ABSTRACT

Synthesis of Protected Amino Thymidines and New Thiol Derivatives of the Vascular Targeting Agent Combretastatin A-4

Daniel Abel Ramirez

Mentor: Robert R. Kane, Ph.D.

Protected amino thymidines were synthesized as part of a project aimed at developing reagents for the site-specific, sequence selective DNA cleavage. Modified DNA oligonucleotides have the capacity to behave like artificial restriction enzymes in addition to being agents for the targeted scission of DNA and RNA in antiviral and gene therapy. Sulfur-containing analogues of the vascular targeting agent (VTA) Combretastatin A4 (CA4) were also synthesized. Vascular targeting agents are a class of anticancer compounds made to target and restrict tumor neovasculature. Disruption of these new tumor-associated capillaries prevents the blood flow necessary to feed the tumor, resulting in tumor cell starvation, the build-up of toxins, and massive necrotic cell death. One of the best characterized small molecule vascular targeting agents is Combretastatin A-4 (CA4), which is currently in Phase II clinical trials.
Synthesis of Protected Amino Thymidines and New Thiol Derivatives of the Vascular Targeting Agent Combretastatin A-4

by

Daniel A. Ramirez, B.S.

A Thesis

Approved by the Department of Chemistry and Biochemistry

David E. Pennington, Ph.D., Chairperson

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Approved by the Enter Dissertation or Thesis Committee

Robert R. Kane, Ph.D, Chairperson

Charles M. Garner, Ph.D

Carlos E. Manzanarez, Ph.D

Christopher M. Kearney, Ph.D

Accepted by the Graduate School

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J. Larry Lyon, Ph.D., Dean
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<td>CA-4P</td>
<td>Combretastatin A-4 Prodrug</td>
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<td>MMTCl</td>
<td>Monomethoxytrityl chloride</td>
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<td>$^{13}$C NMR</td>
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<td>DMAP</td>
<td>N, N-dimethylaminopyridine</td>
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<td>EDTA</td>
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<td>J</td>
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<td>n-Butyllithium</td>
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<td>ppm</td>
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DEDICATION

To my mother, father and brother
CHAPTER ONE

Introduction

Oligonucleotides

An ongoing goal of the Kane research group is to develop reagents for site-specific, sequence selective DNA cleavage. Appropriately modified oligodeoxynucleotides (ODNs) have the potential to act as “active antisense” agents that bind and catalyze the destruction of a target complementary DNA or RNA sequence. An example of their function is as artificial restriction endonucleases (REs), which cut nucleotide polymers at sequence-selective points that can’t be cleaved by natural restriction enzymes. RE’s are enzymes that cut DNA into fragments by recognizing and cutting pallindromic DNA sequences that are 4 to 6 base pairs in length. These enzymes used extensively in chromosomal mapping, gene isolation, and DNA sequencing. One problem with these enzymes is that they are limited in number and scope of recognition sequences and sites. As such, the need for artificial RE’s that are fine tuned in selectivity and recognition sequence size has been widely recognized.1

In preliminary work, the Kane group modified a previous approach developed by Dervan et. al. that utilized a Fe$^{2+}$-EDTA tethered complementary DNA oligomer.2 Our research involved a system utilizing two iminodiaceitic acid (IDA) tethered DNA oligonucleotides (Figure 2).
Figure 1: Structures of Modified Thymidine Phosphoramidites previously developed by the Kane group.

In this system the two IDA oligonucleotides were, attached by an adjacent complementary base pairing to a template oligonucleotide to form a site for metal chelation (Figure 3). The resulting ternary complex could potentially chelate metal ions in an EDTA-like fashion, hopefully resulting in the metal ion catalyzed, hydrolytic or oxidative, sequence selective cleavage of the complementary strand.\(^3\)

![Diagram of DNA Cleavage via an EDTA-linked ODN\(^2\)](image)

The Kane group analyzed the ability of the complex in Figure 3 to afford oxidative DNA cleavage. 5'-IDA modified 13-mer and 3'-IDA modified 12-mer were
bound to 44 and 45-mer DNA substrate templates in the presence of Fe(II) and dithiothreitol (DTT) as described by Dervan et al.

Figure 3: Diagram of the Proposed DNA Cleavage using two IDA oligonucleotides

However, these experiments were inconclusive in demonstrating observable cleavage with the 2xIDA system. In order to study additional structural motifs, compounds 1 and 2 were chosen as synthetic targets, as they would provide useful 3’ and 5’ amino oligonucleotides.

Figure 4: Structures of Modified Thymidine Phosphoramidites
Unfortunately, the synthesis of these phosphoramidite derivatives was more challenging than expected and we were unable to produce useful quantities of the pure compounds. However, the experience gained during these syntheses facilitated progress on a second project.

_Thio-combretastatin Analog_

Cancer is a disease in which abnormal cells divide without control, in many cases producing a mass of tissue called a malignant tumor. The cancer associated with these solid masses spreads when cells break away from the malignant tumor and enter the blood stream. Tumors can be surgically removed or treated with chemotherapeutic agents or radiation. However, complete recovery is often limited to early stage cancers and cures are rare. A major group of antitumor agents that have proven to be effective in the treatment of cancer are the antimitotic drugs.

One target of antimitotic drugs is tubulin, a heterodimeric protein made up of α and β subunits approximately 50 kDa in size. Tubulin polymerizes to form microtubules, cytoskeletal polymers essential for intracellular transport and mitosis in all eukaryotes. Vinca alkaloids, taxoids, colchicine, cryptophycin and combretastatin A-4 are antimitotic agents that are known to have a binding affinity to tubulin. Three well-characterized sites that the antimitotic agents can bind to on tubulin are commonly called the colchicine site, the vinca alkaloid site and the taxol site. The combretastatin prodrugs developed by Professor George R. Pettit (Arizona State University) appear to selectively target the vascular system of tumors by affecting the microtubules that maintain the shape of the endothelial cells lining the tumor vasculature.
The use of such vascular targeting agents is a relatively recent approach to the treatment of solid tumors.\textsuperscript{5,6} This approach to cancer therapy takes advantage of the observation that tumors that enlarge beyond a certain mass can no longer be supported by peripheral blood or by one main blood vessel, and therefore become vascularized. Disruption of new tumor-associated capillaries (neovascularature) prevents the blood flow necessary to feed the tumor, resulting in tumor cell starvation, the build-up of toxins, and massive necrotic cell death. One of the best characterized small molecule vascular targeting agents is Combretastatin A-4 (CA4) (3).\textsuperscript{7,8,9} While this compound is known to be an extremely effective inhibitor of the assembly of tubulin into microtubules,\textsuperscript{10} its potential as a vascular targeting agent was only revealed after its formulation into the water-soluble prodrug, disodium phosphate Combretastatin A-4 (CA4DP) (4).\textsuperscript{11} Although the prodrug 4 is inactive towards tubulin, it is dephosphorylated in the neovascularature of the tumor to reveal the parent tubulin polymerization inhibiting drug 3 (Figure 5). The resulting disruption of the microtubule skeleton of the endothelial cells in the neovascularature causes them to lose their characteristic flat shape and become bloated, thereby occluding the narrow capillaries. The loss of blood flow to the tumor mass results in extensive necrotic cell death.\textsuperscript{12}

The amine analogue (5) of CA4 is also a very active tubulin polymerization inhibitor.\textsuperscript{13} The formulated serine amide 6 shows significant promise as a vascular targeting agent for the treatment of cancer.\textsuperscript{14} As is true for the CA4DP prodrug of CA4, compound 6 is inactive until converted to the parent drug 5. However, the activation chemistry for the two prodrugs is very different (dephosphorylation for 4, 3 compared to deacylation for 6, 5).
This prompted us to investigate the thiol analogue (7) of compounds 3 and 5, and to study the formulation of this novel compound into reductively activated prodrugs.

