nanomolar range, and the compound was tested in the nanomolar range to the millimolar range. Initially, the lower millimolar range was tested as seen in **Figure 9A and 9B**. Since cleavage was seen in this concentration range, both the micromolar and nanomolar ranges were tested, leading to results displayed in **Figure 9C and 9D**. Moreover, we decided to test a different reducing agent, Tris(2-carboxyethyl)phosphine hydrochloride (TCEP). It was found that **4** is not functional in the nanomolar range, but it was able to cleave all DNA in micromolar range. More concentrations were tested in the micromolar range, and **4** could cleave efficiently at a concentration as low 16 μM and as high 1 mM.



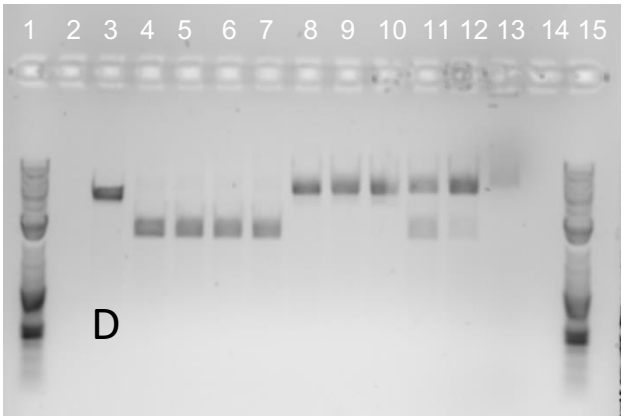
Lane Number	Condition
1	DNA Ladder
2	SKIP
3	Restriction Enzyme
4	DNA Only (Negative Control)
5	2 mM Compound 4 (UV-)(TCEP-)
6	3 mM Compound 4 (UV-)(TCEP -)
7	4 mM Compound 4 (UV-)(TCEP -)
8	2 mM Compound 4 (UV+)(TCEP -)
9	3 mM Compound 4 (UV+)(TCEP -)
10	4 mM Compound 4 (UV+)(TCEP -)
11	2 mM Compound 4 (UV+)(TCEP +)
12	3 mM Compound 4 (UV+)(TCEP +)
13	4 mM Compound 4 (UV+)(TCEP +)
14	SKIP
15	DNA Ladder



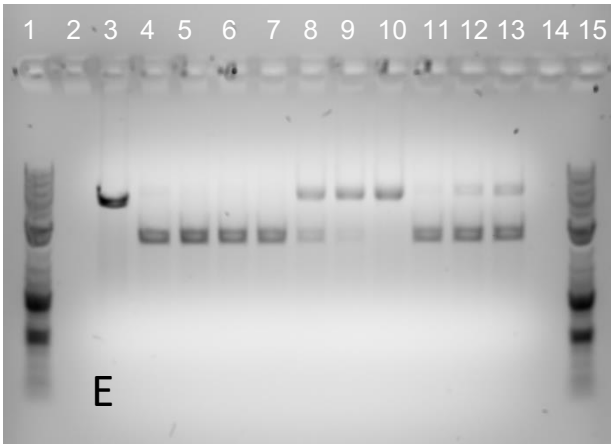
Lane Number	Condition
1	DNA Ladder
2	SKIP
3	Restriction Enzyme
4	DNA Only (Negative Control)
5	6 mM Compound 4 (UV-)(TCEP -)
6	7 mM Compound 4 (UV-)(TCEP -)
7	8 mM Compound 4 (UV-)(TCEP -)
8	6 mM Compound 4 (UV+)(TCEP -)
9	7 mM Compound 4 (UV+)(TCEP -)
10	8 mM Compound 4 (UV+)(TCEP -)
11	6 mM Compound 4 (UV+)(TCEP +)
12	7 mM Compound 4 (UV+)(TCEP +)
13	8 mM Compound 4 (UV+)(TCEP +)
14	SKIP
15	DNA Ladder



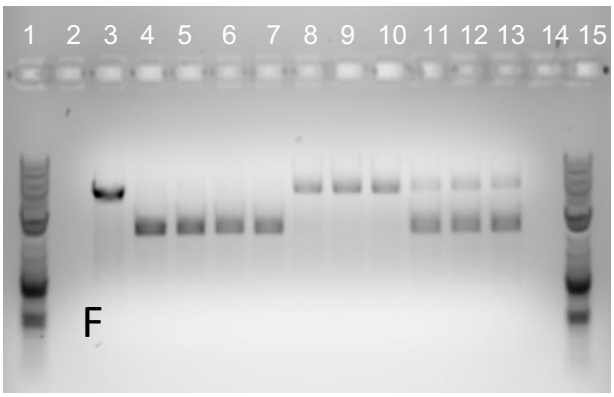
Lane Number	Condition
1	DNA Ladder
2	SKIP
3	Restriction Enzyme
4	DNA Only (Negative Control)
5	100 nM Compound 4 (UV-)(TCEP -)
6	250 nM Compound 4 (UV-)(TCEP -)
7	500 nM Compound 4 (UV-)(TCEP -)
8	100 nM Compound 4 (UV+)(TCEP -)
9	250 nM Compound 4 (UV+)(TCEP -)
10	500 nM Compound 4 (UV+)(TCEP -)
11	100 nM Compound 4 (UV+)(TCEP +)
12	250 nM Compound 4 (UV+)(TCEP +)
13	500 nM Compound 4 (UV+)(TCEP +)
14	SKIP
15	DNA Ladder



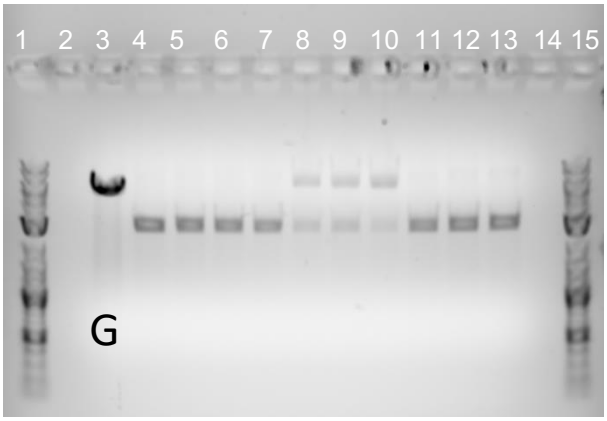
Lane Number	Condition
1	DNA Ladder
2	SKIP
3	Restriction Enzyme
4	DNA Only (Negative Control)
5	100 µM Compound 4 (UV-)(TCEP -)
6	250 µM Compound 4 (UV-)(TCEP -)
7	500 µM Compound 4 (UV-)(TCEP -)
8	100 µM Compound 4 (UV+)(TCEP -)
9	250 µM Compound 4 (UV+)(TCEP -)
10	500 µM Compound 4 (UV+)(TCEP -)
11	100 µM Compound 4 (UV+)(TCEP +)
12	250 µM Compound 4 (UV+)(TCEP +)
13	500 µM Compound 4 (UV+)(TCEP +)
14	SKIP
15	DNA Ladder



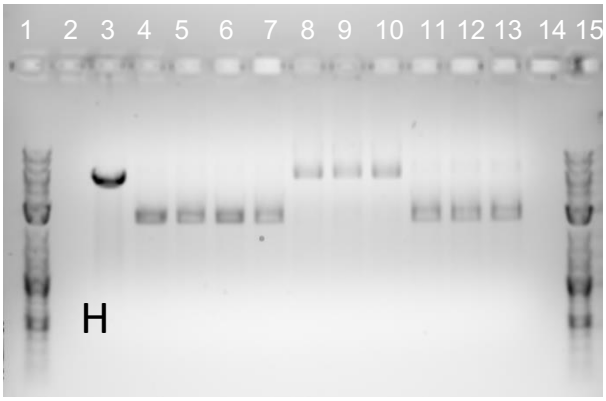
Lane Number	Condition
1	DNA Ladder
2	SKIP
3	Restriction Enzyme
4	DNA Only (Negative Control)
5	10 µM Compound 4 (UV-)(TCEP -)
6	20 µM Compound 4 (UV-)(TCEP -)
7	30 µM Compound 4 (UV-)(TCEP -)
8	10 µM Compound 4 (UV+)(TCEP -)
9	20 µM Compound 4 (UV+)(TCEP -)
10	30 µM Compound 4 (UV+)(TCEP -)
11	10 µM Compound 4 (UV+)(TCEP +)
12	20 µM Compound 4 (UV+)(TCEP +)
13	30 µM Compound 4 (UV+)(TCEP +)
14	SKIP
15	DNA Ladder



Lane Number	Condition
1	DNA Ladder
2	SKIP
3	Restriction Enzyme
4	DNA Only (Negative Control)
5	40 µM Compound 4 (UV-)(TCEP -)
6	50 µM Compound 4 (UV-)(TCEP -)
7	60 µM Compound 4 (UV-)(TCEP -)
8	40 µM Compound 4 (UV+)(TCEP -)
9	50 µM Compound 4 (UV+)(TCEP -)
10	60 µM Compound 4 (UV+)(TCEP -)
11	40 µM Compound 4 (UV+)(TCEP +)
12	50 µM Compound 4 (UV+)(TCEP +)
13	60 µM Compound 4 (UV+)(TCEP +)
14	SKIP
15	DNA Ladder



Lane Number	Condition
1	DNA Ladder
2	SKIP
3	Restriction Enzyme
4	DNA Only (Negative Control)
5	10 µM Compound 4 (UV-)(TCEP -)
6	12 µM Compound 4 (UV-)(TCEP -)
7	14 µM Compound 4 (UV-)(TCEP -)
8	10 µM Compound 4 (UV+)(TCEP -)
9	12 µM Compound 4 (UV+)(TCEP -)
10	14 µM Compound 4 (UV+)(TCEP -)
11	10 µM Compound 4 (UV+)(TCEP +)
12	12 µM Compound 4 (UV+)(TCEP +)
13	14 µM Compound 4 (UV+)(TCEP +)
14	SKIP
15	DNA Ladder

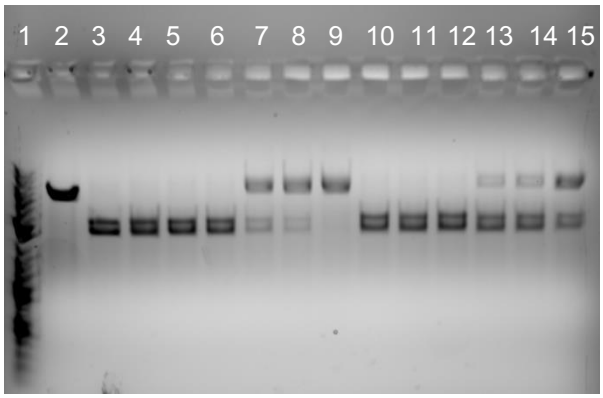


Lane Number	Condition
1	DNA Ladder
2	SKIP
3	Restriction Enzyme
4	DNA Only (Negative Control)
5	16 µM Compound 4 (UV-)(TCEP -)
6	18 µM Compound 4 (UV-)(TCEP -)
7	20 µM Compound 4 (UV-)(TCEP -)
8	16 µM Compound 4 (UV+)(TCEP -)
9	18 µM Compound 4 (UV+)(TCEP -)
10	20 µM Compound 4 (UV+)(TCEP -)
11	16 µM Compound 4 (UV+)(TCEP +)
12	18 µM Compound 4 (UV+)(TCEP +)
13	20 µM Compound 4 (UV+)(TCEP +)
14	SKIP
15	DNA Ladder

Figure 9: These 0.8% agarose gels include TCEP as the reducing agent. (A and B) Testing mM concentrations of **4**. (C-H) Testing µM concentrations of **4**. Completely nicked DNA was observed at the 16 µM concentration and higher.

