DESIGN, SYNTHESIS AND APPLICATION OF CHOLESTEROL-BASED MOLECULAR PROBES AS BIOCHEMICAL TOOLS FOR THE STUDY OF CHOLESTEROL TRAFFICKING

A Dissertation

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DESIGN, SYNTHESIS AND APPLICATION OF CHOLESTEROL-BASED
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Abstract

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The overall goal of this project is to design, synthesize and apply cholesterol-based molecular probes that would aid in the understanding of cholesterol trafficking disorders. One such probe is a novel fluorescent cholesterol mimic that would serve as a general tool to track cholesterol movement in live cells or identify cholesterol localization in fixed cells. The other probes are cholesterol-based cross-linker molecular probes that consist of two cholesterol units are linked from head-to-tail. Our hypothesis behind this probe design is that these cross-linker probes can be used to identify and isolate protein pairs that participate in the transfer of cholesterol. Initially, these probes will be studied in the context of Niemann-Pick type C (NPC) disease since the proteins that are involved in the transport of cholesterol have been well studied. Once we have proven that this probe design works, we can utilize these types of probes in studies of other lipid storage diseases such as Tay-Sachs disease and progressive familial intrahepatic cholestasis.
For my family.
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ABBREVIATIONS

Å .......................................................................................................................... angstrom

ABC .................................................................................................................. ATP-binding cassette

AcOH ........................................................................................................ acetic acid

Asn ........................................................................................................ Asparagine

ATP ........................................................................................................ adenosine triphosphate

BBB ........................................................................................................... blood brain barrier

(S) - BINOL .............................................................................. (S) - (-)-1,1’- Bi(2-naphthol)

Boc₂O .................................................................................. Di-tert-butyl pyrocarbonate

°C ........................................................................................................... degrees Celsius

ca ...................................................................................................................... circa

calcd ........................................................................................................ calculated

CD ............................................................................................................. cyclodextrin

CoA ........................................................................................................ coenzyme A

CM ........................................................................................................ carboxymethyl

d ...................................................................................................................... doublet

DCC .............................................................................................. N,N'-Dicyclohexylcarbodiimide

DCM ...................................................................................................... dichloromethane

DEAE .............................................................................................. diethylaminoethyl

DIBAL-H ................................................................. diisobutylaluminum hydride
DIPEA .......................................................................................... diisopropylethylamine
DMAP .......................................................................................... 4-(dimethylamino)pyridine
DMF .......................................................................................... dimethylformamide
DMSO .......................................................................................... dimethyl sulfoxide
dppf .......................................................................................... 1,1’-bis(diphenylphosphino)ferrocene
DTT .......................................................................................... dithiothreitol
ER .......................................................................................... endoplasmic reticulum
Et2O .......................................................................................... diethyl ether
EtOAc .......................................................................................... ethyl acetate
EtOH .......................................................................................... ethanol
equiv .......................................................................................... equivalents
g .............................................................................................. gram
GC/MS .................................................................................. gas chromatography mass spectrometry
Gln .......................................................................................... Glutamine
Glu .......................................................................................... Glutamic acid
HDL .......................................................................................... high density lipoprotein
HMG-CoA ........................................................................... 3-hydroxy-3-methylglutaryl CoA
HMG-CoAR ........................................................................... 3-hydroxy-3-methylglutaryl CoA reductase
HPLC .................................................................................. high performance liquid chromatography
HRMS ................................................................................... high resolution mass spectrometry
HWE .................................................................................. Horner–Wadsworth–Emmons
Hz .......................................................................................... hertz
IAA .......................................................................................... iodoacetamide
IR ................................................................. infrared

J ................................................................. NMR coupling constant

KOAc ................................................................. potassium acetate

L ................................................................. liter

LAH ................................................................. lithium aluminium hydride

LC/MS ........................................................... liquid chromatography mass spectrometry

LDA ................................................................. lithium diisopropylamide

LDL ................................................................. low density lipoprotein

LE/LY ........................................................... late endosome/lysosome

LXR ................................................................. liver X receptor

MeOH ................................................................. methanol

MHz ................................................................. megahertz

mp ................................................................. melting point

min ................................................................. minute

mL ................................................................. milliliter

mmol ................................................................. millimole

MS ................................................................. mass spectrometry

NaHMDS ............................................................ sodium bis(trimethylsilyl)amide

NH₄OAc ............................................................. ammonium acetate

NMR ................................................................. nuclear magnetic resonance

NPC ................................................................. Niemann-Pick type C disease

NTD ................................................................. N-terminal domain

pdb ................................................................. protein database
Ph ................................................................. phenyl
pyr ........................................................................ pyridine
RMSD ................................................................ root mean square distance
Scap .............................................................. SREBP cleavage activating protein
SDS-PAGE ................................................... sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SP ........................................................................ Sulfopropyl
SREBP ........................................................ sterol regulatory element binding protein
TBAF ........................................................... tetrabutylammonium fluoride
TBDMSiCl/TBSCI ........................................... tert-butyldimethylsilyl chloride
:iBu ....................................................................... tert-butyl
TFA ........................................................................... trifluoroacetic acid
TFAA ..................................................................... trifluoracetic anhydride
THF ...................................................................... tetrahydrofuran
TLC ........................................................................ thin layer chromatography
TMSCl .............................................................. trimethylsilyl chloride
TosCl/TsCl .......................................................... tosyl chloride
pTsOH .............................................................. p-toluene-sulfonic acid
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CHAPTER 1:
INTRODUCTION

1.1 Statement of purpose

Cholesterol is arguably one of the most important sterols in the human body.\textsuperscript{1} Though we know that cholesterol participates in many processes, the mechanisms of cholesterol’s cellular distribution are not fully understood. When cholesterol trafficking is disrupted, accumulation occurs, which leads to a number of diseases.\textsuperscript{2} The overall goal of this dissertation is to design, synthesize and apply two types of cholesterol-based molecular probes that would be used to study cholesterol trafficking disorders.

1.2 Background

Since its discovery in 1789, by French chemist, Francois Poulletier de La Salle, cholesterol has been one of the most studied small molecules in history.\textsuperscript{3} Initially these studies were focused on the interesting structural and biophysical properties of this molecule but lately the focus has been shifted towards its biological properties. Cholesterol is present in 30 to 50 mol % in the plasma membrane of mammalian cells with respect to other lipids.\textsuperscript{4} This sterol is an important component of the eukaryotic cell membrane in which it regulates fluidity and membrane permeability as well as participates in transmembrane trafficking and signaling processes. Additionally,
cholesterol serves as a precursor to various steroidal hormones, vitamins (such as vitamins A and D) and bile acids. Finally, cholesterol has been found to be critical in the formation of “lipid rafts” and caveolae, which are important for processes such as endocytosis. Given that cholesterol serves all of these important functions, the trafficking of cholesterol is tightly regulated.

1.3 Sources of cholesterol

*De novo* synthesized cholesterol accounts for ca. 70% of the total cholesterol in the body. The remaining amount is obtained though dietary consumption. Cholesterol biosynthesis can be separated into three stages: (1) conversion of acetyl CoA to squalene, (2) conversion of squalene to lanosterol and (3) conversion of lanosterol to cholesterol. (Figure 1.1)

![Biosynthetic pathway for cholesterol formation](image)

Figure 1.1 Biosynthetic pathway for cholesterol formation
It is important to note that all of the carbons in cholesterol are derived from acetate.

Also, the rate-determining step occurs in the first stage. This step is the conversion of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) to mevalonate by 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoAR).\(^6\) (Figure 1.2)

![Figure 1.2 Conversion of HMG-CoA to Mevalonate](image)

As a result, HMG-CoAR is targeted by cholesterol-lowering drugs, such as statins, that work to inhibit the enzyme.\(^7\) In the second stage, squalene undergoes an oxidative cyclization, which is catalyzed by lanosterol synthase to form lanosterol. In the final stage, lanosterol is converted to cholesterol through a complex set of reactions.\(^5,8\) Once this process is complete, cholesterol is ready to be transported throughout the body.

1.4 Cholesterol trafficking

1.4.1 Extracellular cholesterol trafficking

Extracellular cholesterol enters the body through dietary consumption, where it enters the small intestine and travels to the liver. The liver then transports the cholesterol to the lymphatic and cardiovascular systems. Also, when intracellular cholesterol concentration is high, cells secrete cholesterol in the blood or lymph. Once in the blood...
or lymph stream, enterocytes and hepatocytes process and package the cholesterol until it is converted to low-density lipoprotein (LDL), which is the primary form of cholesterol that is taken up by cells. When this free cholesterol is secreted from cells, it is esterified and transformed to high-density lipoprotein (HDL) particles. These particles are absorbed in the liver and either processed and returned to the blood stream or secreted from the body.¹

1.4.2 Intracellular cholesterol trafficking

Cholesterol influx into the cell begins with LDL receptors on the outer cellular membrane binding to LDLs (Figure 1.3). This binding event causes endocytosis to occur, which brings the LDL into the cell. Next, the LDL is transported to the sorting endosome and then to the late endosome. From here, the LDL can be transported to various places such as the lysosome, the recycling endosome and the endoplasmic reticulum (ER). Note that the details about cholesterol movement both between and within these organelles are unclear. In addition to the trafficking of cholesterol that is obtained externally, de novo cholesterol, which is made in the ER, is either formed into lipid droplets or is transported to the Golgi apparatus (small amount). The transport of cholesterol out of the cell is facilitated by ATP-binding cassette (ABC) transporters ABCA1 and ABCG1, though the exact mechanism is still unclear.¹
1.4.3 Regulation of intracellular cholesterol concentration

Intracellular cholesterol concentration is tightly regulated through a negative feedback system. This system starts with two proteins on the ER called Scap and HMG-CoAR. Both of these proteins have a sterol-sensing domain that allows them to monitor the amount of cholesterol in the cell. When the cholesterol concentration is low, Scap binds to sterol regulatory element binding proteins (SREBPs) and transports them to the Golgi apparatus. In the Golgi apparatus, SREBPs are fragmented and transported to the nucleus, where they signal cholesterol synthesis. Since HMG CoA reductase is the rate-limiting enzyme in cholesterol biosynthesis, this protein is active when the intracellular cholesterol concentration is low. When the cholesterol concentration in the cell is high,
HMG CoA reductase is rapidly degraded, which ends cholesterol biosynthesis. It is important to note that under these conditions, Scap remains bound to the ER.⁹ (Figure 1.4)


1.5 Niemann-Pick type C disease

Since the movement of cholesterol is tightly regulated, a disruption in cholesterol trafficking will cause an accumulation to occur which would lead to various diseases. The most common disease of this nature is atherosclerosis, which is a hardening of the arterial walls as a result of cholesterol accumulation.² Other diseases, such as lysosomal storage disorders, are also characterized by buildup of metabolites, mainly lipids, in the lysosome. This buildup occurs due to the impaired activity of proteins that either
transport or metabolize the accumulated material. Though these diseases are very rare individually, collectively they occur in ~1:5000 live births.\textsuperscript{10}

One of the lysosomal storage disorders that affect cholesterol trafficking is called Niemann-Pick type C (NPC).\textsuperscript{11} NPC is a rare autosomal recessive disease, which results in the accumulation of free cholesterol, sphingolipids and other lipids in the late endosomes/lysosomes (LE/LY). It is caused by a mutation in either the \textit{npc1} or \textit{npc2} gene, which encodes the NPC1 or NPC2 protein. In fact, 95\% of cases are a result of mutations in NPC1. Though this disease causes a number of hepatological and respiratory problems, the most serious effects are neurological disorders resulting from destruction of the cells in the brain. These effects include dystonia, ataxia, vertical gaze palsy, learning disabilities and seizures. NPC can present itself as early as prenatal development and as late as adulthood. Unfortunately, young patients diagnosed with NPC, die before or during their teenage years.\textsuperscript{11}

1.5.1 Cholesterol trafficking in the context of NPC

Since NPC is characterized by the accumulation of cholesterol in the LE/LY, there is a breakdown in the trafficking of cholesterol in these organelles. Normal cholesterol trafficking in the lysosome commences with LDL being taken up via receptor-mediated endocytosis. Once inside of the lysosome, cholesterol esters are hydrolyzed to free cholesterol by lysosomal acid lipase. Then NPC2 binds to the free cholesterol and transports it to the sterol-sensing domain of NPC1. At this point, NPC1 transports cholesterol out of the lysosome via some unknown mechanism.\textsuperscript{12} (Figure 1.5)
NPC1 is an integral protein that primarily occupies the plasma membrane of late endosomes/lysosomes. In human NPC1, this protein consists of 1278 amino acids. NPC1 also contains a sterol-sensing domain, which is in the lumen of LE/LY. It is believed that this protein binds to the head (3β-hydroxyl end) of cholesterol and transports it out of the lysosome. On the other hand, NPC2 is a 151 amino acid containing soluble protein that is located in the lumen of the lysosome. This protein is believed to bind the isooctyl tail of cholesterol and transports it to NPC1.

1.5.2 Current treatment options

Currently, there is no effective treatment for NPC disease but there are two drugs that are currently in clinical trials. One such treatment is an iminosugar called Miglustat
1.1 (Zavesca®) (Figure 1.6). This drug was originally used for the treatment of Gaucher disease which is characterized by the accumulation of glucocerebroside. It works by inhibiting glucosylceramide synthase, therefore disrupting the glycosphingolipid biosynthetic pathway. Since glycosphingolipids are one of the metabolites that accumulate in NPC disease, Miglustat helps to reduce the amount that is produced, thus slowing the progression of the disease. In 2009, the European Union approved the use of Miglustat for NPC patients, but it has not been approved for use in the United States. Currently, research is being conducted to determine scope of the therapeutic efficacy of this drug.

![Structure of Miglustat](image)

Figure 1.6 Structure of Miglustat

Another treatment under investigation for NPC patients is the use of β-cyclodextrins (CD) 1.2 (Figure 1.7). These compounds are cyclic oligosaccharides that consist of 7 \( \alpha \)-glucopyranosyl units. These compounds contain a hydrophobic core, which allows it to interact with sterols and other non-polar substances and a hydrophilic outer surface.
In terms of NPC disease, CDs have shown to be effective in reducing cholesterol accumulation in NPC mouse models.\textsuperscript{21} Though it is not clear exactly how CDs work, investigators of this potential therapy theorize that CDs are able to perform the function of absent or dysfunctional NPC1 or 2 to allow for cholesterol efflux from the lysosome.\textsuperscript{22} (Figure 1.8) Currently, one of these compounds is in very small clinical trials to determine its effectiveness in NPC patients.
Figure 1.8 Proposed action of cyclodextrin in the lysosome. Reprinted from Rosenbaum, A. I.; Maxfield, F. R. “Niemann-Pick type C disease: molecular mechanisms and potential therapeutic approaches” J. Neurochem. 2011, 116, 789-795 Copyright (2011), with permission from John Wiley and Sons

1.6 Tools for studying cholesterol trafficking

1.6.1 Cholesterol binding molecules

Filipin is the most commonly used tool for cholesterol visualization (Figure 1.9). It is a polyene macrolide that has antibiotic and antifungal properties. Due to its structure, it is both intrinsically fluorescent and has the ability to bind to hydroxy sterols. The basis for using filipin in a visualization assay of cholesterol is that there is a decrease in both the excitation and absorption of cholesterol-bound filipin as compared to
cholesterol-free filipin. In addition to cellular studies, filipin has also been useful in the diagnosis and study of cholesterol accumulation in NPC disease. The drawbacks to this method are the high rate of photobleaching, easy quenchability by a variety of compounds and cytotoxicity of the stain. The latter drawback has limited the use of filipin staining to fixed cells. Also, investigators question whether filipin staining accurately reveals cholesterol localization, especially when cholesterol is located in intracellular compartments such as the binding pocket of a protein due to the large size of filipin. Additionally, there are examples where filipin was ineffective in staining sterol-containing membranes.

Figure 1.9 Structure of Filipin III

1.6.2 Fluorescent cholesterol derivatives

Fluorescent cholesterol analogs have been useful in tracking cholesterol movement, along with identifying and analyzing proteins that bind to cholesterol. Unlike the cholesterol-binding molecules, these compounds have been useful in studying live systems. These analogs can be placed into two categories: 1) analogs that are inherently fluorescent (Figure 1.10 A-B) and 2) analogs that are attached to fluorophores (Figure 1.10 C-D). Fluorescent probes that are in the first category, such as dehydroergosterol,
are analogous to cholesterol but contain conjugated double bonds that allow the compounds to be fluorescent. These probes can move throughout the cell in the same manner as natural cholesterol but they suffer from weak fluorescence and are easily photobleached. On the other hand, analogs in the second category are highly fluorescent but their movement throughout the cell is hindered by the bulky fluorophores.\textsuperscript{30,33} Hence, there is a need for the synthesis of a cholesterol probe that is highly fluorescent yet structurally similar to cholesterol.

![Chemical structures of cholesterol probes](image.png)

**Figure 1.10 Common fluorescent cholesterol probes**

1.6.3 Cholesterol-based photoaffinity probes

Cholesterol-based photoactive probes are useful for directly identifying and studying proteins that bind to cholesterol. These probes contain photolabile groups that, upon irradiation, will form a covalent bond with the protein or other biological
compounds with which cholesterol is associated. In the case that this material is a protein, one can isolate and analyze the stable protein-cholesterol derivative complex. When choosing the appropriate photoaffinity label to place on cholesterol, one must consider the following:\textsuperscript{20}

1. The photoaffinity label must be stable under both storage and experimental conditions. Hence, it is only reactive once irradiation has occurred.

2. The photoaffinity label should have a high extinction coefficient ($\epsilon$). This will ensure for a short irradiation period.

3. The photoaffinity label should have an excitation maximum above 300 nm, in order to avoid interference by amino acid residues such as tryptophan.

4. Finally, the product resulting from photo-induced reaction of the labeled compound with its protein target should be formed in high quantum yield ($\phi$).