In the course of this work a crystalline derivative of compound 8 and its *trans* stereoisomer 9 were isolated and structurally characterized in order to unequivocally confirm their molecular structure.
CHAPTER TWO

Background

Our understanding of DNA and RNA based biomolecules like ribozymes and
DNAzymes has increased due to the considerable amount of research performed. One
major area of study is the development of polynucleotides which can function as
sequence selective chemical nucleases, and particularly, those that derive their activity
from an incorporated metal ion/ligand.²

One common system studied for oligonucleotide cleavage is the iron-mediated
hydroxyl radical generating system often called “Fenton” chemistry.¹⁵ Fenton chemistry
comprises the catalyzed reduction of hydrogen peroxide to generate hydroxide anion and
a reactive hydroxyl radical by a redox active metal (M),

\[
M^{n+} + \text{H}_2\text{O}_2 \rightarrow M^{(n+1)+} + \cdot \text{OH} + \cdot \text{OH}^- \tag{1}
\]

A cyclical reaction will occur in the presence of a metal that can be reduced back to its
active form. This reaction will repeat itself in the presence of hydrogen peroxide and
work as a generator of a hydroxide anion and radical. The superoxide anion is very
effective as a reductant as can be shown in Equation 2.

\[
M^{(n+1)+} + \cdot \text{O}_2^- \rightarrow M^{n+} + \text{O}_2 \tag{2}
\]

The overall reaction of this process can be seen in Equation 3.

\[
\cdot \text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \cdot \text{OH} + \cdot \text{OH}^- \tag{3}
\]

A very good catalytic system for Fenton chemistry was found to be Fe (II)-EDTA
(ethylenediaminetetraacetic acid) because it is easily reduced by superoxides or ascorbic
acid and is oxidized effectively by hydrogen peroxide, while its compact structure restricts reactivity with hydroxyl radicals that it generates. These hydroxyl radicals abstract a deoxyribose hydrogen atom which damages the DNA strand, inducing strand breakage.  

Using this strategy, Dervan and coworkers harnessed the cleavage ability of Fe$^{2+}$-EDTA. Dervan et al illustrated that Fe$^{2+}$-EDTA tethered via modified thymidine ($T^*$) to the center of a 19-mer sequence selectively cleaved a single stranded 167-mer with Fenton like chemistry in the presence of O$_2$ and dithiothreitol (Figure 7). 

In an elegant example of utilizing chemistry for the development of artificial restriction endonucleases (REs), Dervan’s group constructed oligonucleotides, tethered at

---

Figure 7: The Site Specific Cleavage of a 167-mer ODN by a Complementary Base Pairing, Fe$^{2+}$-EDTA tethered 19-Mer.
both ends with Fe$^{2+}$-EDTA that could bind sequence specifically to double stranded DNA by triple helix formation to produce double stranded cleavage.$^2$

Dervan’s group synthesized a 20-mer oligonucleotide with Fe$^{2+}$-EDTA attached to both the 5’ and 3’ ends via the derivatized thymidine phosphoramidite in figure 7. Using this metal-containing oligonucleotide, targeted to a sequence in the 340-kilobase pair chromosome III of the yeast *Saccharomyces cerevisiae*, double-strand cleavage was demonstrated to be limited to a single target site, distinguishing between almost 14 megabase pairs of DNA (Figure 8).$^{17}$ However, the actual cleavage site was distributed over several bases around the target site. This suggests that while the gross cleavage site on the chromosome was well defined, because of the dispersive nature of the hydroxyl
radicals produced and the flexibility of the tether they were unable to achieve extremely precise DNA cleavage. Other problems in achieving sequence specific DNA cleavage is the fact that selecting a target sequence is limited by pH, temperature and nature of the co-solvents. The difference between Dervan’s Fe^{2+} -EDTA linker our previous IDA system was that our 2x IDA system is more rigid and as a result could potentially place a metal ion in such a way that it is able to achieve improved sequence selectivity.
CHAPTER THREE
Materials and Methods

General Section

Chemicals used for synthesis were obtained from Sigma, Aldrich, Fisher (Acros), Crystal Chem. Inc. and used directly as purchased. All solvents such as ethyl acetate, hexanes, methylene chloride, methanol, ethanol, tetrahydrofuran were received from commercial sources and distilled before using. Pyridine and TEA were obtained from commercial sources in DrySolv bottles and used directly as purchased. When dry methylene chloride (CH$_2$Cl$_2$) was used, it was distilled over calcium hydride and used immediately. Tetrahydrofuran (THF) was dried and distilled over potassium metal and benzophenone according to standard procedure prior to use.

Instrumentation

Proton (\(^1\)H), carbon (\(^{13}\)C) and Phosphorous (\(^{31}\)P) NMR were obtained on a Bruker AVANCE 300 NMR running XWIN-NMR 3.1 software at 300 MHz. Chemical shifts are expressed in ppm (\(\delta\)), peaks are listed as singlet (s), doublet (d), triplet (t), or multiplet (m), with the coupling constant (J) expressed in Hz.

High resolution mass spectra were obtained from the University of California, Riverside Mass Spectrometry Facility.
Synthesis of 5’-(Monomethoxytrityl)-3’-(2-cyanoethyl-diisopropylamino) thymidine phosphoramidite

5’-Tosylthymidine (10)$^3$

A solution of thymidine (7.24 g, 30.0 mmol) in 50 ml of dry pyridine was cooled in an ice bath to 0 °C. A solution of p-tolylsulfonyl chloride (9.80 g, 51.5 mmoles) in 50 ml of dry pyridine (also cooled to 0 °C) was added under argon with stirring. The mixture was allowed to react at 0 °C for over 24 hrs, after which it is decomposed with a small piece of ice. The mixture was poured into 500 ml of deionized ice water and extracted three times with 250 mL portions of chloroform. The chloroform was then washed twice with 200 ml portions of saturated aqueous sodium bicarbonate followed by two washings of 300 ml of deionized water. The washed chloroform layer was dried over sodium sulfate and evaporated under reduced pressure to yield a crude brownish solid. The solid was recrystallized from an ethanol and gave 5.52 g of white crystals (total yield 46%). The reaction was followed by TLC (1% triethylamine, 10% methanol, methylene chloride) and visualized by UV and stained with anisaldehyde. $R_f$ thymidine = 0.22, $R_f$ 5’-tosylthymidine = 0.44.