2.10 Evaluating the Necessity of Reducing Agents

As discussed previously, using a reducing agent in the presence of a nitracrine or juglone could help induce the radical mechanism. However, preliminary investigation, as seen in **Figures 8 and 9**, suggests that the reducing agent does not help and may in fact reduce the cleaving activity of **4**. Therefore, since the above results suggest TCEP is not assisting with the cleaving ability of **4**, and additional gel was run at the lower μM concentration to see if DTT would contribute to **4**'s activity. As seen in **Figure 10**, DTT, too, seems to hinder the cleaving activity of **4**, which means that a reducing agent is not needed for **4** to be functional.



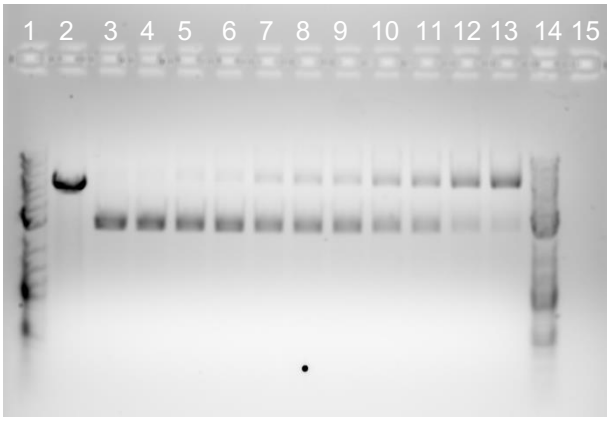
Lane Number	Condition
1	DNA Ladder
2	Restriction Enzyme
3	DNA Only (Negative Control)
4	16 μM Compound 4 (UV-)(DTT -)
5	20 μM Compound 4 (UV-)(DTT -)
6	40 μM Compound 4 (UV-)(DTT -)
7	16 μM Compound 4 (UV+)(DTT -)
8	20 μM Compound 4 (UV+)(DTT -)
9	40 μM Compound 4 (UV+)(DTT -)
10	16 μM Compound 4 (UV-)(DTT +)
11	20 μM Compound 4 (UV-)(DTT +)
12	40 μM Compound 4 (UV-)(DTT +)
13	16 μM Compound 4 (UV+)(DTT +)
14	20 μM Compound 4 (UV+)(DTT +)
15	40 μM Compound 4 (UV+)(DTT +)

Figure 10: This 0.8% agarose gel includes DTT as the reducing agent and checks the effects of the presence of both UV radiation and DTT. Although some cleavage activity is seen for the condition in which both UV radiation and DTT are used, there is more cleavage occurring without DTT and UV radiation alone.

2.11 UV Radiation Time Required for Functionality

Photo crosslinking protocols and experiments have called for a range of UV radiation times from a few minutes to a few hours.[17] However, most protocols call for 15 minutes or less of UV radiation.[18] Our initial tests had be run with 30 minutes of UV incubation (365 nm), and we decided to find the minimum amount of UV radiation that was needed to activate **4**. The

results suggest that **4** needs a minimum of 3 minutes of UV radiation in order to cleave. Based on the results **(Figure 11)**, we decided to keep future incubation times at 30 minutes to ensure maximum cleavage without having too long of an incubation period.



Lane Number	Condition
1	DNA Ladder
2	Restriction Enzyme
3	DNA Only (Negative Control)
4	16 μM Compound 4 (UV: 30 seconds)
5	16 μM Compound 4 (UV: 1 minute)
6	16 μM Compound 4 (UV: 2 minutes)
7	16 μM Compound 4 (UV: 3 minutes)
8	16 μM Compound 4 (UV: 4 minutes)
9	16 μM Compound 4 (UV: 5 minutes)
10	16 μM Compound 4 (UV: 10 minutes)
11	16 μM Compound 4 (UV: 15 minutes)
12	16 μM Compound 4 (UV: 30 minutes)
13	16 μM Compound 4 (UV: 1 hour)
14	DNA Ladder
15	SKIP

Figure 11: This 0.8% agarose gel indicates that a minimum of 3 minutes of UV radiation is needed to see sufficient cleavage activity. However, to ensure most of the DNA is cleaved for testing, future experiments will still have 30 minute incubations.

2.12 Chapter 2 Discussion

This initial project provided an opportunity to explore the effect of quinone-based or quinone-like compounds on DNA. Juglone-derivatives were synthesized with a diazirine moiety with the hope of double-stranded cleaving activity. However, the results of this project suggest that juglones were not effective at cleaving DNA both with the diazirine moiety (**1** and **2**) and without the diazirine (**3**). The DNA Cleaving Assay was developed to effectively test and visualize the activity of the test compounds, but the lack of activity from the juglone compounds could be due to incubation time without UV radiation. If juglones are to be explored further, the DNA Cleaving Assay should be optimized to better suit juglone activity.

However, nitracrine compounds seemed to be a potential route to approach DSBs in DNA, as at least single-stranded cleavage (nicked DNA) was seen in gels containing **4**. This

compound is functional within a micromolar range and can cleave DNA at a concentration as low as 16 μM. Moreover, it was found that reducing agents such as DTT and TCEP inhibit **4**'s cleaving ability, and a 30-minute UV radiation time would be optimal for future experiments using this compound. Using **4** as a basis for DSB-causing compounds could provide potential for the ultimate goal of achieving a DSB in DNA.

Chapter 2 References

(1) Chang, C.H. and Meares, C.F. Light-Induced Nicking of Deoxyribonucleic Acid by Cobalt(III) Bleomycins. *Biochemistry*. **1982,** *21*, 6332-6334.

(2) Gniazdowski, M; Szmigiero, L. Nitracrine and its Congeners- An Overview. *Gen. Pharmac*. **1995,** *26*, 473-481.

(3) Yamashita, M.; Kaneko,M.; Iida, A.; Tokudab, H.; Nishimura, K. *Bioorg. Med. Chem. Lett*. **2007**, *17*, 6417–6420.

(4) Chaker, L.; Pautet, F.; Fillion, H. *Chem. Pharm. Bull.* **1994**, *42*, 2238-2240.

(5) **Lida, A.; Ueda, K.; Yamashita, M. US2012/077986 A1**

(6) Aithal, B.K., Jumar, M.R.S., Rao, B.N., Udupa, N., and Rao, B.S.S. Juglone, a naphthoquinone from walnut, exerts cytotoxic and genotoxic effects against cultured melanoma tumor cells. *Cell Biol. Inter.* **2009,** *33*, 1039-1049.

(7) Ourique, F., Kviecinski, M.R., Felipe, K.B., Correia, J.F.G., Farias, M.S., Castro, L.S.P.W., Grinevicius, V.M.A.S., Valderrama, J., Ríos, D., Benites, J., Calderon, P.B., and Pedrosa, R.C. DNA Damage and Inhibition of Akt Pathway in MCF-7 Cells and Ehrlich Tumor in Mice Treated with 1,4-Naphthoquinones in Combination with Ascorbate. *Oxid. Med. Cell. Long.* **2015,** 1-10.

(8) Stellwagen, N.C. Electrophoresis of DNA in agarose gels, polyacrylamide gels and in free solution. *Electrophoresis*. **2009,** *30*, S188-S195.

(9) Karkare, S., Chung, T.T.H., Collin, F., Mitchenall, L.A., McKay, A.R., Greive, S.J., Meyer, J.J.M., Lall, N., and Maxwell, A. The Naphthoquinone Diospyrin Is an Inhibitor of DNA Gyrase with a Novel Mechanism of Action. *J. Biol. Chem*. **2012,** *288*, 5149-5156.

(10) Farias, M.S., Pich, C.T., Kviecinski, M.R., Bucker, N.C.F., Felipe, K.B., Da Silva, F.O.,

Güthner, T.M.F., Correia, J.F., Ríos, D., Benites, J., Valderrama, J.A., Calderon, P.B., and Pedrosa, R.C. Substituted 3-acyl-2-phenylamino-1,4-napthoquinones intercalate into DNA and cause genotoxicity through the increased generation of reactive oxygen species culminated in cell death. *Mol. Med. Rep.* **2014,** *10*. 405-410.

(11) Aithal, K.B., Kumar,S., Rao, B.N., Udupa, N., and Rao, S.B.S. Tumor Growth Inhibitory Effect of Juglone and Its Radiation Sensitizing Potential: In Vivo and In Vitro Studies. *Int. Can. Ther.* **2012,** *11*. 68-80.

(12) Paulsen, M.T. and Ljungman, M. The natural toxin juglone causes degradation of p53 and induces rapid H2AX phosphorylation and cell death in human fibroblasts. *Tox. App. Pharm.* **2005,** *209*. 1-9.

(13) Wang, M., Yu, Y., Liang, C., Lu, A., and Zhang, G. Recent Advances in Developing Small Molecules Targeting Nucleic Acid. *Int. J. Mol. Sci.* **2016,** *17*. 1-24.

