

## ABSTRACT

BOND, CONSTANCE M. Synthesis of Drug Derivatives Intended for Conjugation to Gold Nanoparticles for the Use in Nanotherapeutics. (Under the direction of Dr. Christian Melander.)

Antibiotic resistance is a serious threat observed around the world. As the number of resistant bacterial strains continue to increase, the introduction of new antimicrobials into clinical use has decreased. The lack of new discovery has led to the growing interest in the synthesis of antibiotic derivatives to reintroduce bioactivity. Several techniques have been employed to produce successful drug analogs, including the incorporation of nanoparticles. Drugs conjugated to nanoparticles provide several advantages over small molecules working alone, and have already led to advances in the areas of cancer, HIV, and antibiotic resistance. For that reason, once highly effective and widely used class of antibiotics, tetracyclines, were investigated to conjugate to gold nanoparticles through thiolated linkers of various lengths. Doxycycline was nitrated upon the completion of two steps to provide 9-aminodoxycycline and several methods for amide coupling were attempted with two different thiolated molecules.

The process of creating an effective nanotherapeutic by attaching a small molecule to a gold nanoparticle was accomplished previously in our lab with the synthesis of SDC-1721. When attached to a gold nanoparticle, SDC-1721 showed equal inhibition of HIV replication as that of the parent compound TAK-779, an antagonist of the co receptor CCR5. To investigate the structural parameters necessary for optimal receptor recognition further, analogs with longer and shorter thiolated-PEG chains were synthesized. The goal is to optimize bioactivity by determining the favorable chain length between the core SDC/TAK

structure and the gold nanoparticle. Four analogs were successfully synthesized and have been submitted for HIV testing.

Synthesis of Drug Derivatives Intended for Conjugation to Gold Nanoparticles for the Use in  
Nanotherapeutics

by  
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## **DEDICATION**

I dedicate this work to my family. We may not be perfect or have a lot; but we have love and each other and that's perfectly enough.

## **BIOGRAPHY**

Constance M. Bond was born on February 15<sup>th</sup> 1988 and raised by her grandparents, Leroy and Suzanna Hardenburger. She is the middle child with an older sister, Nicci, and younger brother, Sam. She grew up in a small Native American town in northern California called Happy Camp. In 2006, she graduated from Happy Camp High School with a class of 17 students, only 5 of which were female. After graduation, Constance attended the University of California, Santa Cruz. In 2008, she joined the lab of Dr. Phillip Crews working on the isolation of natural products from marine organisms. She graduated in 2010 with a Bachelors of Science Degree in Chemistry. Constance decided to continue her education by attending North Carolina State University and completed her graduate research in the lab of Christian Melander.

## ACKNOWLEDGEMENTS

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## TABLE OF CONTENTS

	Page
LIST OF TABLES.....	vii
LIST OF FIGURES.....	viii
LIST OF SCHEMES.....	ix
CHAPTER 1.....	1
Introduction.....	1
1.1    Antibiotic Resistance.....	1
1.1.1    Developing Resistance.....	2
1.1.2    Therapeutic Approaches.....	4
1.2    Gold Nanoparticles.....	6
1.2.1    Synthesis.....	7
1.2.1.1    Head Groups.....	8
1.2.1.2    Ligands.....	8
1.2.2    Nanotherapeutics.....	9
1.3    Progress Towards Nanotherapeutics.....	11
REFERENCES.....	12
CHAPTER 2.....	14
Synthesis of Tetracycline Derivatives for Applications in Nanotherapeutics.....	14
2.1    Tetracyclines.....	14
2.2    Biological Importance.....	16
2.2.2    Mode of Action.....	17

2.2.3	Resistance Mechanism.....	17
2.3	Project Aims.....	18
2.3.1	Hypothesis.....	19
2.4	Synthesis.....	20
2.5	Conclusions.....	26
2.6	Experimental.....	26
REFERENCES.....		28
CHAPTER 3.....		30
Synthesis of SDC-1721.....		30
3.1	Background.....	30
3.1.1	CCR5.....	30
3.1.2	TAK-779.....	31
3.2	SDC-1721.....	32
3.3	Synthesis.....	33
3.4	Conclusion.....	37
3.5	Experimental.....	38
REFERENCES.....		48

## LIST OF TABLES

	Page
Table 1	Major antibiotic families and their mechanisms of action.....5
Table 2	Properties of small molecule drugs versus gold nanoparticle therapeutics.....9
Table 3	Summary of attempts to couple lipoic acid to <b>2.9</b> .....21
Table 4	Summary of attempts to couple mercaptopropionic acid to <b>2.9</b> .....24

## LIST OF FIGURES

	Page
Figure 1	Timeline of antibiotic development and the evolution of antibiotic resistance.2
Figure 2	Example of the five basic antibiotic scaffolds.....6
Figure 3	6-deoxy-6-demethyltetracycline <b>2.1</b> is the simplest tetracycline to display antibiotic activity, labeled A-D and numbered.....14
Figure 4	Examples of tetracycline derivatives: first-generation (row 1), second generation (row 2), and third-generation (row 3).....15
Figure 5	Chemical structure of the tetracycline-Mg <sup>+</sup> that exists under physiological Conditions.....16
Figure 6	9-Aminodoxycycline conjugated to gold nanoparticle via different thiolate linkages.....19
Figure 7	HPLC chromatograms of a) 9-aminodoxycycline <b>2.9</b> ; b) lipoic NHS ester <b>2.13</b> ; c) <b>reaction 4</b> .....23
Figure 8	Structure of TAK-779 and SDC-1721.....32

## LIST OF SCHEMES

	Page
Scheme 1	The Brust-Schiffrin method proceeds in two steps.....7
Scheme 2	Synthesis of 9-aminodoxycycline <b>2.5</b> . ....20
Scheme 3	Attempted Synthesis of <b>2.11</b> .....20
Scheme 4	Preparation and synthesis of activated lipoic acid and <b>2.11</b> .....21
Scheme 5	Preparation and synthesis of activated mercaptopropionic acid and <b>2.11</b> .....24
Scheme 6	Synthesis of <b>2.24</b> .....25
Scheme 7	Synthesis of various thiolated-PEG linkers.....34
Scheme 8	Synthesis of <b>3.24</b> .....36
Scheme 9	Synthesis of SDC-1721 analogs.....37

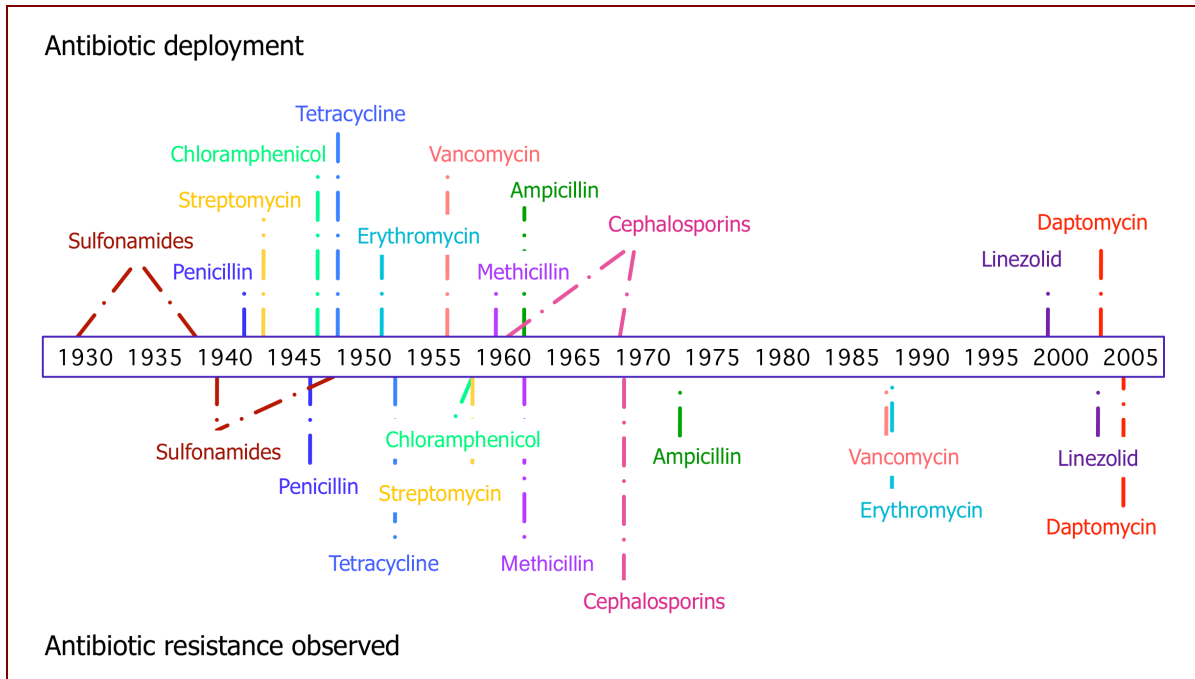
# CHAPTER 1

## Introduction

### 1.1 Antibiotic Resistance

The discovery of penicillin in 1928 by Sir Alexander Fleming marked an important turning point in infectious disease research. Over the next forty years, more than a dozen different classes of drugs were introduced into clinical use.<sup>1</sup> As antibiotics became more accessible and affordable to the public, some began to believe that infectious diseases would be completely eradicated. Fleming tried warning the public in an interview with *The New York Times* in 1945 that the overuse of penicillin could result in resistant mutations within bacteria leading to more infections in the host. This hypothesis was substantiated when the first penicillin-resistant bacterial strains were observed in less than a year.<sup>2</sup>

Today, the evolution of bacteria to form new drug-tolerant strains has perpetuated at an alarming rate with antibiotic resistance discovered for every drug on the market. The need for new therapeutics is apparent. However, in the past forty years only two new classes have been developed (**Figure 1**).<sup>3</sup> In addition to the significant medical impact, the appearance of drug-resistance in bacteria has a substantial economic influence as well. In the United States alone, resistant infections are responsible for \$20 billion in extra health care costs, \$35 billion in societal costs, and 8 million additional hospital days per year.<sup>4</sup> Infectious diseases continue to be the third leading cause of death in the United States and the second leading cause of death worldwide.<sup>5</sup> Without further action, the morbidity and mortality rates from infectious diseases will continue to grow.



**Figure 1.** Timeline of antibiotic deployment and the evolution of antibiotic resistance. (Adapted from Ref 3)

### 1.1.1 Developing Resistance

Organisms must adapt to their environment in order to survive. Bacteria and other single cell organisms have the ability to reproduce at staggering rates giving them a distinct advantage in evolution as compared to more complex organisms. When bacteria develop mutations, the material is encoded and may be passed on through DNA fragments.<sup>6</sup> Bacteria can share genetic material in many ways, the most prevalent methods being transformation, transduction, and conjugation. Transformation is the process in which dead bacterial cells release fragments of DNA into the environment, allowing living bacteria to take up the genetic material and encode into their own DNA. Transduction is the use of virus-containing capsules, i.e., bacteriophages, which infect bacteria with genetic codes for resistance as well as its own viral DNA. As the virus takes over the bacterial replication system, more

bacteriophages are produced until the cell breaks open, releasing them to infect other cells. Conjugation, the most common resistance mechanism, is the sharing of extrachromosomal DNA in the form of plasmids between living bacteria. The plasmids pass through pili, hollow tubes connecting two bacterial cells. Conjugation can occur between bacteria of the same or different genera and species. This transfer between genetically different organisms makes it possible for resistance to spread quickly as one strain can modify a large fraction of the bacterial population.<sup>2,7</sup>

The biological mechanisms of resistance can vary greatly but are typically summarized under three main modes of action: alteration of antibiotics, receptor modification, and active antibiotic efflux. The alteration of antibiotics takes place when bacteria produce enzymes that chemically change or degrade the drug, rendering it inactive. Well-known examples are  $\beta$ -lactamases. The lactamase enzyme works by breaking open the  $\beta$ -lactam ring, destroying all antibacterial properties.<sup>6</sup> These enzymes target penicillins, cephalosporins and other  $\beta$ -lactam containing drugs.  $\beta$ -Lactamases have evolved across bacterial species and are now present in pathogens that did not contain them before.<sup>8</sup> Other examples include chloramphenicol acetyltransferases and aminoglycoside modifying enzymes.<sup>9</sup>

Receptor modification is the alteration of an antibiotic's target site, usually a protein or ribosome, which prevents the drug from interacting with the target, thereby negating its bioactivity. Deactivation can occur with the alteration of a single amino acid at the binding site.<sup>9</sup> Several bacteria have developed a receptor mechanism of resistance and, therefore, can inhibit a range of antibiotics. Such antibiotics include penicillin, due to modified penicillin-binding proteins, and fluoroquinolones by DNA-gyrase modifications.<sup>6</sup>

Efflux pumps are used to actively transport antimicrobials and toxins out of the cell before they reach an active concentration level. In order to be effective, the pumps must be stronger or have a higher binding affinity for the antimicrobial than its site of activation.<sup>10</sup> Efflux pumps are located in the cytoplasm membrane of both Gram negative and positive bacteria. This resistance mechanism was first described for tetracyclines and macrolides but are now active against a wide range of antibiotics.<sup>6</sup>

These simple, yet effective, resistance mechanisms have allowed bacteria to endure the introduction of every antibiotic and even acquire resistance to several unrelated antibiotics. Multi-drug resistant (MDR) bacteria are of substantial concern as they are nearly impossible to treat.<sup>11</sup> The development of resistance, particularly MDR, has stemmed from the overuse of antimicrobials in human medicine as well as consumer products, agriculture, and veterinary medicine. Individually, these applications for antimicrobials offer minimal influence on the development of drug-resistance but the combination of them all expedited the evolution process to occur within a few years of antimicrobial development.<sup>12</sup> It may seem ideal to limit the use of antimicrobials in certain areas, but this will simply slow the process rather than eliminating the threat. New, more cost-effective methods must be developed to aid in the inhibition of MDR bacteria.