Over the years, a variety of cholesterol-based photoactive probes have been obtained. Selected examples are shown in Figure 1.11. The first compounds to be studied as potential cholesterol-based photoactive probes were 3-azido-5-cholestene and 25-azidonorticosterone. In the initial study, these compounds were studied to determine their ability to function like cholesterol in the context of the cholesterol biosynthesis pathway in baby hamster kidney (BHK21) cells.\textsuperscript{20} Normally, cholesterol biosynthesis is subject to feedback regulation, and therefore the presence of cholesterol inhibits its further biosynthesis. This inhibition was exhibited by 25-azidonorticosterone but not by 3-azido-5-cholestene. The authors concluded that 3-azido-5-cholestene was not able to mimic this feedback function of cholesterol because it lacked the 3-\(\beta\)-OH group, which may be important for protein recognition. Based on this initial study, investigators
usually avoid placing photoaffinity labels at the 3 position of cholesterol, although the use of cholesteryl-3-diazoacetate has been reported to be successful in mimicking the interaction of cholesterol in lipid bilayers. In this case, it is believed that the polar group at position 3 can mimic the function of the 3-β-OH group on cholesterol. Although the isoctyl side chain appears to be much more tolerant to modifications, bulky photoaffinity labels such as diazoacetates and aryl azides (Figure 1.11) are not useful for studying systems where there are strict structural requirements for the binding of cholesterol. In these cases, cholesterol derivatives containing diazirines as the photoaffinity labels are used. The most common example is 6-azi-5α-cholestanol. Unlike the previously discussed probes, the photoaffinity label is located on the ring system but away from the 3-β-OH group and isoctyl side chain, which are important for binding. As a result, this probe has been used in a number of cholesterol binding studies. Another probe of this nature is [3H]7-azi-5α-cholestanol, which has been utilized specifically to analyze binding of cholesterol to NPC 1 and 2.
Cholesterol trafficking is a very elaborate process that occurs in the body. When there is a disruption in this process, it results in the accumulation of cholesterol, which leads to a diseased state. Understanding the causes of these diseases has led to the development of biochemical tools that have been used to study this cholesterol trafficking. These tools can be placed into two categories: 1) compounds that are used to visualize cholesterol and 2) cholesterol-based compounds that covalently bind to trafficking substructures. Though current compounds in the first category have been
useful, they suffer from either weak fluorescence or a lack of ability to identify cholesterol in small intracellular compartments such as the binding pocket of a protein. On the other hand, compounds in the second category have been utilized to identify cellular substructures such as proteins and plasma membranes that bind to cholesterol. Note that these compounds are only able to bind to one biological substructure at a given time. Throughout the cholesterol trafficking process, cholesterol is constantly being transferred from one biological substructure to the next. Thus, using the current cholesterol-based photoaffinity probes will only identify one of the biological substructures that is participating in this transfer process. These drawbacks to the current biochemical tools for studying cholesterol trafficking provide an opportunity to create new compounds that would address these challenges. Therefore, this project aimed to synthesize and apply two types of probes for the study of cholesterol trafficking in the context of NPC disease. The first probe that was designed for this purpose in our studies was a fluorescent cholesterol mimic that was to be used to visualize cholesterol localization. The second one was a series of novel cholesterol-based cross-linker probes that was to be used to identify protein pairs that participated in the transfer of cholesterol.
2.1 Concept of cholesterol-based cross-linkers

In the previous chapter, several methods of studying cholesterol trafficking were discussed. Of those methods, the use of cholesterol derivatives containing photolabile groups was the only one that allowed for the isolation and analysis of proteins and other biological material that bind directly to cholesterol. Experimentally, this was achieved by mixing the protein or cells of interest with the photoactive cholesterol derivative. After the appropriate amount of time has elapsed, the mixture is irradiated and the cholesterol derivative becomes covalently attached to the protein. (Figure 2.1)

![Figure 2.1 Mode of action for current photoactive cholesterol derivatives](image)

Figure 2.1 Mode of action for current photoactive cholesterol derivatives
One drawback to current probes of this nature is that they are only able to interact with one protein at a time. In nature, cholesterol may interact with several different proteins in a given pathway, a fact that is especially true when cholesterol is being transported from one place to another. For these situations, there is not a cholesterol-based probe that allows one to analyze the protein-cholesterol interactions that occur when cholesterol is being transported between two proteins. In order to address this issue, we envision creating a cholesterol-based cross-linker where photoaffinity labels are placed on one or both ends of the probe. (Figure 2.2) More specifically, we envision using one or two of the probes in Figure 1.11 and connecting them together via a linker. Conceptually, this cross-linker could isolate protein pairs that participate in the transport of cholesterol in three ways: (1) If the cross-linker contains photoaffinity labels with different photochemical properties, one could mix it with protein A and then irradiate the mixture under conditions that would favor the reaction of only one of the labels. Once the photoreaction is complete and the resulting protein-cross-linker complex is purified, it is mixed with protein B. This mixture would be irradiated under conditions that would favor the reaction of the remaining label. After purification, one should obtain the protein A-cross-linker-protein B complex. (2) If the cross-linker contains photoaffinity labels on each end of the probe with similar photochemical properties, proteins A and B would be mixed with the cross-linker and irradiated in order to isolate a complex. (3) If the cross-linker contains only one photoaffinity label, protein A would be mixed with the cross-linker and the mixture would be irradiated. Upon completion of the photochemical process, the resulting protein A-cross-linker complex would be purified. Then, that complex would be mixed with protein B and allowed to non-covalently interact with the
unlabeled end of the probe. If the non-covalent interaction with protein A is sufficiently strong, then the single photoaffinity label could be placed on the second cholesterol unit for covalent labeling of protein B.

![Diagram of cholesterol-based cross-linker probe](image)

Figure 2.2 Mode of action for cholesterol-based cross-linker probe

2.2 Previous cholesterol-based dimers and oligomers

The cross-linker probes that we envision using are a form of dimeric steroids. Compounds of this nature have only been studied extensively in recent years.\textsuperscript{41–43} Initially these compounds were only considered synthetic byproducts, but when dimeric and oligomeric steroids were discovered in nature,\textsuperscript{44} investigators began to further study these types of compounds. As a result, it was discovered that dimeric and oligomeric steroids could adopt unique physical and biological properties as compared to their monomeric units. Dimeric and oligomeric steroids have been utilized in many capacities,
which include: biomimetic chemistry,\textsuperscript{45} enantioselective receptors,\textsuperscript{46} molecular umbrellas (i.e. A molecule with the ability to transport polar compounds across phospholipid bilayers),\textsuperscript{46} organogelators\textsuperscript{47} and ionophores.\textsuperscript{48–50}

2.2.1 Pharmacological uses of steroid dimers

Dimeric and polymeric steroids have also been studied as both drugs and drug-delivery systems.\textsuperscript{41,51,52,53} The most notable dimeric steroids in these studies are cephalostatins (Figure 2.3). The first cephalostatin was isolated from the marine warm \textit{Cephalodiscus gilchristi} in 1988. It was shown to be very active against murine P388 lymphocytic leukemia cell line with \textit{ED}_{50} values ranging from $10^{-7}$ to $10^{-9}$ µg/ml, which made this compound one of the most potent ones ever tested by the U.S. National Cancer Institute.\textsuperscript{44} Though the high potency of cephalostatins have made them very attractive to investigators, there are only small amounts of them available in Nature. This has led to a
number of formal and total syntheses of cephalostatins and its derivatives. The first total synthesis of cephalostatin 1 was completed in 65 steps by the Fuchs group in 1998.

2.2.2 Structure of Steroid dimers

Due to the variety of applications for steroid dimers, this has led to diversity in the structure of these compounds. Most of the variable structural components of steroid dimers can be placed in three categories: steroid (dimeric unit), linker and point of connection between the dimeric units. All steroid dimers consist of four fused rings, a head region that contains a heteroatom and a tail region, which contains an aliphatic chain. Figure 2.4 labels these regions in the context of cholesterol.

Figure 2.4 A picture of cholesterol labeled with the variable regions of steroid dimers

Below are a few examples of steroid dimers in the literature. The table highlights the key structural components of the dimers and provides the function of these compounds (Table 2.1).
TABLE 2.1
EXAMPLES OF STEROID DIMERS

<table>
<thead>
<tr>
<th>Entry</th>
<th>Steroid</th>
<th>Linker</th>
<th>Point of Connection</th>
<th>Use</th>
<th>Ref.</th>
</tr>
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<tr>
<td>1</td>
<td><img src="image1" alt="Steroid 1" /></td>
<td><img src="image2" alt="Linker 1" /></td>
<td>head-to-head</td>
<td>(3β-OH)</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td><img src="image3" alt="Steroid 2" /></td>
<td><img src="image4" alt="Linker 2" /></td>
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<td>61</td>
</tr>
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<td><img src="image5" alt="Steroid 3" /></td>
<td>None</td>
<td>B ring-to-B ring</td>
<td>Insecticide</td>
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<td>Table 2.1 (Cont.)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td><strong>4</strong></td>
<td>None</td>
<td>C ring-to-C ring</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><img src="image1.png" alt="Chemical Structure 1" /></td>
<td><img src="image2.png" alt="Chemical Structure 2" /></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>5</strong></td>
<td>tail-to-tail</td>
<td>Molecular Umbrella</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><img src="image3.png" alt="Chemical Structure 3" /></td>
<td><img src="image4.png" alt="Chemical Structure 4" /></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>6</strong></td>
<td>tail-to-tail</td>
<td>Steroid Receptor Modulator</td>
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</tr>
<tr>
<td></td>
<td><img src="image5.png" alt="Chemical Structure 5" /></td>
<td><img src="image6.png" alt="Chemical Structure 6" /></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>7</strong></td>
<td>head-to-tail</td>
<td>Bile Acid Reabsorption Regulator</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><img src="image7.png" alt="Chemical Structure 7" /></td>
<td><img src="image8.png" alt="Chemical Structure 8" /></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Notes:**
- **OCH₃**: Methyl group
- **HOAc**: Acetic acid group
- **H**: Hydrogen atom
- **AcO**: Acetoxy group
- **X**: Substituent group
- **(3β-OH)**: 3β-hydroxy group
<table>
<thead>
<tr>
<th>No.</th>
<th>Structure</th>
<th>Reaction</th>
<th>R= O or OAc</th>
<th>66</th>
</tr>
</thead>
<tbody>
<tr>
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<td>head-to-tail</td>
<td>(3β-OH)</td>
<td></td>
</tr>
</tbody>
</table>
2.3 Cross-linker probe design

2.3.1 Experimental justification

Earlier, the idea of using a cross-linker probe to study cholesterol transport between cellular substructures (highlighted in Figure 2.2) was discussed. In order to validate this idea, we have designed cross-linker probes that have been used to study cholesterol trafficking by NPC proteins. More specifically, it was used to stabilize the NPC1/2 complex so that it could be isolated and studied.

The basic concept of a cross-linker probe is that it has two ligands that are separated by a linker. Ideally, a given cellular substructure, i.e. a protein, would have a higher binding affinity for one ligand over the other. In the case of NPC, where cholesterol is the ligand, NPC 1 and 2 preferentially binds to opposite ends of cholesterol. Note that both NPC 1 and 2 have a $K_d$ for cholesterol at 90 nM and 130 nM at 37 °C, respectively. When cholesterol is bound to NPC 2, the isooctyl chain is located deep within the binding pocket and only the $3\beta$-OH group is exposed to the solvent. On the other hand, when cholesterol is bound to NPC 1, the $3\beta$-OH group is located deep within the binding pocket. Based on the crystal structure of the N-terminal domain of NPC 1, the binding pocket contains mainly hydrophobic residues similar to NPC 2, except for the polar groups Asn41 and Gln79 in NPC 1. These amino acids are in close proximity to the $3\beta$-OH group of bound cholesterol, which facilitate hydrogen bonding. Also, the water-mediated interaction formed between the $3\beta$-OH group and Glu30 residue helps both to stabilize sterol binding and dictate the stereospecificity of the binding of sterols to this
protein as demonstrated by the inability of NPC 1 to bind epicholesterol.\textsuperscript{12} Based on this information, we designed head-to-tail cross-linker probes for the present study in order to permit simultaneous binding of both NPC 1 and NPC 2.

2.3.2 Computational justification

Though the Brown and Goldstein groups have provided a working model for the transport of cholesterol between NPC 1 and 2, little is known about the protein-protein interaction during cholesterol transport.\textsuperscript{12,14} In terms of designing the cross-linker probes, it is important to have some information about the approximate distance between the proteins when cholesterol transport occurs. In order to obtain this information, we relied on computational modeling of the cholesterol transport between NPC 1 and 2. In the laboratory of Olaf Wiest, a computational simulation of the transport of cholesterol between NPC1(NTD) and NPC2 was published.\textsuperscript{67} From these studies, Dr. Guillerma Estiu provided a refined structure of the NPC1(NTD)-NPC2 complex formed during cholesterol transport. (Figure 2.5) Using this structure, a docking grid of the binding site of each protein was generated, and cholesterol was docked to both sites using Schrödinger 2010. According to these studies, the cholesterol units in each protein were separated by approximately 4Å. Based on these data, we decided to employ a two-carbon linker, which would place the two steroid units at the appropriate distance from each other.
2.4 Synthesis of cross-linkers

2.4.1 First generation probe

Based on both the experimental and computational data, we envisioned making the cross-linker probe shown in Figure 2.6.
Note that the cholesterol units are linked in a head-to-tail fashion in order to allow preferential binding of NPC 1 and 2 on opposite ends of the probe. Also, we placed a two-carbon linker between the cholesterol units. In order to synthesize this cross-linker probe, we envisioned linking 24-(S)-hydroxycholesterol to cholesterol with the two carbon linker attached between the 24-(S) oxygen and the 3β-oxygen.

2.4.1.1 24-(S)-Hydroxycholesterol

Of the approximately 70% of cholesterol that is de novo synthesized, 25% of the cholesterol is made in the brain. Most of this cholesterol is used in the myelin, which is the material that insulates the axons. Though cholesterol is an important component in the brain, efficiency of de novo cholesterol synthesis is low. Therefore, it is important for the brain to regulate cholesterol influx and efflux. Since cholesterol is unable to cross the blood-brain barrier (BBB), it must be converted into a metabolite that is able pass
through this barrier. Outside of the central nervous system, this metabolite is 27-hydroxycholesterol, which is synthesized from cholesterol by a cytochrome P450 species, CYP27A1, or more commonly known as sterol 27-hydroxylase. A similar process occurs in the brain, where cholesterol is converted by CYP46A1 to 24(S)-hydroxycholesterol, also known as cerebrosterol. 68

In addition to the ability to pass through the BBB, 24(S)-hydroxycholesterol is an agonist of liver X receptors (LXRs). LXRs are transcription factors that are key regulators of cholesterol homeostasis.69 In recent years, studies have been conducted to exploit this relationship in order to develop potential treatments for atherosclerosis.70–72 Studies have also indicated 24(S)-hydroxycholesterol as a possible biomarker for neurodegenerative disorders such as Alzheimer’s disease.66,73 Unfortunately, these studies have been limited by the low amount of 24(S)-hydroxycholesterol that is available from biological sources. This has led chemists to develop a number of syntheses for this compound.74
2.4.1.2 Synthesis of first generation cross-linker

Based on the previous syntheses of 24(S)-hydroxycholesterol, we decided to follow the route similar to the one developed by Zhang and co-workers (Scheme 2.1). The difference in our route is that the cholesterol core was constructed towards the beginning of the synthesis whereas this motif was created towards the end of the synthesis in the literature route. The synthesis of 24-(S)-hydroxycholesterol commenced by converting commercially available hyodeoxycholic acid to the methyl ester in 94% yield. Next, ester 2.1 was converted to ditosylate 2.2 in 91% yield. Then, reacting 2.2 with potassium acetate in refluxing DMF/H₂O provided 3 in 34% yield via a substitution/elimination sequence. After converting the free alcohol to TBS ether 2.4, the resulting compound was transformed to Weinreb amide 2.5. The crude material was converted to ketone 2.6 via reaction with isopropyl magnesium bromide. With 24-ketocholesterol in hand, we tried a number of methods to form 24-(S)-hydroxycholesterol.
Initially, we did a lithium aluminum hydride (LAH) reduction of ketone \(2.6\) to form the diastereomeric mixture of 24-hydroxycholesterol. The purpose of synthesizing this compound was to find the best method to determine the diastereomeric excess \((de)\) for the formation of \(2.7\). Ideally, we wanted to use analytical methods such as LC/MS or GC/MS. Though 3\(\beta\)-OTBS protected 24-hydroxycholesterol has been analyzed by LC in the literature,\(^7\) this study was done using a normal phase column. Since our mass spectrum facility does not have a normal phase column, we tried to separate the diastereomers on a Helic column, which is a hybrid between a reverse and normal phase column. Unfortunately, we were not able to see any separation of the diastereomers with this column. In terms of using GC/MS, we were also unsuccessful. Eventually, the diastereomeric ratio was determined to be 1:1 based on a Mosher’s ester analysis.\(^7\)

While we were searching for analytical methods to resolve the diastereomeric mixture, we also investigated several recrystallization conditions. We were able to grow crystals of 24-hydroxycholesterol via vapor diffusion using a THF/hexanes solvent system. Upon x-ray analysis of the crystals, it was determined that only one diastereomer was present which was the 24-(\(S\))-hydroxycholesterol. Though recrystallization was a successful method to resolve the diastereomers on a small scale (5 mg), we were not able to accomplish it on a large scale. This result led us to investigate synthetic methods to selectively synthesize one diastereomer.