5’-Azidothymidine (11)$^3$

A solution of 0.48 g (1.2 mmoles) 5’-tosylthymidine in 35 mL of deionized water was reacted with 0.24 g (3.7 mmoles) of sodium azide with stirring under argon for over 12 hours until reaction was complete. The product was then extracted with 3 washings of 100mL of ethyl acetate. The ethyl acetate solution was evaporated off and dried overnight. The resulting solid was then dissolved in ethanol to which 5.25 g silica gel was added. The ethanol was then evaporated off to yield crude product adsorbed to the
silica gel. The crude product was then packed into a Biotage FLASH sample injection module (SIM) cartridge which was then eluted through a KP-Si™Silica FLASH 40S cartridge using 3.6% ethanol in methylene chloride using the Biotage FLASH 40 chromatography system. The fractions with product were pooled and evaporated down to yield 0.32 g product shown pure by TLC and NMR (100 % yield). TLC’s were developed in 3.6% ethanol in methylene chloride. Plates were visualized by UV light and with anisaldehyde (5´-tosylthymidine stains black, 5´-azidothymidine stains yellow). Rf 5´-azidothymidine = 0.17

5´-Aminothymidine (12)

A solution of 0.32 g (1.20 mmoles) of 5´-azidothymidine in 70 ml of a pyridine/water mixture (70/30) was reacted with 0.26 ml (2.64 mmoles) 1, 3-propanedithiol and 0.37 ml (2.66 mmoles) triethylamine under a static atmosphere of argon with stirring. It should be noted that when we did the analogous reaction with 3´-azidothymidine that a solvent of 70:30 v/v pyridine-water (ref. 9 in Bayley paper) considerably increased the rate of reaction. The reaction generated tiny bubbles of nitrogen gas and was left stirring for 4 days (we believe that this ensures the oxidation and precipitation of all the excess dithiol, which appears as a white disulfide powder that coats the walls of the reaction vessel). The solution was filtered to remove the disulfide and rotovapped down to give a white precipitate. This was dissolved in deionized water upon which a further amount of disulfide precipitates and was filtered out. The water solution is then washed three times with 150 ml of methylene chloride and then rotovapped to dryness to yield 1.02 g (94 % yield) of an off-white product. The 5´-aminothymidine reaction was followed by TLC with 10% methanol in methylene chloride.
5’-Monomethoxytritylamino thymidine (13)

5’-aminothymidine (1.0 g, 4.1 mmol) was washed three times with pyridine and filtered. Under reduced pressure the material was dried overnight with a drying tube while flushing with nitrogen. The dried 5’-aminothymidine was then dissolved in anhydrous pyridine under nitrogen. At room temperature monomethoxytrityl chloride (1.9 g, 6.2 mmol), triethylamine (4.3 ml, 30.8 mmol) and 4-dimethylaminopyridine (0.025 g, 0.21 mmol) were added to the pyridine mixture, turning the solution a dark green. After stirring for 30 minutes the solution turned black in color. The reaction mixed for additional 3.5 hours and followed by TLC with a methylene chloride/TEA mixture (9.5/0.5, Rf = 0.25). Once the reaction was completed, it was then concentrated under reduced pressure overnight and flashed chromatographed using a Biotage Flash column system. The final product was a light-brown powder (1.8 g, 86% yield):

$^1$H NMR (300 MHz, CDCl$_3$, δ of residual CHCl$_3$ set to 7.26): (δ, ppm): 7.46 (s, 1 H, J = 7.1 Hz), 7.36 (d, 4 H, J = 9.0 Hz), 7.29 (t, 2 H, J = 7.4 Hz), 7.20 (d, 4 H, J = 7.2 Hz), 7.05 (d, 2 H, J = 1.2 Hz), 6.82 (d, 2 H, J = 9.0 Hz), 6.27 (t, H, J = 6.5 Hz), 4.32 (m, H), 3.97 (m, H), 3.78 (s, OCH$_3$), 3.75 (m, H), 2.36 (m, 2H), 2.12 (m, H), 1.85 (d, CH$_3$, J = 1.2 Hz)

$^{13}$C NMR (300 MHz, CDCl$_3$, δ of residual CHCl$_3$ set to 7.26): (δ, ppm): 163.7, 157.9, 150.3, 145.9, 137.7, 135.1, 129.7, 128.4, 127.9, 126.4, 113.2, 111.1, 86.0, 84.3, 72.1, 70.2, 63.2, 55.2, 52.9, 46.1, 45.9, 40.3, 12.5, 9.6 and 7.9.

Synthesis of 5’-(monomethoxytritylamino)-3’-(2-cyanoethyldiisopropyl) thymidine phosphoramidite (I)

5’-(monomethoxytrityl) thymidine was dried in a dessicator with phosphorus pentoxide for two days. The trityl compound (0.185 mmol, 95 mg) was dissolved in
anhydrous CH$_2$Cl$_2$ (4 ml) and cooled to 0°C and left to stir for 10 minutes under nitrogen. Hunig’s base (120 μl) was added followed by 2-cyanoethyl-N, N-diisopropylchlorophosphoramidite (0.268 mmol, 63 mg, 60 μl) was then added. The reaction stirred at 0°C for 15 minutes and then allowed to stir for 1 hour at room temperature. The reaction was followed by TLC and upon completion the reaction was concentrated under reduced pressure and immediately chromatographed (anhydrous ethyl ether containing 2%TEA). The final product was a brown gel (20 mg, 15 % yield):

$^1$HNMR (300 MHz, DMSO-d$_6$, δ of residual DMSO set to 2.48 ppm): (δ, ppm): 7.49 (s, 1H), 7.41 (d, aromatic 4H, J = 7.6 Hz), 7.30 (d, aromatic 2H, J = 8.8 Hz), 7.27 (d, aromatic 2H, J = 7.4 Hz), 7.17 (t, aromatic 2H, J = 7.22 Hz), 6.83 (d, aromatic 2H, J = 8.8 Hz), 6.12 (t, H, J = 6.8 Hz), 4.51 (m, H), 3.98 (m, H), 3.86 (m, 2H), 3.75 (m, 2H), 3.71 (s, CH$_3$), 3.64 (m, H), 3.55 (m, H), 2.82 (t, 2H, J = 5.8 Hz), 2.30 (m, 2H), 1.69 (s, CH$_3$), 0.94 (m, 12H)

$^{13}$C NMR (300 MHz, DMSO-d$_6$, δ of residual DMSO set to 39.43): (ppm): 163.5, 157.3, 150.2, 146.1, 137.6, 136.1, 129.5, 128.2, 127.6, 126.0, 118.7, 112.9, 109.5, 83.6, 73.5, 69.7, 58.3, 55.5, 54.8, 45.6, 42.5, 42.4, 24.2, 19.7 and 11.9.

$^{31}$P NMR (300MHz, DMSO-d$_6$): (ppm): 147.21

**Synthesis of 3´-aminothymidine (14)**

A solution of 0.50 g (1.9 mmoles) of 3´-azidothymidine in 150 ml of methanol mixture (70/30) was reacted with 0.38 ml (3.8 mmoles) of 1, 3-propanedithiol and 0.53 ml (3.8 mmoles) triethylamine under a static pressure of argon with stirring. The reaction generated tiny bubbles of nitrogen gas and was left stirring for 4 days. The solution was filtered to remove the disulfide and rotovapped down to give a white solid. This solid
was then dissolved in deionized water upon which a further amount of disulfide precipitated and was filtered out. The aqueous solution was then washed three times with 150 ml of methylene chloride and then the water was removed by evaporation to yield 0.45 g (98 % yield) of off-white product. The 3’-aminothymidine reaction was followed by TLC with 10% methanol in methylene chloride.