### **1.1.2 Therapeutic Approaches**

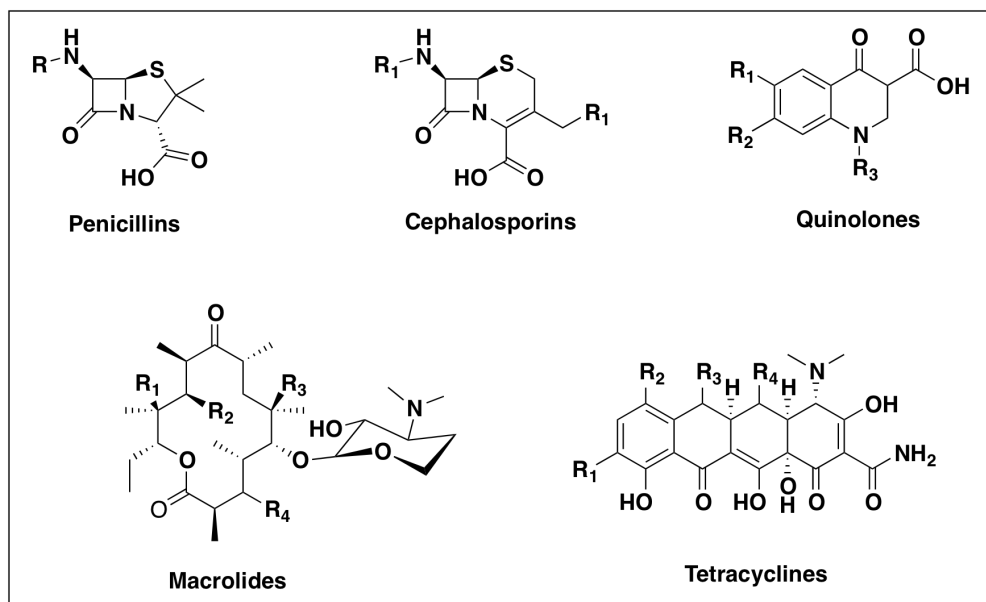
In order to overcome resistance, it is important to understand the specific functions of antibiotics that result in the inhibition of bacterial reproduction (i.e., bacteriostatic) or death of the cell (i.e., bacteriocidal).<sup>3</sup> This selective toxicity works by targeting pathways within a pathogen that are not present or are distinctly different from that of mammalian cells. The

five key modes of action used by the major antibiotic families are shown below in **Table 1**.<sup>13</sup> The discovery of new drugs and/or the alterations of existing drug scaffolds in many antibiotics restored the bioactivity and in specific cases offering novel, exciting approaches to overcoming antimicrobial resistance.

**Table 1.** Major antibiotic families and their mechanisms of action. (Adapted from Ref 13)

Mechanism of Action	Antibiotic families
Inhibition of cell wall synthesis	Penicillins; cephalosporins; carbapenems; daptomycin; monobactams; glycopeptides
Inhibition of protein synthesis	Tetracyclines; aminoglycosides; oxazolidonones; Streptogramins; ketolides; macrolides; lincosamides
Inhibition of DNA synthesis	Fluoroquinolones
Competitive inhibition of folic acid synthesis	Sulfonamides; trimethoprim
Inhibition of RNA synthesis	Rifampin
Other	Metronidazole

Due to the shortage of new findings, scaffold tailoring has been proven to be most successful. Scaffold tailoring involves changing functional groups on the periphery of the antimicrobial while leaving the core structure unaltered to preserve bioactivity. Because of this, most new generations are developed to inhibit the same pathogens in which their parent compounds have become inactive. With only one exception, all of the antibiotics approved for clinical use between the early 1960s and 2000 were derivatives of existing scaffolds.<sup>11</sup> The most widely used core structures of these latter antibiotics came from penicillin, cephalosporins, quinolones, macrolides, and tetracyclines (**Figure 2**).<sup>11,14</sup>



**Figure 2.** Example of the five basic antibiotic scaffolds.

Common alterations of an antibiotic's structure, such as size and sterics, have been effective in reintroducing bioactivity but this process is not a permanent solution. Alternative methods must be explored to compete with the growing threat of bacterial resistance. One such method is with the use of nanotechnology. This relatively new, yet rapidly growing area of research has provided many types of materials for biomedical applications, including those used in the fields of antimicrobials and cancer therapeutics.<sup>15</sup> The use of nanotechnology in medicine has already been shown to both improve bioactivity in certain drug scaffolds as well as provide new applications in biomedical research.<sup>16</sup>

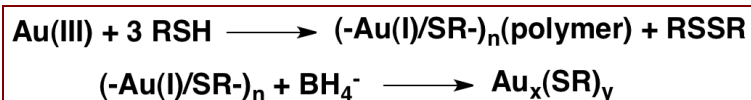
## 1.2 Gold Nanoparticles

Over the past two decades, gold nanoparticles, also known as gold colloids, have been increasingly studied. They are among the most stable metal nanoparticles and their unique properties make them of particular interest in nanotechnology as they can be synthesized in

different shapes (rods, spheres, cubes, and dots), are commercially available in a range of sizes, can be detected at low concentrations, and are rapidly modified through thiol exchange reactions.<sup>17</sup> Gold nanoparticles have been used in a variety of applications including energy, electronics, and medicine. In most recent years, gold nanoparticles have become increasingly important in the diagnosing and treating human disease.

### 1.2.1 Synthesis

In 1857, Michael Faraday successfully reduced the first gold salt into pure colloidal gold. He did so using a two-phase system consisting of an aqueous solution of gold chloride and phosphorous in CS<sub>2</sub>.<sup>18</sup> Today, several different methods are used to synthesize gold nanoparticles, but one of great importance is the Brust-Schiffrin method developed in 1994.<sup>19</sup> Inspired by Faraday's two-phase system, an aqueous solution of chloroauric acid is reacted with a thiol ligand to produce a gold-thiol polymer, which is then reduced with sodium borohydride (**Scheme 1**). The diameter of the nanoparticle (value x), which ranges between 1.5 and 5.2 nm, can be controlled by the amount of thiolate-to-Au(III) used in the first step.<sup>20</sup> The gold nanoparticles produced from the Brust-Schiffrin method are unlike any gold nanoparticle synthesized previously. Their unique properties include water-soluble, synthetic control over particle size, dispersity, and surface functionality as well as extraordinary stability of the thiolate-protected products to air, long-term storage, solvents, temperature, and concentration extremes.<sup>17</sup>



**Scheme 1.** The Brust-Schiffrin method proceeds in two steps. (Adapted from Ref 20)

In 2010, Ackerson et al. optimized the Brust method even further. He found that using 4-mercaptobenzoic acid (*p*-MBA) as the thiolate ligand lead to nanoparticles that are stable for months, yet, are still reactive enough for ligand exchange to occur. Results also show that a reduction time of 16-18hrs with less borohydride lead to monodisperse gold particles of 1.5 nm, 2.0 nm, or 3.0 nm diameter. The 2.0 nm diameter gold nanoparticles have a proposed empirical formula of  $[Au_{144}(SC_6H_4COOH)_{60}]$ . A major benefit to Ackerson's direct synthesis is that no purification is necessary to obtain a monodispersed product.<sup>21</sup>

#### **1.2.1.1 Head Groups**

Three main criteria are needed for successful ligand conjugation to a gold nanoparticle. First, the gold needs to be reduced from its more normal but toxic trivalent state to a safe univalent state. Second, the ligands bound to the gold must be "soft", meaning that the binding atom is of relatively low electronegativity. These atoms include sulfur, phosphorous and carbon, while "hard" ligands contain any oxygen, nitrogen, and halogenated binding atoms. Third, the ligands must not be too stable or tightly bound to the gold to prevent the necessary reactions from occurring. Thiolates most accurately fit all of the criteria and are, therefore, used most often in gold nanoparticle conjugation. Gold(I) thiolates have a structural formula of AuSR where R is the active organic compound or antibiotic.<sup>22</sup>

#### **1.2.1.2 Ligands**

Due to optimized synthetic routes, gold nanoparticles can be functionalized with nearly any thiolate containing ligand. Not only that, but ligand exchange reactions have made it possible to have combinations of two or more chemically distinct ligands present on the nanoparticle.<sup>23</sup> Based on the desired application of the nanoparticle, the ligand(s) can be

chosen and altered for maximum optimization. For example, it has been shown that PEG (polyethylene glycol) linkages bind well to cell membranes. Therefore, gold nanoparticles functionalized with these groups can serve as good drug-carriers.<sup>24</sup> Changing the number of ethylene units provides a simple, yet effective, way to fine tune its activity. As the variety of modifications increase, the applications for gold nanoparticles become increasingly diverse.

### 1.2.2 Nanotherapeutics

The use of gold nanoparticles towards biomedical applications, especially that of drug delivery, have proved quite advantageous. Several reasons have been used to explain why drugs conjugated to gold nanoparticles may be more efficient than small molecule drugs working alone, **Table 2**.<sup>25</sup> Most of these attributes are amplified by the shortcomings of small molecules, but are nonetheless important.

**Table 2.** Properties of Small Molecule Drugs versus Gold Nanoparticle Therapeutics. (Adapted from Ref 25)

<b>Small Molecule Drugs</b>	<b>Nanoparticle Sized Drugs</b>
Limited Size Modifications	Substantial Size Modifications
Unlimited Chemical Diversity	Large Chemical Diversity
Short Circulation Lifetime/Fast Degradation	Long Circulation Lifetime/Slow Degradation
Difficult to Pass Through Cell Membranes	Designed to Pass Through Cell Membranes
Limited Valency (Few contacts must be VERY strong)	High Valency (Many weak contacts can provide activity)
Limited Modes of Action	Possible New Modes of Action

One advantage of nanotherapeutics is the added steric bulk attributed with the conjugation to gold. This is important when exportation out of a cell would disrupt bioactivity. Thus, efflux pumps can be rendered inactive by blocking the necessary mechanism site and allow the antimicrobial to complete their mode of action.<sup>26</sup> It has been

suggested that cancer cells could also benefit from this blocking process. Due to the irregularly shaped vesicles and improper lymphatic flow, nanoparticles can accumulate and become entrapped within cancerous cells at higher concentrations than normal cells. When attached to an anticancer drug, the nanoparticle can also utilize its ability of slow degradation and high valency to apply a large amount of activity for a longer period of time.<sup>27</sup>

Multivalent displays are prevalent in biological systems and have shown to provide enhanced affinity. A popular example is the binding of an influenza virus through multiple hemagglutinin (HA) ligands and sialic acid (SA) surface receptors, which occur with an affinity of  $10^{13} \text{ M}^{-1}$  whereas the  $K_A$  for a single SA-HA interaction is  $10^3 \text{ M}^{-1}$ . The data suggests that biological systems exploit these multivalent interactions to allow an organism to take advantage of an existing set of monovalent ligands without the need to develop new molecules for every required function.<sup>28</sup> The multivalent properties of gold nanoparticles have been shown to be just as advantages as those exhibited in biological systems. This was observed with a weakly active small molecule against CCR5, a co-receptor for HIV that became effective once bound to a gold nanoparticle.<sup>29</sup>

Another benefit for the presence of multiple ligands on a nanoparticle is to generate a high local concentration of binding molecules. With this increased concentration, the binding equilibrium between receptor and ligand may shift to favor more ligand-receptor pairs.<sup>30</sup> The antibiotic vancomycin was attached to a gold nanoparticle to examine the effects of such properties. Bioactivity was regained and it was attributed to an increase of ligand-receptor binding caused by the multivalent nanoparticle.<sup>31</sup> The ability to create mixed ligand combinations on the nanoparticle surface may increase the local concentration and binding

affinity, further improving the superiority of drugs conjugated to gold over the use of standard drugs.<sup>32</sup>

### **1.3 Progress Towards Nanotherapeutics**

Tetracyclines are a class of antimicrobials that showed great potential for inhibiting a broad spectrum of bacteria. The development of active efflux pumps by resistant bacteria quickly limited their use and effectiveness. Gold nanoparticles used in drug delivery have shown the ability to restore bioactivity. With the need for new/improved antibiotics and advances in nanotherapeutics, synthesis of tetracycline derivatives to conjugate to a gold nanoparticle was attempted. This will be discussed further in Chapter Two.

Multivalency has proved to be an important function in nanotherapeutics. This has been previously explored in the Melander lab with Tak-779, a known inhibitor of the co-receptor CCR5 for commonly transmitted strains of HIV-1. When the quaternary ammonium salt at the tail end of Tak-779, which was determined as the site of activity but had poor pharmacological properties, was replaced with a gold nanoparticle bioactivity was retained. To optimize bioactivity, the linkage between the small molecule derivative and gold nanoparticle was altered. Chapter Three details the synthetic routes used to develop the four different analogs.

## REFERENCES

- (1) Conly, J.; Johnston, B. *The Canadian journal of infectious diseases & medical microbiology* **2005**, *16*, 159.
- (2) Vogt, D. U.; Jackson, B. A. *Antimicrobial resistance : an emerging public health issue*; Novinka Books: Huntington, NY, 2001.
- (3) Clatworthy, A. E.; Pierson, E.; Hung, D. T. *Nature chemical biology* **2007**, *3*, 541.
- (4) Bush, K.; Courvalin, P.; Dantas, G.; Davies, J.; Eisenstein, B.; Huovinen, P.; Jacoby, G. A.; Kishony, R.; Kreiswirth, B. N.; Kutter, E.; Lerner, S. A.; Levy, S.; Lewis, K.; Lomovskaya, O.; Miller, J. H.; Mobashery, S.; Piddock, L. J.; Projan, S.; Thomas, C. M.; Tomasz, A.; Tulkens, P. M.; Walsh, T. R.; Watson, J. D.; Witkowski, J.; Witte, W.; Wright, G.; Yeh, P.; Zgurskaya, H. I. *Nature reviews. Microbiology* **2011**, *9*, 894.
- (5) Nathan, C. *Nature* **2004**, *431*, 899.
- (6) Alanis, A. J. *Archives of medical research* **2005**, *36*, 697.
- (7) Croft, A. C.; D'Antoni, A. V.; Terzulli, S. L. *Medical science monitor : international medical journal of experimental and clinical research* **2007**, *13*, RA103.
- (8) Jacoby, G. A.; Munoz-Price, L. S. *New England Journal of Medicine* **2005**, *352*, 380.
- (9) Sefton, A. M. *Drugs* **2002**, *62*, 557.
- (10) Hooper, D. C. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **2005**, *40*, 1811.
- (11) Fischbach, M. A.; Walsh, C. T. *Science* **2009**, *325*, 1089.
- (12) Spellberg, B.; Guidos, R.; Gilbert, D.; Bradley, J.; Boucher, H. W.; Scheld, W. M.; Bartlett, J. G.; Edwards, J., Jr. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **2008**, *46*, 155.
- (13) Levy, S. B.; Marshall, B. *Nature medicine* **2004**, *10*, S122.
- (14) Newman, D. J.; Cragg, G. M. *Journal of Natural Products* **2007**, *70*, 461.
- (15) Cui, Y.; Zhao, Y.; Tian, Y.; Zhang, W.; Lü, X.; Jiang, X. *Biomaterials* **2012**, *33*, 2327.