In order to selectively synthesize of 24-(\(S\))-hydroxycholesterol, we chose two routes based on methods found in the literature. (Scheme 22) The first route involves a LAH reduction of \(2.6\) in the presence of (\(S\))-BINOL as a chiral ligand.\(^7\) After several attempts, we were not able to isolate 3\(\beta\)-OTBS protected 24-(\(S\))-hydroxycholesterol as
the major diastereomer. Instead, we obtained either the starting ketone or 2.7 as a 1:1 mixture of diastereomers. Note that to perform this reaction, the chiral complex had to be formed prior to the addition of the ketone, which was accomplished by adding 1 equivalent of (S)-BINOL and ethanol to a solution of LAH in THF. The synthesis of this complex proved to be difficult, where the main issue was precipitate formation once all of the reagents were mixed together. When this happened, the procedure would have to be repeated. The problems with the synthesis of the chiral complex most likely attributed to the lack of diastereoselectivity in this reaction.

Scheme 2.2 Routes for the synthesis of 24-(S)-hydroxycholesterol

The second route for the diastereoselective synthesis of 24-(S)-hydroxycholesterol was the isopropylation of aldehyde 2.8 in the presence of a chiral sugar derivative. Unfortunately, we were not able to get this reaction to work in our hands. At this point, we decided to complete the synthesis of the cross-linker probe with the diastereomeric mixture of 24-hydroxycholesterol.
2.4.1.3 Endgame for first generation probe

Initially, we envisioned linking the two cholesterol units together via an etherification. After several failed attempts, we switched to linking the units together via an amide coupling. (Figure 2.7)

In order to execute the amide coupling, we needed to synthesize the corresponding 24-aminocholesterol and 3β-O-cholesteryl acetic acid. We were able to synthesize 24-aminocholesterol from 2.7 by first converting the alcohol to the corresponding azide via a Mitsunobu reaction. Then azide 2.8 was converted to amine 2.9 under Staudinger reduction conditions. Finally, 2.9 was linked to 3β-O-cholesteryl acetic acid via a DCC coupling. (Scheme 2.3)
2.4.2 Second generation probes

The synthesis of the first generation probe proved to be difficult due to the inability to set the stereochemistry on C-24. Therefore, we decided to simplify the probe. Docking studies indicated that the isopropyl group on C-24 of the left side of the probe was not necessary for binding. These studies were performed by Dr. Guillerma Estiu in Schrödinger 2010. This eliminated the need to induce stereochemistry at that point.

(Figure 2.8)
The synthesis of the second-generation probe commenced by reducing ester 2,4 to the alcohol via a LAH reduction (Scheme 2.4). Then, 2.11 was converted to azide 2.12 via a Mitsunobu reaction. Next, 2.12 was subjected to Staudinger reduction conditions in order to yield the primary amine, which was isolated as the crude product and used in the next reaction without further purification. The amine was coupled to carboxylic acid 2A via an amide linkage between the two cholesterol-based units. Finally, 2.13 was desilylated with tetrabutylammonium fluoride in THF to yield the first of the second-generation cross-linkers. Note that under these conditions, there was significant decomposition of the starting material over the course of the reaction. Therefore, on average, 30% of the product was isolated.

Scheme 2.4 Synthesis of second-generation cross-linker probe

Once we had the first second-generation cross-linker in hand, we had to synthesize a derivative that contained a photoaffinity label. As mentioned earlier in the chapter, the photoaffinity label would allow the cross-linker to be convalently attached to
the protein or biological substructure of interest. Ideally, we would like to have two
different photoaffinity labels attached to opposite ends of the cross-linker. For our initial
studies, we plan to use these cross-linkers to stabilize the NPC1/2 protein complex. In
order to accomplish this goal, each end of the cross-linker must reside deep within the
binding pocket of each protein. Therefore, the photoaffinity label that is used must be
small because there is not much available space when cholesterol is bound to these
proteins. Of the known photoaffinity labels that are both small and contain good
photochemical properties, a suitable choice is the use of a diazirine. Since we did not
want to place the same photoaffinity label on each end of the cross-linker, we decided to
only use one affinity label. Conceptually, this cross-linker would be covalently attached
to the first protein. Then the other end of the linker would interact with the second
protein via non-covalent interactions. The photoaffinity label was placed on C-7 of the
end of the cross-linker that would preferentially interact with NPC 2. (Figure 2.9) Though
the label could have also been placed on the opposite end to the cross-linker, we decided
against this because there was more NPC 2 available for the biological studies.

![Figure 2.9 Structure of the second-generation cross-linker probe with diazirine](image)

Figure 2.9 Structure of the second-generation cross-linker probe with diazirine
Fortunately, we were able to synthesize the photoaffinity labeled cross-linker without much deviation from the route of the unlabeled cross-linker. The only difference was to place a handle on the carboxylic acid coupling partner that can later be converted to the diazirine.

![Scheme 2.5 Synthesis of 7-keto-3β-O-cholesteryl acetic acid](image)

With this in mind, we decided to synthesize 7-keto-3β-O-cholesteryl acetic acid. (Scheme 2.5) Starting with cholesterol, we were able to synthesize \(2.14\) in 41% yield by treating cholesterol with tert-butyl bromoacetate in the presence of potassium tert-butoxide in toluene. Then, \(2.14\) was oxidized at the 7 position to yield \(\alpha,\beta\)-unsaturated ketone \(2.15\). The double bond in \(2.15\) was removed under hydrogenation conditions to yield \(2.16\) in 45%. Finally, the ester was converted to the acid \(2.17\) in quantitative yield.
With acid 2.17 in hand, we were able to complete the synthesis of the diazirine derivative of the cross-linker by first coupling the acid with free amine derived from 2.12. (Scheme 2.6) Note that previously, this amine was formed via a Staudinger reduction of azide 2.12. Next, cross-linker 2.17 was desilylated using HF-pyridine in THF at room temperature. Unlike the TBAF conditions used in the previous synthesis, the product did not decompose during the course of the reaction. Therefore, the yield over three steps (from the Staudinger reduction to the O-deprotection) improved from 50% to 80%. Finally, ketone in 2.18 was converted to the diazirine using a literature procedure.39

Once we synthesized both cross-linkers 2.13 and 2.19, we were able to use these compounds to study the NPC 1/2 interactions. These experiments have been initiated in the laboratory of Professor Suzanne Pfeffer at Stanford University.
CHAPTER 3:
PURIFICATION, DELIPIDATION AND PARTIAL PROTEOLYSIS OF NPC 2:
PREPARATION FOR BIOLOGICAL STUDIES WITH CHOLESTEROL-BASED CROSS-LINKERS

3.1 Plan of action

In the previous chapter, we hypothesized that cholesterol-based cross-linkers in Figure 3.1, could be used to stabilize the NPC 1/2 complex (Figure 3.1). In order to test this hypothesis, we decided to design our experiments around the use of 2.19. The photoaffinity label on this compound would allow for covalent binding to NPC 2. The resulting complex would be more stable for subsequent interaction with NPC 1 than a complex where the cross-linker and protein were not covalently attached. Before cross-linker 2.19 could be utilized, we had to purify bovine NPC 2 from raw milk.

Figure 3.1 Second generation cholesterol-based cross-linkers
3.2 Purification of bovine NPC 2

NPC 2 was isolated and purified from raw milk according to the procedure from the Pfeffer group. A brief description of the procedure is the following: First, the raw milk was acidified to pH 3.5. Once the milk began to curdle, the precipitate was removed and the supernatant was subjected to a 60% ammonium acetate precipitation. The resulting precipitate was dialyzed and purified via the following ion exchange columns: DEAE Sepharose, CM Sepharose and SP-Sepharose. Following the SP-Sepharose column, we were able to determine which fractions contained NPC 2 by SDS-PAGE gel electrophoresis (Figure 3.2).

![Figure 3.2 SDS-PAGE gel of fractions collected from SP-Sepharose column](image)

According to the gel above, NPC 2 is in fractions 20-26. Note: Only fractions containing impurities with a lower molecular weight than NPC 2 were collected. The low molecular weight impurities are β-casein fragments that have been removed by either purifying the
NPC 2 containing fractions on a HiTrap Q HP column or by concentrating these fractions using Amicon® tubes with a cutoff of 10 K. We found that we could isolate pure NPC 2 by subjecting the fractions from the SP column to a series of ammonium sulfate precipitations. First, the fractions were combined and treated with 50% ammonium sulfate to induce precipitation. Upon centrifugation, the resulting pellet was set aside. The precipitation was repeated by treating the supernatant with 90% ammonium sulfate. Pellets from both precipitations were run on a SDS-PAGE gel (Figure 3.3). As shown in the gel, the lower molecular weight contaminant was found in the first pellet, and NPC 2 was found in the second pellet.

Figure 3.3 SDS-PAGE gel of pellets collected from the 50% and 90% ammonium sulfate precipitation
The pellet containing NPC 2 obtained from the 90% ammonium sulfate precipitation was resuspended in 12.5 mM NH₄OAc, pH= 4.5 and dialyzed in the same buffer to remove the excess ammonium sulfate. Bradford assay was used to determine the amount of protein that was obtained from the purification. On average, we were able to isolate 1 to 1.5 mg of NPC 2 from 2 L of raw milk. With purified NPC 2 in hand, we focused our attention to determining the difference between cholesterol-bound and unbound forms of NPC 2.

3.3 Efforts to distinguish difference between lipidated and delipidated NPC 2

It is important that we have a method to distinguish the difference between apo NPC 2 and cholesterol-bound NPC 2. This is true because cholesterol in the binding pocket of NPC 2 would affect the binding of the cholesterol-based cross-linker. Also, we can use this process to determine the binding efficiency of the cross-linker to NPC 2. The method that we decided to use was developed by Ann Stock and co-workers where they used cation-exchange chromatography to determine the difference between apo and cholesterol-bound NPC 2. Cholesterol-bound NPC 2 eluded from the column faster than apo NPC 2. We loaded approximately 100 µg of purified bovine NPC 2 onto a HiTrap Fast Flow SP column and eluted the sample with a linear gradient of 12.5 to 512.5 mM of ammonium acetate, pH 4.5. Note: It is assumed that the bovine NPC 2 is bound to cholesterol because it was purified from milk, which is cholesterol-rich. (Figure 3.4)
Figure 3.4 Graph of FPLC analysis of NPC 2
Fractions from each peak were ran on SDS-PAGE gels to determine which peak contains NPC 2. The SDS-PAGE gel in Figure 3.5 shows that NPC 2 is in fractions 40-46.

(Figure 3.5)

![SDS-PAGE gel of fractions from SP chromatography](image)

Figure 3.5 SDS-PAGE gel of fractions from SP chromatography

Once we successfully used cation-exchange chromatography to analyze the lipidated NPC 2, we wanted to apply this method to apo NPC 2. Bovine NPC 2 was delipidated by acetone precipitation\(^83\) and then analyzed on the HiTrap Fast Flow SP column. (Note: In our hands, at least 50% of NPC 2 was lost after the acetone precipitation.) The data from this analysis were inconclusive and optimization of chromatography conditions is required. Moreover, we were also able to delipidate NPC 2 upon treatment with 1% Chaps detergent. Though the use of Chaps detergent is not ideal
for samples that are analyzed on the HiTrap Fast Flow SP column, this delipidation method can be useful in our future experiments.

3.4 Partial proteolysis of NPC 2

With both lipidated and apo NPC 2 in hand, we focused our attention on initiating studies to bind NPC 2 to cross-linker 2.19. We decided to first develop proteolysis conditions for NPC 2. Our reasoning for doing this is that we can use this process to determine where the cross-linker is attached to NPC 2. The experiment would go as follows: First, cross-linker 2.19 would bind to NPC 2. Then the mixture would be irradiated with light so that the cross-linker would covalently attached to NPC 2. Next, the resulting cross-linker-NPC 2 complex would be subjected to proteolysis. Finally, SDS-PAGE gels, mass spectrometry and peptide sequencing would be used to determine where the cross-linker is attached to NPC 2.

The proteolysis experiments commenced with trying to digest NPC 2 using trypsin. Note that this enzyme has 15 cleavage sites in bovine NPC 2. First, we treated NPC 2 with trypsin and incubated the mixture at 37 °C for the following time points: 0.5 hour, 1 hour, 2 hour, 3 hour and overnight. After each time point, trypsin inhibitor was added to stop the digestion. The samples were run on a Tricine-SDS-PAGE gel. According to the gel, there was no proteolysis observed. (Figure 3.6)
<table>
<thead>
<tr>
<th>Lane</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ladder</td>
</tr>
<tr>
<td>2</td>
<td>Trypsin Inhibitor</td>
</tr>
<tr>
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<td>Trypsin</td>
</tr>
<tr>
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<td>NPC overnight w/ Trypsin</td>
</tr>
<tr>
<td>5</td>
<td>3 hour incubation w/ Trypsin</td>
</tr>
<tr>
<td>6</td>
<td>2 hour incubation w/ Trypsin</td>
</tr>
<tr>
<td>7</td>
<td>1 hour incubation w/ Trypsin</td>
</tr>
<tr>
<td>8</td>
<td>0.5 hour incubation w/ Trypsin</td>
</tr>
<tr>
<td>9</td>
<td>NPC 2 w/o Trypsin</td>
</tr>
</tbody>
</table>

Figure 3.6 Tricine-SDS-PAGE gel of the trypsin digestion of NPC
We hypothesized that trypsin did not digest NPC 2 because it was not able to access the appropriate cleavage sites of the protein. In order to rectify this situation, we decided to treat NPC 2 with dithiothreitol (DTT) and iodoacetamide (IAA) prior to the digestion with trypsin. DTT is a reagent that cleaves disulfide bonds in proteins and IAA is reagent that alkylates the resulting thiols to prevent the disulfide bonds from reforming. Note that NPC 2 contains three disulfide bonds. Unfortunately, this did not help in the trypsin digestion of NPC 2. Finally, in addition to treating NPC 2 with DTT and IAA, we also added 4 M urea, which helps to denature the protein. When using urea in a proteolysis protocol, there is a concern that the urea would also denature the digestive enzyme. According to the supplier (Promega), trypsin maintains its activity in the presence of 4 M urea. Like the first experiment, trypsin was added to treated NPC 2 and the mixture was incubated at 37 °C for varying time points. After running the samples on a Tricine-SDS-PAGE gel, it shows that partial digestion was observed for the sample that was incubated overnight. (Figure 3.7)
<table>
<thead>
<tr>
<th>Lane</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ladder</td>
</tr>
<tr>
<td>2</td>
<td>NPC 2</td>
</tr>
<tr>
<td>3</td>
<td>1 hour incubation w/ Trypsin</td>
</tr>
<tr>
<td>4</td>
<td>2 hour incubation w/ Trypsin</td>
</tr>
<tr>
<td>5</td>
<td>3 hour incubation w/ Trypsin</td>
</tr>
<tr>
<td>6</td>
<td>Overnight incubation w/ Trypsin</td>
</tr>
</tbody>
</table>

Figure 3.7 Tricine-SDS-PAGE gel of the trypsin digestion of NPC 2 treated with DTT, IAA and 4 M urea
Though we achieved some success in the proteolysis of NPC 2 using trypsin, we decided to test different digestive enzymes in an effort to find the optimal conditions. First, we tried using chymotrypsin but no digestion was observed with this enzyme, even under the conditions where NPC 2 was treated with DTT, IAA and 4 M urea. The next enzyme we tried was endoproteinase Arg-C, which has 3 cleavage sites in bovine NPC 2. For these experiments, Arg-C was used to digest NPC 2 that was subjected to varying conditions and incubated at 0 °C overnight. Below is the Tricine-SDS-PAGE gel that was run on the samples after the incubation period. (Figure 3.8)
<table>
<thead>
<tr>
<th>Lane</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ladder</td>
</tr>
<tr>
<td>2</td>
<td>NPC 2</td>
</tr>
<tr>
<td>3</td>
<td>NPC 2 w/Arg-C</td>
</tr>
<tr>
<td>4</td>
<td>NPC 2 in 1% Chaps detergent w/Arg-C</td>
</tr>
<tr>
<td>5</td>
<td>NPC 2 (incubated w/DTT and IAA) w/Arg-C</td>
</tr>
<tr>
<td>6</td>
<td>NPC 2 (incubated w/DTT and IAA and treated w/ 4M urea) w/Arg-C</td>
</tr>
</tbody>
</table>

Figure 3.8 Tricine-SDS-PAGE gel of the Arg-C digestion of NPC
Lane 3 shows the attempt to digest NPC 2 with Arg-C without the use of denaturing reagents. This resulted in no observed digestion. In lane 4, NPC 2 was treated with 1% Chaps detergent prior to the addition of Arg-C. As mentioned earlier, treating NPC 2 with 1% Chaps detergent is a method to remove cholesterol from the binding pocket. The gel suggests that apo NPC 2 is digested with Arg-C but the results are distorted by the presence of the Chaps detergent. This can be remedied by digesting apo NPC 2 that is obtained via acetone-precipitation. If Arg-C is really digesting apo NPC 2, that would suggest that apo NPC 2 is more susceptible to digestion than cholesterol-bound NPC 2 because the use of denaturing reagents was not necessary to achieve digestion. Lane 5 shows the Arg-C proteolysis fragments of NPC 2, which had been treated with DTT and IAA. Here, there is definitely partial digestion of NPC 2. Finally, lane 6 shows the proteolysis results for NPC 2, which had been treated with DTT and IAA in the presence of 4 M urea. No digestion was observed.