(14) Gniazdowski, M. and Szmigiero, L. Nitracrine and its Congeners- An Overview. *Gen. Pharmac*. **1995,** *26*. 473-481.

(15) Pawlak, J.W., Pawlak, K., and Konopa, J. The Mode of Action of Cytoxic and Antitumor 1-Nitroacridines. II. In Vivo Enzyme-mediated Covalent Binding of a 1-Nitroacridine Derivative, Ledakrin or Nitracrine, with DNA and Other Macromolecules of Mammalian or Bacterial Cells. *Chem. Biol. Inter.* **1983,** *43*. 151-173.

(16) Aloisi, G.G., Amelia, M., Barbafina, A., Latterini, L., Elisei, F., dall'Acqua, F., Vedaldi, D., Faccio, A., and Viola, G. DNA Cleavage Induced by Photoexcited Antimalarial Drugs: A Photophysical and Photobiological Study. *Photochem. Photobiol.* **2007,** *83*. 664-674.

(17) Xing, Y., Qian, M., Wang, G., Zhang, G.,Guo, D., and Wu, J. UV irradiation induced

conductivity improvement in poly(3,4 ethylenedioxythiophene):poly(styrenesulfonate) film. *Sci. China Tech. Sci.* **2014,** *57*. 44-48.

(18) MacKinnon, A.L. and Taunton, J. Target Identification by Diazirine Photo-Cross-linking and Click Chemistry. *Curr. Protoc. Chem. Biol.* **2009,** *1*. 55-73.

Chapter 3: Designing a Double Strand Breaking Compound

3.1 Double Strand Breaking Compound

The goal of this project is to develop a compound that can cleave both of DNA's strands, creating linear DNA. The juglone derivatives did not successfully cleave DNA, but the nitracrine compound **4** did cleave once, resulting in nicked DNA. This compound's performance was used to develop a set of parameters that the target compound must achieve.

3.2 Design Goals for Target Compound A

This compound will:

-Double-strandedly cleave DNA

-Cleave within an hour incubation time

-Cleave better without a reducing agent such as 1,4-Dithiothreitol (DTT)

-Cleave in the micromolar scale

-Be comparable to Compound **4**'s cleaving ability

Double-Strandedly Cleave DNA

For my target compound to meet this specification, it must be able to cleave both strands of DNA. As discussed above, a large portion of DNA cleaving agents can cleave only one strand of DNA. This project uses DNA from the pcDNA 3.1 (+) Mach 1 strain with ampicillin resistance from ThermoFischer. Single-stranded cleavage creates nicked DNA while double-stranded cleavage creates linear DNA. In order to visualize this difference and determine the type of cleavage my desired compound achieved, gel electrophoresis was used. This technique allows for the separation of different types of DNA by weight and shape, distinguishing linear and nicked DNA as shown in the **Figure 12** below.[1]

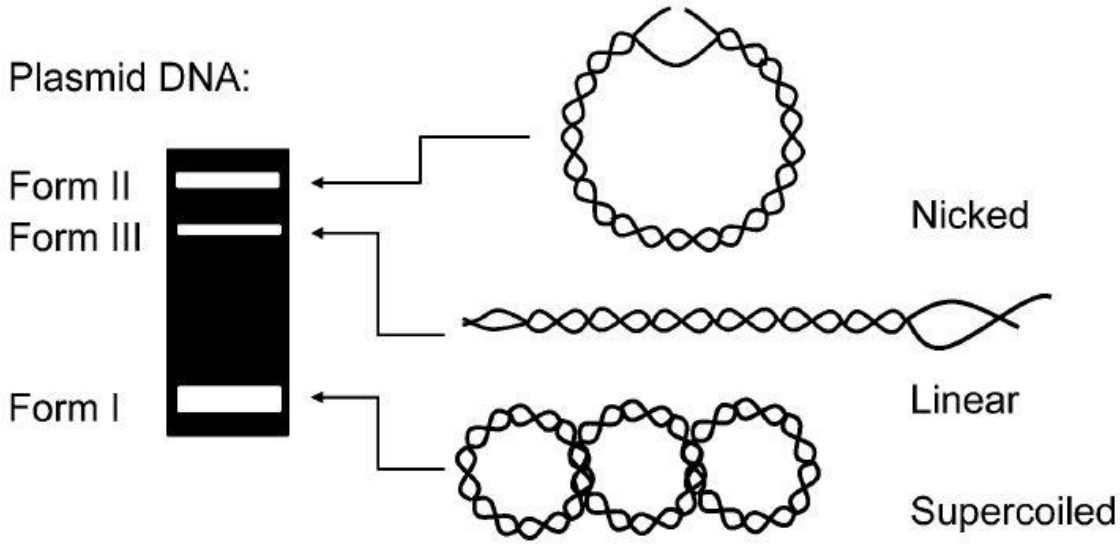


Figure 12[1]: Different structures of DNA travel varying distances in an agarose gel. Supercoiled DNA travels the furtherest from its starting point because it is densely packed. Nicked DNA travels the smallest distance of the three DNA structures pictured due to the large surface area whereas linear DNA is more compact.

Cleave within an hour incubation time

The desired double-strand break compound would be able to effectively cleave DNA within a total incubation time of one hour: 30 minutes of incubation in the dark and 30 minutes of incubation under 365 nm UV light. This wavelength of UV light allows for activation of the desired cleaving compound without causing damage to the DNA from the irradiation alone; UV incubation times required for proper activation are usually around 10 to 15 minutes long.[2] With this in mind, a maximum of 30 minutes of UV incubation would be within a reasonable time range for activation. The most optimal time of total incubation time would be 45 minutes but up to 60 minutes will be tolerated.

The above requirements were selected based on previous drug screenings and research on drug compounds. UV crosslinking protocol is used universally in biological research, and this compound is designed to be functional at the current UV radiation times since in previous

experiments done in our lab and in other papers, diazirine moieties crosslink after 10-15 minutes of UV radiation at 365 nm.

Cleave better without a reducing agent such as 1,4-Dithiothreitol (DTT)

Reducing agents such as 1, 4-Dithiothreitol have been used to initiate radical mechanisms in compounds and have sometimes been paired up with drug compounds to enhance a compound's performance.[3] However, some research has shown that reducing agents can produce false negatives and positives when screening the efficacy of drug compounds.[3] In addition to preventing the accurate assessment of drug compounds, reducing agents are also an additional component to the medication; keeping the medicine as simple as possible can be beneficial to the production of the medicine. Therefore, the compound should be able to function well without a reducing agent.

Cleave in the micromolar scale

A potent compound is desired for double-strand breaks, which means the compound needs to cleave effectively at low concentrations. Many drugs are screened at the micro- and nanomolar scale, meaning that the minimum requirement for the desired compound is to have it cleave effectively in the micromolar scale.

*Comparable to Compound **4**'s Cleaving Ability*

This new compound will use **4**'s structure as a foundation to design, synthesize, and test a DSB-inducing candidate. The addition of a diazirine to the primary amine could potentially introduce a second nucleophilic site, allowing for both the secondary amine and the diazirine to cause damage to the DNA double helix.

3.3 Target Compound A

The figure below shows the structure of **4** compared to the designed structure of the target compound **A**.

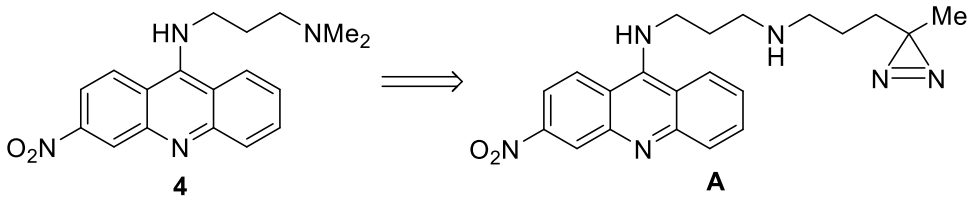


Figure 13: Using **4** as an inspiration, the target compound of this thesis, **A**, was designed. The tertiary amine in 4 is replaced by a secondary amine. This could provide the chain with more flexibility (less steric hinderance). Moreover, the diazirine moiety is added to crosslink to the DNA upon UV radiation. The planar nature of compound will allow the compound to slide between base pairs for optimal positioning near the DNA backbone.

Chapter 3 References

(1) Li, Z. and Grant, K.B. DNA photo-cleaving agents in the far-red to near-infrared range- a review. *RSC Adv*. **2016,** *6*, 24617-34.