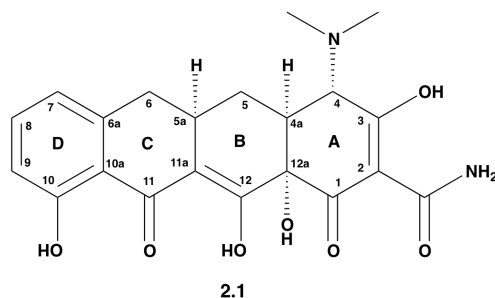
- (16) Caruthers, S. D.; Wickline, S. A.; Lanza, G. M. *Current opinion in biotechnology* **2007**, *18*, 26.
- (17) Daniel, M. C.; Astruc, D. *Chemical reviews* **2004**, *104*, 293.
- (18) Faraday, M. *Philosophical Transactions* **1857**, *147*, 145.
- (19) Brust, M. B., D.; Schriffirin, D. J.; Whyman, R. J. *Journal of the Chemical Society, Chemical Communications* **1994**, 801.
- (20) Ackerson, C. J.; Jadzinsky, P. D.; Kornberg, R. D. *Journal of the American Chemical Society* **2005**, *127*, 6550.
- (21) Ackerson, C. J.; Jadzinsky, P. D.; Sexton, J. Z.; Bushnell, D. A.; Kornberg, R. D. *Bioconjugate Chemistry* **2010**, *21*, 214.
- (22) Parish, R. V.; Cottrill, S. *Gold Bulletin* **1987**, *20*, 3.
- (23) Hostetler, M. J.; Templeton, A. C.; Murray, R. W. *Langmuir* **1999**, *15*, 3782.
- (24) Tiwari, P.; Vig, K.; Dennis, V.; Singh, S. *Nanomaterials* **2011**, *1*, 31.
- (25) Kudgus, R. A., North Carolina State University, 2010.
- (26) Tshikhudo, T. R. W., Z.; Brust, M. *Materials Science and Technology* **2004**, *20*, 980.
- (27) Wang, S. C., K. J.; Wu, T. H.; Lin, W. Y.; Ohashi, M.; Chiou, P. Y.; Tesng, H. R. *Angewandte Chemie International Edition* **2010**, *122*, 3865.
- (28) Mammen, M. C., S. K.; Whitesides, G. M. *Angewandte Chemie International Edition* **1998**, *37*, 2755.
- (29) Bowman, M.-C.; Ballard, T. E.; Ackerson, C. J.; Feldheim, D. L.; Margolis, D. M.; Melander, C. *Journal of the American Chemical Society* **2008**, *130*, 6896.
- (30) Lytton-Jean, A. K. R.; Mirkin, C. A. *Journal of the American Chemical Society* **2005**, *127*, 12754.
- (31) Gu, H.; Ho, P. L.; Tong, E.; Wang, L.; Xu, B. *Nano Letters* **2003**, *3*, 1261.
- (32) Bresee, J.; Maier, K. E.; Boncella, A. E.; Melander, C.; Feldheim, D. L. *Small* **2011**, *7*, 2027.

## CHAPTER 2

### Synthesis of Tetracycline Derivatives for Applications in Nanotherapeutics

#### 2.1 Tetracyclines

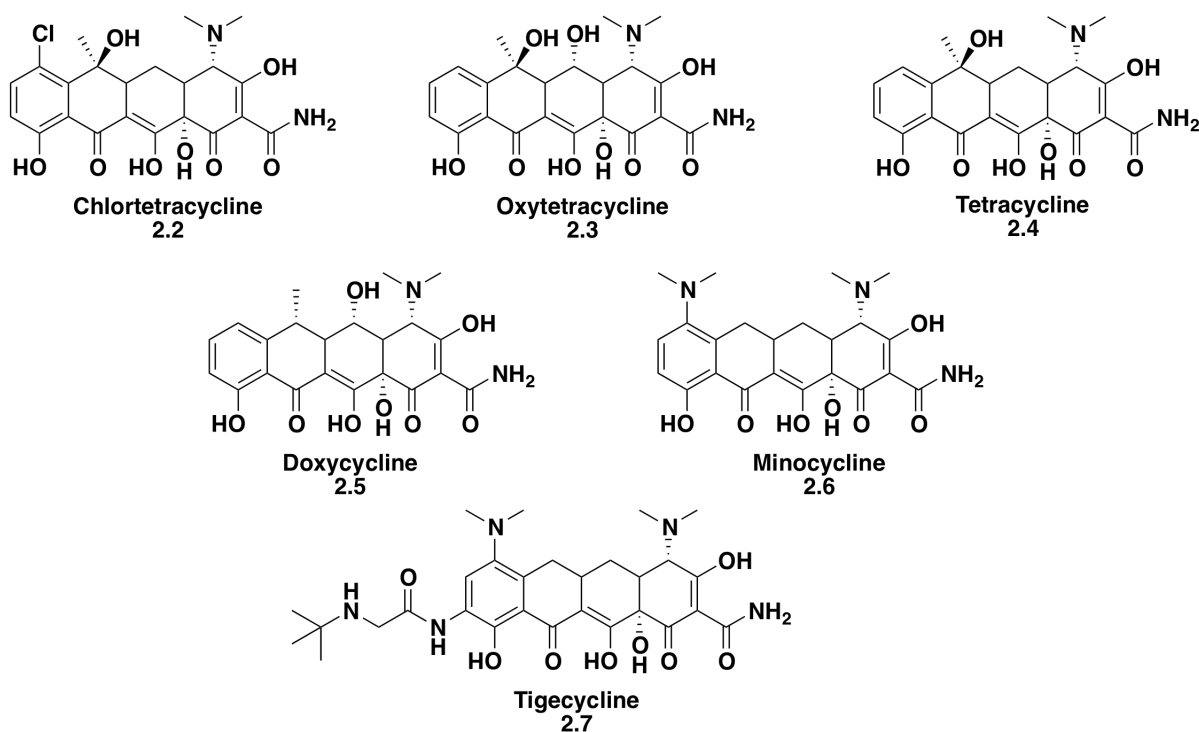
Tetracyclines are a class of antibiotics originally isolated from soil bacteria of the genus *Streptomyces*. Tetracyclines consist of four linear fused rings labeled A-D and are a member of the naphthacene ring family of organic polycyclic hydrocarbons. Numbering of tetracycline rings begins at the C1 position of ring A and continues counterclockwise.<sup>1</sup> Years of research have led to a detailed understanding of the structural features required for antibiotic activity.<sup>2</sup> The simplest tetracycline to display activity is 6-deoxy-6-demethyltetracycline **2.1**. Thus, the minimal pharmacophore structure must contain the linear-fused tetracyclic nucleus, the dimethylamino functionality at C4, the naturally occurring ( $\alpha$ ) stereochemistry at 4a, 12a, and 4 positions, and the keto-enol system (positions 11, 12, and 12a) in close proximity to the phenolic D ring, as represented in **Figure 3**.<sup>3</sup>



**Figure 3.** 6-deoxy-6-demethyltetracycline **2.1** is the simplest tetracycline to display antibiotic activity, labeled A-D and numbered.

Chlortetracycline **2.2** and oxytetracycline **2.3** were first in the class to be discovered.<sup>4,5</sup> The parent structure, tetracycline **2.4**, was discovered five years later by

Conover.<sup>6</sup> Over the next two decades, tetracyclines were extensively studied with a primary focus on the isolation of novel compounds from microorganisms. Synthetic efforts have led to the evolution of second-generation tetracycline derivatives, such as doxycycline **2.5** and minocycline **2.6**. More recently, a third-generation of tetracyclines has been developed, including tigecycline **2.7**, a derivative of minocycline.<sup>1</sup> Since their discovery, over a dozen principal tetracyclines have been produced.



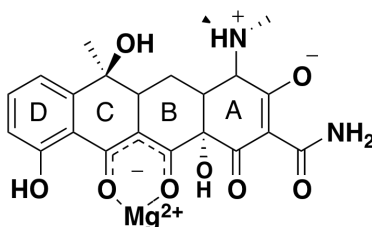
**Figure 4.** Examples of tetracycline derivatives: first-generation (row 1), second-generation (row 2), and third-generation (row 3).

Tetracyclines affect the growth and physiology of both Gram-positive and Gram-negative bacteria.<sup>1</sup> Their antimicrobial properties and lack of major side effects resulted in tetracyclines becoming one of the most widely used drugs for the treatment of both human

and animal infections. The expansion of their use has continued into other areas including animal feed to act as growth promoters.<sup>7</sup> Though tetracyclines are still effective, bacterial resistance has been widely observed.<sup>8</sup> The development of third-generation tetracyclines are aimed at overcoming this resistance by utilizing the structure activity relationships and installing functional groups that lead to restored bioactivity. One such example that is proving quite successful is tigecycline 2.7.<sup>9</sup>

## 2.2 Biological Importance

Tetracyclines are strong chelating agents due to the large number of heteroatoms on the periphery of the molecule. These chelation sites consist of the keto-enol system (positions 11 and 12) and the enol (positions 1 and 3) with carboxamide (position 2).<sup>10</sup> The metal ions available for chelation within cells are magnesium and calcium, both of which are present in blood plasma at concentrations of approximately 1 mM.<sup>11</sup> Extensive research has shown that magnesium complexes more readily to tetracyclines than calcium and X-ray analysis suggests that the magnesium primarily coordinates at the keto-enol moiety (**Figure 5**).<sup>12</sup> This chelation process is necessary for tetracyclines to exhibit biological relevance.



**Figure 5.** Chemical structure of the tetracycline-Mg<sup>2+</sup> that exists under physiological conditions.

### 2.2.2 Mode of Action

Tetracyclines are known protein synthesis inhibitors.<sup>7</sup> Tetracyclines elicit their activity by blocking the A-site of the 30S ribosomal subunit. This binding prevents aminoacylated tRNA from binding thereby inhibiting transcription.<sup>13</sup> Tetracyclines enter bacterial cells by passively diffusing through membrane porin channels. Once inside, they chelate to the divalent metal ions due to the increased metal concentration and pH within the cell. The tetracycline-Mg<sup>+</sup> complex then reversibly binds, via the chelation site<sup>13</sup>, to the only high-affinity acceptor site (A-site) in the ribosomal 30S unit.<sup>14</sup>

### 2.2.3 Resistance Mechanism

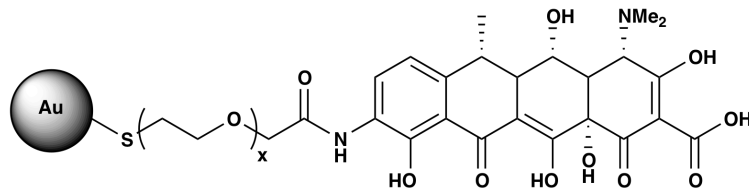
Gram negative and positive bacteria have developed inner membrane proteins, Tet proteins, to protect themselves against tetracyclines.<sup>15</sup> Most commonly, gram-negative bacteria utilize the membrane embedded efflux protein, TetA, to export tetracycline-Mg<sup>+</sup> against equimolar intake of a proton.<sup>3</sup> Overexpression of TetA will kill the bacteria, therefore, it is regulated by the repressor protein, TetR. TetR exists as a homodimer that blocks the operator sites, *tetO*<sub>1</sub> and *tetO*<sub>2</sub>, preventing transcription of the appropriate genes required for the efflux mechanism to occur. When the tetracycline-Mg<sup>+</sup> enters the cell, it binds to TetR due to higher affinity ( $K_a \sim 10^9 \text{ M}^{-1}$ ) compared to that of the 30S ribosomal unit ( $K_a \sim 10^6 \text{ M}^{-1}$ ). In addition, the affinity of TetR for *tetO* (normally  $K_a \sim 10^{11} \text{ M}^{-1}$ ) is decreased by an order of 2 or 3 upon binding to the first tetracycline-Mg<sup>+</sup> and further decreases by orders between 4 and 7 after binding to the second tetracycline-Mg<sup>+</sup>.<sup>16</sup>

As tetracycline-Mg<sup>+</sup> enters the binding pocket of TetR, leading with the A-ring, it becomes anchored by hydrogen bonds formed between functional groups on the ring and

amino acids within the protein. A sequence of conformational changes occurs as TetR recognizes and binds to the chelated magnesium site. As binding finishes, the end terminus of the protein swings up in a pendulum-like or “sliding door”<sup>17</sup> motion behind the D-ring, thus, locking tetracycline-Mg<sup>+</sup> within and dissociating from one of the *tetO* sites. The same process occurs on the other side of the dimer as well, releasing a TetR(tetracycline-Mg<sup>+</sup>)<sub>2</sub> complex to be ejected from the cell by the TetA protein. This resistance mechanism continues until the concentration of tetracycline-Mg<sup>+</sup> in the cell is too low for bioactivity.<sup>12</sup>

### 2.3 Project Aims

The goal of this project is to reestablish antibiotic activity of a tetracycline derivative through attachment of a gold nanoparticle. Commercially available doxycycline **2.5** is nitrated at the 9 position. After reduction of the nitro group to the amine, 9-aminodoxycycline **2.9** is coupled via amide bond formation to various thiolated linkages. Our collaborators, the Feldheim research group at University of Colorado, Boulder, will conjugate the thiolated-doxycycline derivatives to gold nanoparticles via conditions outlined in Chapter 1 and as previously reported<sup>18</sup> (**Figure 6**). The conjugated systems will be subjected to biological testing in our laboratory against tetracycline resistant *Escherichia coli*. As previously shown by tigecycline, substitution at the 9 position can restore bioactivity.<sup>19</sup> By utilizing the size and multivalency of gold nanoparticles, it may be possible to promote bioactivity more than with the antibiotic alone.



**Figure 6.** 9-Aminodoxycycline conjugated to gold nanoparticle via different thiolate linkages.

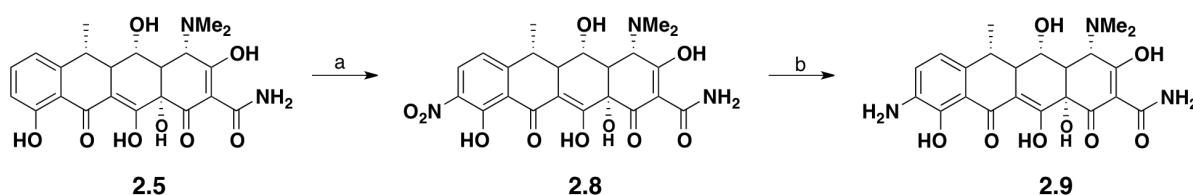
### 2.3.1 Hypothesis

We believe the doxycycline-nanoparticle will be able to continue a normal mode of action by blocking the 30S ribosome. It is also hypothesized that the conjugated nanoparticles will inhibit the efflux resistance mechanism. Due to the natural binding orientation of tetracycline derivatives to the ribosome, the linker and nanoparticle should not get in the way of the active site. In fact, the gold nanoparticle may even assist in blocking the aminoacylated tRNA, further preventing bacterial transcription from occurring. Also, as discussed in Chapter 1, multivalency may promote binding affinity to the ribosome over that of the repressor protein TetR.

We have predicted that the doxycycline-nanoparticle complex may stop the production of efflux pumps in two ways. First, the steric bulk of the linker and nanoparticle may prevent the conjugated antibiotic from entering the binding site of the TetR protein. Therefore, hydrogen bonding and conformational changes within the protein cannot occur. Second, the pendulum-like or “sliding-door” motion may be blocked by the combination of the linker and nanoparticle. In both instances, the overall size and multivalency of the doxycycline-nanoparticle are hypothesized to inhibit TetR from dissociating from the *tetO* sites. If the protein cannot activate the operators then the efflux mechanism will not be initiated.