*Synthesis of 3’-Monomethoxytrityl amino thymidine (15)*

In a similar reaction to produce compound 13, 3’-aminothymidine (1.86 mmol, 0.450 g) was washed three times with pyridine and dried overnight with a drying tube while flushing with nitrogen and co-evaporating with pyridine. The material was then dissolved in anhydrous pyridine under nitrogen. At room temperature monomethoxytrityl chloride (0.861 g, 2.79 mmol), triethylamine (14.0 mmol, 1.96 mL) and 4-dimethylaminopyridine (0.011 g, 0.093 mmol) were added to the mixture turning the solution a dark green. After stirring for 30 minutes the solution turned black in color. The reaction was left to mix for additional 3.5 hours and followed by TLC with a methylene chloride/ TEA mixture (9.5/0.5, Rf = 0.25). Upon completion the reaction was then concentrated under reduced pressure overnight and flashed chromatographed using a Biotage Flash column system. The final product was a light brown powder (0.600 g, 86% yield):

\[ ^1H \text{NMR (300 MHz, CDCl}_3, \delta \text{ of residual CHCl}_3 \text{ set to 7.26): (δ, ppm): 7.51(d, 1 H, J = 8.2 Hz), 7.41(d, 4 H, J = 6.8 Hz), 7.29 (t, 2 H, J = 7.4 Hz), 7.21 (d, 4H, J = 6.11 Hz), 7.17 (s, H), 6.82 (d, 2H, J = 8.94 Hz), 6.03 (t, H, J = 6.14 Hz, 6.68 Hz), 3.86 (dd, H, J = 12.0, 2.2), 3.78 (s, OCH}_3), 3.66 (dd, H, J = 12.0, 3.4), 3.33 (m, 3H), 2.08 (s, OH), 1.96 (s, NH), 1.83 (s, CH}_3) \]
$^{13}$C NMR (300 MHz, CDCl$_3$, δ of residual CHCl$_3$ set to 7.26): (δ, ppm): 163.5, 158.2, 150.2, 146.3, 138.1, 136.0, 129.8, 128.5, 128.1, 126.7, 113.4, 110.8, 86.6, 84.9, 70.7, 62.1, 55.2, 53.2, 40.0 and 12.5.

Scheme 1: Synthesis of 5’-(monomethoxytritylamo)-3’-(2-cyanoethyl-diisopropyl) thymidine phosphoramidite
Scheme 2: Synthesis of 3\textsuperscript{'}-(Monomethoxytritylamino)-5\textsuperscript{'}-(2-cyanoethyldiisopropyl) thymidine phosphoramidite, 2

86\% yield
CHAPTER FOUR

Results and Conclusion

The goal of my oligonucleotide research was to synthesize compounds 1 and 2, which could be useful in the development of novel functional oligonucleotides single base specific cleavage.

![figure 9: compounds 1 and 2](image)

Different changes to the original synthesis were attempted to increase the overall yield. One problem that was prevalent in the original synthesis of 5’-azidothymidine was the use of DMF as the solvent. Because of the nature of DMF it was difficult to remove all the solvent from the 5’-azidothymidine product. As a result DMF could be seen by NMR throughout the rest of the synthesis. In order to avoid this problem an azeotrope
method was incorporated which produced a more pure product but with a lower yield. Despite the low yield, in that step, the biggest setback in the overall synthesis was the decomposition of the final product and the instability of the reagent-2-cyanoethyldiisopropyl phosphorus chloride. One of the problems was that the reagent would decompose rapidly even when kept in the freezer. Additionally, the final product would further decompose while in the column which made it difficult to get any measurable quantity to characterize or to proceed further. Even when the column was ran at a faster rate the decomposition was still a problem. We were able to get enough of the 5’-(Monomethoxytritylamino)-3’-(cyanoethyldiisopropyl) thymidine phosphoramidite to characterize by NMR but unfortunately not enough of the product was isolated to proceed with the synthesis of modified oligonucleotides. In the end we learned a variety of chromatography techniques, as well as synthetic techniques that could be applied to other synthesis.
CHAPTER FIVE

Background

In a recent paper in Nature Review, Mary Ann Jordon wrote “Highly dynamic mitotic-spindle microtubules are among the most successful targets for anticancer therapy”.\textsuperscript{18} The major component of microtubules is tubulin, which is a heterodimeric protein that consists of two polypeptides subunits $\alpha$-tubulin and $\beta$-tubulin with dimensions of 4nm x 5nm x 8nm and approximately 100,000 daltons.\textsuperscript{4} The structures of these subunits are similar, as can be seen in Figure 1.

Figure 10: Tubulin dimer\textsuperscript{19}

Polymerization of tubulin results in microtubules that are the essential cytoskeleton polymers that effect cell shape, cell transport and cell division. The initial
stage of microtubule formation is called nucleation, where the alpha and beta tubulin molecules join to form a heterodimers. In the second stage (elongation) the heterodimers then attach to other dimers in a head to tail fashion to form protofilaments which then join together to form a microtubule. Each microtubule contains 13 protofilaments and is 20-25 nm in diameter (Figure 11).4

Figure 11: Microtubule Structure and Subunits20

The rate of polymerization differs at the two ends of the microtubule, as assembly and disassembly occurs at both the plus and minus ends of the microtubule. This behavior is known as dynamic instability, which is a process that allows the ends of the microtubule to switch phases between growth and shortening. The plus end is where the microtubule rate of growth is the fastest. The assembly and disassembly of the microtubule is a result of the binding and hydrolysis of guanosine triphosphate (GTP) by the tubulin subunits. Each α/β-tubulin subunit is bound together by one molecule of GTP which is non-exchangeable. However, the GTP that is attached to the beta monomer is exchangeable. Due to this exchangeability when the tubulin subunits bind to the microtubule the GTP is hydrolyzed to guanosine diphosphate (GDP). Upon release of the
GTP-tubulin subunits, depolymerization results and the tubulin subunits exchange GDP, on beta tubulin, for GTP and can undergo another round of polymerization.

Prevention of the formation of microtubules is the focus of a significant body of cancer research. There are three well characterized ligand binding sites found in microtubules; the taxane site, the vinca alkaloid site, and the colchicine binding site. It has been shown that each binding site has an influence on a different part of mitosis. The taxane site effects the depolymerization of microtubules, whereas the vinca alkaloid and colchicine sites effects the polymerization of microtubules. By preventing polymerization or depolymerization the ability for cancer cells to reproduce is affected, thereby causing a reduction in cellular division.

Figure 12: Role of Tubulin in Cell Division and Interaction with Tubulin Ligands Colchicine Binding Site

There have been many drugs developed or discovered in nature that disrupt microtubule polymerization by binding to tubulin (Figure 13). These antimitotic drugs
bind to either of the three binding sites of tubulin; the colchicine site, the vinca alkaloid site, or the taxoid site.