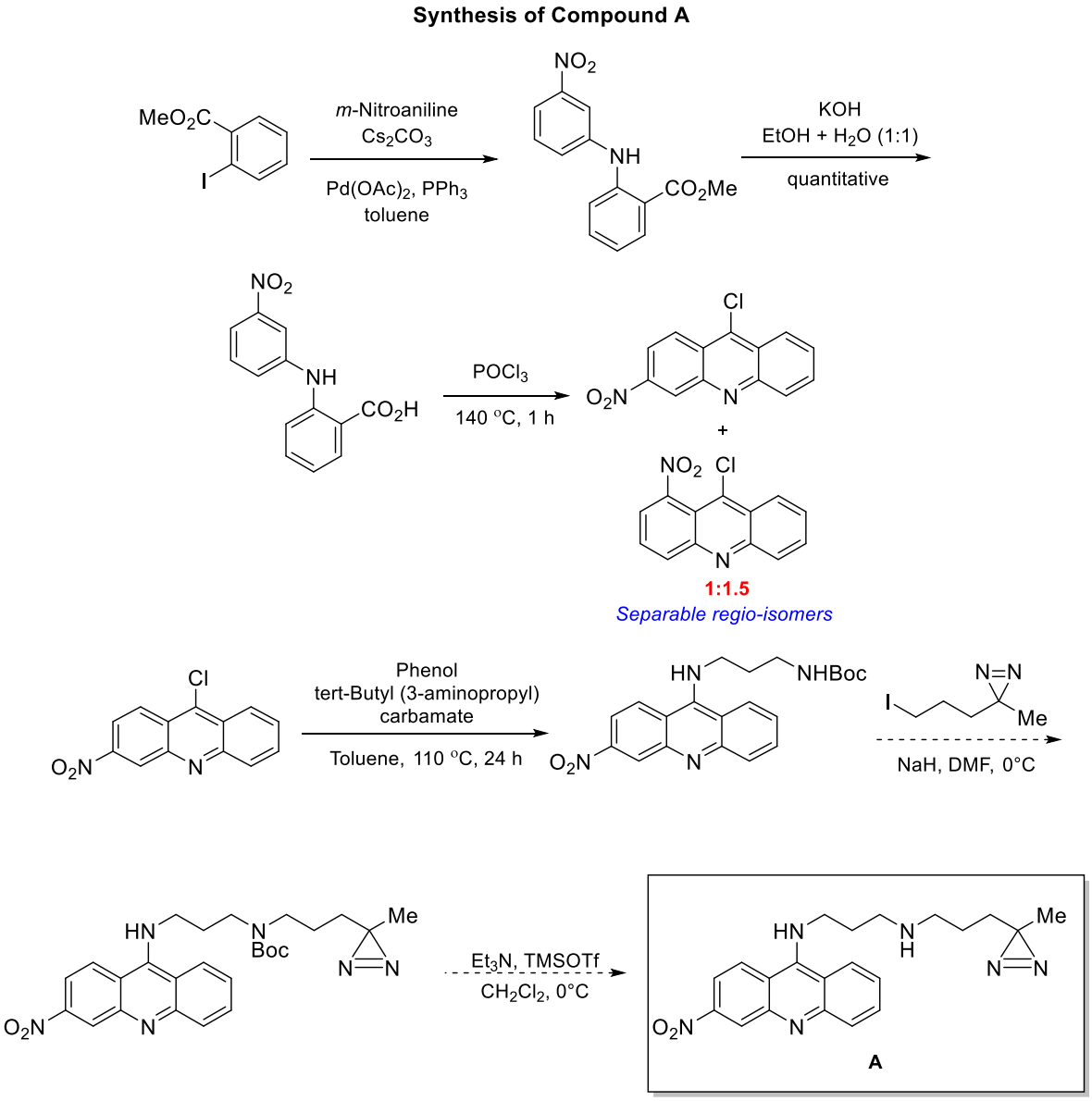
(2) MacKinnon, A.L. and Taunton, J. Target Identification by Diazirine Photo-Cross-linking and Click Chemistry. *Curr Protoc Chem Biol*. **2009,** *1*, 55-73.

(3) Lee, H., Torres, J., Truong, L., Chaudhuri, R., Mittal, A., and Johnson, M.E. Reducing agents affect inhibitory activities of compounds: Results from multiple drug targets. *Anal Biochem*. **2011,** *423*, 46-53.

Chapter 4: Synthesis and Biological Evaluation of Target Compound A

4.1 Synthetic Route to Target Compound A

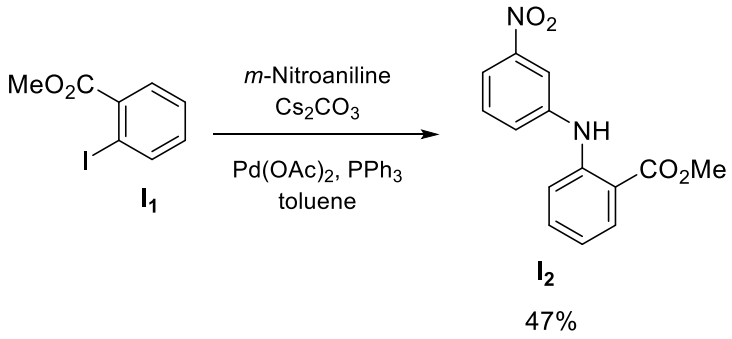
With the assistance of Dr. Praveen Kokkonda, **Scheme 3** was developed to synthesize **A**. Many factors were considered during this synthesis, especially the number of steps to achieve the final compound. Dr. Kokkonda had done similar reactions to develop other derivatives of **4** and found that some of the steps in this synthesis would require small scale set up or would be low yielding. With all of these considerations, this final scheme was decided as the best approach to making **A**.



Scheme 3: The scheme describes the planned synthetic route to **A**.

4.2 Synthesis of 2-(3-Nitrophenyl) Amino Benzoic Acid Methyl Ester

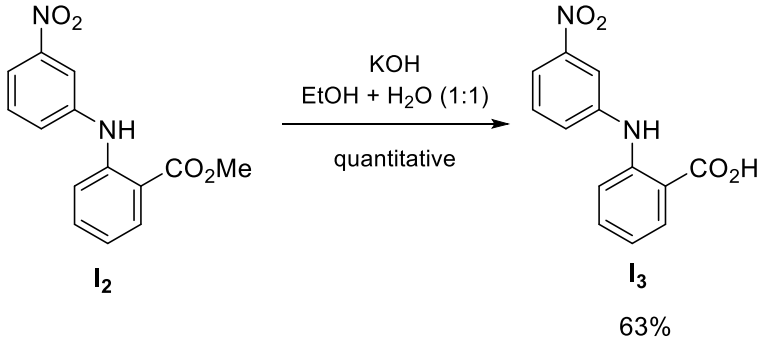
As discussed previously, a majority of this synthesis had already been developed by Dr. Kokkonda. Using the same protocol as he did, I synthesized the amino benzoic acid methyl ester using a palladium catalyst in a Buchwald-Hartwig Cross Coupling Reaction.



Scheme 4: Buchwald-Hartwig Cross Coupling Reaction

4.3 Synthesis of 2-(3-Nitrophenyl) Amino Benzoic Acid

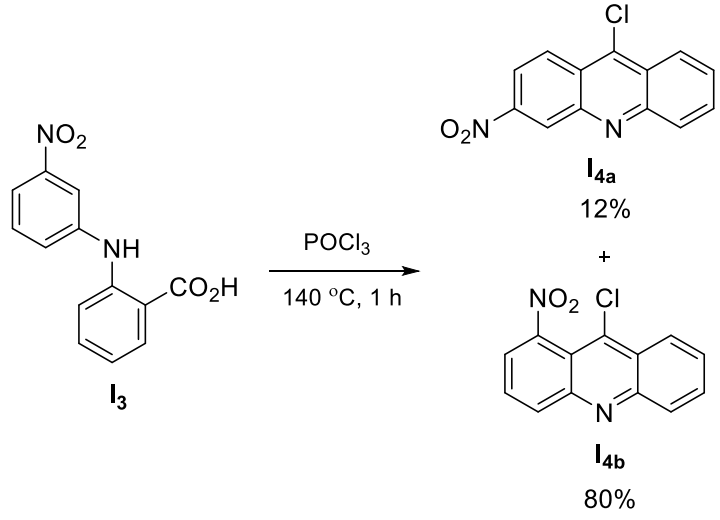
The amino benzoic acid methyl ester was then treated with potassium hydroxide to generate the amino benzoic acid. This reaction yielded 1.2 g of a yellow powder, allowing for multiple reaction set ups of the next step.



Scheme 5: Synthesis of 2-(3-Nitrophenyl) Amino Benzoic Acid

4.4 Cyclization of 2-(3-Nitrophenyl) Amino Benzoic Acid

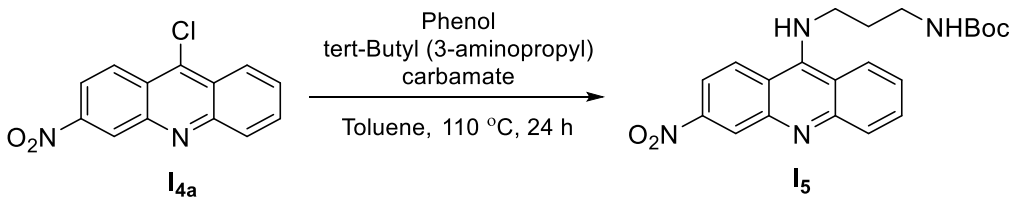
The amino benzoic acid was treated with phosphorous oxychloride to cyclize the compound. Based on Dr. Kokkonda's previous work, it was found that this reaction can only be run on a smaller scale (300 mg), so this reaction was repeated as more product was needed. The reaction was repeated four times, and 0.9663 g of **I4b** was yielded while about 150 mg of **I4a** was yielded; this product was pushed to the next step, and the total produced mass was not taken. This reaction yields two regioisomers, and the desired product was separated with column chromatography.



Scheme 6: Cyclization of Amino Benzoic Acid yields two regioisomers separated by column chromatography.

4.5 Synthesis of Nitracrine

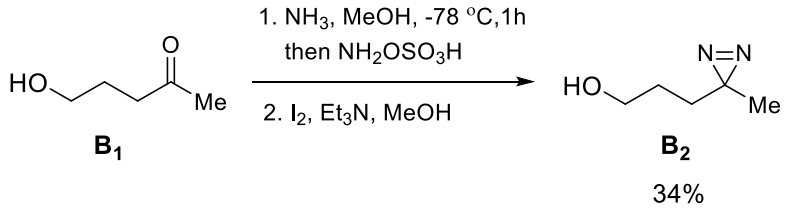
The cyclization product with the nitro group in the meta position was reacted with phenol and tert-Butyl (3-aminopropyl) carbamate to yield the nitracrine compound. This compound is similar in structure to **4**.



Scheme 7: Synthesis of Nitracrine

4.6 Synthesis of Diazirine with Alcohol

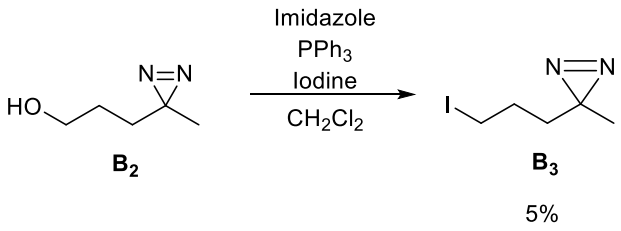
The diazirine moiety added to the **I4a** was synthesized using the same protocol used throughout the lab for diazirine synthesis. Every step of the synthesis required a dry environment. The reaction mixture was filtered through Celite before the second step of synthesis.