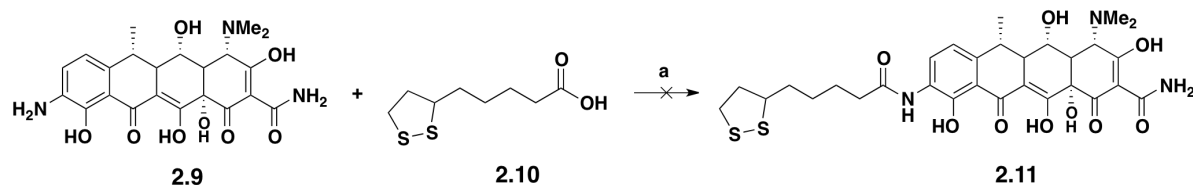
## 2.4 Synthesis

9-aminodoxycycline **2.9** was synthesized using well-known nitration and reduction procedures<sup>20</sup> (**Scheme 2**). Nitration with NaNO<sub>3</sub> and H<sub>2</sub>SO<sub>4</sub> produces a 5:1 mixture of products at C9 and C7 respectively. The methyl substituent at the 6 position has been identified to suppress nitration at C7, thus favoring the 9 position.<sup>21</sup> Separation of the two isomers is reportedly accomplished at the end of the reaction sequence, so purification was not attempted at this point.<sup>22</sup>



**Scheme 2.** Synthesis of 9-aminodoxycycline **2.5**. Reagents and conditions: (a) NaNO<sub>3</sub>, H<sub>2</sub>SO<sub>4</sub>; (b) H<sub>2</sub>/10%, Pd/C, 50 psi.<sup>20</sup>

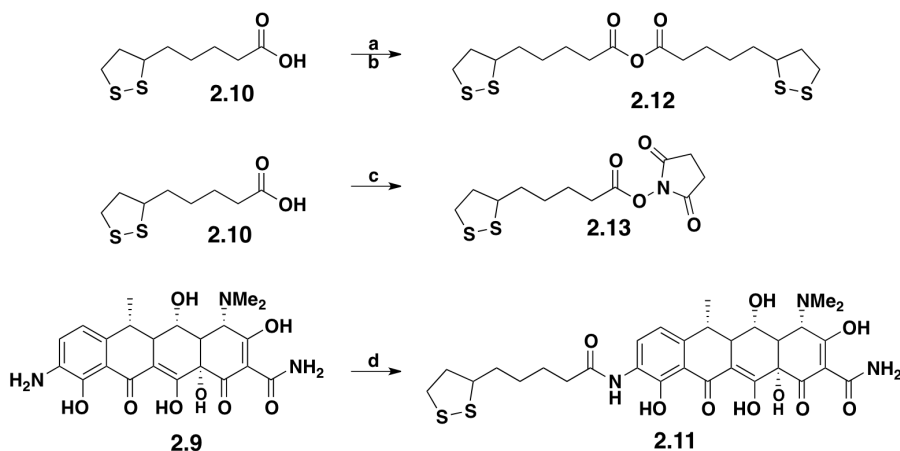
Amino acids have been successfully attached to **2.9** using direct coupling techniques.<sup>22</sup> Utilizing similar reaction conditions, attempts were made with commercially available lipoic acid. However, the desired product could not be synthesized (**Scheme 3**).



**Scheme 3.** Attempted synthesis of **2.11**. Reagent and conditions: (a) HOBT, BOP, NMP, DMF, r.t., 3 days.<sup>22</sup>

An alternate coupling procedure was then attempted. Berens et al. used *N*-*N'*-dicyclohexylcarbodiimide (DCC) to convert different carboxylic acid linkers into

symmetrical anhydrides and reacted them with **2.9**.<sup>23</sup> Several attempts using similar reaction conditions are shown below with lipoic anhydride **2.12** as well as the activated NHS ester of lipoic acid **2.13** (Scheme 4 and Table 3).



**Scheme 4.** Preparation and Synthesis of activated lipoic acid and **2.11**. Reagents and conditions: (a) DCC, DCM, r.t., 45 min.; (b) PS-EDC, DCM, r.t., 45 min.;<sup>23</sup> (c) EDC·HCl, DIPEA, N-hydroxysuccinimide (84%);<sup>24</sup> (d) presented in table below

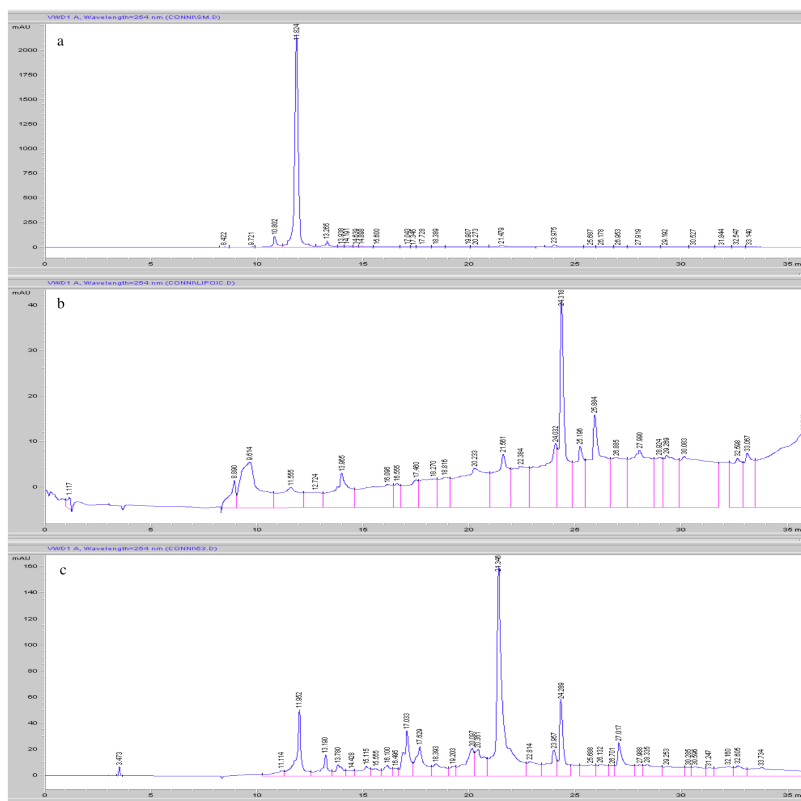
**Table 3.** Summary of attempts to couple lipoic acid to **2.9**

Reaction	Linker	Conditions (d)	Result
1	<b>2.12</b> (2 eq) via DCC	NMP, 4.5 hrs	N/A
2	<b>2.12</b> (2 eq) via PS-EDC	NMP, 4.5 hrs	N/A
3	<b>2.13</b> (1.02 eq)	TEA (pH 8.5), DCM, overnight	No Reaction
4	<b>2.13</b> (2 eq)	NaHCO <sub>3</sub> (2 eq), DMF, 3.5 hrs	Product
5	<b>2.13</b> (4 eq)	NaHCO <sub>3</sub> , (2 eq), DMF, 3.5 hrs	Product
6	<b>2.13</b> (2 eq)	NaHCO <sub>3</sub> , (2 eq), DMF, overnight	Product
7	<b>2.13</b> (4 eq)	NaHCO <sub>3</sub> , (2 eq), DMF, overnight	Product
8	<b>2.13</b> (2 eq)	DBU (10 eq), acetonitrile, 3.5 hrs	Product

Product formation with crude material was often difficult to establish using NMR techniques. Therefore, other characterization tools were used including HPLC, LC/MS, and

HRMS. When **reaction 1** was subjected to HPLC analysis, pressure began to build and the chromatogram was too messy to determine product formation. It was concluded that a side product of the reaction, water-insoluble DCC urea, was contaminating the HPLC. **Reaction 2** was attempted with polymer-supported EDC, in which the formation of urea stays bound to the polymer support. Again, the HPLC chromatograms presented multiple, broad peaks high above the baseline that were too difficult to analyze.

Product formation was achieved with **reaction 4**, utilizing the activated lipoic ester **2.13** over the symmetrical anhydride **2.12**. Using combined HPLC and LC/MS chromatograms, it was determined that the largest peak at approximately 21.3 min was indeed lipoic acid conjugated to 9-aminodoxycycline **2.11**. The peaks at approximately 11.8 min and 24.3 min were excess starting materials, correlating to **2.9** and **2.13** respectively, **Figure 7**. Increasing the equivalences of linker, base, and/or reaction time, **reactions 3** and **5-8**, did not reduce the amount of excess starting materials. It has been determined that **reaction 4** has the best conditions thus far.

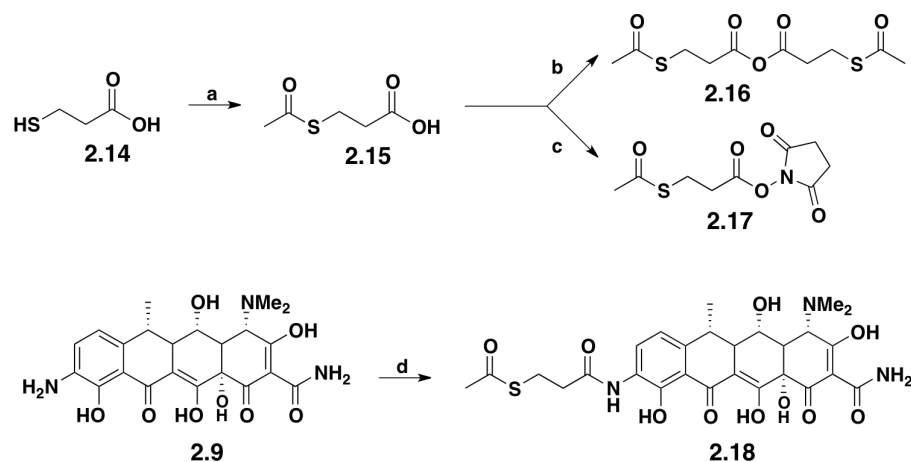


**Figure 7.** HPLC chromatograms of (a) 9-aminodoxycycline **2.9**; (b) lipoic NHS ester **2.13**; (c) reaction **4**

Pure product was not obtained as further complications arose when attempting to purify the material on a large preparatory column. Switching from the analytical column caused large differences in the chromatograms. Though gradient and run conditions were altered for optimization, none could replicate earlier spectra. These modifications included lengthening and shortening the run times, altering the flow rate, and changing the start and end concentrations of the solvents.

Mercaptopropionic acid **2.14**, a commercially available thiol-containing carboxylic acid, was chosen to examine the differences between thiol and disulfide linkages on bioactivity. Acid **2.14** was first protected with acetyl chloride<sup>25</sup> to produce the thioester **2.15**,

which was then activated via two separate pathways as described earlier (**Scheme 5**). The first used PS-EDC to form the symmetrical anhydride **2.16**. The second was accomplished by forming the N-hydroxysuccinimide ester **2.17**.<sup>24</sup> Conjugation of the activated linkers and **2.9** was again attempted with several different reaction conditions (**Table 4**).



**Scheme 5.** Preparation and Synthesis of activated mercaptopropionic acid and **2.11**. Reagents and conditions: (a) AcCl, AcOH, DCM, 36 hrs (97%),<sup>25</sup> (b) PS-EDC, DCM, r.t., 45 min.; (c) EDC•HCl, DIPEA, N-hydroxysuccinimide (70%),<sup>24</sup> (d) presented in table below

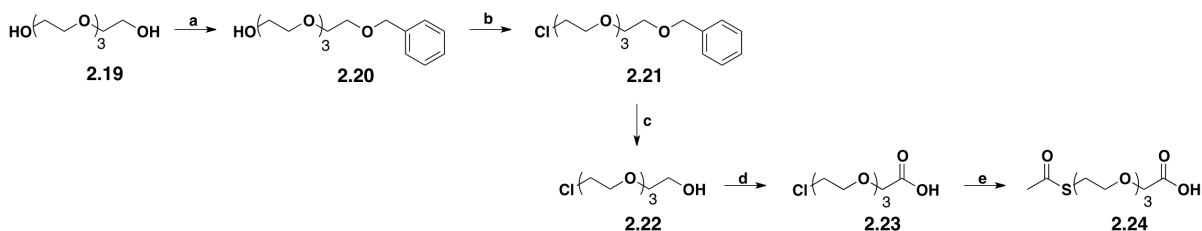
**Table 4.** Summary of attempts to couple mercaptopropionic acid to **2.9**

Reaction	Linker	Conditions (d)	Result
9	<b>2.16</b> (2 eq)	DMF, 4.5 hrs	N/A
10	<b>2.17</b> (1.5 eq)	DMF, DCM, reflux, overnight	No Product
11	<b>2.17</b> (2 eq)	DBU (10 eq), acetonitrile, 3.5 hrs	N/A
12	<b>2.17</b> (4 eq)	DBU (10 eq), acetonitrile, 3.5 hrs	N/A
13	<b>2.17</b> (2 eq)	NaHCO <sub>3</sub> (2 eq), DMF, 3.5 hrs	Product

The chromatogram for **reaction 10** did not show any new peak formation in comparison with the starting materials of **2.9** and **2.17**. The chromatograms of reactions **9** and **11-12** were too messy and high above the baseline to determine product formation.

Crude material for **reaction 13** showed possible product peaks in the  $^1\text{H}$  NMR and was recently confirmed by HRMS. **Reaction 13** has not yet been subjected to HPLC. Again, it has been shown that a reaction of 2 equivalences of linker with 2 equivalences of  $\text{NaHCO}_3$  for several hours can lead to the formation of desired product.

A thiolated-tetraethylene-PEG chain **2.24** was synthesized beginning with tetraethylene glycol and employing known reactions over 5 steps. The glycol was benzyl protected using sodium hydride and benzyl bromide.<sup>26</sup> After purification, the monobenzyl-protected alcohol **2.20** was chlorinated with thionyl chloride and pyridine for a maximum of 4 hrs, as degradation of the product **2.21** would begin to occur.<sup>27</sup> The benzyl-protecting group was removed via hydrogenolysis with 10% palladium on carbon at 1 atm hydrogen afforded **2.22**.<sup>28</sup> The Jones reagent, a 2.5 M solution of chromium oxide and concentrated sulfuric acid, was used to oxidize the free alcohol to the carboxylic acid **2.23**.<sup>29</sup> Chloro-displacement with potassium thioacetate resulted in the final compound [2-(2-(2-Acetylsulfanylethoxy)ethoxy)ethoxy]acetic acid **2.24** in good yield (90%).<sup>30</sup> Conjugation with **2.9** was never attempted due to the lack of pure product obtained from the previous coupling reactions.



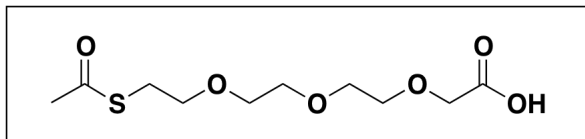
**Scheme 6.** Synthesis of **2.24**. Reagents and conditions: (a) NaH, BrBn, TBAI, THF (88%);<sup>26</sup> (b)  $\text{SOCl}_2$ , Pyridine, 4 hrs (54%);<sup>27</sup> (c)  $\text{H}_2/10\%$ , Pd/C, 1 atm (96%);<sup>28</sup> (d)  $\text{CrO}_3$ , 2.5 M  $\text{H}_2\text{SO}_4$ , acetone (30%);<sup>29</sup> (e) KSAc, TBAI, DMF (90%).<sup>30</sup>

## 2.5 Conclusions

We hypothesize that bioactivity can be restored to doxycycline analogs via conjugation to a gold nanoparticle at the 9 position. The coupling of two commercially available alkyl thiols using various reaction conditions has been attempted thus far. Although pure products have yet to be obtained, data from HPLC chromatograms, LC/MS, HRMS and crude NMR suggests product formation of the two different thiolated-9-aminodoxycycline derivatives. These products await further purification and will then be coupled to a gold nanoparticle for biological testing.