3.5 Conclusion/Future directions

While at Stanford University, we successfully purified bovine NPC 2 from raw milk. Then we were able to differentiate between apo and cholesterol-bound NPC 2 by using FPLC analysis. Finally, we were able to develop partial proteolysis conditions for NPC 2 using trypsin or endoproteinase Arg-C. The next step for this project is to covalently attach cross-linker 2.19 to NPC 2. At the same time, we need to optimize the FPLC analysis of apo NPC 2. Then, we can covalently attach NPC 2 to the cross-linker and digest the complex. The digestion mixture would then be analyzed by SDS-PAGE gels and mass spectrometry to determine where the cross-linker is covalently attached to
NPC 2. Finally, NPC1-NTD will be incubated with the NPC 2-cross-linker complex and analyzed to determine if there is an interaction between the NPC 1-NTD and the complex.
CHAPTER 4:
FLUORESCENT CHOLESTEROL MIMIC

4.1 Introduction

In the introduction, we talked about the use of fluorescent cholesterol derivatives in the study of cholesterol trafficking. There are two properties each derivative of this nature must have to be effective: 1) they must have a high structural similarity to cholesterol so that they mimic cholesterol movement, and 2) they must be inherently fluorescent so that these compounds can be visualized. Balancing these two properties has proven to be difficult where the most common fluorescent derivative has a high structural similarity of cholesterol yet has poor fluorescent properties. On the other hand, the cholesterol derivatives with the best fluorescent properties lack structural resemblance to cholesterol, which led us to design and synthesize a fluorescent cholesterol derivative that would balance good fluorescent properties and structural similarity to cholesterol.20 (Figure 1.10)
4.2 Design of cholesterol mimic

In order to design the cholesterol mimic, an analysis of the crystal structure of NPC2 (pdb 2HKA) with cholesterol was performed. This analysis revealed that only the hydroxyl group and the alkyl side chain (without the methyl group on carbon 20) on the steroid core were necessary for cholesterol binding. (Figure 4.2)

![Cholesterol and Cholesterol Mimic Comparison](image)

**Figure 4.2 Comparison between cholesterol and the designed cholesterol mimic**

We included an extensive conjugated π system, which would make the compound highly fluorescent. Also, the methyl group on carbon 20 was excluded, thus simplifying the synthesis of the cholesterol mimic. Finally, we replaced carbon 15 with nitrogen in order to establish a push-pull π-electron system, which would enhance the fluorescent properties of the molecule.\(^{84-86}\) Both the free hydroxyl group and the alkyl side chain were conserved in the cholesterol mimic.

After the cholesterol mimic was designed, further computational studies were conducted to determine how the structural changes in the cholesterol mimic would effect its binding to NPC 2 as compared to cholesterol. A docking grid of the NPC2 binding site was generated from pdb code 2HKA, and docking studies were performed by Dr. Guillermira Estiu using Schrödinger 2010. (Figure 4.3) As shown in Figure 4.3, the
cholesterol and the cholesterol mimic exhibit good overlay with both compounds binding to NPC 2 in a highly similar fashion with a low RMSD.

Figure 4.3 A computational model of cholesterol (light blue) and the cholesterol mimic (magenta) in the binding pocket of NPC 2

4.3 Other azasteroids

Naturally occurring steroids such as cholesterol have been compounds of interest for the past couple of centuries in areas ranging from biophysics and chemistry to biology and medicine.³ When one or more of the carbons are replaced with a heteroatom, the chemical and biological properties of the compound are changed. Within the last century, scientists have exploited these properties to access unnatural heterosteroids that have both synthetic and biological significance.⁸⁷–⁸⁹ Azasteroids, in particular, have emerged as compounds of medicinal interest due to their antimicrobial, antibiotic and anticancer properties.⁹⁰–⁹² Also, these compound have been studied as potential treatment
options for hypocholesterolemia\textsuperscript{93} and benign prostatic hyperplasia (5\(\alpha\)-reductase inhibitors).\textsuperscript{87}

4.3.1 15-azasteroids as antimicrobial agents

In 1976, Chesnut and co-workers studied the antimicrobial properties of a series of steroids.\textsuperscript{92} (Figure 4.4)

At molar concentrations as low as 10\(^{-5}\), they found that azasteroids A and B were able to significantly inhibit the growth of \textit{B. subtilis} and \textit{E. coli}. Also, when B was combined with known antibacterial agents, the potency was greatly improved. For example, the authors found that the growth of \textit{P. fluorescens} was completely inhibited when the bacterium was incubated in medium containing B (6.4 \times 10\(^{-5}\) M) and polymyxin (5.6 \times 10\(^{-7}\) M). Note that no growth inhibition was detected when \textit{P. fluorescens} was incubated with only B (6.4 \times 10\(^{-5}\) M) or polymyxin (5.6 \times 10\(^{-7}\) M). Though our initial studies are focused on using the cholesterol mimic as a fluorescent derivative of cholesterol, future studies can include testing for antimicrobial activity.
4.4 Synthesis

4.4.1 First generation synthesis

The previous syntheses of compounds similar to the cholesterol mimic were at least 12 linear steps in length. Consequently, we envisioned a convergent synthesis of the compound where an advanced intermediate could be reached by doing a tandem asymmetric conjugate addition/alkylation (CAA) reaction with 3-methyl-\(N\)-Boc-pyrrol-2(5\(H\))-one. The remaining steps of the synthesis could be completed through known chemistry. This approach would allow us to synthesize our compound in only four steps, which is a drastic improvement over previous syntheses. (Scheme 4.1) Therefore, we had to develop a tandem asymmetric CAA reaction of pyrrolinones in order to apply it to the synthesis of the cholesterol mimic.

![Scheme 4.1 Retrosynthetic analysis of cholesterol mimic](image)

Scheme 4.1 Retrosynthetic analysis of cholesterol mimic
4.4.1.1 Efforts to synthesize 4.1

The synthesis commenced with trying to synthesize 4.1. Initial efforts were focused on making this compound through a literature synthesis, but we were unsuccessful. Then we tried to make 4.1 via the route in Scheme 4.2.

First we converted commercially available glycine methyl ester hydrochloride to the Boc-protected compound in 96% yield. Then ester 4.3 was reduced to the aldehyde with DIBAL-H in 61% yield. Next, an HWE olefination was done using 4.4 and the phosphonate of 4.6, which yielded a 3:1 mixture of Z/E products in 62%. Since we only expected the Z isomer to cyclize to our desired product, we decided not separate to the isomers. For the final cyclization, we treated 4.7 with AlMe3 in toluene to yield 4.1 in 11%. Though we were able to synthesize 4.1 via the route in Scheme 4.2, we found a simpler procedure in the literature. (Scheme 4.3)
Scheme 4.3 Simplified route for making 4.1

3-Methyl-2-pyrrolidinone was Boc-protected to form 4.8 in 74% yield. Then, 4.8 was treated with phenyl selenium chloride and lithium TMP to form 4.9 in 59%. Finally, the phenyl selenium group was oxidatively eliminated by treating 4.9 with hydrogen peroxide (30 wt.% in water) in the presence of acetic acid to yield 4.1 in 52%.

4.4.1.2 Development of the tandem asymmetric conjugate addition-alkylation of pyrrolinones

While we were having trouble synthesizing 4.1, the unsubstituted pyrrolinone was easily made in one step starting from pyrrole.102 (Scheme 4.4)

After protecting the pyrrolinone with a Boc group, we used 4.11 to do some preliminary conjugate addition experiments. According to the literature, Ben Feringa’s group had done asymmetric conjugate addition reactions on 4.11 using both diethyl zinc and triethyl...
aluminum albeit with low yields and enantiomeric excess. We decided to initially focus on the zinc reactions because there is literature precedent for making dialkyl zinc reagents which would be necessary for the synthesis of the cholesterol mimic. Therefore, we repeated the conjugate addition of 4.11 with diethyl zinc according to the Feringa paper. (Scheme 4.5)

![Scheme 4.5 Conjugate addition of 4.11 with diethyl zinc and copper (II) triflate in toluene](image)

Our results were comparable to those reported even though we used 0.5 equivalents of copper versus 0.1 equivalents used in the paper. Though this reaction has a high percent conversion (>90%), the yield is still low. The authors suggested that the Boc group was being removed during the column purification. In order to circumvent this problem, we tried to basify the column with triethylamine. Unfortunately, this made it very difficult to identify the fractions that contained product because all of the spots turned yellow when stained with KMnO₄. Hence, this resulted in a lower yield of the reaction. In the Feringa paper, the authors addressed this problem by converting 4.11 to the N-benzyl derivative. When the authors ran the same reaction in Scheme 4.5, where they use triethyl aluminum instead of diethyl zinc, they obtained the N-benzyl derivative of 4.12 in a 25% yield.

This information points to the conclusion that the starting material may be consumed by some side reaction that is taking place faster than the desired conjugate addition. We
were not able to isolate any side products from this reaction, thus the fate of the unaccounted starting material is still a mystery.

Next, we focused our attention on improving the reaction in Scheme 4.5. (Table 4.1) Initially, we tried to use different copper(II) catalysts such as Cu(II) thiophene-2-carboxylate and CuSO₄-5H₂O, but only starting material was recovered. Finally, we ran the reaction with copper iodide, but only a very small amount of product formed, and mostly starting material remained (Table 4.1, entry 2). These results led us to investigate the use of different organometallic reagents in order to improve the yield of the conjugate addition reaction. First, we tried using n-butyllithium and copper iodide as our reagents to perform this reaction. (Table 4.1, entry 3) Under these conditions, we obtained a complex mixture of products. Next, we tried the previous reaction again with TMSCl. (Table 4.1, entry 4) TMSCl has been shown to accelerate the rate of conjugate additions and to trap the enolate intermediate. Under these conditions, we were able to obtain the desired product in 47% yield. The use of BF₃-OEt in conjugate additions has been shown to form reactive cuprates that are able to perform these reactions when Grignard-based and Gilman-type cuprates fail. Therefore, we tried this reagent as an additive in the conjugate addition of 4.11 with n-butyllithium, and we obtained the desired product in 30% yield. (Table 4.1, entry 5) Though the conditions in entry 4 and 5 provided the product in moderate yield, they also required a stoichiometric amount of copper. These conditions are not suitable for further development because use of a stoichiometric amount of copper is not attractive for the development an asymmetric variant of the conjugate addition reaction.
TABLE 4.1 CONDITIONS FOR CONJUGATE ADDITION OF N-BOC-PYRROL-2(5H)-ONE

<table>
<thead>
<tr>
<th>Entry</th>
<th>Organometallic Reagent</th>
<th>Additive</th>
<th>Copper (Eq)</th>
<th>Temp</th>
<th>Solvent</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Et₂Zn</td>
<td>-</td>
<td>Cu(OTf)₂ (0.5)</td>
<td>-78 to 0 °C</td>
<td>Toluene</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>Et₂Zn</td>
<td>-</td>
<td>CuI (1)</td>
<td>-78 to 0 °C</td>
<td>Toluene</td>
<td>Trace</td>
</tr>
<tr>
<td>3</td>
<td>nBuLi</td>
<td>-</td>
<td>CuI (1)</td>
<td>-78 °C</td>
<td>THF complex mixture</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>nBuLi</td>
<td>TMSCl</td>
<td>CuI (1.5)</td>
<td>-78 °C</td>
<td>THF</td>
<td>47</td>
</tr>
<tr>
<td>5</td>
<td>nBuLi</td>
<td>BF₃-OEt</td>
<td>CuI (1.5)</td>
<td>-78 °C</td>
<td>THF</td>
<td>30</td>
</tr>
<tr>
<td>6</td>
<td>EtMgBr</td>
<td>-</td>
<td>CuI (0.1)</td>
<td>-78 to 0 °C</td>
<td>Et₂O</td>
<td>47</td>
</tr>
<tr>
<td>7</td>
<td>EtMgBr</td>
<td>-</td>
<td>CuBr-SMe₂ (0.1)</td>
<td>-78 to 0 °C</td>
<td>Et₂O</td>
<td>36</td>
</tr>
</tbody>
</table>

Consequently, we turned our attention to using Grignard reagents for the conjugate addition. We treated 4.11 with ethyl magnesium bromide and 0.1 equivalent of copper iodide and got the desired product in 47% yield. Note that there are currently no examples in the literature of Grignard-based copper-catalyzed conjugate additions of pyrrolinones. We were also able to get this reaction to work using CuBr-SMe₂ to yield the product in 36% yield. While we were working to optimize this reaction, we tried the
tandem CAA of 4.11 using the conditions in entry 4. Instead of capturing the enolate intermediate with TMSCl, we added 4-methoxybenzyl bromide to form N-Boc-4-butyl-3-(4-methoxybenzyl)-2-pyrrolidinone in 7% yield. We did not try this reaction using the condition in entry 6. After varying the different copper sources, solvent and temperature, we were not able to improve the yield of the conjugate addition of 4.11. These results led us to move on to a different route for the synthesis of the cholesterol mimic.

4.4.2 Second generation synthesis

For the second-generation synthesis, we decided to follow the strategy in Scheme 4.6.

![Scheme 4.6 Revised retrosynthetic analysis of cholesterol mimic](image)

Like the previous synthesis, the key intermediate is the tri-substituted pyrrolidinone. Since we were unable to develop sufficient conditions for the asymmetric CAA reaction, we decided to synthesize this compound in a stepwise fashion. We envisioned alkylating the 3,4-di-substituted pyrrolinone with the 1-iodo-2-naphthylethane derivative to form the key intermediate. Therefore, this route started with the synthesis of these two coupling partners. (Scheme 4.7 and 4.8)
Scheme 4.7 Synthesis of 3,4-di-substituted pyrrolinone

The attempted synthesis of 4.17 commenced with converting N-Boc glycine to pyrrolinone 4.13 in 62% yield. Next, the free hydroxyl group was activated with tosyl chloride in the presence of diisopylethylamine to form 4.14 in 74% yield. Then, 4.15 was obtained in 99% yield via a Suzuki coupling between 4.14 and the boronic acid synthesized from 5-methylhex-1-ene. 4.15 was reduced in the presence of hydrogen gas and palladium on carbon to form pyrrolidinone 4.16. Finally, 4.16 was methylated by treating it with sec-BuLi and methyl iodide in an attempt to obtain 4.17. A product was obtained in 40% yield, but there were ambiguities in the NMR and MS data. Although the desired mass was observed, and a doublet was seen for the supposed α-methyl group, there were two new carbonyl carbons seen in the 13C NMR spectrum. One of them appeared at 214 ppm, which would suggest formation of a ketone. Further characterization was not attempted. On the other hand, the corresponding α-methylation of the N-Ts derivative of 4.16 was clearly successful as indicated by unambiguous NMR and MS data.
The starting 6-methoxy-1-tetralone, was subjected to an HWE reaction with the carbanion of triethyl phosphonoacetate to form a 2.5:1 ratio of endocyclic/exocyclic olefin as determined by the ratio of vinyl protons in the $^1$H NMR spectrum. This mixture was refluxed with 10% palladium on carbon in triglyme to form 4.21 in 55% yield over two steps. An LAH reduction of 4.21 resulted in the formation of alcohol 4.22 in 90% yield. Finally, 4.22 was transformed to the iodo derivative in 52% yield upon treated with diphosphorous diiodide in carbon disulfide.

With 4.17 and 4.23 in hand, we attempted to couple the two units together based on a literature precedent but we were unsuccessful. (Scheme 4.9) We only recovered the starting materials.
In order to find optimal conditions for the second alkylation of 4.17, we used 3-methyl-2-pyrrolidinone as a simplified version of 4.17 to run the test reactions. (Scheme 4.10) When we planned our test reactions, we had to consider a couple of factors that would affect the outcome. One factor was the nature of the protecting group on the nitrogen of the pyrrolidinone. Since we used a Boc protecting group in Scheme 4.9, which is somewhat electron-withdrawing, we used a very strong base to facilitate the reaction. In order to get the reaction to work, we initially looked to replacing the Boc group with a stronger electron-withdrawing group. Replacing the protecting group would make the α-proton more acidic, thus enhancing the formation of the enolate. Therefore, we synthesized N-tosyl-3-methyl-2-pyrrolidinone\textsuperscript{110} and tried to methylate this compound via a literature procedure.\textsuperscript{111} (Scheme 4.10, equation 1) Unfortunately, we were only able to isolate starting material from this reaction.
This result led us to return to the use of the Boc group, which would have the additional advantage of being easier to remove later in the synthesis. With this plan in mind, we tried to methylate $N$-Boc-3-methyl-2-pyrrolidinone (Scheme 4.10, equation 2). Also, we tried to methylate the $N$-Boc-3-methyl-2-thiopyrrolidinone because thiolactams are more reactive than their corresponding lactams. (Scheme 4.10, equation 3) Interestingly, we obtained the 1,2 addition of sec-BuLi instead of the desired gem-dimethyl pyrrolidinone. Our reasoning for this result is that the sec-BuLi was too bulky to access the $\alpha$-proton on the pyrrolidinone for enolate or thioenolate formation, and therefore it underwent a 1,2-addition instead. In order to remedy the problem, we used bases such as...
LDA and NaHMDS that were less nucleophilic. Since we were using weaker bases, these reactions were run on the thiolactams. (Scheme 4.10, equations 4 and 5) We were able to acquire the desired product but only in low yields using LDA (9%) and NaHMDS (20%). At this point, we decided to synthesize the cholesterol mimic through an alternative route.

4.5 Conclusion

In conclusion, we designed a compound that would serve as a fluorescent mimic of cholesterol. Though we are still working the on synthesis of this compound, we made some interesting observations of pyrrolinone and pyrrolidinones through the failed routes. First, we saw that changing the organometallic reagent from an alkylzinc to a Grignard reagent increased the yield of the copper-catalyzed conjugate addition reaction of pyrrolidiones from 17% to 47% respectively. The use of a Grignard reagent in conjugate addition of pyrrolinones provides a method of conducting these reactions that has not been shown in the literature. Also, we observed that α-methylation of 3-methyl-2-pyrrolidinone proved to be very problematic even though the literature would suggest otherwise.