Scheme 8: Synthesis of Diazirine with Alcohol

4.7 Synthesis of Diazirine-Iodide Derivative

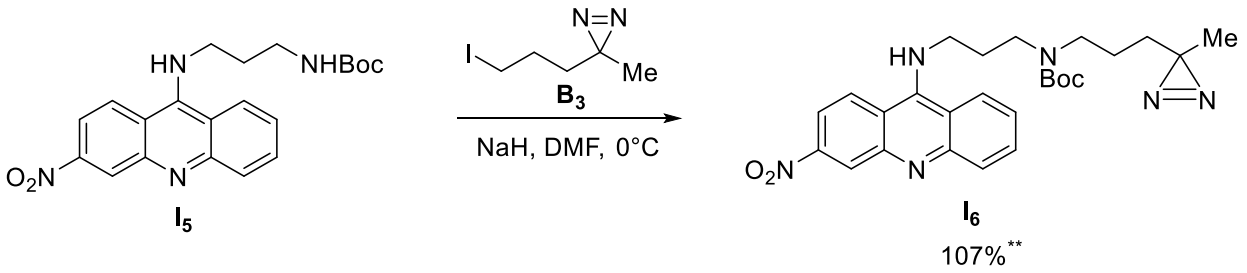
The diazirine compound was reacted with imidazole, iodine, and triphenylphosphine to yield a diazirine-iodide derivate that will be reacted with the nitracrine compound to reach the target compound. The compound was worked up and purified through column chromatography.



Scheme 9: Synthesis of diazirine-iodide derivative for conjugation to the nitracrine.

4.8 Synthesis of Nitracrine-Diazirine

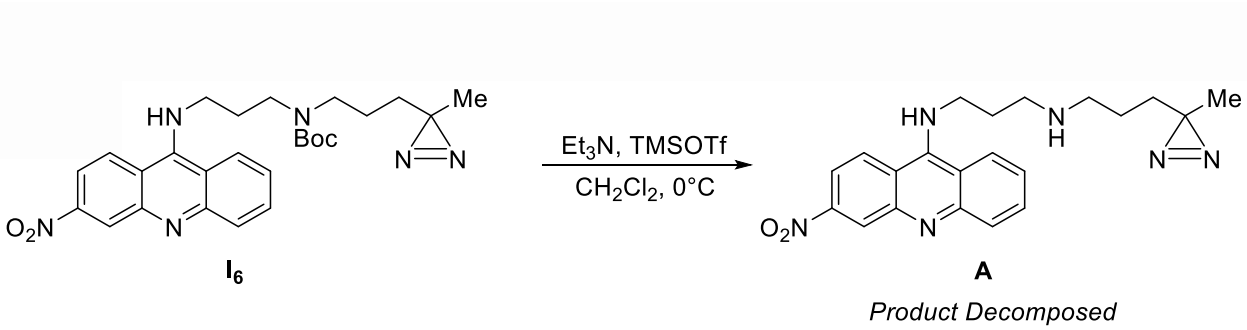
I5 and **B3** were reacted with sodium hydride to yield the nitracrine-diazirine compound, which still contains the Boc protecting group.



Scheme 10: Synthesis of nitracrine-diazirine compound. **Post column chromatography, some impurities and dimethylformamide (DMF) were still present.

4.9 Synthesis of Target Compound A- Deprotection

The nitracrine-diazirine compound **I6** was reacted with triethylamine and trimethylsilyl trifluoromethanesulfonate to yield **A**. An aqueous work up could not be used due to potential hydrolyzation of the diazirine chain from the acridine. However, this product decomposed after the work up; the product, therefore, was unable to be characterized or used for testing in a DNA Cleaving Assay.



Scheme 11: Synthesis of Target Compound **A**

4.10 Discussion of Synthesis

The synthesis of A did run to completion as seen in the thin layer chromatography (TLC) in **Figure 14**. The first spot is the starting material or nitracrine-diazirine and the third spot is the reaction mixture. The product did not travel in a 3:97 Methanol/Dichloromethane (DCM) solvent system. Given that the polar spot was not present in the starting material, product very likely formed. The reaction mixture was worked up, but the compound degraded after concentration under reduced pressure; the proton NMR did not have any characteristic peaks. If there was more time to continue the project, this reaction would need to be optimized to find the best way to isolate the product. Therefore, since **A** could not be used for the DNA Cleaving Assay, the penultimate compound $\mathbf{I_6}$ was used instead for the DNA Cleaving Assay.

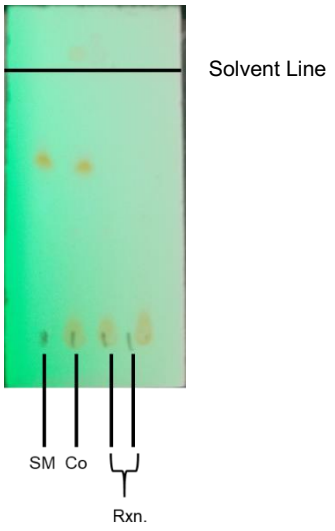
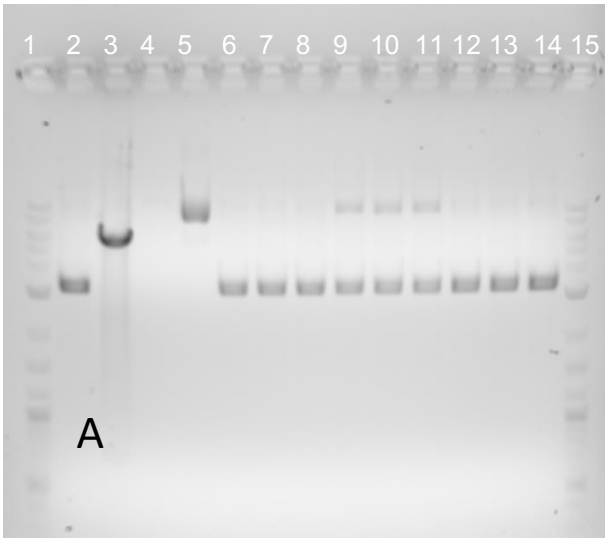


Figure 14: TLC of **A** reaction mixture. The starting material (SM) is less polar than the reaction mixture (Rxn.) and ran further on the TLC plate. Since the reaction mixture did not have any starting material left, it was determined that the reaction ran to completion.

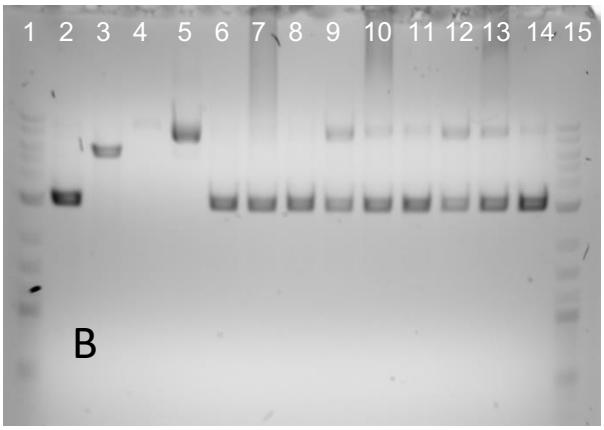
4.11 DNA Cleaving Assay with Nitracrine-Diazirine

As discussed previously, the goal of this project is to design and synthesize a compound that can create DSBs in DNA. Given that **A** was not isolated, the DNA Cleaving Assay was run with $\mathbf{I_6}$. The Boc protecting group is a large protecting group, and this could have affected the efficacy of $\mathbf{I_6}$. $\mathbf{I_6}$ was tested in both the millimolar and micromolar range, and the samples were

incubated for 30 minutes in the dark and 30 minutes under UV radiation (365 nm). The results (**Figure 15**) suggest that **I$_6$** can cleave DNA by creating single-strand breaks, seen as nicked DNA in the gel. Although the testing of **A** was not possible, the results with **I$_6$** suggest that nitracrine derivatives have potential in being used for DNA cleavage and could possibly be developed to create DSBs.



Lane Number	Condition
1	DNA Ladder
2	DNA Only (Negative Control)
3	Restriction Enzyme
4	Nicked DNA Reference (Decomposed)
5	16 µM Compound 4 (UV+)(DTT-)
6	100 µM Compound **I$_6$** (UV-)(DTT-)
7	250 µM Compound **I$_6$** (UV-)(DTT-)
8	500 µM Compound **I$_6$** (UV-)(DTT-)
9	100 µM Compound **I$_6$** (UV+)(DTT-)
10	250 µM Compound **I$_6$** (UV+)(DTT-)
11	500 µM Compound **I$_6$** (UV+)(DTT-)
12	100 µM Compound **I$_6$** (UV+)(DTT+)
13	250 µM Compound **I$_6$** (UV+)(DTT+)
14	500 µM Compound **I$_6$** (UV+)(DTT+)
15	DNA Ladder



Lane Number	Condition
1	DNA Ladder
2	DNA Only (Negative Control)
3	Restriction Enzyme
4	Nicked DNA Reference (Decomposed)
5	16 µM Compound 4 (UV+)(DTT-)
6	1 mM Compound **I$_6$** (UV-)(DTT-)
7	5 mM Compound **I$_6$** (UV-)(DTT-)
8	10 mM Compound **I$_6$** (UV-)(DTT-)
9	1 mM Compound **I$_6$** (UV+)(DTT-)
10	5 mM Compound **I$_6$** (UV+)(DTT-)
11	10 mM Compound **I$_6$** (UV+)(DTT-)
12	1 mM Compound **I$_6$** (UV+)(DTT+)
13	5 mM Compound **I$_6$** (UV+)(DTT+)
14	10 mM Compound **I$_6$** (UV+)(DTT+)
15	DNA Ladder

Figure 15: The nicked DNA reference decomposed, and Compound 4 at 16 µM was used as a nicked DNA reference. (A) **I$_6$** was tested in the micromolar scale, and it was found that nicked DNA was produced as a result of **I$_6$**'s cleaving activity. In the micromolar scale, it seems that the reducing agent reduced the activity of **I$_6$**. (B) In the millimolar scale, about equal nicking activity was seen in both the presence and absence of DTT.

4.12 Chapter 4 Discussion

The final product **A** was not isolated and due to time constraints, **Scheme 11** could not be repeated or optimized to yield more material for characterization and testing in the DNA Cleaving Assay. This led to the use of the penultimate intermediate $\mathbf{I_6}$ to be used for testing in the biological assay. The results suggest that even with the large Boc protecting group, $\mathbf{I_6}$ can still cleave DNA. Although there is no linear DNA formed, hence no DSBs, $\mathbf{I_6}$ is able to nick DNA, and it can do so in the micromolar scale. Thus, this compound can be further explored to find an optimal function group modification to create DSBs.