## 2.6 Experimental

All  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra were recorded at 25.0 °C on a Varian Mercury spectrometer. Chemical shifts ( $\delta$ ) are given in ppm relative to the tetramethylsilane or respective NMR solvent; coupling constants ( $J$ ) are in hertz (Hz). Abbreviations used are s = singlet, bs = broad singlet, d = doublet, t = triplets, and m = multiplet. Mass spectra were obtained at the NCSU Department of Chemistry Mass Spectrometry Facility. Chromatography was performed using 60 mesh standard grade silica gel from Sorbtech (Alanta, GA, USA). All other reagents were used as purchased from commercial sources unless otherwise noted.



**13-oxo-3,6,9-trioxa-12-thiatetradecan-1-oic acid (2.24):**<sup>30</sup> 2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)acetic acid (**2.23**) (268 mg, 1.18 mmol) and tetrabutylammonium iodide (44 mg, 0.12 mmol) were dissolved in anhydrous DMF (5 mL) at room temperature and stirred. Potassium thioacetate (405 mg, 3.55 mmol) was added and the reaction mixture was allowed to stir overnight. After completion, 1 N HCl (5 mL) and ethyl acetate (20 mL) were added. The organic layer was washed with 1 N HCl (5 mL). The organic layer was extracted with saturated NaHCO<sub>3</sub> (20 mL x 2). The basic aqueous layer was washed with DCM (30 mL x 3). After the layer was acidified with 2 N HCl, it was extracted with DCM (50 mL x 3). The organic extracts were combined, dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated. Purification by column chromatography (eluent: hexanes/ethyl acetate (v/v) 1:1) gave 282 mg of **2.24** (90%) as dark yellow oil: <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 2.32 (s, 3 H), 3.07 (t, =6.42 Hz, 2 H), 3.55 - 3.77 (m, 10 H), 4.07 (s, 2 H) ppm; <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 29.02, 30.82, 70.00, 70.50 70.84, 71.77, 195.82, 200.53 ppm; HRMS (FAB) m/z, ([M + H]<sup>+</sup>, C<sub>10</sub>H<sub>18</sub>O<sub>6</sub>S): calcd. 289.0716, found. 289.0713.

## REFERENCES

- (1) Nelson, M. L. *Tetracyclines in Biology, Chemistry and Medicine*; Nelson, M. L., Ed.; Birkhauser: Basel; Boston; Berlin, 2001, p 1.
- (2) Dax, S. L. *Antibacterial chemotherapeutic agents*; Blackie Academic and Professional London, United Kingdom, 1997.
- (3) Mitscher, L. A. B., A. C.; Sokoloski, T. D. In *Antimicrobial Agents and Chemotherapy* American Society for Microbiology: Bethesda, 1968.
- (4) Atkinson, B. A.; Abu-Al-Jaibat, A.; Leblanc, D. J. *Antimicrobial Agents and Chemotherapy* **1997**, *41*, 1598.
- (5) Banai, M.; LeBlanc, D. J. *Journal of Bacteriology* **1983**, *155*, 1094.
- (6) Barbosa, T. M.; Scott, K. P.; Flint, H. J. *Environmental Microbiology* **1999**, *1*, 53.
- (7) Chopra, I.; Roberts, M. *Microbiology and Molecular Biology Reviews* **2001**, *65*, 232.
- (8) Roberts, M. C. *FEMS Microbiology Reviews* **1996**, *19*, 1.
- (9) Johnson, A. P. *Current Opinion in Anti-Infective Investigational Drugs* **2000**, *2*, 164.
- (10) Asleson, G. L. S., L. J., Newman, E. C.; Frank, C. W. *Journal of Pharmaceutical Sciences* **1974**, *63*, 111.
- (11) Schneider, S. In *Tetracyclines in Biology, Chemistry and Medicine*; Nelson, M. L., Ed.; Birkhauser: Basel; Boston; Berlin, 2001, p 1.
- (12) Hinrichs, W. K. C. D., M.; Muller, A.; Tovar, K.; Hillen, W.; Saenger, W. *Science* **1994**, *264*, 418.
- (13) Pioletti, M.; Schlunzen, F.; Harms, J.; Zarivach, R.; Gluhmann, M.; Avila, H.; Bashan, A.; Bartels, H.; Auerbach, T.; Jacobi, C.; Hartsch, T.; Yonath, A.; Franceschi, F. *EMBO Journal* **2001**, *20*, 1829.
- (14) Schnappinger, D.; Hillen, W. *Archives of microbiology* **1996**, *165*, 359.
- (15) Levy, S. B.; McMurry, L. *Biochemical and biophysical research communications* **1974**, *56*, 1060.
- (16) Orth, P.; Saenger, W.; Hinrichs, W. *Biochemistry* **1998**, *38*, 191.

- (17) Orth, P.; Cordes, F.; Schnappinger, D.; Hillen, W.; Saenger, W.; Hinrichs, W. *Journal of Molecular Biology* **1998**, *279*, 439.
- (18) Bowman, M.-C.; Ballard, T. E.; Ackerson, C. J.; Feldheim, D. L.; Margolis, D. M.; Melander, C. *Journal of the American Chemical Society* **2008**, *130*, 6896.
- (19) Noskin, G. A. *Clinical Infectious Diseases* **2005**, *41*, S303.
- (20) Boothe, J. H.; Hlavka, J. J.; Petisi, J. P.; Spencer, J. L. *Journal of the American Chemical Society* **1960**, *82*, 1253.
- (21) Rogalski, W. In *Handbook of Experimental Pharmacology, The Tetracyclines*; Hlavka, J. J. B., J. H., Ed.; Springer Berlin, 1985; Vol. 78, p 179.
- (22) Barden, T. C.; Buckwalter, B. L.; Testa, R. T.; Petersen, P. J.; Lee, V. J. *Journal of Medical Chemistry* **1994**, *37*, 3205.
- (23) Berens, C.; Lochner, S.; Loeber, S.; Usai, I.; Schmidt, A.; Drueppel, L.; Hillen, W.; Gmeiner, P. *ChemBioChem* **2006**, *7*, 1320.
- (24) Dougan, J. A.; Karlsson, C.; Smith, W. E.; Graham, D. *Nucleic Acids Research* **2007**, *35*, 3668.
- (25) Kratz, F.; Haag, R.; Calderon, M.; Freie Universitaet Berlin, Germany; KTB Tumorforschungsgesellschaft mbH . 2009, p 51.
- (26) Pilkington-Miksa, M. A.; Sarkar, S.; Writer, M. J.; Barker, S. E.; Shamlou, P. A.; Hart, S. L.; Hailes, H. C.; Tabor, A. B. *European Journal of Organic Chemistry* **2008**, 2900.
- (27) Mirviss, S. B. *Journal of Organic Chemistry* **1989**, *54*, 1948.
- (28) Bonnet, N.; O'Hagan, D.; Haehner, G. *Chemical Communications (Cambridge, U. K.)* **2007**, 5066.
- (29) LaBell, R. Y.; Jacobsen, N. E.; Gervay-Hague, J.; O'Brien, D. F. *Bioconjugate Chemistry* **2002**, *13*, 143.
- (30) Nolting, B.; Yu, J.-J.; Liu, G.-Y.; Cho, S.-J.; Kauzlarich, S.; Gervay-Hague, J. *Langmuir* **2003**, *19*, 6465.

## CHAPTER 3

### Synthesis of SDC-1721 Analogs

#### 3.1 Background

The first report of acquired immune deficiency syndrome (AIDS) was observed in 1981. By the end of that year, of the 270 reported cases, 121 of the infected patients had died due to HIV-related complications.<sup>1</sup> In a 1983 *Science* journal, two research groups each published findings of a virus that reacted on a host's T cells similarly but less severe to that of AIDS and therefore, predicted to be a milder form or caustic of the disease.<sup>2,3</sup> It wasn't until 1986, that the viruses were deemed the same, thus given the name human immunodeficiency virus (HIV), and proved to be an early stage of AIDS.<sup>4</sup> HIV works by gradually destroying T cells, or more specifically T helper cells called CD4<sup>+</sup> T. These cells belong to a group of white blood cells known as lymphocytes and are an important component in the immune system.<sup>4</sup> HIV inevitably develops into AIDS, a point at which the CD4<sup>+</sup> T cell count is below 200 cells per  $\mu\text{L}$ .<sup>1</sup> Today, there is still no cure for this disease though treatment has progressed significantly. Advances in research and medicine have extended the lifetime of the infected, but without a true cure, the virus will result in fatality.

##### 3.1.1 CCR5

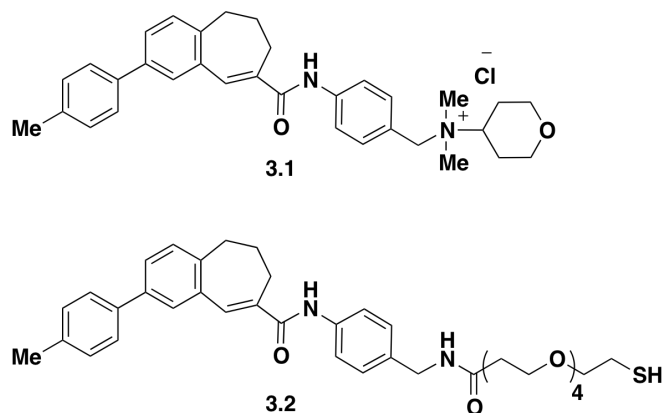
In order for HIV to infect a host cell, its envelope glycoprotein (gp120) must first bind to the CD4 receptor; a protein located on T cells. Further binding to one of the co-receptors, CCR5 or CXR5, causes a structural change in another viral protein, which allows the virus to fuse with the host's cell membrane.<sup>5</sup> Early on in infections, binding primarily

occurs at the CCR5 co-receptor. However, as the disease progresses and the virus turns from non-syncitium-inducing to syncitium-inducing strains, a switch to the CXCR4 co-receptor occurs in approximately 50% of patients.<sup>6,7</sup>

The importance of the two co-receptors was discovered in 1998.<sup>8</sup> A 32-base pair (bp) deletion mutation in the CCR5 gene was discovered to be associated with resistance against HIV. Furthermore, hosts exhibiting a homozygous deletion of the base pair (D32/D32) exhibit immunity against HIV while heterozygous deletion (CCR5/D32) would result in slower progression of the disease.<sup>9</sup> Thus, CCR5 is considered a great target receptor for new antivirals as it does not appear to be necessary for normal immune function.<sup>10</sup>

### 3.1.2 TAK-779

A small molecule, TAK-779 **3.1** shown in **Figure 8**, has proven to be an effective antagonist of the CCR5 co-receptor site. Discovered in 1999, it is the first small molecule reported with high potency and selectivity against CCR5. However, it is not effective against the second co-receptor CXCR4.<sup>11</sup> The preferential binding of TAK-779 blocks the envelope gp120 protein, thus, inhibiting HIV replication. A number of structure-activity relationship studies have shown that the quaternary ammonium salt is necessary for TAK-779 bioactivity.<sup>12</sup> The quaternary ammonium center allows for high-affinity binding to CCR5; without this feature, the molecule would be inactive. In addition, the use of this drug can cause unfavorable side effects including irritation at the injection site.<sup>13</sup> This observation inspired the Melander lab to develop a new, safer alternative to TAK-779.



**Figure 8.** Structure of TAK-779 **3.1** and SDC-1721 **3.2**.

### 3.2 SDC-1721

In 2008, our lab successfully synthesized SDC-1721 **3.2** as a TAK-779 analog (**Figure 8**). SDC-1721 has the same core structure of TAK-779, up to the location of the quaternary ammonium salt, at which point it is substituted with a thiolated PEG chain. The PEG group aids in solubility while the thiol allows the molecule to be conjugated to a gold nanoparticle. The gold nanoparticle is used as a multivalent drug display platform. Through conjugation to the nanoparticle, SDC-1721 is able to retain bioactivity without the presence of a quaternary ammonium salt. The development of this novel molecule provided evidence of a biologically inactive small molecule that becomes biologically active through the incorporation of nanotherapeutics. When conjugated to a 2.0 nm gold nanoparticle through simple ligand exchange methods at a ratio of 12:1 (SDC-1721:gold), HIV replication was inhibited with an  $IC_{50}$  value of 10 nM almost identical to the  $IC_{50}$  value of TAK-779.

The bioactivity attributed to SDC-1721 conjugated to the gold nanoparticle was due to the multivalence effect provided by the nanoparticle. This was proven with further testing

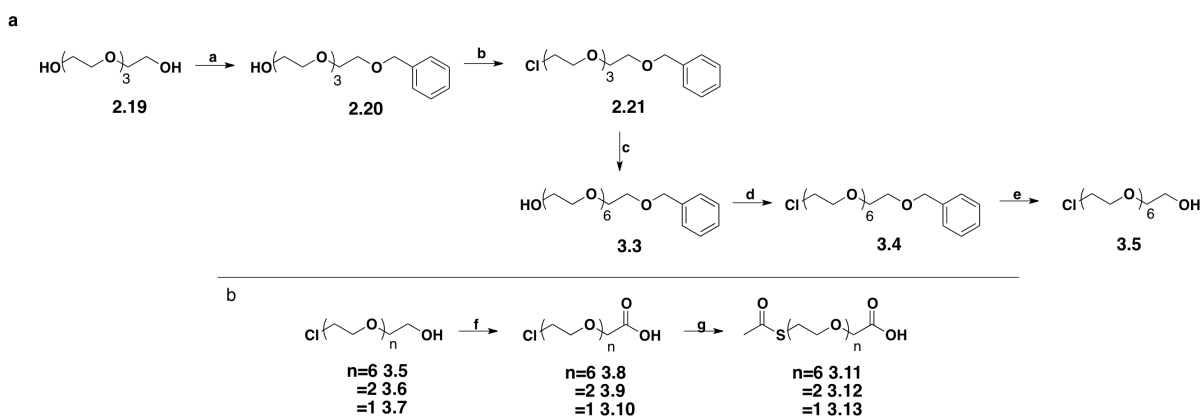
of the conjugated system by limiting the ratio to 0.93:1 (SDC-1721:gold). Inhibition of viral replication was not observed up to 300  $\mu$ M. This suggests that multiple SDC-1721 molecules must be conjugated to a single gold nanoparticle to trigger HIV inhibition. To rule out the bioactivity of gold in this process, a control experiment was run in which a glutathione-modified gold nanoparticle was constructed and tested for inhibition of viral replication. As expected, this functionalized nanoparticle did not inhibit replication, further supporting the necessity of the SDC-1721 moiety.<sup>14</sup>

### 3.3 Synthesis

The previous successes of the SDC-1721 nanotherapeutic inspired us to expand the library of analogs with varying linker lengths and test the effect on the inhibition of HIV replication. We believe there is an optimal linker length that will maximize binding with the CCR5 co-receptor. If the linker is too short, the active moiety may not be able reach the co-receptor site or exhibit minimal binding. A length that is too long may result in the molecule having additional range of motion and, therefore, lose affinity for the co-receptor. For these reasons, one longer and two shorter thiolated-PEG chains as compared to the original derivative were synthesized and used to create SDC-1721 analogs. Lipoic acid was also attached to examine the effects of a short, alkyl disulfide linker.