4.6 Future directions

4.6.1 Third generation synthesis of cholesterol mimic

In terms of the revised strategy for the cholesterol mimic, we envisioned retrosynthetic disconnection of the imine bond leading to the precursor nitroketone A.
Application of the Michael transform to intermediate A leads to intermediates B and C. (Scheme 4.11)

Scheme 4.11 Revised retrosynthetic analysis of cholesterol mimic

With this revised route in mind, we initially focused our attention on the synthesis of intermediates B and C. Since there was a literature procedure for the synthesis of intermediate B,94 we based our synthesis on this precedent. (Scheme 4.12) For our synthesis, we modified the Reformatsky reaction to form 4.25 and the subsequent aromatization reaction from the literature procedure because these changes improved both the yield and the ease of synthesis. To finish the synthesis of B, we will need to perform an α-methylation of ketone 4.28 at the end of the synthesis as opposed to using methyl 4-bromo-2-methylcrotonate in the first step because we were unable to synthesize this compound in pure form. We formed an inseparable mixture of 4- and 2-subsituted bromo isomer when we followed a literature procedure for the synthesis.112
We are currently synthesizing intermediate C via the route in Scheme 4.13. First, alcohol 4.29\textsuperscript{112} would be oxidized to the corresponding aldehyde using pyridinium dichromate.\textsuperscript{113} Then, aldehyde 4.30 would be used in a Henry reaction with nitromethane to form intermediate C.\textsuperscript{114}

While we are synthesizing intermediates B and C, we plan to run a series of test reactions for the Michael addition. According to the literature, the Michael addition between an enamine\textsuperscript{115} or lithium enolate\textsuperscript{116} (derived from cyclohexanone) and E-nitropropene will proceed with anti-diastereoselectivity, which is the desired outcome for our synthesis. (Scheme 4.14)
Based on this literature precedent, we want to try the test reactions in Scheme 4.15.

If we are successful, we plan to apply this methodology to the Michael addition using intermediates B and C.

4.6.2 Proposed biological experiments using the cholesterol mimic

Once the synthesis of the cholesterol mimic is completed, we initially plan to measure the photochemical properties of the compound. Most important will be the determination of the compound’s fluorescence. If the photochemical properties are favorable for biological studies, we would provide the compound to collaborators to determine how well our compound mimics cholesterol and to determine whether it exhibits any undesirable toxicity. Our collaborators would then run cellular uptake and intracellular distribution studies and compare these results to the data that is available for
cholesterol. Finally, the ability of the compound to perform the function of cholesterol would be tested in the context of the SREBP-SCAP system. If the cholesterol mimic successfully passes these tests, this compound would be used to study cholesterol localization in the context of NPC.
5.1 Materials

All reactions were performed in flame or oven-dried glassware under an atmosphere of argon unless otherwise specified. THF was distilled from sodium/benzophenone ketyl radical prior to use. Alternatively, THF or toluene was passed through a solvent purification system (Innovative Technology, Inc) and stored over 4 Å molecular sieves. Triglyme, acetone, chloroform and 1,4-dioxane were all dried over 4 Å molecular sieves for at least 3 days prior to use. DCM and DMF were purchased from Acros in “extra dry” quality over molecular sieves. Anhydrous pyridine was purchased from Alfa Aesar in a ChemSeal™ bottle. Column chromatography was done using either flash chromatography with EMD silica gel 20-400 mesh or a Biotage® Isolera Prime automated chromatography system employing KP-Sil SNAP cartridges which contain normal phase silca gel. Glass and alumina-backed EMD plates with a fluorescent indicator were used to perform TLC analysis. Both sequencing grade trypsin and endoproteinase Arg-C were obtained from Promega.
5.2 Instrumentation

\(^1\)H NMR (600 MHz) and \(^{13}\)C NMR (150 MHz) spectra were obtained on a Varian INOVA 600 spectrometer. \(^1\)H NMR (500 MHz) and \(^{13}\)C NMR (125 MHz) spectra were obtained on a Bruker AVANCE III HD 500 spectrometer. \(^1\)H NMR (300 MHz) spectra were obtained on a Varian INOVA 300 spectrometer and a Bruker AVANCE III HD 400 spectrometer. Every spectrum was recorded in CDCl\(_3\) or DMSO where the solvent was used as the reference point (\(^1\)H NMR = 7.27 ppm, \(^{13}\)C NMR = 77.23 ppm in CDCl\(_3\)) or (\(^1\)H NMR = 2.50 ppm, \(^{13}\)C NMR = 39.5 ppm in DMSO). Infrared spectra were obtained on a Perkin Elmer Paragon 1000 FT-IR spectrometer or a Jasco FT/IR-6300 both equipped with an attenuated total reflection (ATR) probe. Most of the mass spectra were obtained on a Bruker microTOFII instrument. The mass spectra of compounds 2.9, 2.12, 2.13*, 2.18* and 2.18 were obtained via LC/MS using a Dionex RSLC coupled to a Bruker microTOF Q II. The samples were analyzed on an Agilent Zorbax RX-C8 column with the dimensions of 2.1x150 mm, 5 \(\mu\)m. The mobile phase was isocratic 100% acetonitrile for compounds 2.9, 2.12 and 2.18 and isocratic 2:1 acetonitrile:methylene chloride with 0.1% formic acid for compounds 2.13* and 2.18*.

Preparative liquid chromatography on a Waters Alliance e2695 HPLC with a photodiode array detector was used to purify some compounds. Melting points were recorded on a Thomas Hoover Uni-Melt\textsuperscript{®} apparatus and are uncorrected.
5.3 Synthesis

5.3.1 Experimental for Chapter 2

Methyl hyodeoxycholate (2.1) A mixture of hyodeoxycholic acid (10.01 g, 25.51 mmol) and p-toluenesulfonic acid (1.213 g, 6.38 mmol) was dissolved in methanol (100 mL) and allowed to sit unstarred for 24 h at 25 °C. Afterwards, most of the excess solvent was removed under reduced pressure. Then the residue was dissolved in ethyl acetate, washed with brine and dried over anhyd Na2SO4. Once the excess solvent was removed under reduced pressure, the resulting residue was dissolved in a small amount of ethyl acetate. Then an excess of hexanes was added that caused the product to precipitate out of solution. The mixture was filtered and yielded 10.27 g (99%) of 2.1 as a white solid. 1H NMR (CDCl3) δ 4.05 (m, 1H), 3.67 (s, 3H), 3.63 (m, 1H), 2.42-0.98 (m, 30H), 0.92 (d, 3H, J= 6.2 Hz, 21-CH3), 0.91 (s, 3H, 19-CH3), 0.64 (s, 3H, 18-CH3) (lit. 1H NMR)
3α,6α-Ditosyl-hyodeoxycholic methyl ester (2.2) Pyridine (65 mL) was added to a mixture of 2.1 (7.43 g, 18.26 mmol) and tosyl chloride (10.44 g, 54.78 mmol) at 25 °C under argon. The mixture was stirred for 4 h at 25 °C. Then ice chips were added to the reaction mixture, and a precipitate formed. The mixture was filtered and the precipitate was dissolved in DCM. This solution was washed with 1M HCl, water and brine and dried over anhyd NaSO₄. Once the excess solvent was removed, 11.26 g (86%) of 2.2 was isolated as a white amorphous solid and was used without further purification.

$^1$H NMR (CDCl₃) δ 7.78 (dd, 4H, $J = 11.6, 8.1$ Hz), 7.35 (m, 4H), 4.78 (m, 1H), 4.31 (m, 1H), 3.67 (s, 3H), 2.47 (s, 6H), 2.38-0.91 (m, 34H), 0.90 (d, 3H, $J = 6.1$ Hz 21-CH₃), 0.81 (s, 3H, 19-CH₃), 0.59 (s, 3H, 18-CH₃) (lit$^{118}$ $^1$H NMR)
**3β-Hydroxychol-5-en-24-oic acid methyl ester (2.3)** A 4:1 mixture of DMF/water (120 mL) was added to a mixture of 2.2 (7.953 g, 11.12 mmol) and potassium acetate (1.644 g, 16.69 mmol) at 25 °C, and the cloudy mixture was heated to reflux for 12 h. Upon cooling to 25 °C, the mixture was extracted with ethyl acetate, the organic layer was washed with brine and dried over anhyd NaSO₄, and the solvent was evaporated under reduced pressure. The resulting residue was purified via flash chromatography (3:1 hexanes/ethyl acetate) to yield 1.45 g (34%) of 2.3 as a white solid.

**1H NMR (CDCl₃)** δ 5.33 (m, 1H), 3.64 (s, 3H), 3.49 (m, 1H), 2.45-1.02 (m, 32H), 0.98 (s, 3H, 19-CH₃), 0.95 (d, 3H, J= 6.3 Hz, 21-CH₃), 0.66 (s, 3H, 18-CH₃) (lit. 118 1H NMR)

![Methyl 3β-(dimethyl-t-butyldimethyloxyl)chol-5-en-24-oate (2.4)](image)

**Methyl 3β-(dimethyl-t-butyldimethyloxyl)chol-5-en-24-oate (2.4)** Dry pyridine (0.67 mL) and DMF (14.8 mL) were added to a mixture of 2.3 (1.1 g, 2.60 mmol), tert-butyldimethylsilyl chloride (1.29 g, 8.58 mmol) and imidazole (2.43 g) under argon at 25 °C. The mixture was stirred for 1.5 h before it was diluted with ethyl acetate and washed with water and brine. The organic layer was dried over anhyd NaSO₄, and excess solvent was removed under reduced pressure. The crude residue was purified via flash chromatography (3:1 hexanes/ethyl acetate) to yield 1.27 g (97%) of 2.4 as a white solid.

**1H NMR (CDCl₃)** δ 5.33 (m, 1H), 3.67 (s, 3H), 3.49 (m, 1H), 2.40-1.04 (m, 32H), 1.00 (s,
3H, 19-CH₃), 0.94 (d, 3H, J= 6.3 Hz, 21-CH₃), 0.87 (s, 9H, SiC(CH₃)₃), 0.68 (s, 3H, 18-CH₃), 0.06 (s, 6H, CH₃SiCH₃) (lit.¹¹⁹¹H NMR)

\[
\text{\(\beta\)-\text{(Dimethyl-\(t\)-butylsilyloxy)chol-5-en-24-\(N\)-methoxy-\(N\)-methylamide (2.5)\)}}
\]

A 1 M solution of dimethylaluminum chloride in hexanes (20.5 mL, 18.41 mmol) was added dropwise over 5 min to a solution of \(N,O\)-dimethylhydroxylamine (1.8 g, 18.41 mmol) in dry DCM (10 mL) under argon at 0 °C. The mixture was stirred for 1 h while the temperature was allowed to rise to 25 °C. Then, a solution of 2.4 (1.83 g, 3.65 mmol) in DCM (10 mL) was added to the mixture dropwise. The solution was stirred for 4 h before it was recooled to 0 °C and quenched with a phosphate buffer (pH 8.5). After the solution was stirred for an additional 20 min, the mixture was diluted with chloroform and filtered through Celite. The filtrate was extracted with chloroform, washed with brine and dried over anhyd NaSO₄. Once the excess solvent was removed, the resulting brown residue was used without further purification.
**3β-(Dimethyl-t-butyldimethylsiloxy)chol-5-en-24-one (2.6)** A 2.2 M solution of isopropylmagnesium bromide (10.0 mL, 22.0 mmol) was added dropwise to a solution of 2.5 (0.59g, 1.10 mmol) in THF (8 mL) under argon at 0 °C. After the addition, the mixture was stirred at 0 °C until the reaction was complete as determined by TLC analysis (3:1 hexanes/ethyl acetate). Once the reaction was complete, the mixture was quenched with sat aq NH₄Cl solution. Then the mixture was extracted with diethyl ether, washed with brine and dried over anhyd NaSO₄. Excess solvent was removed under reduced pressure, and the resulting residue was purified via flash chromatography (3:1 hexanes/ethyl acetate) to yield 0.34g (60%) of 2.6 as a white solid. mp 79-85°C; ¹H NMR (CDCl₃), δ 5.32 (m, 1H), 3.49 (m, 1H), 2.64-1.13 (m, 37H), 1.10 (d, 6H, J = 6.9 Hz, -CH(CH₃)₂), 1.00 (s, 3H, 19-CH₃), 0.93 (d, 3H, J = 6.5 Hz, 21-CH₃), 0.89 (s, 9H, SiC(CH₃)₃), 0.68 (s, 3H, 18-CH₃), 0.06 (s, 6H, CH₃SiCH₃); ¹³C NMR (CDCl₃) δ 215.48, 141.52, 121.12, 72.61, 56.75, 55.86, 50.15, 42.79, 42.35, 40.82, 39.76, 37.37, 37.22, 36.56, 35.38, 32.07, 31.90, 31.88, 30.30, 29.83, 29.69, 28.13, 25.93, 24.25, 22.69, 21.04, 19.42, 18.50, 18.37, 18.31, 18.27, 11.85, -4.60; HRMS (ESI) calcd for C₃₃H₅₉O₂Si (M+H)⁺ 515.4279, found for 515.4280; IR (solid) 2929, 2857, 1713
A solution of 2.6 (0.12 g, 0.237 mmol) in THF (1.5 mL) was added dropwise to a solution of lithium aluminum hydride (0.014 g, 0.356 mmol) in THF (1.5 mL) under argon at 0 °C. The mixture was stirred at 0 °C for 1.5 h before 0.1 mL of water was added to the mixture. Then 0.1 mL of 1N NaOH and 0.3 mL of water was added to the mixture. Next, the mixture was filtered, and the filtrate was extracted with diethyl ether, washed with brine and dried over anhyd NaSO₄. Excess solvent was removed under reduced pressure, and the resulting residue was purified via flash chromatography (3:1 hexanes/ethyl acetate) to yield 0.088g (72%) of 2.7 as a white solid. mp 104-107°C; ¹H NMR (CDCl₃), δ 5.33 (m, 1H), 3.48 (m, 1H), 3.32 (s, br, 1H), 2.28-1.07 (m, 41H), 1.04 (s, 3H, 19-CH₃), 1.00 (s, 6H, -CH(CH₃)₂), 0.98-0.92 (m, 15H), 0.90 (d, 3H, J= 4.3 Hz, 21-CH₃), 0.89 (s, 9H, SiC(CH₃)₃), 0.68 (s, 3H, 18-CH₃), 0.06 (s, 6H, CH₃SiCH₃); ¹³C NMR (CDCl₃), δ 141.54, 121.13, 72.62, 68.95, 56.77, 55.99, 55.90, 54.67, 50.17, 42.80, 42.33, 39.78, 37.37, 36.57, 35.91, 35.68, 33.54, 33.12, 32.07, 31.89, 30.68, 30.55, 28.27, 28.20, 26.04, 25.93, 24.27, 21.05, 19.42, 19.06, 18.90, 18.27, 17.22, 16.68, 11.85,-4.43, -4.59; HRMS (FAB) calcd for C₃₃H₆₀O₂Si (M+H)+ 517.4435, found for 517.4479; IR (solid) 3386, 2931
3β-(tert-Butyldimethylsilyloxy)chol-5-en-24-al (2.8) A 1M solution of diisobutylaluminium hydride in hexanes (3.18 mL, 3.18 mmol) was added dropwise to a solution of 2.4 (0.799 g, 1.59 mmol) in dry DCM (22 mL) at -78 °C. After stirring for 1 h at -78 °C, the mixture was quenched with ethyl acetate and methanol. Then, aq Rochelle’s salt was added, and the mixture was stirred overnight. Afterwards, the mixture was extracted with ethyl acetate, and the extract was dried over anhyd NaSO₄. Excess solvents were removed under vacuum, and the resulting residue was purified via flash chromatography (3:1 hexanes/ethyl acetate) to yield 0.493 g (74%) of 2.8 as a beige solid. Note that corresponding alcohol was also isolated as the major impurity. ¹H NMR (CDCl₃) δ 9.78 (s, 1H), 5.31 (m, 1H), 3.49 (m, 1H), 2.52-1.04 (m, 35H), 1.00 (s, 3H, 19-CH₃), 0.97-0.92 (m, 6H), 0.91 (d, 3H, J= 4.4 Hz, 21-CH₃), 0.90 (s, 9H, SiC(CH₃)₃), 0.68 (s, 3H, 18-CH₃), 0.06 (s, 6H, CH₂SiCH₃); (lit.¹¹¹H NMR)
3β-(Dimethyl-t-butylsilyloxy)-24-azidochole-5-en (2.9) Diisopropyl azodicarboxylate (0.11 mL, 0.554 mmol) and diphenyl phosphoryl azide (0.12 mL, 0.554 mmol) were added to a solution of 2.7 (0.186 g, 0.359 mmol) and triphenylphosphine (0.141 g, 0.539 mmol) in THF (3 mL) under argon at 0 °C. The mixture was stirred at 0 °C for 10 min before it was allowed to warm to 25 °C and stirred for an additional 22 h. Afterwards, the solvent was removed under reduced pressure and the resulting residue was purified via flash chromatography (9:1 hexanes/ethyl acetate) to yield 0.193 g (99%) of 2.8 as a white solid. mp 68-72°C; ^1H NMR (300 MHz, CDCl₃), δ 5.33 (m, 1H), 3.49 (m, 1H), 3.05 (m, 1H), 2.30-1.04 (m, 38H), 1.01 (s, 3H, 19-CH₃), 0.98-0.91 (m, 12H), 0.90 (s, 9H, SiC(CH₃)₃), 0.69 (s, 3H, 18-CH₃), 0.06 (s, 6H, CH₃SiCH₃); ^13C NMR (125 MHz, CDCl₃), δ 141.56, 121.13, 72.64, 69.96, 69.69, 56.79, 55.91, 50.20, 42.83, 39.80, 37.40, 36.59, 35.85, 35.55, 32.70, 32.64, 32.28, 32.10, 31.93, 28.21, 28.09, 25.94, 21.07, 19.69, 19.53, 19.43, 18.83, 18.63, 18.14, 17.62, 11.87, 11.86, -4.58; LC/MS calcd for C₂₈H₅₉N₅NaO₃Si (M+Na)+ 564.4279, found for 564.4278; IR (film) 2929.8, 2854.1, 1471.0, 1253.2
A 10:1 THF/water solution (2 mL) was added to a mixture of 2.9 (0.150 g, 0.277 mmol) and triphenylphosphine (0.152 g, 0.579 mmol) at 25 °C under argon. This mixture was heated to reflux for 3 h and then allowed to cool to 25 °C. Then the mixture was diluted with diethyl ether, washed with brine and dried over anhyd NaSO₄. Excess solvent was removed under reduced pressure, resulting in the isolation of the crude amine. Then 2A (0.148 g, 0.332 mmol) was added to the flask, and the mixture was placed under an atmosphere of argon. Next dry DCM (6 mL) was added to the flask, and it was cooled to 0 °C. Next, N,N'-dicyclohexylcarbodiimide (0.080 g, 0.388 mmol) and 4-(dimethylamino)pyridine (0.003 g, 0.0277 mmol) were added to the reaction flask. After stirring for 10 min at 0 °C, the reaction mixture was allowed to warm 25 °C and stir for 1.5 h. Then the mixture was diluted with DCM, and the mixture was filtered. The filtrate was concentrated at reduced pressure, and the resulting residue was purified via flash chromatography (95:5 hexanes/ethyl acetate) to yield 0.107 g (41% over 2 steps) of 2.10 as a white solid. mp 191-193°C; ¹H NMR (CDCl₃), δ 6.38 (d, 0.5H, J= 10 Hz), 6.33 (d, 0.5H, J= 10 Hz), 5.35 (m, 1H), 5.30 (m, 1H), 3.98 (m, 2H), 3.75 (m, 1H), 3.47 (m, 1H), 3.20 (m, 1H), 2.35-1.03 (m, 55H), 1.01 (s, 3H, 19-CH₃), 0.99 (s, 3H, 19'-CH₃), 0.97-0.89 (m, 17H), 0.88 (s, 9H, -C(CH₃)₃), 0.87 (d, 3H, J= 2.6 Hz, 26 or 27-CH₃), 0.85 (d, 3H, J= 2.7 Hz, 26 or 27-CH₃), 0.67 (s, 3H, 18-CH₃), 0.66 (s, 3H, 18'-CH₃), 0.05 (s, 6H, CH₃SiCH₃); ¹³C NMR (CDCl₃), δ 170.03, 141.54, 140.00, 122.29, 121.11, 80.18, 72.61, 67.60, 56.79, 56.71, 56.14, 55.84, 55.80, 54.05, 53.78, 50.18, 50.12, 50.04, 42.79, 42.30, 39.81, 39.72, 39.49, 39.16, 39.00, 37.38, 37.09, 36.98, 36.77, 36.56, 36.17, 35.89, 35.77, 35.29, 32.18, 32.06, 31.90, 31.84, 31.44, 28.53, 28.41, 28.21, 28.00, 26.04, 25.93, 25.64, 24.27, 23.85, 22.81, 22.55, 21.05, 19.42, 19.36, 19.20, 18.87, 18.72, 18.60, 18.26, 17.92, 84
17.47, 11.85, -3.59, -4.60; HRMS (FAB) calcd for C_{62}H_{107}NO_{3}Si (M+Na)^+ 964.7912, 
found for 964.7928; IR (film) 2931, 1676, 1519, 1463