Chapter 5: Discussion and Future Directions

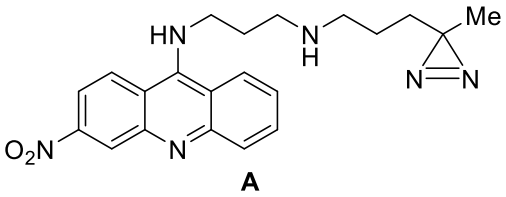
5.1 Thesis Discussion

The purpose of this thesis project was to design and synthesize a compound that could create DSBs in DNA for use in cancer treatment. This compound could have potential application in gene editing and treatment of cancer. Ultimately, this compound would have the potential to be added to ADCs, which can specifically deliver the cytotoxic compound to malignant tumor cells with the corresponding antibody receptors. Finding and optimizing cytotoxic but targeted cancer treatments is extremely important: in 2016 alone, about 1, 685, 210 cases of cancer were diagnosed and 595, 690 patients were expected to die because of it.[1] Many cancer treatments now, such as chemotherapy and radiation, are somewhat effective at ridding patients of their tumor cells. However, the side effects of these treatments are painful and emotionally tolling on the patients. ADCs provide a new approach to giving patients the potency of chemotherapy with the specificity of antibody binding.

To synthesize a double-stranded cleaving agent for ADCs, a juglone-derivative approach was taken since juglones have been seen to target DNA directly (can intercalate between base pairs) and cleave it. Since our lab often uses diazirines for covalent bonding, it was hypothesized that adding a diazirine moiety to a juglone, such as **1** and **2**, the diazirine would be able to cleave one strand of DNA while the radical mechanism of the juglone would target the other strand. The results seen in **Figure 5** suggest that the juglone derivatives were not able to cleave DNA at all. One potential reason for this could be that the diazirine may have somehow interfered with the juglone's activity. If one was particularly interested in using juglones, this DNA Cleaving Assay would need to be optimized for juglones and further troubleshooted.

Dr. Kokkonda synthesized **4**, and we decided to test its activity in the DNA Cleaving Assay to see how well nitracrines could cleave DNA. As seen in **Figure 9**, **4** is able to nick

DNA, creating a single-strand break. This interested me, and I decided I would like to use the nitracrine as a foundation to design a compound that could potentially create DSBs. This led to the design of **A**, my target compound (**Figure 16**).



A

Figure 16: Target Compound **A**

The design parameters for this compound were as follows: create double-strand breaks in DNA, cleave DNA within 1 hour incubation time, cleave better without a reducing agent, cleave in the micromolar scale, and have a similar potency as **4**. A six-step synthesis was developed (**Scheme 3**), and **A** was synthesized, but it degraded during the work up. This then led to the use of $\mathbf{I_6}$, the penultimate compound, to be used in the DNA Cleaving Assay. $\mathbf{I_6}$ was able to nick DNA without a reducing agent in the micromolar scale. A summary of all the DNA Cleaving Assay results are pictured in **Table 1**.

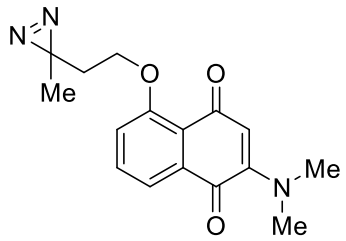
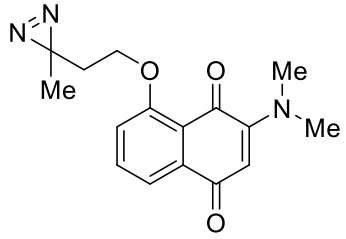
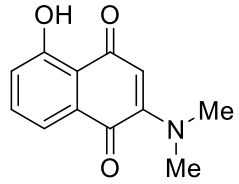
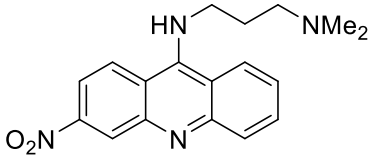
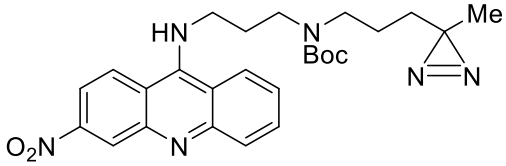
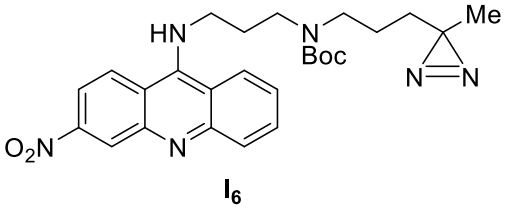
Summary of Compound Results				
Compound	**Cleavage Type**	**UV Radiation Time Minimum**	**Lowest Functional Concentration**	**Reducing Agent Required**
 **1**	None Observed	30 minutes	N/A	N/A
 **2**	None Observed	30 minutes	N/A	N/A
 **3**	None Observed	30 minutes	N/A	N/A
 **4**	Nicked (Single Strand Break)	3 minutes (tests were at 30 minutes)	16 μM	No; both DTT and TCEP reduced **4**'s performance
 **I6**	Nicked (Single Strand Break)	30 minutes	100 μM	Based on current results, no. Further testing required.

Table 1: The table above summarizes the results of all the compounds tested in the DNA Cleaving Assay developed and optimized in this thesis.

5.2 Discussion of Design

Target compound **A** was not stable enough for purification, and **I6** was used to test DNA cleaving efficacy. The same design parameters were applied to **I6**, and this compound met all the design parameters except having the ability to produce DSBs. **Table 2** evaluates each design parameter. Overall, given that **I6** was not the target compound, it is promising that it was still able to cleave DNA, allowing for an opportunity to further develop this compound.



I6

Design Parameter	Design Parameter Met?	How was it met?	Improvements
Create Double Strand Breaks	**No.**	Nicked DNA	Modify functional group and optimize synthesis for **A**
Cleave with 1 hour Incubation	Yes.	30 min. Dark + 30 min. UV	Could test minimum time required for UV radiation
Cleave better without Reducing Agent	Yes.	More cleavage was seen with just UV radiation	Could test different reducing agent like TCEP to see if anything else would help.
Cleave in micromolar range	Yes.	Can cleave at 100 μM	Test lower limits of concentration
Cleave Similarly to **4**	Yes.	Cleaves in μM range and can cleave similar amounts of DNA	Improve compound's ability such that all DNA in sample is cleaved.

Table 2: The table above summarizes **I6**'s ability to meet the design parameters specified for **A**.

5.3 Conclusions and Future Directions

This project was able to design, synthesize, and test a novel compound **I_6** that was seen as biologically active. This compound, however, was not the target compound **A**. Optimizing the synthesis for **A** could allow for a better workup protocol that does not degrade the product. Moreover, different moieties other than a diazirine could be explored as alternatives and tested to check for DSB-inducing activity. Testing these compounds in cells may also provide helpful insight as to the bio-compatibility of the compounds and whether or not they are able to maintain their structure or functionality in cells. If single-stranded cleaving could effectively kill cells, **I_6** could be further developed to include a handle for conjugation to a linker. This linker could be attached to an antibody and tested as a complex in cells. **I_6** and possibly other nitracrine-derivatives show potential for DNA cleaving compounds, and further development of their synthesis, design, and biological testing could lead to the creation of sufficiently potent drug compounds that can be used in ADCs for cancer treatment.

Chapter 5 References

(1) National Cancer Institute. https://www.cancer.gov/about-cancer/understanding/statistics. (accessed March 10, 2018). Cancer Statistics Page.

Materials, Methods, and Experimentals

DNA Cleaving Assay (Reference: Maria Brouard Benchling)

Enzyme References

Nicked DNA Reference was prepared using Nt.BspQI enzyme with NEB Buffer 3.1 using the protocol from New England Biolabs (NEB). Thus, 1µg of DNA (10 µL of 100 ng/µL solution), 5 µL of 10X NEB Buffer 3.1, 5 µL of enzyme, and 30 µL of water were combined and incubated for 1 hour at 50°C. The restriction enzyme digest for linear DNA reference was slightly modified from the original protocol in order to have complete cleavage of DNA. 5 µL of EcoRI-HF enzyme and 8 µL CutSmart Buffer (both from NEB) were combined with 5µg of DNA (50 µL of 100 ng/µL solution) and 17 µL of water to make the enzyme solution. This solution was incubated at 37°C for 3 hours.

Agarose Gel Preparation (0.8% Agarose Gel)

480 mg of agarose (Invitrogen UltraPure Agarose) was added to 60 mL of Tris-acetate-EDTA (TAE) buffer and heated and swirled until solution was clear. 6 µL of ethidium bromide was added to this solution and casted in a agarose gel mold for 1 hour.

Bacterial Prep and DNA Retrieval

Bacterial stock of pcDNA 3.1 (+) Mach 1 strain with ampicillin resistance from ThermoFischer were plated on Ampicillin (+) plates and incubated for 24 hours. Single colonies were inoculated into 1% Ampicillin Lysogeny broth (LB) media and grown for 48 hours. These cultures were then centrifuged, and plasmids were extracted using the QIAprep Spin Miniprep Kit. The absorbance of the samples was found using a NanoDrop (ThermoFisher). Gel samples were prepared (4 µL DNA sample and 6 µL phosphate-buffered saline (PBS) buffer), and these samples were run in an 0.8% agarose gel at 120V for 30 minutes in a Biogen gel electrophoresis system. DNA samples that did not run at the same length as the majority of the samples were

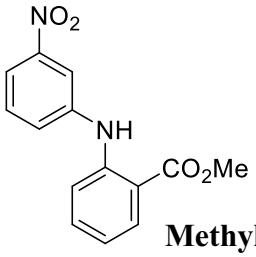
discarded. All samples were then combined, and absorbance was recalculated to create one DNA stock solution.

DNA Cleaving Assay

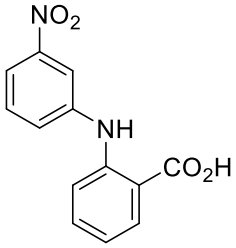
All samples for incubation contained 400 ng of pcDNA (4 μL of 100 ng/1 μL). The negative control had 6 μL of PBS buffer to result in a total volume of 10 μL. Solutions for compounds tested were prepared such that 3 μL of the compound solution would create the desired concentration of the compound in a 10 μL total sample volume. Whenever a reducing agent was used, the solution was created such that there would be a concentration of 500 μM of reducing agent in the 10 μL when 3 μL of the solution were added. If the volume of any sample was not 10 μL, PBS buffer was added to fill the remaining volume. The samples were then all centrifuged and covered with foil to incubate at 25 °C for 30 minutes. Samples that were subject to UV radiation were taken after this period to be incubated under 365 nm UV light (UVP Handheld UV Lamp, LW, 160W, B-100SP) for 30 minutes while the other samples incubated for another 30 minutes under foil.