![Chemical structures of ligands binding to tubulin](image)

Figure 13: Natural and Synthetic ligands that bind to tubulin

Of special interest is the colchicine binding site. Colchicine (Figure 14) is a natural product that can be extracted from the meadow saffron, and which has historically
been used as a treatment for gout. Although each molecule of colchicine binds tightly to tubulin and prevents polymerization, once tubulin has polymerized into a microtubule colchicine will not have an effect upon it. The rapid disappearance of the mitotic spindle caused by colchicine indicates a chemical equilibrium between the spindle microtubules and the free tubulin.\textsuperscript{4} The site on \(\beta\)-tubulin which colchicine binds is termed the “colchicine binding site” and is located at the interface of the \(\alpha\) / \(\beta\) subunits of tubulin. Antimitotic drugs that bind to the colchicine site block tubulin polymerization.\textsuperscript{18} The fact that colchicine is a powerful antitumor agent its use in the clinic is limited because of its toxicity.

![Figure 14: Structure of Colchicine](image)

A novel “footprinting” method developed by Chaudhuri\textsuperscript{30} and coworkers allows the drug-binding sites as well as the domain of tubulin affected by drug-induced conformational changes to be determined. In this method, tubulin is treated with concentrations of urea low enough to allow the ligand to bind to tubulin, yet high enough to loosen the conformation of the protein so that any cysteine residue not affected by the ligand can react with unlabeled N-ethylmaleimide. Afterwards, the ligand is removed and tubulin is reacted with labeled N-ethylmaleimide to identify the residues which were blocked when the ligand was originally bound. It was determined in this study that
colchicine binds to tubulin at the α/β interface with the B-ring on the α-subunit and the A and C-rings on the β-subunit. In addition, the B-ring of the colchicine plays a major role in the stability of colchicine with little effect on the A and C-rings.

In a similar study done by Hamel and coworkers, cysteine 354 of β-tubulin was determined to be the binding site for the A ring of colchicine (Figure 14). In this study 3-chloroacetyl-3-dimethylthiocolchicine (3CTC), an analog of colchicine and a competitive inhibitor of the binding of colchicine to tubulin, was reacted with β-tubulin containing radiolabeled peptides. Once these peptides were cleaved from the intact protein and isolated it was determined that a strong covalent reaction between [14C]3CTC and β-tubulin occurred at cysteine 354. Conveniently, the calculated length of the chloroacetyl moiety in 3CTC is 3 Å, and it was determined that there is a 3 Å distance between the C-3 oxygen of colchicine and the Cys-354 sulfur atom. This suggests that the colchicine A ring lies somewhere between Cys-354 and Cys-239 which is known to have a distance of 9 Å.

**Combretastatins**

Combretastatins are antimitotic agents isolated and characterized by Pettit and coworkers in 1982 from the bark of the South Afican bush willow *Combretum caffrum.* A series of naturally occurring combretastatins (Figure 15) were isolated and shown to function as antimitotic agents. The combretastatin A series has been the focus of much research, especially the combretastatins A-1, A-2 and A-4. These simple cis-stilbenes differ according to various substituents on the A and B-rings. The combretastatin B series consist of two aryl rings connected by a saturated hydrocarbon bridge.
Combretastatin C is a single compound which is similar to combretastatin A-1 with a unique tricyclic core. The D series has an ether linkage that connects the two aryl rings which are joined together by a macrocyclic lactone. Even though these compounds are similar in structure to colchicine they are more attractive as anti-cancer targets because of their decreased toxicity to normal healthy cells.

Among the most potent compounds in the combretastatin family is the combretastatin A-4 (CA-4). This compound inhibits the polymerization of tubulin into microtubules by binding to the colchicine site on β-tubulin. CA-4 is similar to colchicine in that it contains two aromatic rings connected by a hydrocarbon bridge in which one aryl ring has a 3, 4, 5-trimethoxyphenyl moiety. CA-4 has shown to have excellent activity as an anti-tubulin agent with an inhibition constant (IC50) of 2-3uM for inhibition of tubulin assembly. The cytotoxic properties of CA4 can be seen in the following table.

---

Figure 15: A Diagrammatic Representation of the Proposed Interaction of Colchicine with Tubulin

---
Extensive SAR studies have demonstrated that one of the requirements for these compounds is to have the Z-geometry for the stilbenoid system. The E-configuration shows a considerable drop in antitubulin activity and cancer cell inhibition.\textsuperscript{33}

In addition, the 3, 4, 5-trimethoxyphenyl substitution and the 3-hydroxy-4-methoxy rings are both vital parts of CA-4 binding to the colchicine site on tubulin.\textsuperscript{37}

From molecular recognition studies performed by Dr. Pinney’s group at Baylor
University, it was determined that the distance between the aryl rings of CA-4 is important for tubulin polymerization inhibition.

Table 1: In Vitro Human Tumor Cell Line Assay for Combretastatin A-4.23

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Cell Line</th>
<th>GI50 (g/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovarian</td>
<td>OVCAR-3</td>
<td>&lt;1.0 x 10^{-3}</td>
</tr>
<tr>
<td>CNS</td>
<td>SF-295</td>
<td>&lt;1.0 x 10^{-3}</td>
</tr>
<tr>
<td>Lung-NSC</td>
<td>NCI-H460</td>
<td>5.6 x 10^{-4}</td>
</tr>
<tr>
<td>Colon</td>
<td>KM20L2</td>
<td>6.1 x 10^{-2}</td>
</tr>
<tr>
<td>Melanoma</td>
<td>SK-MEL-5</td>
<td>2.0 x 10^{-4}</td>
</tr>
<tr>
<td>Pancreas</td>
<td>BXPC-3</td>
<td>3.9 x 10^{-1}</td>
</tr>
<tr>
<td>Pharynx</td>
<td>FADU</td>
<td>6.5 x 10^{-4}</td>
</tr>
<tr>
<td>Thyroid</td>
<td>SW1736</td>
<td>7.1 x 10^{-4}</td>
</tr>
<tr>
<td>Prostate</td>
<td>DU-145</td>
<td>7.6 x 10^{-3}</td>
</tr>
</tbody>
</table>

Prodrugs

Despite the exciting antitubulin activity of CA-4, its availability in aqueous environments is unfortunately low. Therefore, modifications to CA-4 were necessary to achieve an appropriate prodrug. The prodrug form of CA-4, combretastatin A-4 phosphate disodium salt (CA-4P)\(^{11}\) was developed by the functionalization of the free hydroxyl moiety. Prodrugs are agents that are transformed after administration, either by metabolism or spontaneous chemical breakdown, to form a pharmacologically active species.\(^{36}\) Once in the bloodstream enzymes convert the water-soluble prodrug into the fat-soluble active form of the drug. This activation chemistry for the CA4 prodrug prompted us to investigate the thiol analogue (8) CA4, and to study the formulation of this novel compound into reductively activated prodrugs. In the course of this work a crystalline derivatives of compound 8 and its trans stereoisomer 9 were isolated and structurally characterized in order to unequivocally confirm their molecular structure.
CHAPTER SIX
Materials and Methods

General Section

Chemicals used for synthesis were obtained from Sigma, Aldrich, Fisher (Acros), BACHEM and used directly as purchased. All solvents such as ethyl acetate, hexanes, methylene chloride, methanol, ethanol, tetrahydrofuran were received from commercial sources and distilled before using. When dry methylene chloride (CH$_2$Cl$_2$) was used it was distilled over calcium hydride and used immediately. Tetrahydrofuran (THF) was dried and distilled over potassium metal and benzophenone according to standard procedure prior to use.