![Chemical Structure](image)

2.11

(20R)-3β-(tert-Butyldimethylsilyloxy)chol-5-en-24-ol (2.11) A solution of 2.4 (1 g, 1.99 mmol) in THF (4.5 mL) was added dropwise to a solution of lithium aluminum hydride (0.151 g, 3.98 mmol) in THF (4.5 mL) under argon at 0 °C. The mixture was stirred at 0 °C for 1.5 h before 0.1 mL of water was added to the mixture. Then 0.1 mL of 1N NaOH and 0.3 mL of water was added to the mixture. Next, the mixture was filtered, and the resulting solution was extracted with diethyl ether, washed with brine and dried over anhyd NaSO₄. Excess solvent was removed under reduced pressure, and the resulting residue was purified via flash chromatography (3:1 hexanes/ethyl acetate) to yield 0.633g (67%) of 2.11 as a white solid. ¹H NMR (300 MHz, CDCl₃) δ 5.33 (m, 1H), 3.62 (m, 2H), 3.49 (m, 1H), 2.31-1.04 (m, 29H), 1.01 (s, 3H, 19-CH₃), 0.96 (d, 3H, J= 6.5 Hz, 21-CH₃), 0.90 (s, 9H, SiC(CH₃)₃), 0.69 (s, 3H, 18-CH₃), 0.06 (s, 6H, CH₂SiCH₃); (lit.¹²¹¹H NMR)
(20R)-24-Azido-3β-(tert-butyldimethylsiloxy)chol-5-ene (2.12) Diisopropyl azodicarboxylate (0.33 mL, 1.654 mmol) and diphenylphosphoryl azide (0.36 mL, 1.654 mmol) were added to a solution of 2.12 (0.510 g, 1.074 mmol) and triphenylphosphine (0.423 g, 1.611 mmol) in THF (12 mL) under argon at 0 °C. The mixture was stirred at 0 °C for 10 min before it was allowed to warm to 25 °C and stirred for an additional 22 h. Afterwards, the solvent was removed under reduced pressure, and the resulting residue was purified via flash chromatography (9:1 hexanes/ethyl acetate) to yield 0.500 g (99%) of 2.12 as a white solid. mp 68-72°C; ¹H NMR (300 MHz, CDCl₃), δ 5.33 (m, 1H), 3.49 (m, 1H), 3.23 (m, 2H), 2.30-1.04 (m, 44H), 1.00 (s, 3H, 19-CH₃), 0.99-0.91 (m, 10H), 0.90 (s, 9H, SiC(CH₃)₃), 0.69 (s, 3H, 18-CH₃), 0.06 (s, 6H, CH₃SiCH₃); ¹³C NMR (125 MHz, CDCl₃), δ 141.58, 121.10, 72.63, 56.79, 55.88, 51.98, 50.20, 42.83, 42.37, 39.80, 37.40, 36.59, 35.46, 35.45, 32.94, 32.10, 31.92, 31.77, 28.74, 28.21, 26.05, 25.94, 25.51, 24.26, 21.06, 21.06, 21.06, 19.43, 18.65, 18.58, 11.86, -4.58; LC/MS calcd for C₃₀H₅₃N₃NaOSi (M+Na)+ 522.3850, found for 522.3874; IR (solid) 2934, 2856, 2097
2.13* A 10:1 THF/water solution (2.4 mL) was added to a mixture of 2.12 (0.118 g, 0.236 mmol) and triphenylphosphine (0.130 g, 0.494 mmol) at 25 °C under argon. This mixture was heated to reflux for 3 h and then allowed to cool to 25 °C. Then the mixture was diluted with diethyl ether, washed with brine and dried over anhyd NaSO₄. Excess solvent was removed under reduced pressure and this resulted in the isolation of the crude amine. Then 3β-O-cholesteryl acetic acid (0.126 g, 0.284 mmol) was added to the flask, and the mixture was placed under an atmosphere of argon. Next dry DCM (4 mL) was added to the flask, and it was cooled to 0 °C. Next, N,N’-dicyclohexylcarbodiimide (0.068 g, 0.331 mmol) and 4-(dimethylamino)pyridine (0.003 g, 0.0236 mmol) were added to the reaction flask. After stirring for 10 min at 0 °C, the reaction mixture was allowed to warm to 25 °C and stir for 1.5 h. Then the mixture was diluted with DCM, and the mixture was filtered. The filtrate was concentrated at reduced pressure, and the resulting residue was purified via the Biotage chromatography system (3% to 10% methanol in DCM) to yield 0.169g (79% over 2 steps) of 2.13* as a white solid. Note that the compound could be further purified by recrystallization in THF and water. mp 139-142 °C; ¹H NMR (300 MHz, CDCl₃), δ 6.62 (br s, 1H, NH), 5.38 (m, 1H), 5.32 (m, 1H), 3.97 (s, 2H), 3.49 (m, 1H, 3β-H), 3.23 (m, 3H), 2.38-1.04 (m, 68H), 1.02
(s, 6H, 19-CH₃), 0.99-0.91 (m, 14H), 0.90 (s, 9H, SiC(CH₃)₃), 0.87 (dd, 6H, J= 1.7, 4.9 Hz, (CH₃)₂CH)), 0.69 (s, 6H, 18-CH₃), 0.07 (s, 6H, CH₃SiCH₃); °C NMR (125 MHz, CDCl₃), δ 170.07, 141.58, 140.07, 122.27, 121.08, 80.17, 72.63, 67.65, 56.81, 56.75, 56.19, 55.93, 50.23, 50.15, 42.84, 42.36, 42.33, 39.77, 39.52, 39.18, 39.05, 37.40, 37.03, 36.80, 36.59, 36.20, 35.77, 35.39, 33.06, 32.10, 31.92, 31.89, 28.38, 28.20, 28.00, 26.12, 26.03, 25.93, 24.28, 23.84, 22.78, 22.54, 21.08, 19.41, 19.34, 18.72, 18.67, 11.86, -4.58; LC/MS calcd for C₅₉H₁₀₂NO₃Si (M+H)+ 900.7620, found for 900.7583; IR (solid) 3321.8, 2927.9, 1625.2, 1572.7

2.13 A 1M solution of TBAF in THF (0.059 mL, 0.00589 mmol) was added dropwise to a solution of 2.13* (0.053g, 0.00589 mmol) in dry THF (2 mL) at 25 °C. The mixture was stirred for 24 h at 25 °C. Afterwards, the reaction was quenched with water and extracted with chloroform. The organic layer was washed with water and dried over anhyd NaSO₄. Excess solvent was removed under reduced pressure, and the resulting residue was purified via Biotage chromatography system (0% to 7% methanol in DCM) to yield 0.0368 g (80%) of 2.13 as a white solid. mp 93-95°C; °H NMR (300 MHz, CDCl₃), δ 6.62 (br s, 1H, NH), 5.36 (m, 2H), 3.97 (s, 2H), 3.53 (m, 1H), 3.30 (m,
1H), 3.22 (m, 2H), 2.37-1.05 (m, 66H), 1.01 (s, 6H, 19-CH₃), 0.92 (t, 6H, J= 6.6 Hz), 0.87 (dd, 6H, J= 2.8, 3.8 Hz, (CH₃)₂CH)), 0.68 (s, 6H, 18-CH₃); ¹³C NMR (125 MHz, CDCl₃), δ 170.12, 140.75, 140.03, 122.29, 121.62, 80.14, 71.75, 67.59, 56.72, 56.70, 56.12, 55.85, 50.08, 50.07, 42.31, 42.29, 42.27, 39.72, 39.49, 39.17, 39.01, 37.23, 36.99, 36.77, 36.48, 36.16, 35.77, 35.39, 33.84, 33.02, 31.90, 31.86, 31.84, 31.63, 28.34, 28.21, 28.00, 26.08, 25.56, 24.89, 24.27, 24.25, 23.81, 22.81, 22.55, 21.05, 19.39, 19.35, 18.70, 18.65, 11.85; HRMS (FAB) calcd for C₅₃H₈₈NO₃ (M+H)+ 786.6759, found for 786.6453; IR (solid) 3416, 2934, 1661, 1542, 1464

2.14

*tert*-Butyl cholest-5-en-3β-yloxyacetate (2.14) A solution of cholesterol (3 g, 7.76 mmol) in toluene (40 mL) was added to a mixture of potassium *tert*-butoxide (2.61 g, 23.28 mmol) in toluene (20 mL) at 25 °C under argon. The mixture was stirred for 3 h before *tert*-butyl-bromoacetate (2.29 mL, 15.52 mmol) was added dropwise to the solution. Then the mixture was stirred for 14 h. Afterwards, the mixture was diluted with toluene, washed water and brine and dried over anhyd NaSO₄. Excess solvent was removed under reduced pressure, and the resulting residue was purified via flash chromatography (DCM) to yield 1.60 g (41%) of 2.14 as a white solid. ¹H NMR (CDCl₃) δ 5.37 (m, 1H), 4.01 (s, 2H), 3.24 (m, 1H), 2.43-1.50 (m, 13H), 1.48 (s, 9H, ³Bu), 1.44-
tert-Butyl cholest-7-keto-5-en-3β-yloxyacetate (2.15)\textsuperscript{80} A 70% solution of aq tert-butylhydrogen peroxide (2.6 mL) was added slowly to a suspension of 2.14 (1.5 g, 3.0 mmol), pyridinium dichromate (3.49 g, 9.29 mmol) and Celite (3 g) in benzene (23 mL) at 0 °C under argon. After the addition, the cooling bath was removed, and the mixture was stirred for 24 h at 25 °C. Then the mixture was filtered through Celite, and the filtrate was concentrated under reduced pressure. The resulting residue was purified via Biotage chromatography system (5% to 50% ethyl acetate in hexanes) to yield 0.794 g (52%) of 2.15 as a light brown solid. mp 55-57°C; \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}), \( \delta \) 5.68 (m, 1H), 4.01 (s, 2H), 3.36 (m, 1H), 2.61-1.51 (m, 17H), 1.47 (s, 9H), 1.37-1.21 (m, 10H), 1.18 (s, 3H, 19-CH\textsubscript{3}), 1.15-0.95 (m, 10H), 0.92 (d, 3H, \( J = 6.6 \) Hz, 21-CH\textsubscript{3}), 0.85 (dd, 6H, \( J = 2.8 \), 3.8 Hz), 0.67 (s, 3H, 18-CH\textsubscript{3}); \textsuperscript{13}C NMR (125 MHz, CDCl\textsubscript{3}), \( \delta \) : 202.22, 169.72, 164.85, 126.20, 81.69, 78.44, 66.28, 54.76, 49.93, 49.89, 45.37, 43.07, 39.45, 38.68, 38.63, 38.55, 36.16, 35.69, 28.53, 28.09, 27.98, 27.80, 26.29, 23.80, 22.80, 22.54, 21.18, 18.85, 17.25, 11.96; HRMS (FAB) calcd for C\textsubscript{33}H\textsubscript{54}NaO\textsubscript{4} (M+Na\textsuperscript{+}) 537.3914, found for 537.3918; IR (solid) 2950, 1742, 1668.
tert-Butyl cholestan-7-keto-3β-yloxyacetate (2.16) 10% Palladium on carbon (0.090 g) was added to a degassed solution of 2.15 (0.907 g, 1.76 mmol) in 1:1 methanol/DCM (20 mL) at 25 °C. The mixture was degassed again before it was placed under an atmosphere of hydrogen gas. After stirring 14 h, the mixture was filtered through a pad of Celite, and the filtrate was evaporated to dryness. The resulting residue was purified via Biotage chromatography system (6% to 50% ethyl acetate in hexanes) to yield 0.560 g (62%) of 2.16 as a light brown solid. $^1$H NMR (300 MHz, CDCl$_3$), δ 3.95 (s, 2H), 3.28 (m, 1H), 2.36-1.67 (m, 10H), 1.50 (m, 3H), 1.44 (s, 9H, -C(CH$_3$)$_3$), 1.42-1.08 (m, 12H), 1.04 (s, 3H, 19-CH$_3$), 1.03-0.90 (m, 4H), 0.88 (d, 3H, J= 6.5 Hz, 21-CH$_3$), 0.83 (dd, 6H, J= 1.8, 4.9 Hz, -CH(CH$_3$)$_2$), 0.62 (s, 3H, 18-CH$_3$); $^{13}$C NMR (125 MHz, CDCl$_3$), δ 212.34, 170.23, 81.73, 78.86, 66.36, 55.48, 55.22, 50.20, 49.09, 47.04, 46.40, 42.71, 39.69, 38.96, 36.46, 36.35, 36.20, 35.87, 34.54, 28.63, 28.33, 28.22, 27.91, 25.19, 23.98, 23.03, 22.78, 22.05, 19.00, 18.88, 12.28, 12.00; HRMS (FAB) calcd for C$_{33}$H$_{57}$O$_4$ (M+H)$^+$ 517.4251, found for 517.4233; IR (solid) 2933, 2869, 1749, 1709
Cholestan-7-keto-3β-yloxyacetic acid (2.17) \(^{78}\) Formic acid (12 mL) was added to a solution of 2.16 (0.615 g, 1.20 mmol) in diethyl ether (12 mL). This mixture was then heated to 65 °C for 4 h. Then the solvents were removed under reduced pressure. This yielded 0.542 g (99%) of 2.17 as a beige solid. mp 111-113°C; \( ^1H \) NMR (300 MHz, CDCl\(_3\)), \( \delta \) 4.14 (s, 2H), 3.41 (m, 1H), 2.40-1.11 (m, 35H), 1.10 (s, 3H), 1.08-0.94 (m, 4H), 0.92 (d, 3H, \( J = 6.6 \) Hz, 21-CH\(_3\)), 0.87 (dd, 6H, \( J = 2.8, 3.8 \) Hz, -CH(CH\(_3\))\(_2\)), 0.66 (s, 3H, 18-CH\(_3\)); \( ^{13}C \) NMR (125 MHz, CDCl\(_3\)), \( \delta \) 212.22, 173.62, 79.48, 65.47, 55.35, 55.21, 50.19, 49.05, 46.86, 46.22, 42.69, 39.67, 38.90, 36.39, 36.34, 36.04, 35.85, 34.47, 28.61, 28.21, 27.79, 25.16, 23.97, 23.02, 22.77, 22.05, 18.99, 12.27, 12.00; HRMS calcd for C\(_{29}\)H\(_{49}\)O\(_4\) (M+H\(^+\)) 461.3625, found for 461.3605; IR (solid) 3736, 2934, 2866, 1740, 1700.