Preparation of Samples for Loading to Agarose Gel

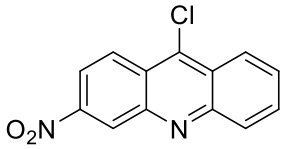
2 μL of Quick-Load® Purple 2-Log DNA Ladder (0.1 -10.0 kb) from NEB was used as a ladder in all the gels (wherever ladder is present). The samples from the DNA Cleaving Assay were mixed with 2 μL of GelPilot® DNA Loading Dye by Qiagen and loaded into the agarose gel. All gels were run at 120V for 30-minute intervals as specified. Gels were imaged using an Azure c400 and Azure c600 by Azure Biosystems.



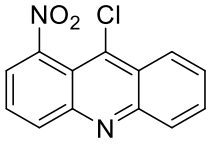
Methyl 2-((3-nitrophenyl)amino)benzoate (I_2): In a round bottom flask, methyl-2-Iodobenzoate (2.0 g, 7.63 mmol, 1.0 equiv.), m-nitroaniline (1.26 g, 9.15 mmol, 1.2 equiv.), palladium(II) acetate (0.34 g, 1.52 mmol, 0.2 equiv.), triphenylphosphine (0.8 g, 3.05 mmol, 0.4 equiv.), and cesium carbonate (3.72 g, 11.44 mmol, 1.5 equiv.) were added to toluene (48 mL). The reaction mixture was degassed for 45 minutes under argon atmosphere at 24 °C. After degassing, the reaction mixture was stirred at 120 °C for 15 hours. The reaction mixture was cooled and diluted with ethyl acetate (300 mL), and the organic layer was extracted with ethyl acetate (1×300 mL). The organic layer was washed with 1M hydrochloric acid (2x40mL) and later washed with brine (40 mL). The organic layer was then dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The crude product purified by column chromatography on a silica gel column eluting with 1:2 hexane/ethyl acetate using Combiflash system to afford the title compound (2.0 g, 7.34 mmol, 74%). ^{1}H NMR: Matched literature (Adeniji, A. O.; Twenter, B. M.; Byrns, M. C.; Jin, Y.; Chen, M.; Winkler, J. D.; Penning, T. M. *J. Med. Chem.* **2012**, *55*, 2311−2323.)



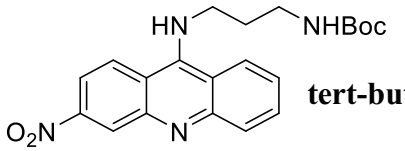
2-((3-nitrophenyl)amino)benzoic acid (I_3): In a round bottom flask, methyl 2-((3-nitrophenyl)amino)benzoate **I_2** (2.0 g, 7.35 mmol, 1.0 equiv.) and potassium hydroxide (0.82 g, 14.69 mmol, 2.0 equiv.) were added to ethanol (14 mL) and water (14 mL). The reaction mixture was refluxed for 1 hour at 110 °C. The reaction mixture was cooled to 24 °C, and solvent was evaporated under reduced pressure. The residue was acidified with 1M HCl (pH=3) at 0 °C and kept at the same temperature for 10 minutes. The residue solids were filtered, washed with cold water (50 mL) and dried under vacuum for 3 hours (1.2 g, 4.64 mmol, 63%). ^{1}H NMR: Matched literature (Adeniji, A. O.; Twenter, B. M.; Byrns, M. C.; Jin, Y.; Chen, M.; Winkler, J. D.; Penning, T. M. *J. Med. Chem.* **2012**, *55*, 2311−2323.).



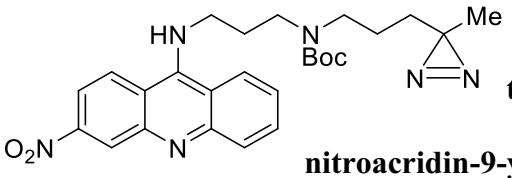
9-chloro-3-nitroacridine (I_{4a}): In a round bottom flask, a solution of 2-((3-nitrophenyl)amino)benzoic acid **I_3** (0.300 g, 1.16 mmol, 1.0 equiv.) and phosphorous oxychloride (3 mL) was heated for 1 hour at 110 °C. The reaction mixture was cooled to 24 °C and poured into an ice cold ammonium hydroxide solution (30 mL). The aqueous layer was extracted with chloroform (2x50 mL). The combined organic layers were then dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The solid was purified by column chromatography, first eluted with 1:10 ethyl acetate/hexane solution and then with 3:10 ethyl acetate/hexane solution to yield a yellow solid (After four reactions 150 mg, 0.580 mmol, 12%). ^{1}H NMR: Matched literature (US 2009/0226940 A1 and Yu, J. *et a*l. *New J. Chem*. **2017**, *41*, 4087-4095.).



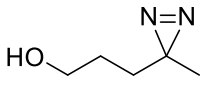
9-chloro-1-nitroacridine (I_{4b}): In a round bottom flask, a solution of 2-((3-nitrophenyl)amino)benzoic acid **I_3** (0.300 g, 1.16 mmol, 1.0 equiv.) and phosphorous oxychloride (3 mL) was heated for 1 hour at 110 °C. The reaction mixture was cooled to 24 °C and poured into an ice cold ammonium hydroxide solution (30 mL). The aqueous layer was extracted with chloroform (2x50 mL). The combined organic layers were then dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The solid was purified by column chromatography, first eluted with 1:10 ethyl acetate/hexane solution and then with 3:10 ethyl acetate/hexane solution to yield a yellow solid (After four reactions 0.9663 g, 3.74 mmol, 80%). ^{1}H NMR: Matched literature (US 2009/0226940 A1 and Yu, J. *et a*l. *New J. Chem*. **2017**, *41*, 4087-4095.).



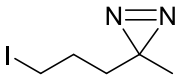
tert-butyl (3-((3-nitroacridin-9-yl)amino)propyl)carbamate (I_5): In a round bottom flask, a solution of 9-chloro-3-nitroacridine **I_{4a}** (0.100 g, 0.38 mmol, 1.0 equiv.) and phenol (0.203 mL, 1.38 mmol, 6.0 equiv.) in toluene (4 mL) was heated for 40 minutes at 110 °C. At this time a solution of tert-Butyl (3-aminopropyl)carbamate in toluene (1 mL) was added at 110 °C and stirred at the same temperature for 18 hours. The reaction mixture was cooled to 24 °C, diluted with dichloromethane (60 mL) and quenched with water (10 mL). The combined organic layers were then dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The solid was purified by column chromatography, first eluted with 0.5% methanol/dichloromethane solution gradually increasing to a 1.5% methanol/dichloromethane solution to yield a dark brown substance (60 mg, 0.151 mmol, 66%). ^{1}H NMR: Matched Dr. Kokkonda's spectrum (Experiment Number Reference: PK172).



tert-butyl (3-(3-methyl-3H-diazirin-3-yl)propyl)(3-((3-nitroacridin-9-yl)amino)propyl)carbamate (I_6): In a round bottom flask, sodium hydride (0.00252 g, 0.105 mmol, 2.1 equiv.) was dissolved in dimethylformamide (DMF) (0.5 mL). tert-butyl (3-((3-nitroacridin-9-yl)amino)propyl)carbamate I_5 (0.020 g, 0.050 mmol, 1.0 equiv.) was dissolved in DMF (1 mL) and added to the reaction mixture, changing the mixture's color from orange-red to dark green. After 10 minutes, 3-(3-iodopropyl)-3-methyl-3H-diazirine **B_3** dissolved in DMF (1.5 mL) and added to the reaction mixture which stirred for 15 hours. The reaction mixture was then quenched with ethyl acetate (2 mL) and distilled water (4 mL). At this time, the reaction mixture was diluted with water (10 mL), and the aqueous layer was extracted with ethyl acetate (2 x 25 mL). The combined organic layers were then washed with brine, dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The solid was purified by column chromatography, first eluted with 0.5% methanol/dichloromethane solution gradually increasing to a 2% methanol/dichloromethane solution to yield a red solid (25.6 mg, 0.053 mmol, 107%- All impurities were not removed). ^{1}H NMR (500 MHz; $CDCl_3$): δ 1.13 (s, 3H, H_{23}), 1.26 (s, 9H,$H_{29,30,31}$), 1.67 (t, *J*= 5 Hz, H_{21}), 1.72-1.76 (m, 2H, $H_{17,20}$), 1.95 (m, 2H, $H_{17,20}$), 3.38 (m, 2H, H_{16}), 3.98 (m, 2H, $H_{18,19}$), 4.10 (m, 2H, $H_{18,19}$), 7.13 (m, 1H, $H_{12,13}$), 7.54-7.55 (m, 1H, $H_{6,11,14}$), 7.79-7.80v(m, 1H, $H_{6,11,14}$), 7.92 (m, 1H, $H_{6,11,14}$), 8.13 (m, 1H, $H_{1,3}$), 8.27 (m, 1H, $H_{1,3}$); ^{13}C NMR (125 MHz; $CDCl_3$): δ 162.51, 156.08, 149.11, 142.06, 140.07, 129.40, 127.78, 120.31, 115.72, 114.33, 113.92, 113.08, 108.30, 105.79, 31.36, 29.69, 29.69, 28.47, 27.07, 25.22, 22.68, 20.81, 20.02, 14.11.