Instrumentation

Proton ($^1$H), carbon ($^{13}$C) and Phosphorous ($^{31}$P) NMR were obtained on a Bruker AVANCE 300 NMR running XWIN-NMR 3.1 software at 300 MHz. Chemical shifts are expressed in ppm (δ), peaks are listed as singlet (s), doublet (d), triplet (t), or multiplet (m), with the coupling constant (J) expressed in Hz.

3-(N, N-dimethylthiocarbamoyloxy)-4-methoxybenzaldehyde$^{38}$ (16)

A solution of N, N-dimethylcarbamoyl chloride (4.07 g, 32.9 mmol) in THF was added dropwise at 0° C to a solution of 3-Hydroxy-4-methoxybenzaldehyde (5.03 g, 32.9 mmol) and potassium hydroxide (1.84 g, 32.9 mmol) in water (22 mL). A white precipitate was filtered using suction filtration, washed with water and dried by vacuum.
Yield 6.72 g (85 %) of 3-(N, N-dimethylthiocarbamoyloxy)-4-methoxybenzaldehyde.

$^1$H NMR (300 MHz, CDCl₃, δ of residual CHCl₃ set to 7.26 ppm): (δ, ppm): 9.86 (s, 1H, CHO), 7.77 (dd, H, J = 8.5, 2.0 Hz), 7.57 (d, H J = 2.0 Hz), 7.06 (d, H J = 8.5 Hz), 3.90 (s, OCH₃), 3.45 and 3.35 (2s, N(CH₃)₂).

$^{13}$C NMR (300 MHz, CDCl₃, δ of residual CHCl₃ set to 77.0 ppm): (ppm): 38.8, 43.5, 56.3, 112.2, 124.9, 129.8, 143.2, 156.8, 187.2 and 190.1.

3-(N, N-dimethylcarbamoylthio)-4-methoxybenzaldehyde

A solution of 3-(N, N-dimethylcarbamoylthio)-4-methoxybenzaldehyde (4.93 g, 20.6 mmol) in 200 mL of diphenyl ether was heated to 245 -250 °C under argon for 2 hr 10 min. The mixture was cooled to room temperature then added to 1L of hexanes and the precipitate formed was collected by suction filtration, washed with hexanes and dried by vacuum. Yield 3.77 g (76.5 %) of 3-(N, N-dimethylcarbamoylthio)-4-methoxybenzaldehyde as a light brown precipitate.

$^1$H NMR (300 MHz, CDCl₃, δ of residual CHCl₃ set to 7.26 ppm): (δ, ppm): 9.87 (s, 1H CHO), 7.99 (d, 1H J = 2.1 Hz), 7.94 (dd, 1H J = 8.53, 2.16 Hz), 7.06 (d, 1H J = 8.54 Hz), 3.95 (s, OCH₃), 3.08 (2 broad s, N(CH₃)₂).

$^{13}$C NMR (300 MHz, CDCl₃, δ of residual CHCl₃ set to 77.0 ppm): (ppm): 190.1, 165.4, 164.8, 140.3, 133.2, 130.0, 118.4, 111.5, 56.6 and 37.0.

3, 4, 5-Trimethoxybenzyl-triphenylphosphonium bromide

To a solution of CBr₄ (22.9 g, 69.0 mmol) in anhydrous acetone (170 mL) at 0 °C under argon, 3, 4, 5-trimethoxybenzyl alcohol (10.0 g, 50.4 mol) and triphenylphosphine (17.99 g, 68.0 mmol) were added. The reaction was left overnight and then the solvent
was removed under reduced pressure to obtain 3, 4, 5-trimethoxybenzyl bromide crude as a brown gel. The crude product was dissolved in toluene (200 mL) and triphenylphosphine (14.54 g, 55 mmol) was added. The reaction was refluxed for 2 hours. After refluxing the reaction was allowed to stir overnight, and a sticky orange precipitate was formed. The solvent was removed under reduced pressure and the precipitate was dried under vacuum. Once the material was dry, it was recrystallized using ethanol in the cold overnight. The solvent was then removed under reduced pressure and the white crystals were dried under vacuum. Yield 17.64 g (66.8%) of 3, 4, 5-trimethoxybenzyltriphenylphosphonium bromide.

\[(E/Z)-1-(3'-N, N\text{-dimethylthiocarbamoyl}-4'-methoxy)-2-(3'', 4'', 5''-\text{trimethoxyphenyl})\text{ethane (3 and 4)}\]

A well stirred solution of 3, 4, 5-trimethoxybenzyltriphenylphosphonium bromide (4.37 g, 8.35 mmol) and n-BuLi (0.535 g, 8.36 mmol) in THF, 3-(N,N-dimethylcarbamoylthio)-4-methoxybenzaldehyde (1.00 g, 4.18 mmol) was added drop wise at -15 °C. Once at room temperature the reaction was followed by thin-layer chromatography. When reaction was complete the mixture was quenched with ice-cold water. The products were then extracted with ether. The etherate solution was then washed with ice-cold water and dried over Na\textsubscript{2}SO\textsubscript{4}. Ether was removed in vacuo and the resulting brownish gel was subjected to flash chromatography (silica gel, 20% EtOAc/60% hexanes), in order to obtained the E-isomer as a pale-yellow crystalline solid. \((Z: E, 2:1\text{ as determined by integration of }^1\text{H NMR signals})\). Yield 0.71 g (42%) of Z-1-(3'-N, N-dimethylthiocarbamoyl-4'-methoxy)-2-(3'', 4'', 5''-trimethoxyphenyl)
ethane and 0.34 g (21%) of E-1-(3'-N, N-dimethylthiocarbamoyl-4'-methoxy)-2-(3'', 4'', 5''-trimethoxyphenyl) ethane.

\[ ^1H \text{ NMR Z-isomer (300 MHz, CDCl}_3, \delta \text{ of residual CHCl}_3 \text{ set to 7.26 ppm):} \]
\[
\delta, \text{ ppm): 7.44 (d, 1H, J = 2.3 Hz), 7.31 (dd, 1H, J = 8.6, 2.3 Hz), 6.82 (d, 1H, J = 8.6 Hz), 6.51 (s, 2H), 6.45 (d, 1H, J = 12.4 Hz), 6.40 (d, 1H, J = 12.2 Hz), 3.84 (s, 3H), 3.82 (s, 3H), 3.70 (s, 6H), 3.03 (broad, N(CH}_3)_2). \]

\[ ^13C \text{ NMR Z-isomer (300 MHz, CDCl}_3, \delta \text{ of residual CHCl}_3 \text{ set to 77.0 ppm): (ppm): 14.2, 21.1, 37.0, 56.0, 56.2, 60.4, 60.9, 105.8, 111.1, 116.6, 128.6, 129.2, 130.0, 132.3, 132.6, 137.1, 138.5, 153.0, 159.3, and 165.9.} \]

\[ ^1H \text{ NMR E-isomer (300 MHz, CDCl}_3, \delta \text{ of residual CHCl}_3 \text{ set to 7.26 ppm):} \]
\[
\delta, \text{ ppm): 7.64 (d, 1H, J = 2.3Hz), 7.51 (dd, 1H, J = 8.6 Hz, J = 2.3 Hz), 6.94 (d, 1H, J = 8.6 Hz), 6.93 (d, 1H, J = 16.1 Hz), 6.89 (d, 1H, J = 16.1 Hz), 6.69 (s, 2H), 3.90 (s, 6H), 3.89 (s, 3H), 3.85 (s, 3H), 3.11 (broad, N(CH}_3)_2). \]

\[ ^13C \text{ NMR Z-isomer (300 MHz, CDCl}_3, \delta \text{ of residual CHCl}_3 \text{ set to 77.0 ppm): (ppm): 37.1, 56.1, 56.3, 61.0, 103.4, 111.7, 117.3, 127.0, 127.4, 129.5, 130.5, 133.3, 136.0, 137.8, 153.4 and 159.6.} \]