2.18*

A 10:1 THF/water solution (11 mL) was added to a mixture of 2.12 (0.145 g, 0.290 mmol) and triphenylphosphine (0.159 g, 0.607 mmol) at 25 °C under argon. This mixture was heated to reflux for 3 h and then allowed to cool to 25 °C. Then the mixture was diluted with diethyl ether, washed with brine and dried over anhyd Na\(_2\)SO\(_4\).
Excess solvent was removed under reduced pressure, resulting in the isolation of the crude amine. Then 2.17 (0.160 g, 0.349 mmol) was added to the flask, and the mixture was placed under an atmosphere of argon. Next dry DCM (14 mL) was added to the flask, and it was cooled to 0 °C. Next, \(N,N'\)-dicyclohexylcarbodiimide (0.084 g, 0.407 mmol) and 4-(dimethylamino)pyridine (0.004 g, 0.0290 mmol) were added to the reaction flask. After stirring for 10 min at 0 °C, the reaction mixture was allowed to warm to 25 °C and stir for 1.5 h. Then the mixture was diluted with DCM, and the mixture was filtered. The filtrate was concentrated at reduced pressure, and the resulting residue was purified via Biotage chromatography system (3% to 10% methanol in DCM) to yield 0.202 g (76% over 2 steps) of 2.18* as a beige solid. The compound was further purified by recrystallization in THF and water. mp 164-166 °C; \(^1\)H NMR (300 MHz, CDCl\(_3\)), \(\delta\) 6.57 (br s, 1H, NH), 5.32 (m, 1H), 3.95 (s, 2H), 3.48 (m, 1H), 3.28 (m, 2H), 3.22 (m, 1H), 2.38-1.11 (m, 76H), 1.09 (s, 6H), 1.08-1.01 (m, 6H), 1.00 (s, 6H, 19-CH\(_3\)), 0.99-0.94 (m, 4H), 0.93 (d, 3H, \(J = 6.5\) Hz, 21-CH\(_3\)), 0.91 (d, 3H, \(J = 6.6\) Hz, 21'-CH\(_3\)), 0.89 (s, 9H, SiC(CH\(_3\))\(_3\)), 0.87 (dd, 6H, \(J = 2.8, 3.8\) Hz, (CH\(_3\))\(_2\)CH)), 0.67 (s, 3H, 18-CH\(_3\)), 0.65 (s, 3H, 18'-CH\(_3\)), 0.06 (s, 6H, CH\(_3\)SiCH\(_3\)); \(^{13}\)C NMR (125 MHz, CDCl\(_3\)), \(\delta\) 212.09, 170.16, 141.91, 121.44, 79.27, 72.97, 67.99, 57.13, 56.17, 55.46, 55.34, 50.50, 50.32, 49.19, 46.94, 46.39, 43.15, 42.83, 42.67, 40.11, 39.80, 39.52, 39.04, 37.71, 36.91, 36.55, 36.53, 36.47, 36.18, 35.98, 35.71, 34.97, 34.64, 34.26, 33.34, 32.41, 32.22, 28.74, 28.58, 28.33, 28.11, 26.40, 26.29, 25.31, 24.60, 24.11, 23.15, 22.90, 22.30, 22.18, 21.38, 19.77, 19.12, 19.01, 18.62, 12.40, 12.20, 12.14, -4.24; LC/MS calcd for C\(_{59}\)H\(_{102}\)NO\(_4\)Si (M+H\(^+\)) 916.7573, found for 916.7654; IR (solid) 2900, 2852, 1707, 1683, 1539
2.18 HF-pyridine (0.30 mL) was added to a solution of 2.18* (0.140 g, 0.153 mmol) in THF (4 mL) at 25 °C and stirred for 2 d. (Note: Reaction was run in a plastic container due to the use of HF-pyridine!) Afterwards, the reaction was diluted, n DCM and carefully quenched with sat aq NaHCO₃. Next, the mixture was extracted with DCM, and the organic layer was dried over anhyd Na₂SO₄. Excess solvent was removed under reduced pressure, and the resulting residue was purified via Biotage chromatography system (0% to 7% methanol in DCM) to yield 0.115 g (94%) of 2.18 as a beige solid. mp 85-88°C; ¹H NMR (300 MHz, CDCl₃), δ 6.57 (br s, 1H, NH), 5.36 (s, 1H), 3.95 (s, 2H), 3.53 (m, 1H), 3.31 (m, 2H), 3.23 (m, 1H), 2.38-1.15 (m, 63H), 1.09 (s, 6H), 1.01 (s, 6H, 19-CH₃), 1.00-0.95 (m, 4H), 0.94 (d, 3H, J = 6.5 Hz, 21-CH₃), 0.92 (d, 3H, J = 6.5 Hz, 21'-CH₃), 0.86 (dd, 6H, 3H, J = 2.8, 3.8 Hz, (CH₃)₂CH), 0.68 (s, 3H), 0.66 (s, 3H); ¹³C NMR (125 MHz, CDCl₃), δ 212.09, 170.16, 141.11, 121.98, 79.27, 72.12, 68.00, 57.09, 56.19, 55.46, 55.35, 50.42, 50.32, 49.20, 46.95, 46.40, 42.83, 42.67, 42.63, 40.09, 39.81, 39.52, 39.04, 37.58, 36.83, 36.54, 36.47, 36.18, 35.99, 35.72, 34.97, 33.34, 32.22, 31.99, 28.74, 28.58, 28.34, 28.11, 26.42, 25.31, 24.60, 24.11, 23.15, 22.90, 22.19, 21.40, 19.75, 19.13, 19.01, 12.41, 12.22, 12.14; LC/MS calcd for C₅₃H₈₇NNaO₄ (M+Na)⁺ 824.6527, found for 824.6485; IR (film) 3416, 2933, 1707, 1670, 1539
According to a general method for diazirine synthesis, anhyd ammonia was bubbled through a solution of 2.18 (0.0584 g, 0.0728 mmol) in 2:1 dry THF/methanol (3 mL) for 2 h at 0 °C. Afterwards, a solution of hydroxylamine-O-sulfonic acid (0.0387 g, 0.342 mmol) in methanol (1 mL) was added dropwise to the mixture at 0 °C, over 10 min. Next, the mixture was stirred for 1 h at 0 °C and then at 25 °C for 14 h. The mixture was then filtered, and the excess solvent was removed from the filtrate under reduced pressure. The resulting white residue was the diaziridine intermediate, which was quickly dissolved in dry methanol (5 mL) and triethylamine (0.20 mL). Then a solution of iodine (0.080 g) in dry methanol (1 mL) was added to the stirring solution until the solution remained light brown. At this point, the excess iodine was reduced by slow addition of sodium dithionite. The mixture was then diluted with chloroform, washed with brine and dried over anhyd NaSO₄. Excess solvent was removed under reduced pressure, and the resulting residue was purified via preparative HPLC to yield 0.006 g (10%) of 2.19 as a beige solid.
Conditions for prep HPLC

Solvent A = water

Solvent B = methanol

Flow rate = 4 mL/min

Column: Waters xBridge Prep C 18 5µm 10 x 50 mm reversed-phase column

1. The separation was initiated using 5% A/95% B which was held for 10 min
2. Solvent B was increased to 100% over 1 min
3. Solvent B was held at 100% for 4 min
4. Then the column was reequilibrated

$^1$H NMR (300 MHz, CDCl$_3$), $\delta$ 6.57 (m, 1H), 5.37 (m, 1H), 3.94 (s, 2H), 3.53 (m, 1H), 3.34 (m, 2H), 3.22 (m, 1H), 2.24 (m, 4H), 2.05-1.80 (m, 12H), 1.84 (m, 12H), 1.55 (s, H$_2$O), 1.52-1.04 (m, 39H), 1.26 (m, 38H), 1.02 (s, 3H), 0.93 (s, 3H), 0.85 (dd, 6H, $J$=2.6, 4 Hz), 0.68 (s, 3H, 18-CH$_3$), 0.59 (s, 3H, 18-CH$_3$); $^{13}$C NMR (125 MHz, CDCl$_3$), $\delta$ 170.32, 141.10, 122.01, 79.46, 76.99, 72.15, 67.95, 57.09, 56.19, 54.81, 52.69, 50.42, 50.17, 43.54, 42.68, 42.64, 40.09, 39.79, 39.50, 39.14, 37.59, 37.53, 36.84, 36.69, 36.40, 36.37, 36.32, 35.76, 35.73, 34.30, 33.35, 32.43, 32.22, 32.00, 30.06, 28.58, 28.34, 28.27, 26.42, 25.96, 25.19, 24.60, 24.08, 23.15, 22.89, 21.41, 21.35, 19.75, 19.14, 19.01, 12.31, 12.22, 11.79; HRMS (FAB) calcd for C$_{53}$H$_{87}$N$_3$O$_3$ (M+H)$^+$ 814.6820, found for 814.6773; UV abs. 349.5 and 366.5 nm; IR (solid) 3384, 1652, 1635, 1455
5.3.2 Experimental for chapter 3

5.3.2.1 Typical procedure of trypsin digestion of NPC 2

Preparation of stock solutions for digest

*Preparation of trypsin stock solution:*

50 µl of 1 mM HCl was added to the 25 µg of sequencing grade trypsin in order to make a 0.5 µg/µl solution. Note: Since the optimal pH for trypsin activity is between 7.5 and 8.5, storage under acidic conditions will help to maintain the activity of this enzyme.

*Preparation of NPC 2 stock solution:*

The frozen stock of NPC 2 had a concentration of 0.5 µg/µl in 12.5 mM NH₄OAc, pH= 4.5. Therefore, 60 µl of the stock solution was set aside for this experiment. The pH of the solution was changed to ~7.5 by adding 1M Tris buffer, pH= 8.

*Preparation of trypsin inhibitor (chicken egg white):*

1 mg of trypsin inhibitor was dissolved in 2 ml of PBS buffer. Therefore the solution had a concentration of 0.5 mg/ml or 0.5 µg/µl.

*1 M DL-dithiothreitol (DTT):*

0.0771 g of DTT was dissolved in 500 µl of 50 mM Tris, pH=8

*1 M Iodoacetamide (IAA):*
0.0740 g of IAA was dissolved in 400 µL of 50 mM Tris, pH=8

Trypsin digestion of NPC 2

1. 37 mg of urea was added to the stock solution of NPC 2. (Note: At this point, the concentration of urea is 8 M and the volume increased to 78 µL.)

2. Next, 3.9 µL of 1 M DTT was added to the NPC 2 solution and the mixture was incubated for 1 h at 25 °C. The final concentration of DTT in the tube was 50 mM

3. Afterwards, 15.6 µL of 1 M IAA was added to the tube containing NPC 2 and it was incubated for 1 h at 25 °C.

4. 22.5 µL of 50 mM Tris pH=8 was added to the mixture to change the concentration of urea to 4 M.

5. 20 µL of the NPC 2 solution was transferred to another eppendorf and set aside (0 time point).

6. 5 µL of the trypsin solution was added to the NPC 2 tube. Then 1 M Tris, pH=8 was used to adjust the pH of the mixture to approximately pH= 7.5. After the pH adjustment, the tube was incubated at 37 °C.

7. 20 µL aliquots of the NPC 2 digestion was transferred to an eppendorf after the following incubation periods: 0.5 h, 1 h, 2 h, 3 h and 14 h. Following each transfer, 1 µl of trypsin inhibitor (Egg White) added to the extracted digestion mixture. Afterwards, the sample were stored in the refrigerator until the next day. This was done with all of the samples with the exception of the 14-h incubation sample.

8. The following day, the digestion was analyzed on a Tricine gel.123
5.3.2.2 Typical procedure for endoproteinase Arg-C digestion of NPC 2

Preparation of stock solutions for digest
- 1 M DTT, 1 M IAA and NPC 2 stock solution were prepared using the procedure from the trypsin digest. Activation buffer was provided by the supplier of the protease.

*Incubation buffer:*

100 mM Tris-HCl, 10 mM CaCl₂, pH= 7.6

*2% Chaps detergent in Incubation buffer:*

1 mg of Chaps was dissolved in 50 µL of Incubation buffer

Preparation of tubes for digest

*Tube 1:*

1. 10 µL of NPC 2 solution
2. 10 µL of Incubation buffer

*Tube 2:*

1. 10 µL of NPC 2 solution
2. 75 µL of Incubation buffer
3. 10 µL of Activation buffer

*Tube 3:*

99
1. 10 µL of NPC 2 solution
2. 50 µL of Incubation buffer with 2% Chaps
3. 10 µL of Incubation buffer
4. 10 µL of Activation buffer

Preparation of tubes 4 and 5

1. 10 µL NPC 2 solution was added to tubes 4 and 5
2. Then 40 µL of Incubation buffer was added to each tube.
3. 2.5 µL of 1 M DTT was added to each tube. Then 0.024 mg of urea was added to tube 5. Both tubes were incubated at 25 °C for 1h. (Note: the concentration of DTT = 50 mM and the concentration of urea = 8 M.)
4. Next, 10 µL of 1 M IAA was added to each tube. The tubes were incubated at 25 °C for 1h.
5. After the incubation period, 22.5 µL of Incubation buffer and 10 µL of Activation buffer was added to the tubes.

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<th>Tube</th>
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<td>2</td>
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<td>3</td>
<td>NPC 2 in 1% Chaps detergent w/Arg-C</td>
</tr>
<tr>
<td>4</td>
<td>NPC 2 (incubated w/DTT and IAA) w/Arg-C</td>
</tr>
</tbody>
</table>
Arg-C digestion of NPC 2

1. 5 µL of Arg-C was added to tubes 2-5.
2. All of the tubes were incubated at 37 °C for 14 h.
3. The digestion in tubes 2-4 was stopped by boiling the samples at 95 °C for 10 min.
4. For tube 5, which contains urea, formic acid was added to the mixture.
   (Concentration of formic acid in the tube = 0.5%)
5. The digestion was analyzed on a Tricine gel

5.3.3 Experimental for chapter 4

\[
\text{N-Boc-3-methyl-2-pyrrolidinone (4.8)}
\]

Di-tert-butyl dicarbonate (0.550 g, 2.52 mmol) and DMAP (0.216 g, 1.77 mmol) were added sequentially to a solution of 3-methy-2-pyrrolidinone (0.250 g, 2.52 mmol) in dry DCM at room temperature. The brown solution was stirred overnight. Then the mixture was washed with 1M HCl and brine and dried over anhyd MgSO₄. Excess solvent was removed under reduced pressure, and the resulting solution was purified by flash chromatography (1:1
hexanes/ethyl acetate). This yielded 0.36 g (72%) of 4.8 yield as a colorless oil. \(^1\)H NMR (300 MHz, CDCl\(_3\)), \(\delta\) 3.75 (m, 1H), 3.55 (m, 1H), 2.54 (m, 1H), 2.20 (m, 1H), 1.61 (m, 1H), 1.52 (s, 9H), 1.22 (d, 3H, \(J = 7.1\) Hz) (lit.\(^{101}\) \(^1\)H NMR)

\[
\text{N-Boc-3-methyl-3-phenylselenyl-2-pyrrolidinone (4.9)}
\]

A 2.5 M solution of \(n\)-butyllithium (5.22 mL, 10.04 mmol) in hexanes was added to a solution of 2,2,6,6-tetramethylpiperidine (2.20 mL, 13.05 mmol) in dry THF (5 mL) at 0 °C under argon. After stirring for 20 min, the mixture was cooled to -78 °C, and a solution of 4.8 (2 g, 10.04 mmol) in THF (10 mL) was added. This mixture was stirred for 1 h at -78 °C. Then a solution of phenyl selenium chloride (1.92 g, 10.04 mmol) in THF (5 mL) was added to the mixture and it was stirred for an additional hour. Next, the mixture was allowed to warm to 25 °C and quenched with sat aq NH\(_4\)Cl. The mixture was extracted with ethyl acetate, washed with brine and dried over anhyd MgSO\(_4\). Once the excess solvents were removed under vacuum, the yellow oil was purified by flash chromatography (1:1 hexanes/ethyl acetate). This yielded 2.11 g (59%) of 4.9 as a yellow solid. \(^1\)H NMR (300 MHz, CDCl\(_3\)), \(\delta\) 7.63 (d, 2H, \(J = 8\) Hz), 7.37 (t, 1H, \(J = 7.2\) Hz), 7.28 (t, 2H, \(J = 7.4\) Hz), 3.55 (t, 2H, \(J = 8.9\) Hz), 3.32 (m, 1H), 2.19 (dd, 1H, \(J = 6.8, 7.3\) Hz), 1.55 (s, 3H), 1.48 (s, 9H) (lit.\(^{101}\) \(^1\)H NMR)
N-Boc-3-methyl-2-5-dihydro-pyrrolinone (4.1) Glacial acetic acid (0.12 mL) was added to a solution of 4.9 (0.20 g, 0.565 mmol) in THF (2 mL) under argon. Then 30% aq. w/w H₂O₂ (1.08 mL) was added to the mixture, and it was stirred for 1 h. Afterwards, the mixture was quenched with 1M NaHCO₃ and extracted with ethyl acetate. The organic layer was washed with brine and dried over anhyd MgSO₄. Removal of excess solvents yielded 0.058 g (52%) of 4.1 as a white solid. ¹H NMR (300 MHz, CDCl₃), [δ 6.80 (m, 1H), 4.20 (m, 2H), 1.89 (m, 3H), 1.56 (s, 9H) (lit. ¹H NMR)]

N-Boc-4-ethyl-2-pyrrolidinone (4.12) A 3 M solution of ethylmagnesium bromide (0.25 mL, 0.764 mmol) in hexanes was added to a suspension of copper(I) iodide (0.010 g, 0.0546 mmol) in diethyl ether (1 mL) at -20 °C under argon. After stirring for 15 min, the mixture was cooled to -78 °C. Then a solution of 4.11 (0.100 g, 0.546 mmol) in diethyl ether (1 mL) was added to the mixture. After stirring for 1.5 h, the mixture was quenched with sat aq NH₄Cl and allowed to warm to 25 °C. Then the mixture was extracted with diethyl ether, and the organic layer was dried over anhyd MgSO₄. Once the excess solvents were removed, the residue was purified via flash
chromatography (1:1 hexanes/ethyl acetate). This yielded 0.055 g (47%) of 4.12 in as a beige solid. $^1$H NMR (300 MHz, CDCl$_3$), δ 3.86 (dd, 1H, J = 3.3, 7.3 Hz), 3.30 (dd, 1H, J = 3, 7.7 Hz), 2.59 (dd, 1H, J = 11.4, 9 Hz), 2.20 (m, 2H), 1.50 (s, 9H), 1.46 (m, 2H), 0.92 (t, 3H, J = 7.4 Hz); (lit.$^{103}$$^1$H NMR)

Table 4.1, entry 4

$N$-Boc-4-ethyl-2-pyrrolidinone (Table 4.1, entry 4) A 1.6 M solution of $n$-butyllithium (1.02 mL, 1.64 mmol) in hexanes was added to a suspension of copper(I) iodide (0.156 g, 0.819 mmol) and TMSCl (0.104 mL, 0.819 mmol) in THF (1 mL) at -20 °C under argon. After stirring for 15 min, the mixture was cooled to -78 °C. Then a solution of 4.11$^{124}$ (0.100 g, 0.546 mmol) in THF (1 mL) was added to the mixture. After stirring for 1.5 h, the mixture was quenched with sat aq NH$_4$Cl and allowed to warm to 25 °C. Then the mixture was extracted with diethyl ether, and the organic layer was dried over anhyd MgSO$_4$. Once the excess solvents were removed, the residue was purified via flash chromatography (1:1 hexanes/ethyl acetate). This yielded Table 4.1, entry 4 in 47% (0.062 g) as a beige solid. $^1$H NMR (300 MHz, CDCl$_3$), δ 3.86 (dd, 1H, J = 3.1, 7.6 Hz), 3.29 (dd, 1H, J = 3.1, 7.7 Hz), 2.61 (dd, 1H, J = 9, 6.6 Hz), 2.22 (m, 2H), 1.51 (s, 9H), 1.42 (m, 2H), 1.30 (m, 4H), 0.89 (t, 3H, J = 6.6 Hz); (lit.$^{125}$$^1$H NMR)
tert-Butyl 4-hydroxy-2-oxo-2,5-dihydro-1H-pyrrole-1-carboxylate (4.13)\textsuperscript{106}

A 1 M solution of isopropyl chloroformate in toluene (21.4 mL, 42.81 mmol) was added dropwise to a mixture of N-Boc-glycine (5 g, 28.54 mmol), Meldrum’s acid (4.96 g, 34.25 mmol) and DMAP (8.72 g, 71.36 mmol) in dry DCM (50 mL) at 0 °C under argon. After stirring for 3 h at 0 °C, the mixture was washed with 15% KHSO\textsubscript{4} and dried over anhyd Na\textsubscript{2}SO\textsubscript{4}. Excess solvent was removed under reduced pressure. Next, the resulting crude acylated Meldrum’s acid was heated at reflux in ethyl acetate (400 mL) for 1 h. Afterwards, the solvent was evaporated under reduced pressure, and the crude compound was recrystallized from ethyl acetate to yield 3.54g (62%) of 4.13 as a light yellow solid.