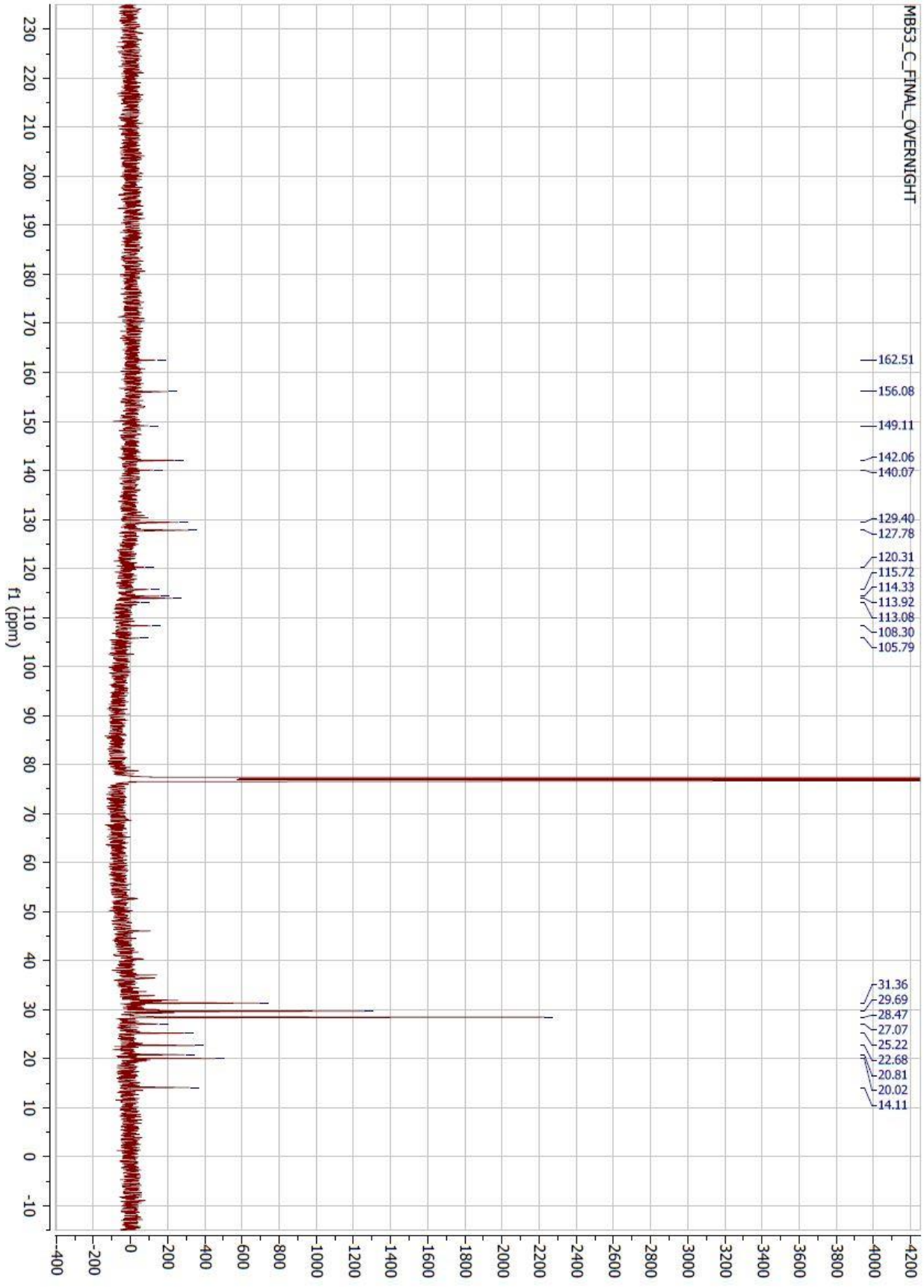


3-(3-methyl-3H-diazirin-3-yl)propan-1-ol (B_2): In a round bottom flask, 5-hydroxypentan-2-one **B_1** (1.986 mL, 19.58 mmol, 1.0 equiv.) dissolved in dry methanol (3 mL) was added. Gaseous ammonia (25 mL) was condensed into the flask at -78 °C, and the reaction was stirred for 3 hours. At this time, a solution of hydroxylamine-O-sulfonic acid (3.32 g, 29.37 mmol, 1.5 equiv.) in methanol (10 mL) was added at -78 °C. The reaction was slowly warmed to 24 °C, and the reaction was stirred at this temperature for 17 hours. Next, the reaction mixture was filtered through a short pad of celite and washed with methanol (25 mL). The mixture was concentrated under reduced pressure. Triethylamine (5.45 mL, 39.16 mmol, 2.0 equiv.) in methanol was added to the reaction mixture at 0 °C followed by dropwise addition of a solution of iodine (7.46 g, 29.37 mmol, 1.5 equiv.) in methanol (20 mL). The reaction was stirred for 2 hours and the solvent was evaporated. The residue was diluted with water (20 mL) and extracted with diethyl ether (2 x 250 mL). The organic layers were combined, washed with saturated sodium thiosulfate solution (80 mL), dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The compound was purified using column chromatography eluting with a solution gradually increasing from 5% ethyl acetate/hexane to 20 % ethyl acetate/hexane to yield a yellow liquid (0.7566 g, 6.6 mmol, 34%). ^{1}H NMR: Matched literature (Shi, Wei; Nacev, Benjamin A.; Aftab, Blake T.; Head, Sarah; Rudin, Charles M.; Liu, Jun O.; *J. Med. Chem.* **2011**, *54*, 7363- 7374.).

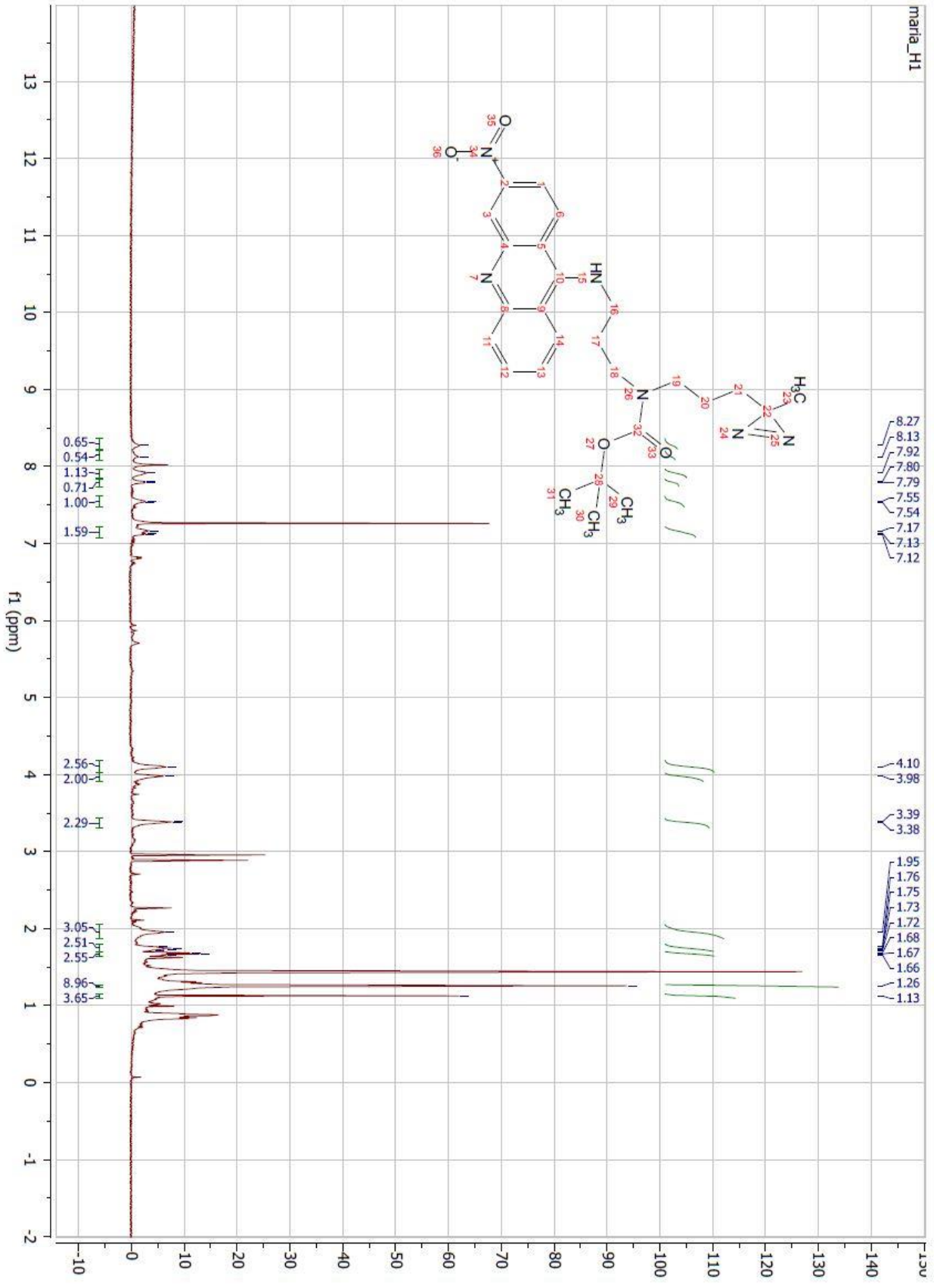


3-(3-iodopropyl)-3-methyl-3H-diazirine (B_3): In a round bottom flask, a solution of 3-(3-methyl-3H-diazirin-3-yl)propan-1-ol **B_2** (1.1175 g, 9.79 mmol, 1.0 equiv.) in dichloromethane (2 mL) was added to a stirring solution of imidazole (1.33g, 19.57 mmol, 2.0 equiv.), triphenylphosphine (3.081 g, 11.74 mmol, 1.2 equiv.), and iodine (2.98 g, 11.747 mmol, 2.0 equiv.) in dichloromethane (50 mL) at 0 °C and stirred for 1 hour. The ice bath was removed, and the reaction was stirred at 24 °C for one hour. The solvent was evaporated, and the residue was diluted with water (15 mL). The aqueous layer was extracted with diethylether (2 x 50 mL). The combined organic layers were washed with brine (25 mL), dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. Column purification was run on the residue, and the column was eluted with a solution gradually increasing from 1% diethylether/pentane to 3% diethylether pentane to yield a yellow oil (110 mg, 0.49 mmol, 5 %). ^{1}H NMR: Matched literature (Durek, T.; Zhang, J.; He, C.; Kent, B.H**.** S. *Org. Lett*. **2007**, *9*, 5497–5500.).

^{13}C NMR for **I$_6$**



^{1}H NMR for **I$_6$**


maria_H1
f1 (ppm)
0.65
0.54
1.13
0.71
1.00
1.59
2.56
2.00
2.29
3.05
2.51
2.55
8.96
3.65
8.27
8.13
7.92
7.80
7.79
7.55
7.54
7.17
7.13
7.12
4.10
3.98
3.39
3.38
1.95
1.76
1.75
1.73
1.72
1.68
1.67
1.66
1.26
1.13