(Z/E)-1-(3'-thiol-4'-methoxy)-2-(3'', 4'', 5''-trimethoxyphenyl) ethane (9)

A solution of 3 (0.640 g, 1.58 mmol) in THF was collected to -42 °C and allowed to mix for 15 minutes. To the cooled mixture LiAlH\textsubscript{4} (0.36 g, 9.48 mmol) was added in portions over a 10 min period. As the LiAlH\textsubscript{4} was added the mixture began to bubbled and turn to a dull grayish color. The reaction was followed by TLC and was determined after 2 ½ hours that no change was observed. The reaction was then diluted with ether and allowed to mix for 15 minutes. The mixture was then added to a flask containing
HPLC water to neutralize any remaining LiAlH₄. While adding the mixture to water a white precipitate (lithium hydroxide) was formed. The precipitate was filtered off and the remaining solution was washed with 3 portions of methylene chloride. The methylene chloride mixture was then washed with water and dried overnight by vacuum to give an off-white crude product. Yield 0.22 g (40%) of (Z/E)-1-(3'-thiol-4'-methoxy)-2-(3", 4", 5"-trimethoxyphenyl) ethane.

¹H NMR E-isomer (300 MHz, CDCl₃, δ of residual CHCl₃ set to 7.26 ppm): 7.24 (d, H, J = 2.4 Hz), 7.04 (dd, H, J = 6.4, 2.1 Hz), 6.72 (d, H, J = 8.5 Hz), 6.51 (s, 2H), 6.42 (s, 2H), 3.86 (s, 3H), 3.84 (s, 3H), 3.70 (s, 6H).

¹³C NMR Z-isomer (300 MHz, CDCl₃, δ of residual CHCl₃ set to 77.0 ppm): (ppm):
206.9, 154.0, 153.0, 129.8, 129.3, 128.7, 127.2, 110.3, 106.1, 61.0, 56.0, 56.0 and 31.0.

Acetal Protected 3-(N, N-dimethylcarbamoylthio)-4-methoxybenzaldehyde (19)

To a well stirred solution of 17 (4.00 g, 16.7 mmol) and ethylene glycol (8.89 g, 8 mL, 143 mmol) in benzene, para- toluenesulphonic acid (0.066 g, 0.347 mmol) was added. The temperature was then increased to 120 °C and allowed to react for 2hrs. The reaction was monitored by thin-layer chromatography and determined that it was complete. The benzene was removed in vacuo to give a brownish crude. The crude was subjected to flash chromatography (silica gel, 50% EtOAc/50% hexanes, Rf = 0.23), in order to obtain the acetal protected as a pale-yellow solid (yield 4.40 g).

¹H NMR (300 MHz, CDCl₃, δ of residual CHCl₃ set to 7.26 ppm): (δ, ppm): 7.58 (d, H, J = 2.2 Hz), 7.51 (dd, H, J = 2.2, 8.5 Hz), 6.96 (d, H, J = 8.5 Hz), 5.79 (s, H), 4.05 (m, 4H), 3.88 (s, 3H), 3.11 (b, 3H), 3.01 (b, 3H)
3-Thio-4-methoxybenzaldehyde (20)

To a well stirred solution of 19 (2.00 g, 7.06 mmol) in methanol, sodium hydroxide (2.24 g, 3.50 mol) was added and allowed to reflux overnight. The reaction was monitored by thin-layer chromatography (50% EtOAc/50% hexanes, Rf = 0.65). The mixture was acidified to a pH 6.0 and allowed to mix an additional 30 minutes. The methanol was removed by vacuum filtration and the product was dried overnight to give a white solid (yield 1.45 g).

$^1$H NMR (300 MHz, CDCl$_3$, δ of residual CHCl$_3$ set to 7.26 ppm): (δ, ppm): 9.84 (s, COH), 7.81 (d, 1H, J = 2.02 Hz), 7.66 (dd, 1H, J = 2.05, 8.44 Hz), 6.97 (d, 1H, J = 12.05 Hz), 3.99 (s, 3H), 3.89 (s, SH)

3-(3’-carboxypropyl)-4-methoxybenzaldehyde disulfide (21)

To a well stirred solution of 17 (2.00 g, 7.06 mmol) in methanol, sodium hydroxide (2.24 g, 3.50 mol) was added and allowed to reflux overnight. The 3-carboxypropyl disulfide was added in excess and allowed to mix for 1 hour. At this time the reaction was followed by TLC until it was determined that the reaction had gone to completion. The mixture was then acidified to a pH 6.0 and allowed to stir for 30 minutes. The methanol was removed by vacuum filtration to leave a crude white-brownish solid. The crude product was subjected to flash chromatography (silica gel, 50% EtOAc/50% hexanes, Rf = 0.25) to give a white solid (yield 1.25 g).

$^1$H NMR (300 MHz, CDCl$_3$, δ of residual CHCl$_3$ set to 7.26 ppm): (δ, ppm): 9.91 (s, COH), 8.25 (d, 1H, J = 2.0 Hz), 7.75 (dd, 1H, J = 2.02, 8.38 Hz), 6.97 (d, 1H, J = 8.4 Hz), 3.99 (s, 3H), 2.79 (t, 2H, J = 7.10 Hz), 2.50 (t, 2H, J = 7.2 Hz), 2.04 (m, 2H, J = 7.0, 7.1, 7.3 Hz)
$^{13}$C NMR (300 MHz, CDCl$_3$, δ of residual CHCl$_3$ set to 77.0 ppm): (ppm): 190.6, 177.9, 161.2, 130.8, 130.3, 128.5, 127.1, 110.4, 56.4, 37.3, 32.1, 29.7 and 23.7
CHAPTER SEVEN
Results and Discussion

The goal of this research was to develop sulfur derivatives of Combretastatin A-4. The thiol derivatives would then be converted to several novel disulfide prodrug formulations, as well as reductively- and hydrolytically-activated compounds and the antitumor activity of these new sulfur derivatives would be examined.

The initial problem was how we would put a thiol in the 3 position of the A-ring. This problem was resolved by using a transformation based on the “Newman-Kwart Rearrangement”.\textsuperscript{38}

This rearrangement was the key step in placing the thiol on C-3 of the A-ring. There are two parts to this step that had a dramatic effect on the yield. If the temperature
was not kept within 245 °C – 250 °C the rearrangement would not be as effective which would affect the yield and if the solution was added too rapidly to hexanes precipitation would not occur efficiently. To allow as much of the product to precipitate out the solution was added in portions over a 30 minute period. The next step that posed a challenge was whether or not the Wittig reaction would give us our cis isomer in a high enough yield. Two of the reactions can be seen below.