From Chapter 2, molecule **2.19** was used to synthesize the longer linker. **2.19** was first benzyl protected as described earlier with an 88% yield.<sup>15</sup> The monobenzyl alcohol **2.20** was then chlorinated with thionyl chloride and pyridine for a maximum of 4 hrs to produce **2.21**.<sup>16</sup> Substitution of the chlorine with triethylene glycol in the presence of 50% NaOH in water produced the nearly doubled in length monobenzyl alcohol **3.3** in 91% yield.<sup>17</sup>

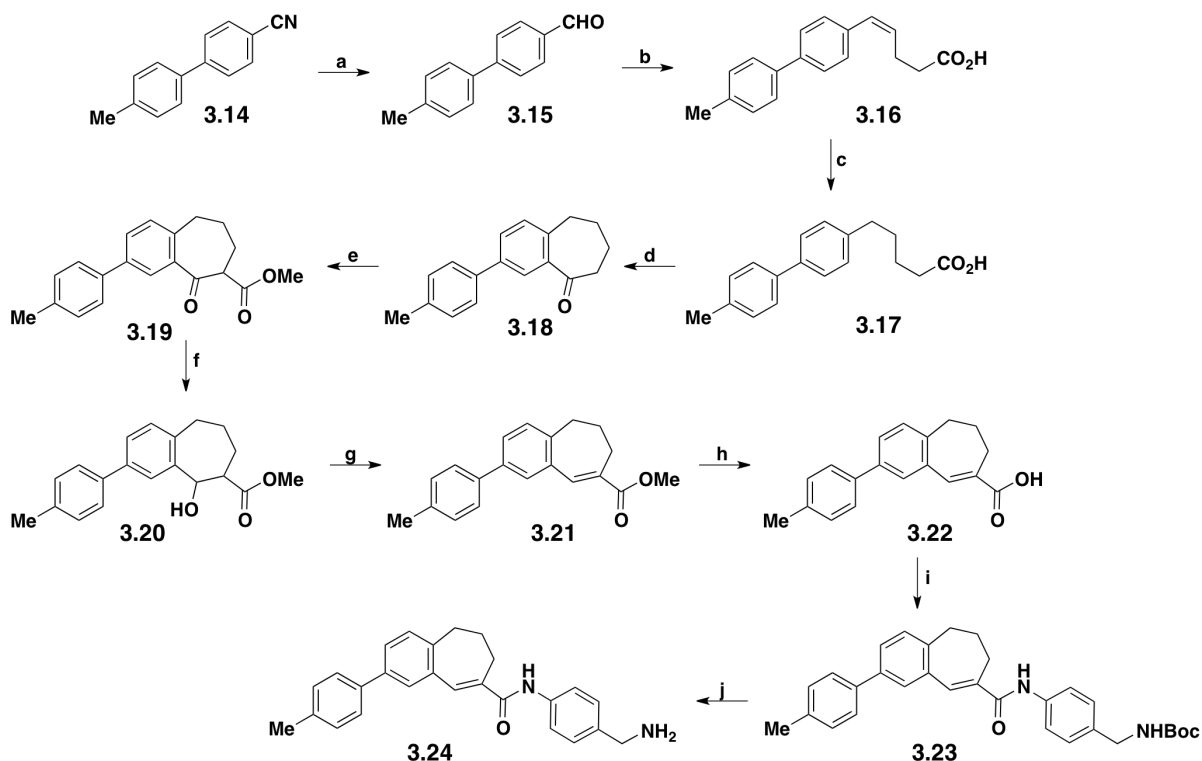
Similar reaction conditions from Chapter 2 and above were employed to chlorinate the free alcohol using thionyl chloride and pyridine in moderate yield of 71%.<sup>16</sup> Removal of the benzyl-protecting group was done via hydrogenolysis with 10% palladium on carbon at 1 atm hydrogen afforded **3.5** (**Scheme 7a**).<sup>18</sup> Two commercially available molecules were introduced this point; 2-(2-chloroethoxy)ethanol **3.6** and 2-[2-(2-chloroethoxy)ethoxy]ethanol **3.7**, and the three linkers of varying lengths were subjected to the same set of reaction conditions. The free alcohols were oxidized to the carboxylic acids using 2.5 M Jones reagent, a solution of chromium oxide and concentrated sulfuric acid.<sup>19</sup> Chloro-displacement of the linkers with potassium thioacetate was carried out in the presence of tetrabutylammonium iodide in DMF to produce the final products **3.11-13** (**Scheme 7b**).<sup>20</sup>



**Scheme 7a.** Synthesis of various thiolated-PEG linkers. Reactions and conditions: (a) NaH, BrBn, TBAI, THF (88%),<sup>15</sup> (b) SOCl<sub>2</sub>, Pyridine, 4 hrs (54%);<sup>16</sup> (c) Triethylene glycol, 50% NaOH, Toluene, 100 °C (91%);<sup>17</sup> (d) SOCl<sub>2</sub>, Pyridine (71%);<sup>16</sup> (e) H<sub>2</sub>/10%, Pd/C, 1 atm (Quant.);<sup>18</sup> **Scheme 7b.** Reaction conditions: (f) CrO<sub>3</sub>, 2.5 M H<sub>2</sub>SO<sub>4</sub>, acetone;<sup>19</sup> (g) KSAc, TBAI, DMF;<sup>20</sup>

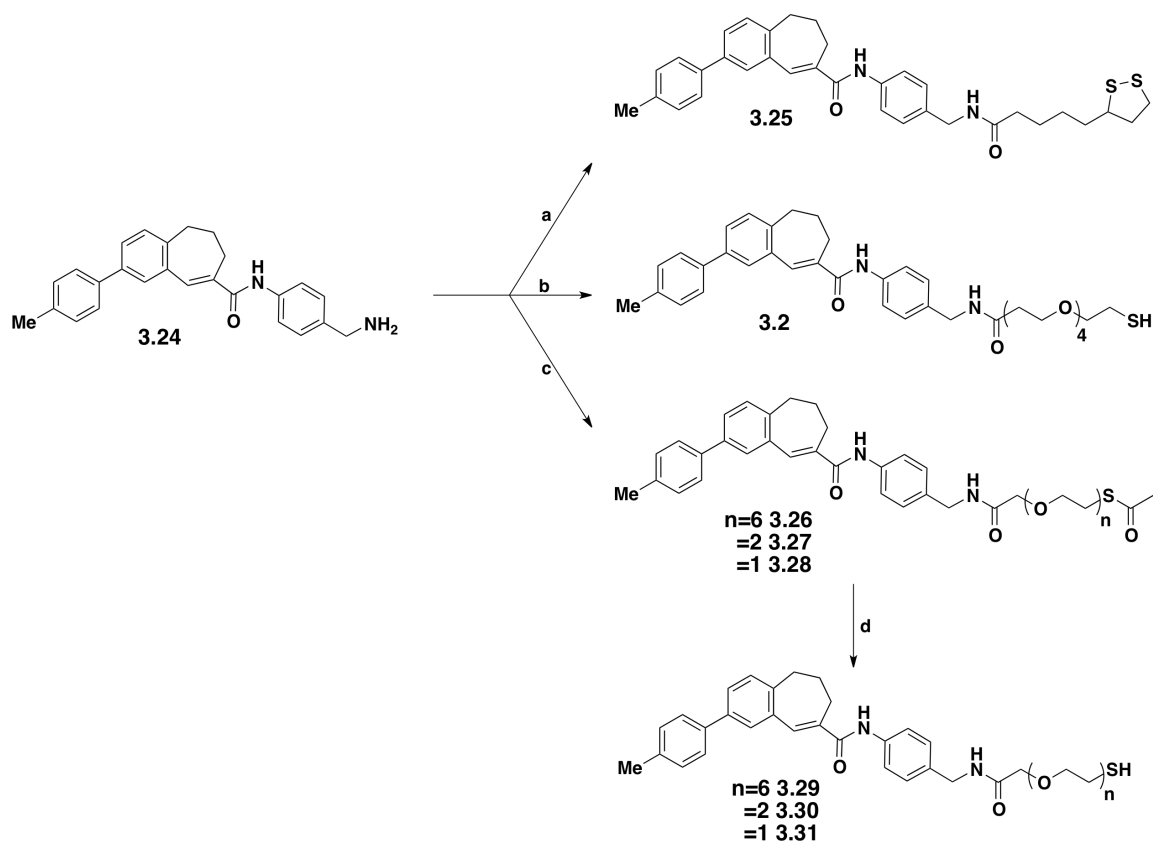
The precursor amine of SDC-1721 **3.25** was synthesized as previously reported and shown in **Scheme 8**.<sup>14,21</sup> Commercially available 4-(4-methylphenyl) benzonitrile **3.14** was reduced to the aldehyde **3.15** using sodium bis(2-methoxyethoxy)aluminum hydride, also

known as Red-Al. A Wittig reaction with (3-carboxypropyl) triphenylphosphonium bromide followed by hydrogenolysis with 10% palladium on carbon at 20 psi was carried out in good yield 97% of **3.17**. The 7-membered ring closure to provide **3.18** in 95% yield was accomplished using polyphosphoric acid at 105 °C stirring overnight with a mechanical stirrer. Failed deprotonation with the Claisen reaction required an alteration of the reported procedure where sodium methoxide was traditionally used, sodium hydride was employed providing a comparable yield of 82% to produce **3.19**. Reduction of the  $\beta$ -ketoester was successful with sodium borohydride **3.20**. Elimination of the alcohol to give **3.21** followed by saponification to provide **3.22** prepared the molecule for amide coupling to 4-[(N-Boc)-amino methyl] aniline. Deprotection of the Boc group with trifluoroacetic acid in dichloromethane in a 1:2 ratio overnight produced **3.24** in 76.5% yield over 10 steps.<sup>21,14</sup>



**Scheme 8.** Synthesis of **3.24**. Reactions and conditions (a) Red-Al, THF, -15 °C (77%); (b) (3-carboxypropyl) triphenylphosphonium bromide, NaOMe/MeOH, THF, 60 °C (86%); (c) H<sub>2</sub>/10%, Pd/C, 20 psi (94%); (d) polyphosphoric acid, 105 °C (95%); (e) NaH, DMC, 110 °C (82%); (f) NaBH<sub>4</sub>, THF/H<sub>2</sub>O, -10 °C (94%); (g) (i) MsCl, TEA; (ii) DBU, 3 hrs (67%); (h) 1 N NaOH, MeOH, THF, 60 °C, 2 hrs (83%); (i) (i) (COCl)<sub>2</sub>, DMF, DCM; (ii) 4-[(N-Boc)-amino methyl] aniline, THF (24%); (j) TFA/DCM, overnight (63%).<sup>14,21</sup>

**3.24** was first coupled to commercially available lipoic acid **2.10** to produce **3.25** in 37% yield. The reaction utilized EDC to turn the carboxylic acid into a symmetric anhydride and was further activated with HOBT. **3.24** was coupled with each of the three PEG linkers **3.11-13** using a similar procedure as above.<sup>14</sup> However, polymer-supported EDC was used to simplify the removal of urea produced from the reaction. Final molecules **3.28-30** were obtained after deprotection of the acetyl group with 0.5 M sodium methoxide in methanol.<sup>20</sup> SDC-1721 was also resynthesized for more biological testing, therefore, **3.24** was coupled with commercially available thiol-dPEG®4-acid in a low yield 50%,<sup>14</sup> (**Scheme 9**).



**Scheme 9.** Synthesis of SDC-1721 analogs. Reactions and conditions (a) **2.10**, EDC•HCl, HOBT, NMM, THF, DMF (37%); (b) thiol-dPEG@4-acid, DCC, HOBT, DMF (50%);<sup>14</sup> (c) **3.11-13**, PS-EDC, Pyridine, DCM, DMF;<sup>20</sup> (d) NaOMe, MeOH, 2 hrs.<sup>20</sup>

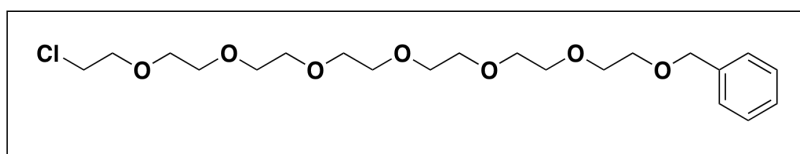
### 3.4 Conclusion

Four analogs of SDC-1721 were successfully synthesized and sent off to our collaborators, the Feldheim research group at the University of Colorado, Boulder. There, the analogs will be conjugated to gold nanoparticles as previously reported<sup>14</sup>. After conjugation, the nanoparticles will be sent to another set of collaborators at Chapel Hill, the Margolis laboratory, for HIV testing. Similar biological studies to those done previously will be employed to compare the effectiveness of the various linkers at inhibiting HIV replication to that of SDC-1721. Although SDC-1721 conjugated to a gold nanoparticle already showed

important inhibition against viral replication, a linker of a varied length may exhibit optimized activity for its analog.

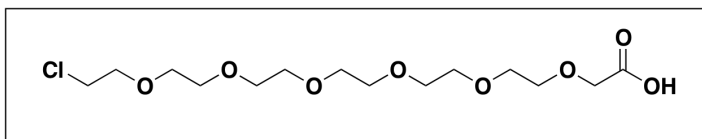
### 3.5 Experimental

All  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra were recorded at 25.0 °C on a Varian Mercury spectrometer. Chemical shifts ( $\delta$ ) are given in ppm relative to the tetramethylsilane or respective NMR solvent; coupling constants ( $J$ ) are in hertz (Hz). Abbreviations used are s = singlet, bs = broad singlet, d = doublet, dd= doublet of doublets, t = triplet, and m = multiplet. Mass spectra were obtained at the NCSU Department of Chemistry Mass Spectrometry Facility. Chromatography was performed using 60 mesh standard grade silica gel from Sorbtech (Alanta, GA, USA). All other reagents were used as purchased from commercial sources unless otherwise noted.