Figure 18: Wittig reaction using Sodium Hydride

Figure 19: Wittig reaction using Butyllithium
A comparison of the crude NMR’s of the reactions revealed that the *cis* to *trans* ratio for the n-BuLi reaction was the most favorable (which is normally the best reagent for this purpose) as it provided the products in a ratio of 2 to 1. The two isomers were then carefully separated by column chromatography and each isomer was crystallized. To verify that the NMR-based isomer assignments were correct, crystal structures of each isomer were determined. It was found that crystals of these compounds could be grown by dissolving the crude product in methylene chloride and initiating crystallization with hexanes. These crystals had a yellow film around them which was washed off with cold MeCl₂, which left a clear crystalline solid. The yellow film was dried and crystallized using the same procedure and produced a clear yellowish solid. Crystals of both isomers were taken and crystal structures were determined by X-ray crystallography. The structures are shown below (Figure 20 & 21) along with a summary of the crystallographic data (Table 2) determined in our department by Dr. Kevin Klausmeyer.\textsuperscript{41}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure20.png}
\caption{Thiol *cis*-isomer crystal structure\textsuperscript{41}}
\end{figure}

\textsuperscript{41}
Once the crystal structures of both the cis and trans isomers were determined and the purity confirmed by HPLC we proceeded to the next step. After characterizing the cis-isomer the next step was to convert the thiol-carbamate to a thiol. This step became the most difficult to accomplish.
Figure 22: Purity of Cis-Isomer determined by HPLC

Figure 23: Thiocarbamate deprotection

Many variables that needed to be controlled during the deprotection step included the dryness of the THF solution, temperature, isomerization and the deprotonation of the thiol isomer during column chromatography. The one complication that we were not able to avoid was the decomposition of the product and as a result the yield was very low. A potential reason for this is that thiols can possibly react with the ethylene linker, potentially forming polymeric products. After trying numerous different solvents for column chromatography we determined that we would not be able to obtain the desired
product in a suitable enough yield. However, we were able to obtain enough of the pure product in order to obtain an NMR spectrum and to test the blood flow shutdown.

<table>
<thead>
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<th>Table 3: Blood flow in % of the control</th>
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<tbody>
<tr>
<td>CA4 (control)</td>
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<tr>
<td>Oxi-com-224 100mg/kg</td>
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<td>Oxi-com-224 10mg/kg</td>
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The above results showed us that the thiol analog of CA-4 was not as effective as CA4. However, the limited activity could possibly be due to the decomposition of the thiol analog.

At this point it was decided to try a different synthetic target. A variety of other synthetic approaches were explored but the one compound that showed the most promise was a disulfide combretastatin analog. The new synthetic scheme can be seen below,

![Figure 24: Acetal Protection of Aldehyde](image)

Once the aldehyde was protected the carbamate was removed using sodium hydroxide in methanol under refluxing conditions, producing a protected thiol salt. The thiol salt was then reacted with 3-carboxypropyl disulfide and acidified to a pH of 6.0.
The Wittig reaction was then performed on the deprotected aldehyde.

Despite many attempts we were unable to get complete separation of the *cis* and *trans* products of this reaction.
CHAPTER EIGHT

Conclusion

Various design and synthetic procedures were performed throughout both research projects. During the oligonucleotide project much was learned through the synthetic process. We were able to develop a clean reaction for 5’-(Monomethoxytritylamino)-3’-(cyanoethyl-diisopropyl) thymidine phosphoramidite using an azeotrope method which produced a more pure product. We were also able to place a MMT group on both the 5’- amine and 3’- amine ends of the thymidine compound. Running the final product through the column at a faster rate allowed us to get enough of the 5’-(Monomethoxytritylamino)-3’-(cyanoethyl-diisopropyl) thymidine phosphoramidite to characterize it by NMR and determine that we were able to obtain the product but not in enough yield to proceed. In the end we learned a variety of separation techniques such as reverse-, normal phase chromatography and preparatory TLC. As well as synthetic techniques that could be applied to other synthesis such as an azetrope method, using phosphoramidates.

Obtaining the thiol derivative of CA4 through a new route for a potential new prodrug was developed. A variety of synthetic, separation and crystallization techniques were learned while developing the CA4 thiol analog. The Newman-Kwart rearrangement was successfully performed and was the key step in placing the thiol at the C-3 position. Using X-ray crystallography it was determined that the CA4 thiol carbamate analog was made. We were able to deprotect the thiol carbamate to give the desired thiol product.
and obtain the blood flow shutdown data. Due to possible decomposition of the thiol product a disulfide analog was explored. The disulfide Wittig reaction was attempted but problems occurred that prevented us from obtaining the final disulfide product, despite several approaches. Another approach that was not explored was to determine if n-BuLi has more of an affinity to react with a disulfide or an aldehyde. If n-BuLi has a higher affinity to the disulfide then a different reactant will be needed. Once this disulfide is made this will allow a library of other CA4 disulfides to be developed. With this library of disulfides there can be other compounds that will open the door to explore of research areas.
APPENDICES
APPENDIX A

$^1\text{H NMR for 3'-(Monomethoxytrityl) thymidine}$
APPENDIX B

$^{13}$C NMR for 3’-(Monomethoxytrityl) thymidine
APPENDIX C

$^1$H NMR for 5'-(Monomethoxytrityl) thymidine

![Chemical structure](image)

![NMR spectrum](image)
APPENDIX D

$^{13}$C NMR for 5’-(Monomethoxytrityl) thymidine
APPENDIX E

$^1$H NMR for 5’-(Monomethoxytrityl)-3’-(2-cyanoethyldiisopropylamino) thymidine
APPENDIX F

$^{13}$C NMR for 5’-(Monomethoxytrityl)-3’-(2-cyanoethyl-diisopropylamino) thymidine
APPENDIX G

$^{31}$P NMR for 5’-(Monomethoxytrityl)-3’-(2-cyanoethyldiisopropylamino) thymidine

![Chemical structure of 5’-(Monomethoxytrityl)-3’-(2-cyanoethyldiisopropylamino) thymidine]

![NMR spectrum with peak at 147.216 ppm]
APPENDIX H

1H NMR for CA4 Carbamate Cis-Isomer
APPENDIX I

1H NMR (expanded) for CA4 Carbamate Cis-Isomer
APPENDIX J

1H NMR for CA4 Carbamate Trans-Isomer
APPENDIX K

1H NMR for CA4 thiol Cis-Isomer
APPENDIX L

1H NMR for CA4 thiol Cis-Isomer (expanded)
APPENDIX M

13C NMR for CA4 thiol Cis-Isomer
APPENDIX N

1H NMR for Acetal Protected 3-(N, N-dimethylcarbamoylthio)-4-methoxybenzaldehyde
APPENDIX O

3-Thiol-4-methoxybenzaldehyde

![Chemical Structure]

### 

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</table>

Impurity from CDCl3
APPENDIX P

3-(3’-carboxypropyl)-4-methoxybenzaldehyde disulfide
REFERENCES

3. Jonklass, M. D.; The synthesis of novel phosphate diester prodrugs of combretastatin A4: DNA cleavage with a metal ion-iminodiacetic acid linked deoxyoligonucleotide system or by photoreaction with 4-amino-1, 8-naphthalimides, Baylor University. 2003, 1-62.