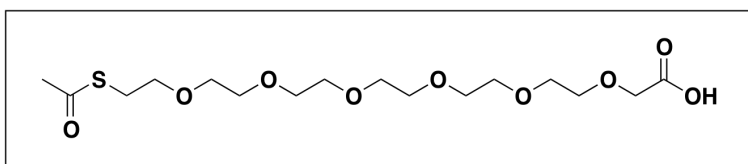
**22-chloro-1-phenyl-2,5,8,11,14,17,20-heptaoxadocosane (3.4):**<sup>16</sup> To a solution of 1-phenyl-2,5,8,11,14,17,20-heptaoxadocosan-22-ol **3.3** (2.32 g, 5.46 mmol) and pyridine (0.44 mL, 0.43 g, 5.46 mmol) was added 0.424 mL (0.69 g, 5.84 mmol) of  $\text{SOCl}_2$  dropwise over 45 min at room temperature. The solution was heated at 65 °C for 4 hrs. The reaction was cooled to room temperature and DCM (20 mL) was added. The organic layer was washed with  $\text{H}_2\text{O}$  (20 mL x 2), dried over  $\text{Na}_2\text{SO}_4$ , and concentrated. Purification by column chromatography (eluent: DCM/MeOH (v/v) 95:5) gave 1.85 g (3.86 mmol, 71%) as an orange oil:  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  3.60 - 3.80 (m, 28 H), 4.58 (s, 2 H), 7.28 - 7.38 (m, 5 H) ppm;

$^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  42.72, 69.38, 70.57, 70.61, 71.31, 71.37, 73.20, 127.57, 127.71, 128.32 ppm; HRMS (FAB)  $m/z$ , ( $[\text{M} + \text{H}]^+$ ,  $\text{C}_{21}\text{H}_{35}\text{ClO}_7$ ): calcd. 435.2144, found. 435.2145.



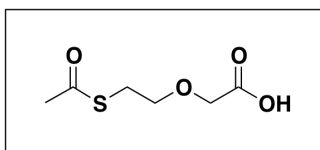
**20-chloro-3,6,9,12,15,18-hexaoxaicosan-1-oic acid (3.8):**<sup>19</sup> A mixture of 20-chloro-3,6,9,12,15,18-hexaoxaicosan-1-ol **3.5** (1.624 g, 4.18 mmol) in acetone (30 mL) was stirred at room temperature as 1.84 mL of 2.5 M Jones reagent\* (4.59 mmol) was added dropwise over 15 min. The reaction was stirred for an additional 30 min followed by an addition of 3 drops of 2-propanol.  $\text{H}_2\text{O}$  (20 mL) was added to the mixture followed by the removal of acetone in vacuo. Saturated brine (10 mL) was added and the aqueous layer was washed with DCM (15 mL x 5). The combined organic layers were dried over  $\text{Na}_2\text{SO}_4$  and concentrated. The product **3.8** was obtained in 91% yield and was a light orange oil (1.36 g, 3.91 mmol):  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  3.62 - 3.82 (m, 24 H), 4.18 (s, 2 H), ppm;  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  42.99, 61.96, 68.99, 70.43, 70.65, 70.86, 71.71, 177.16 ppm; HRMS (FAB)  $m/z$ , ( $[\text{M} + \text{H}]^+$ ,  $\text{C}_{14}\text{H}_{27}\text{ClO}_8$ ): calcd. 381.1287, found. 381.1280.

\*(Jones reagent was made by dissolving 2.5 g  $\text{CrO}_3$  in 10 mL  $\text{H}_2\text{SO}_4$ )

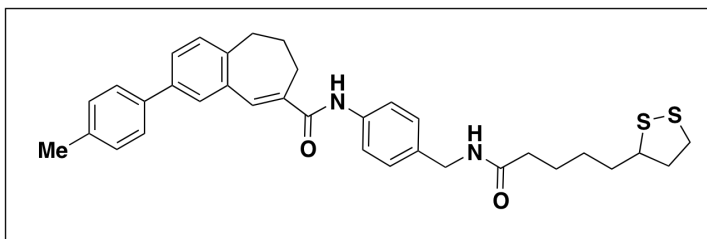


**22-oxo-3,6,9,12,15,18-hexaoxa-21-thiatricosan-1-oic acid (3.11):**<sup>20</sup> 20-chloro-3,6,9,12,15,18-hexaoxaicosan-1-oic acid (**3.8**) (268 mg, 1.18 mmol) and

tetrabutylammonium iodide (44 mg, 0.12 mmol) was dissolved in anhydrous DMF (5 mL) at room temperature and stirred. Potassium thioacetate (405 mg, 3.55 mmol) was added and the reaction mixture was allowed to stir overnight. After completion, 1N HCl (5 mL) and EtOAc (20 mL) were added. The organic layer was washed with 1 N HCl (5mL) and then extracted with saturated NaHCO<sub>3</sub> (20 mL x 2). The basic aqueous layer was washed with DCM (30 mL x 3). After the layer was acidified with 2 N HCl, it was extracted with DCM (50 mL x 3). The organic extracts were combined, dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated. Purification by column chromatography (eluent: hexanes/EtOAc (v/v) 1:1) gave 282 mg (0.71 mmol, 90%) as dark yellow oil: <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 2.34 (s, 3 H) 3.09 (t, *J*=6.42 Hz, 2 H), 3.57 - 3.79 (m, 35 H), 4.09 (s, 2 H), 4.18 (s, 2 H) ppm; <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 28.93, 30.73, 69.92, 70.42, 70.76, 71.69, 76.44, 188.46, 195.74 ppm; HRMS (FAB) *m/z*, ([M + H]<sup>+</sup>, C<sub>16</sub>H<sub>30</sub>O<sub>9</sub>S): calcd. 421.1503, found. 421.1519.



**2-(2-(acetylthio)ethoxy)acetic acid (3.13):**<sup>20</sup> Compound **3.13** (0.36g, 2.02 mmol) a light orange oil was obtained from 2-(2-chloroethoxy)acetic acid (**3.10**) (0.602 g, 4.35 mmol) in 46% yield performing the same procedure as **3.11**: <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 2.29 (s, 3 H), 3.07 - 3.11 (m, 2 H), 3.62 - 3.67 (m, 2 H), 3.97 (s, 2 H) ppm; <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 28.91, 30.84, 68.03, 70.53, 70.88, 71.56, 76.04, 195.46, 199.58 ppm; HRMS (FAB) *m/z*, ([M + H]<sup>+</sup>, C<sub>6</sub>H<sub>10</sub>O<sub>4</sub>S): calcd. 201.0192, found. 201.0192.

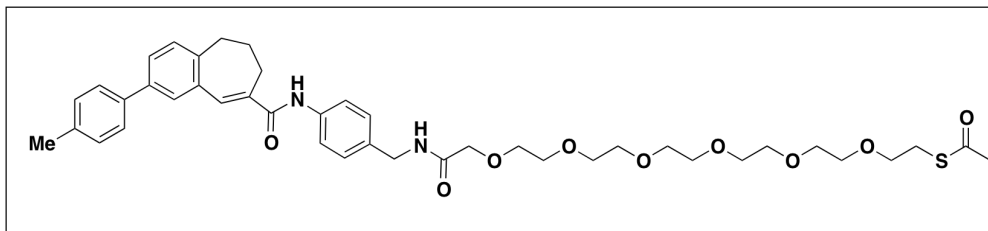


***N*-(4-((5-(1,2-dithiolan-3-yl)pentanamido)methyl)phenyl)-2-(*p*-tolyl)-6,7-dihydro-5*H*-**

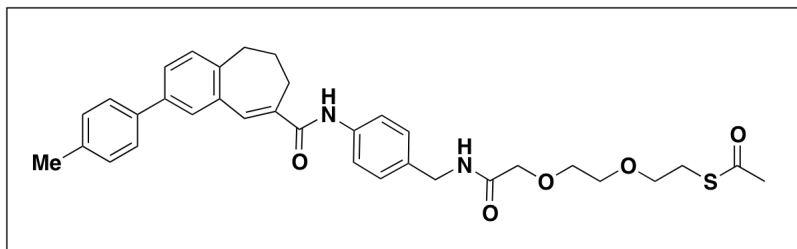
**benzo[7]annulene-8-carboxamide (3.25):** *N*-(4-(aminomethyl)phenyl)-2-(*p*-tolyl)-6,7-

dihydro-5*H*-benzo[7]annulene-8-carboxamide **3.24** (70 mg, 0.183 mmol) was dissolved in THF (3 mL) and cooled to 0 °C. Lipoic acid (38 mg, 0.183 mmol), EDC•HCl (70 mg, 0.366 mmol), HOBT (70 mg, 0.549 mmol), and NMM (0.04 mL, 36.8 mg, 0.366 mmol) were added consecutively. DMF (1 mL) was added and the reaction mixture was warmed to room temperature and stirred overnight. EtOAc (10 mL), DCM (20 mL), and H<sub>2</sub>O (10 mL) were added. Organic layer was washed with 1 N HCl (20 mL x 2), H<sub>2</sub>O (20 mL), saturated sodium bicarbonate (20 mL x 2), H<sub>2</sub>O (20 mL), and saturated brine (20 mL). Organics were dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated. Gradient column chromatography eluting with 0-5%

MeOH/DCM resulted in 39 mg (0.068 mmol) of **3.25** as a white solid with 37% yield: <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 1.31 - 1.88 (m, 16 H), 2.03 - 2.10 (m, 4 H), 2.15 (t, =7.33 Hz, 4 H), 2.32 (s, 3 H), 2.34 - 2.44 (m, 3 H), 2.63 (t, =6.05 Hz, 4 H), 2.76 - 2.85 (m, 4 H), 2.98 - 3.14 (m, 4 H), 3.41 - 3.52 (m, 2 H), 4.33 (d, =5.87 Hz, 3 H), 5.80 (bs, 2 H), 7.12 - 7.24 (m, 12 H), 7.33 - 7.45 (m, 10 H), 7.49 (d, =8.43 Hz, 4 H), 7.75 (s, 2 H) ppm; <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 21.31, 25.61, 28.07, 29.09, 30.62, 34.80, 36.65, 38.67, 40.43, 43.31, 56.61, 120.70, 126.91, 128.73, 130.07, 130.89, 134.53, 134.81, 137.33, 137.63, 137.80, 139.32, 141.44, 168.34, 172.77 ppm; HRMS (FAB) *m/z*, ([*M* + *H*]<sup>+</sup>, C<sub>34</sub>H<sub>38</sub>N<sub>2</sub>O<sub>2</sub>S<sub>2</sub>): calcd. 593.2267, found. 593.2260.



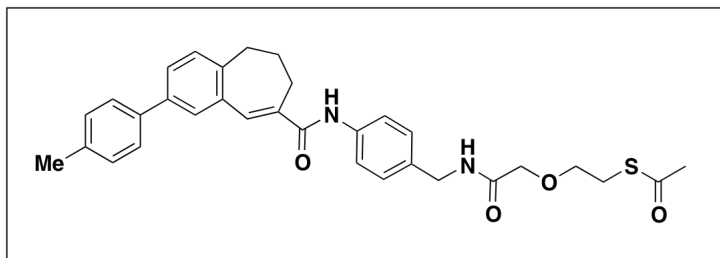
***S*-(3-oxo-1-(4-(2-(*p*-tolyl)-6,7-dihydro-5*H*-benzo[7]annulene-8-carboxamido)phenyl)-5,8,11,14,17,20-hexaoxa-2-azadocosan-22-yl) ethanethioate (3.26):** <sup>20</sup> PS-EDC (15 mg, 1.01 mmol/g, 0.04 mmol) was dissolved in anhydrous DCM (0.25 mL) and stirred for 5 min. 22-oxo-3,6,9,12,15,18-hexaoxa-21-thiatriacosan-1-oic acid **3.11** (23.4 mg, 0.06 mmol) was dissolved in anhydrous DCM (0.25 mL) and added to the stirring solution. After an additional 5 min, *N*-(4-(aminomethyl)phenyl)-2-(*p*-tolyl)-6,7-dihydro-5*H*-benzo[7]annulene-8-carboxamide **3.24** (15 mg, 0.04 mmol) was dissolved in a 1:1 mixture of anhydrous DMF and pyridine (0.5 mL each) and added to the suspension. The reaction was allowed to stir for 2 days at room temperature under inert conditions. The resin was filtered off and washed with MeOH and DCM. The filtrate was concentrated and the material was purified by gradient column chromatography eluting with 0-5% MeOH/DCM. The desired product **3.26** (21 mg, 0.028 mmol) was obtained in 70% yield as a light orange amorphous solid: <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 2.08 (d, *J*=12.10 Hz, 2 H), 2.18 - 2.35 (m, 6 H), 2.63 (d, *J*=6.23 Hz, 2 H), 2.78 (s, 1 H), 2.87 (bs, 1 H), 2.90 - 2.97 (m, 1 H), 3.02 (t, *J*=6.23 Hz, 2 H), 3.35 - 3.73 (m, 17 H), 3.97 (s, 1 H), 4.08 - 4.22 (m, 2 H), 4.39 (d, *J*=5.50 Hz, 2 H), 7.11 - 7.24 (m, 6 H), 7.30 - 7.46 (m, 4 H), 7.52 (d, *J*=8.43 Hz, 2 H), 7.89 (bs, 1 H) ppm; <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 21.26, 28.87, 30.57, 30.75, 34.74, 42.52, 70.82, 72.05, 76.27, 120.51, 126.87, 128.60, 129.70, 130.04, 134.62, 137.28, 137.29, 141.40, 170.18, 185.23, 195.90 ppm; HRMS (FAB) *m/z*, ([*M* + *H*]<sup>+</sup>, C<sub>42</sub>H<sub>54</sub>N<sub>2</sub>O<sub>9</sub>S): calcd. 785.3442, found. 785.3446.



***S*-(2-(2-(2-oxo-2-((4-(2-(*p*-tolyl)-6,7-dihydro-5*H*-benzo[7]annulene-8-**

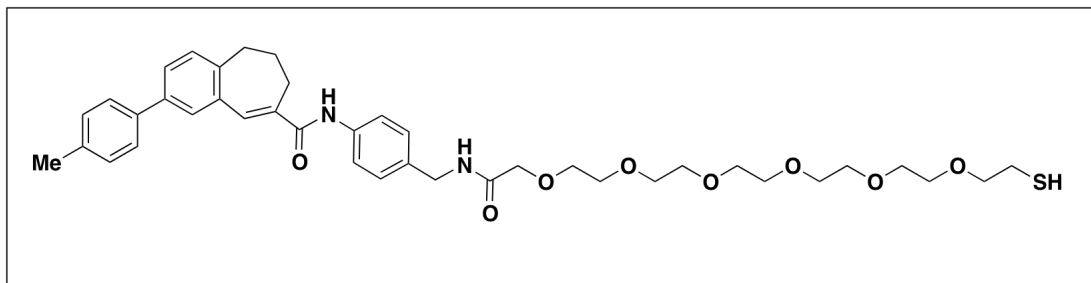
**carboxamido)benzyl)amino)ethoxy)ethoxy)ethyl) ethanethioate (3.27):**<sup>20</sup> Compound **3.27**

(24 mg, 0.04 mmol) as a light yellow soft powder was obtained from *N*-(4-(aminomethyl)phenyl)-2-(*p*-tolyl)-6,7-dihydro-5*H*-benzo[7]annulene-8-carboxamide **3.24** (81 mg, 0.21 mmol), 2-(2-(acetylthio)ethoxy)acetic acid **3.13** (71mg, 0.32 mmol), and PS-EDC (230 mg, 1.4 mmol/g, 0.32 mmol) in 18% yield performing the same procedure as **3.36**: <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 1.89 - 1.98 (m, 2 H), 2.03 - 2.10 (m, 2 H), 2.19 - 2.25 (m, 3 H), 2.32 (s, 3 H), 2.64 (t, *J*=6.60 Hz, 2 H), 2.76 - 2.82 (m, 2 H), 2.87 (t, *J*=6.42 Hz, 2 H), 3.43 (t, *J*=6.42 Hz, 2 H), 3.49 - 3.53 (m, 2 H), 3.55 - 3.60 (m, 2 H), 3.97 (s, 2 H), 4.40 (d, =5.87 Hz, 2 H), 7.11 - 7.26 (m, 7 H), 7.32 - 7.45 (m, 5 H), 7.52 (d, *J*=8.43 Hz, 1 H), 7.84 (s, 1 H) ppm; <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 21.36, 28.14, 28.84, 30.66, 30.82, 34.83, 42.68, 69.93, 70.05, 70.62, 71.23, 76.88, 77.30, 77.73, 120.64, 126.95, 127.09, 128.73, 129.79, 130.12, 130.90, 134.21, 134.72, 134.90, 137.36, 137.70, 137.76, 138.01, 139.35, 141.48, 168.42, 170.18 ppm; HRMS (FAB) *m/z*, ([*M* + *H*]<sup>+</sup>, C<sub>34</sub>H<sub>38</sub>N<sub>2</sub>O<sub>5</sub>S): calcd. 609.2394, found. 609.2393.



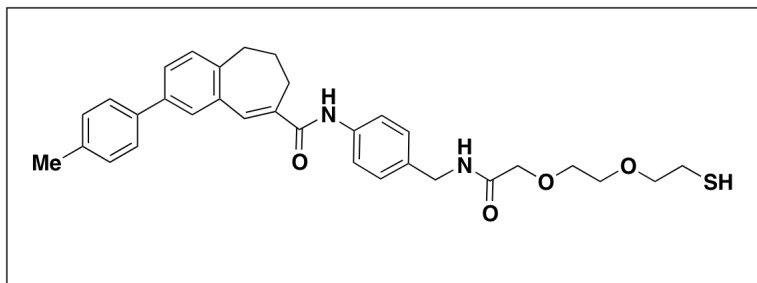
***S*-(2-(2-oxo-2-((4-(2-(*p*-tolyl)-6,7-dihydro-5*H*-benzo[7]annulene-8-**

**carboxamido)benzyl)amino)ethoxy)ethyl) ethanethioate (3.28):**<sup>20</sup> Compound **3.28** (25 mg, 0.046 mmol) as a white soft powder was obtained from *N*-(4-(aminomethyl)phenyl)-2-(*p*-tolyl)-6,7-dihydro-5*H*-benzo[7]annulene-8-carboxamide **3.24** (86 mg, 0.22 mmol), [2-(2-(2-(acetylthio)ethoxy)ethoxy)acetic acid **3.13** (60mg, 0.34 mmol), and PS-EDC (241 mg, 1.4 mmol/g, 0.34 mmol) in 20% yield performing the same procedure as **3.36**: <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 2.01 - 2.12 (m, 4 H), 2.21 (s, 4 H), 2.32 (s, 3 H), 2.63 (t, *J*=6.42 Hz, 3 H), 2.79 (d, *J*=5.13 Hz, 3 H), 3.01 (t, *J*=6.05 Hz, 2 H), 3.49 - 3.58 (m, 3 H), 3.93 (s, 2 H), 4.39 (d, *J*=5.87 Hz, 2 H), 7.10 - 7.23 (m, 8 H), 7.31 - 7.45 (m, 6 H), 7.53 (d, *J*=8.07 Hz, 2 H), 7.83 (s, 1 H) ppm; <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 21.04, 27.77, 28.65, 30.36, 30.50, 34.52, 42.33, 70.07, 70.11, 76.58, 77.00, 77.43, 120.39, 126.63, 128.40, 129.47, 129.81, 130.59, 133.67, 134.42, 134.56, 137.04, 137.37, 137.57, 137.68, 139.01, 141.17, 168.22, 169.14, 195.13 ppm; HRMS (FAB) *m/z*, ([*M* + *H*]<sup>+</sup>, C<sub>32</sub>H<sub>34</sub>N<sub>2</sub>O<sub>4</sub>S): calcd. 543.2312, found. 543.2315.



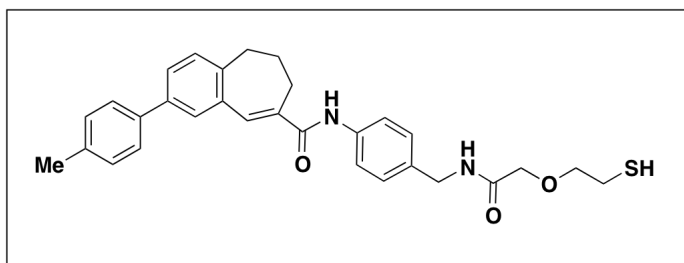
**20-mercapto-*N*-(4-(2-(*p*-tolyl)-6,7-dihydro-5*H*-benzo[7]annulene-8-**

**carboxamido)benzyl)-3,6,9,12,15,18-hexaoxaicosan-1-amide (3.29):**<sup>20</sup> Compound **3.28** (21 mg, 0.028 mmol) was dissolved in MeOH (1 mL) and catalytic amount of NaOMe (0.5 M in MeOH) was added. The reaction was allowed to stir for 30 min at room temperature, at which point acidic DOWEX-ion-exchange resin was added and stirred for 5 min. The resin was filtered off and washed with MeOH (10 mL x 3). The filtrate was concentrated and **3.29** was obtained a light orange amorphous solid in 50% yield (10 mg, 0.014 mmol): product **3.26** (21 mg, 0.028 mmol) was obtained in 70% yield as a light orange amorphous solid: <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 1.53 (br. s., 1 H), 1.95 (d, *J*=5.87 Hz, 1 H), 2.04 (d, *J*=5.87 Hz, 2 H), 2.31 (br. s., 2 H), 2.57 - 2.93 (m, 9 H), 3.38 - 3.73 (m, 18 H), 3.75 - 3.83 (m, 1 H), 3.92 - 4.00 (m, 1 H), 4.32 - 4.42 (m, 1 H), 7.10 - 7.24 (m, 4 H), 7.29 - 7.47 (m, 5 H), 7.52 (d, =8.43 Hz, 2 H), 8.05 (s, 1 H) ppm; <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 21.31, 30.62, 34.79, 38.43, 42.52, 69.66, 70.60, 71.24, 120.57, 126.88, 128.52, 129.73, 130.05, 130.83, 134.63, 137.64, 141.44, 183.64, 192.58 ppm; HRMS (FAB) *m/z*, ([*M* + *H*]<sup>+</sup>, C<sub>40</sub>H<sub>52</sub>N<sub>2</sub>O<sub>8</sub>S): calcd. 743.3337, found. 734.3313.



***N*-(4-((2-(2-(2-mercaptoethoxy)ethoxy)acetamido)methyl)phenyl)-2-(*p*-tolyl)-6,7-**

**dihydro-5*H*-benzo[7]annulene-8-carboxamide (3.30):**<sup>20</sup> Compound **3.30** (24 mg, 0.04 mmol) a white powder was obtained from **3.27** (22 mg, 0.04 mmol) in 91% yield performing the same procedure as **3.29**: <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 1.94 (br. s., 1 H), 2.02 (br. s., 1 H), 2.09 (bs, 1 H), 2.28 - 2.35 (m, 2 H), 2.54 - 2.68 (m, 3 H), 2.72 - 2.85 (m, 2 H), 3.45 - 3.71 (m, 7 H), 3.93 - 4.01 (m, 1 H), 4.26 - 4.44 (m, 3 H), 7.10 - 7.25 (m, 5 H), 7.28 - 7.57 (m, 7 H), 7.66 (dd, *J* = 5.87, 3.30 Hz, 1 H), 8.02 (s, 1 H) ppm; <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 21.06, 23.97, 27.67, 30.38, 34.53, 37.93, 42.36, 69.15, 69.63, 69.82, 70.25, 70.90, 76.58, 77.00, 77.43, 120.37, 126.61, 128.41, 129.47, 129.81, 130.61, 133.80, 134.45, 134.57, 137.03, 137.35, 137.62, 138.96, 141.19, 168.39, 169.93 ppm; HRMS (FAB) *m/z*, ([*M* + *H*]<sup>+</sup>, C<sub>32</sub>H<sub>36</sub>N<sub>2</sub>O<sub>4</sub>S): calcd. 567.2288, found. 567.2286.



***N*-(4-((2-(2-(2-mercaptoethoxy)ethoxy)acetamido)methyl)phenyl)-2-(*p*-tolyl)-6,7-dihydro-5*H*-**

**benzo[7]annulene-8-carboxamide (3.31):**<sup>20</sup> Compound **3.31** (13 mg, 0.02 mmol) a white

powder was obtained from **3.28** (15 mg, 0.028 mmol) in 93% yield performing the same procedure as **3.29**:  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  1.51 - 1.68 (m, 3 H), 1.94 (s, 2 H), 2.04 (bs, 3 H), 2.32 (s, 4 H), 2.61 (bs, 3 H), 2.76 (d,  $J=5.50$  Hz, 5 H), 3.48 (d,  $J=5.50$  Hz, 1 H), 3.66 (bs, 2 H), 3.94 (s, 2 H), 4.38 (d,  $J=5.50$  Hz, 2 H), 7.07 - 7.21 (m, 11 H), 7.29 - 7.44 (m, 7 H), 7.49 (d,  $J=8.07$  Hz, 2 H), 7.98 (s, 1 H) ppm;  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  21.04, 27.76, 28.64, 30.36, 30.48, 34.51, 42.32, 70.06, 76.58, 77.00, 77.42, 120.39, 126.63, 126.76, 128.40, 129.47, 129.81, 130.59, 133.68, 134.43, 134.55, 137.04, 137.37, 137.55, 137.67, 139.02, 141.17, 168.21, 169.12 ppm; HRMS (FAB)  $m/z$ , ( $[\text{M} + \text{H}]^+$ ,  $\text{C}_{30}\text{H}_{32}\text{N}_2\text{O}_3\text{S}$ ): calcd. 523.2026, found. 523.2027.

## REFERENCES

- (1) Levy, J. A. *Microbiological reviews* **1993**, *57*, 183.
- (2) Gallo, R. C.; Sarin, P. S.; Gelmann, E. P.; Robert-Guroff, M.; Richardson, E.; Kalyanaraman, V. S.; Mann, D.; Sidhu, G. D.; Stahl, R. E.; Zolla-Pazner, S.; Leibowitch, J.; Popovic, M. *Science* **1983**, *220*, 865.
- (3) Barre-Sinoussi, F.; Chermann, J. C.; Rey, F.; Nugeyre, M. T.; Chamaret, S.; Gruest, J.; Dauguet, C.; Axler-Blin, C.; Vezinet-Brun, F.; Rouzioux, C.; Rozenbaum, W.; Montagnier, L. *Science* **1983**, *220*, 868.
- (4) Weiss, R. A. *Science* **1993**, *260*, 1273.
- (5) Labrecque, J.; Metz, M.; Lau, G.; Darkes, M. C.; Wong, R. S. Y.; Bogucki, D.; Carpenter, B.; Chen, G.; Li, T.; Nan, S.; Schols, D.; Bridger, G. J.; Fricker, S. P.; Skerlj, R. T. *Virology* **2011**, *413*, 231.
- (6) Kamp, C. *BMC Evolutionary Biology* **2009**, *9*.
- (7) Locher, C. P.; Witt, S. A.; Kassel, R.; Dowell, N. L.; Fujimura, S.; Levy, J. A. *Journal of General Virology* **2005**, *86*, 1171.
- (8) Moore, J. P.; Kitchen, S. G.; Pugach, P.; Zack, J. A. *AIDS Research and Human Retroviruses* **2004**, *20*, 111.
- (9) Lu, Y.; Nerurkar, V. R.; Dashwood, W. M.; Woodward, C. L.; Ablan, S.; Shikuma, C. M.; Grandinetti, A.; Chang, H.; Nguyen, H. T.; Wu, Z.; Yamamura, Y.; Boto, W. O.; Merriwether, A.; Kurata, T.; Detels, R.; Yanagihara, R. *International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases* **1999**, *3*, 186.
- (10) Safarian, D.; Carnece, X.; Tsamis, F.; Kajumo, F.; Dragic, T. *Virology* **2006**, *352*, 477.
- (11) Konno, H.; Aimoto, S.; Smith, S. O.; Nosaka, K.; Akaji, K. *Bioorganic and Medicinal Chemistry* **2009**, *17*, 5769.
- (12) Shiraishi, M.; Aramaki, Y.; Seto, M.; Imoto, H.; Nishikawa, Y.; Kanzaki, N.; Okamoto, M.; Sawada, H.; Nishimura, O.; Baba, M.; Fujino, M. *Journal of Medicinal Chemistry* **2000**, *43*, 2049.
- (13) Barbaro, G.; Scozzafava, A.; Mastrolorenzo, A.; Supuran, C. T. *Current Pharmaceutical Design* **2005**, *11*, 1805.

- (14) Bowman, M.-C.; Ballard, T. E.; Ackerson, C. J.; Feldheim, D. L.; Margolis, D. M.; Melander, C. *Journal of the American Chemical Society* **2008**, *130*, 6896.
- (15) Pilkington-Miksa, M. A.; Sarkar, S.; Writer, M. J.; Barker, S. E.; Shamlou, P. A.; Hart, S. L.; Hailes, H. C.; Tabor, A. B. *European Journal of Organic Chemistry* **2008**, 2900.
- (16) Mirviss, S. B. *Journal of Organic Chemistry* **1989**, *54*, 1948.
- (17) Moreau, J.; Marchand-Brynaert, J. *European Journal Organic Chemistry* **2011**, 1641.
- (18) Bonnet, N.; O'Hagan, D.; Haehner, G. *Chemical Communications (Cambridge, U. K.)* **2007**, 5066.
- (19) LaBell, R. Y.; Jacobsen, N. E.; Gervay-Hague, J.; O'Brien, D. F. *Bioconjugate Chemistry* **2002**, *13*, 143.
- (20) Nolting, B.; Yu, J.-J.; Liu, G.-Y.; Cho, S.-J.; Kauzlarich, S.; Gervay-Hague, J. *Langmuir* **2003**, *19*, 6465.
- (21) Ikemoto, T.; Ito, T.; Hashimoto, H.; Kawarasaki, T.; Nishiguchi, A.; Mitsudera, H.; Wakimasu, M.; Tomimatsu, K. *Organic Process Research and Development* **2000**, *4*, 520.