\textsuperscript{1}H NMR (DMSO) \(\delta\) 12.18 (br s, 1H), 4.92 (s, 1H), 4.18 (s, 2H), 1.48 ( s, 9H); (lit.\textsuperscript{106} \textsuperscript{1}H NMR)

N-Boc-4-OTs-pyrrolinone (4.14)\textsuperscript{106} DIPEA (2.19 mL, 12.57 mmol) was added to a solution of 4.13 (1.25 g, 6.29 mmol) and tosyl chloride (1.20 g, 6.29 mmol) in dry DCM (60 mL) at 25 °C. The resulting solution was stirred for 6 h. Afterwards, the
solution was washed with 1M HCl, sat NaHCO₃ and brine. Then the mixture was dried over anhyd Na₂SO₄ and excess solvent was removed under reduced pressure. The crude residue was purified via flash chromatography (3:1 hexanes/ethyl acetate) to yield 1.94 g (87%) of **4.14** as an orange-yellow solid. ¹H NMR (CDCl₃) δ 7.86 (d, 2H, J = 8.4 Hz), 7.42 (d, 2H, J= 8 Hz), 5.75 (s, 1H), 4.22 (d, 2H, J= 1.3 Hz), 2.49 (s, 3H), 1.52 (s, 9H); (lit.¹⁰⁶ ¹H NMR)

![Chemical Structure](4.15)

**N-Boc-4-(5-methylhexyl)-pyrrolinone (4.15)**¹²⁶ A 0.5 M solution of 9-BBN in THF (9.9 mL, 4.95 mmol) was added dropwise to a solution of 5-methyl-1-hexene (0.35 mL, 3.48 mmol) in dry THF (0.1M) under argon at 25 °C. Afterwards, the mixture was stirred for 3 h. Then 1 N NaOH (3 eq) was added to the mixture and it was stirred for an additional 30 min at 25 °C. In a separate flask, dry THF (0.1 M) was added to a mixture of **4.14** (0.583 g, 1.65 mmol) and Pd(dppf)Cl₂ (0.241 g, 0.33 mmol) under argon. Next, the organoborane was added, via cannulation, to the palladium mixture. This solution was stirred at 25 °C for 1 h. The mixture was then diluted with ethyl acetate and filtered through a 1:1 celite/silica mixture. The filtrate was washed with 1N NaOH, water and brine and dried over anhyd Na₂SO₄. Once the excess solvent was removed, the resulting residue was purified via flash chromatography (3:1 hexanes/ethyl acetate) to yield 0.422 g (91%) of **4.15** as an orange solid. mp 46-48°C; ¹H NMR (300 MHz, CDCl₃), δ 0.88 (d, 2H, J= 8 Hz), 5.75 (s, 1H), 4.22 (d, 2H, J= 1.3 Hz), 2.49 (s, 3H), 1.52 (s, 9H); (lit.¹⁰⁶ ¹H NMR)
(±) N-Boc-4-(5-methylhexyl)-pyrrolidinone (4.16) 10% Palladium on carbon (0.028 g) was added to a degassed solution of 4.15 (0.275 g, 0.98 mmol) in methanol at 25 °C. The mixture was degassed again before it was placed under an atmosphere of hydrogen gas. After stirring for 22 h, the mixture was filtered through a pad of celite, and the filtrate was evaporated to dryness. This resulted in 4.16 (0.275 g, 99%) as a light brown solid that was used without further purification. mp 33-36°C, 1H NMR (300 MHz, CDCl3), δ 0.84 (d, 6H, J= 6.6 Hz), 1.15 (m, 2H), 1.27 (m, 4H), 1.43 (m, 2H), 1.50 (s, 9H), 1.67 (m, 1H), 2.21 (m, 2H), 2.57 (m, 1H), 3.29 (m, 1H), 3.84 (m, 1H); 13C NMR (125 MHz, CDCl3) δ 22.02, 22.58, 27.25, 27.42, 27.55, 27.88, 28.00, 30.90, 34.05, 38.78, 39.54, 52.08, 70.92, 72.04, 82.73, 150.15 174.03; HRMS (FAB) calcd for C16H29NO3 (M+H)+ 284.2220, found for 284.2201, IR (solid) 2928.0, 2858.0, 1754.5, 1707.2, 1311.7, 1155.0
(±) N-Boc-3-methyl-4-(5-methylhexyl)-pyrrolidinone (4.17) A 1.3 M solution of sec-butyllithium in 92:8 cyclohexane/hexane (1.26 mL, 1.64 mmol) was added dropwise to a solution of 4.16 (0.209 g, 1.023 mmol) in THF (9.5 mL) at -78 °C under argon. The mixture was stirred at -65 °C for 3 h. Then, a solution of methyl iodide (0.08 mL, 1.28 mmol) in THF (0.5 mL) was added dropwise to the mixture at -65°C. Next, the mixture was allowed to warm to 0°C over 2 h before it was quenched with sat aq NH₄Cl. After extracting the mixture with diethyl ether, the organic layer was washed sequentially with sat NaS₂O₃, water and brine and anhyd Na₂SO₄. Once the excess solvent was removed under reduced pressure, the resulting residue was purified via flash chromatography (3:1 hexanes/ethyl acetate). This reaction yielded a product (0.048 g, 42%) as a light yellow oil. The identity of this product is questionable, but it is clear that this product was not the desired one. ¹H NMR (300 MHz, CDCl₃), δ 4.60 (br s, 1H), 3.11 (m, 1H), 2.99 (m, 1H), 2.41 (m, 2H), 2.10 (m, 1H), 1.67 (m, 2H), 1.48 (m, 4H), 1.42 (s, 9H), 1.34 (qt.d, 2H, J= 2.5, 4.7 Hz), 1.25 (m, 6H), 1.14 (m, 2H), 1.06 (d, 3H, J= 6.9 Hz), 0.86(d, 6H, J= 6.6 Hz); ¹³C NMR (125 MHz, CDCl₃), δ 214.72, 156.14, 79.02, 48.22,
Trifluoroacetic acid (0.19 mL, 2.42 mmol) was added to a solution of 4.16 (0.275 g, 0.969 mmol) in dry DCM (3 mL) under argon at 0 °C. After the mixture was stirred for 1 h at 0 °C, the excess solvent was removed under reduced pressure. The resulting residue was dissolved in ethyl acetate and washed with sat aq NaHCO₃ and brine. The organic layer was dried over anhyd Na₂SO₄ and the excess solvent was removed under reduced pressure. This yielded 0.166 g (93%) of 4.18 as a light yellow solid, which was used without any further purification.

(±)-4-(5-Methylhexyl)pyrrolidinone (4.18) Trifluoroacetic acid (0.19 mL, 2.42 mmol) was added to a solution of 4.16 (0.275 g, 0.969 mmol) in dry DCM (3 mL) under argon at 0 °C. After the mixture was stirred for 1 h at 0 °C, the excess solvent was removed under reduced pressure. The resulting residue was dissolved in ethyl acetate and washed with sat aq NaHCO₃ and brine. The organic layer was dried over anhyd Na₂SO₄ and the excess solvent was removed under reduced pressure. This yielded 0.166 g (93%) of 4.18 as a light yellow solid, which was used without any further purification.

1H NMR (300 MHz, CDCl₃), δ 6.30 (br s, 1H), 3.48 (t, 1H, J = 7.7 Hz), 3.02 (dd, 1H, J = 6.6, 2.7 Hz), 2.41 (m, 2H), 2.01 (m, 2H), 1.47 (m, 4H), 1.28 (m, 5H), 1.17 (m, 2H), 0.87 (d, 6H, J = 6.6 Hz); 13C NMR (125 MHz, CDCl₃), δ 178.53, 70.88, 48.16, 38.82, 36.63, 34.96, 34.62, 32.04, 27.89, 27.72, 27.29, 26.20, 22.59, 21.98; HRMS (FAB) calcd for C₁₇H₃₂NO₃ (M+H)+ 298.2377, found for 298.2360; IR (KBr)
(±) N-Ts-4-(5-methylhexyl)-pyrrolidinone (4.19) A 1M solution of sodium hexamethyldisilyamide (1.4 mL, 1.44 mmol) was added dropwise to a solution of 4.18 (.239 g, 1.31 mmol) in THF (8 mL) at -40 °C under argon. After the solution stirred for 30 minutes at -40 °C, tosyl chloride (0.274 g, 1.44 mmol) in THF (3 mL) was added dropwise to the reaction mixture. Then the mixture was allowed to stir at -40 °C for an additional 30 min before it was quenched with 1N HCl. Afterwards, the reaction was allowed to 25 °C. Next, the mixture was extracted with ethyl acetate and washed with sat aq NaHCO₃ and brine. Then the organic layer was dried over anhyd Na₂SO₄, and the excess solvent was removed under reduced pressure. The resulting residue was purified via flash chromatography (3:1 hexanes/ethyl acetate) to yield 0.202 g (46%) of 3.6 as a light yellow solid. mp 42-44 °C; ¹H NMR (300 MHz, CDCl₃), δ 7.91 (d, 2H, J = 8.3 Hz), 7.32 (d, 2H, J = 8 Hz), 4.02 (dd, 1H, J = 7.6, 2.2 Hz), 3.42 (dd, 1H, J = 7.4, 2.5 Hz), 2.52 (dd, 1H, J = 9.1, 8.2 Hz), 2.42 (s, 3H), 2.32 (quintet, 1H, J = 8.3 Hz), 2.08 (dd, 1H, J = 8.6, 8.7 Hz), 1.85 (m, 1H), 1.63 (m, 1H), 1.48 (m, 2H), 1.37 (m, 2H), 1.23 (m, 4H), 1.12 (m, 2H), 0.85 (d, 6H, J = 6.6 Hz); ¹³C NMR (125 MHz, CDCl₃), δ 173.12, 145.27, 135.37, 129.82, 128.19, 71.10, 52.79, 39.02, 38.91, 33.94, 32.21, 32.13, 28.04, 27.54, 27.35, 26.38, 22.75, 22.16, 21.86; HRMS (FAB) calcd for C₁₉H₂₈NO₃S (M+H)+ 338.1784, found for 338.1764; IR (solid) 2924.1, 1731.2, 1356.3, 1168.1

110
(±) \(N\)-Ts-3-methyl-4-(5-methylhexyl)-pyrrolidinone (4.20) A 1M solution of sodium hexamethyldisilyamide (0.34 mL, 0.343 mmol) was added dropwise to a solution of 4.19 (0.109 g, 0.324 mmol) in THF (3 mL) at -78 °C under argon. The mixture was stirred for 1 h at -78 °C before methyl iodide (0.030 mL, 0.486 mmol) was added dropwise. After the addition, the reaction was stirred for and additional 1.5 h. Then the mixture was quenched at -78 °C with sat aq NH₄Cl and allowed to warm to 25 °C. Next, the mixture was extracted with ethyl acetate, and the organic layer was washed with brine and dried over anhyd Na₂SO₄. Once the excess solvent was removed under reduced pressure, the resulting residue was purified via flash chromatography (3:1 hexanes/ethyl acetate). This yielded 0.048 g (42%) of 4.20 as a light yellow solid. mp 47-49 °C; \(^1\)H NMR (CDCl₃), \(\delta\) 7.93 (d, 2H, \(J= 8.4\) Hz), 7.34 (d, 2H, \(J= 8.6\) Hz), 4.06 (dd, 1H, \(J= 2.1, 7.6\) Hz), 3.26 (t, 1H, \(J= 9.6\) Hz), 2.44 (s, 3H), 2.05 (m, 1H), 1.90 (m, 1H), 1.60 (m, 1H), 1.52 (m, 1H), 1.27 (m, 6H), 1.13 (d, 3H, \(J= 6.9\) Hz), 0.87 (d, 6H, \(J= 6.6\) Hz); \(^{13}\)C NMR (CDCl₃), \(\delta\) 175.54, 145.03, 135.23, 129.63, 128.01, 50.93, 44.45, 40.49, 38.74, 32.55, 27.86, 27.35, 27.27, 22.60, 22.56, 21.67, 13.67; HRMS (FAB) calcd for C₁₉H₃₀NO₃S (M+H)\(^+\) 352.1941, found for 352.1931; IR (solid) 2928.7, 2855.1, 1724.9, 1360.9, 1173.1.
Triethyl phosphonoacetate (18.6 mL, 94.0 mmol) was added dropwise to a suspension of sodium hydride (60% suspension in mineral oil, 3.76 g, 94.0 mmol) in toluene (130 mL) at 0 °C under argon. The mixture was then allowed to warm to 25 °C and stirred for 15 min. Then the mixture was cooled to 0 °C, and a solution of 6-methoxy-tetralone (8 g, 45.4 mmol) in toluene (20 mL) was added dropwise to the mixture. Next, the resulting brown solution was heated to reflux for 14 h. Afterwards, the reaction mixture was allowed to cool to 25 °C and diluted with ethyl acetate. The organic solution was washed with brine and dried over anhyd Na2SO4. Once the excess solvent was removed under reduced pressure, the resulting residue was purified with the Biotage system (5% to 50% ethyl acetate in hexanes gradient). This yielded 7.11 g (64%) of approximately a 2.5:1 (endo/exo) mixture of 4.21* as a yellow oil. $^1$H NMR (CDCl$_3$) δ 7.67 (dd, 1H, $J$ = 8.7, 5.5 Hz), 7.11 (m, 0.4H), 6.73 (m, 2H), 6.65 (m, 2H), 6.25 (m, 0.4H), 5.87 (m, 0.4H), 5.72 (s, 0.6H), 4.16 (2 overlapping q, 4H, $J$ = 7.3 Hz), 3.81 and 3.80 (2 overlapping s, 6H), 3.41 (s, 1H), 3.19 (t, 1H, $J$ = 5.3 Hz), 2.85 (t, 2H, $J$ = 6.6 Hz), 2.78 (m, 2H), 2.49 (m, 2H), 2.31 (m, 1H), 1.96 (m, 2H), 1.85 (m, 1H), 1.28 (2 overlapping t, 6H, $J$ = 7.1 Hz); (lit.$^{127}$ $^1$H NMR)
Ethyl 6-methoxy-1-naphtheneneacetate (4.21) 10% palladium on carbon (0.300 g, 1.21 mmol) was added to a degassed solution of 4.21* (3 g, 12.18 mmol) in triglyme (30 mL). The mixture was heated to reflux under argon for 2 h before it was allowed to cool to 25 °C. Next, the mixture was filtered through a pad of celite and the filtrate was concentrated. The resulting oil was purified via the Biotage system (2% to 20% ethyl acetate in hexanes). This yielded 1.36 g (46%) of 4.21 as a yellow oil. ¹H NMR (CDCl₃) δ 7.95 (d, 1H, J = 9.1 Hz), 7.71 (d, 1H, J = 8.2 Hz), 7.41 (t, 1H, J = 8 Hz), 7.27 (d, 1H, J = 6.3 Hz), 7.22 (d, 1H, J = 2.6 Hz), 7.18 (m, 1H), 4.17 (q, 2H, J = 7.2 Hz), 4.04 (s, 2H), 3.94 (s, 3H), 1.24 (t, 3H, J = 7.2 Hz); (lit. ¹H NMR)

6-methoxy-1-naphtheneneethanol (4.22) A solution of 4.21 (0.515 g, 2.11 mmol) in THF (3 mL) was added dropwise to a solution of LAH (0.160 g, 4.22 mmol) in THF (2 mL) at 0 °C under argon. After the addition, the mixture was stirred for 10 min at 0 °C, and then the mixture was allowed to warm to 25 °C and was stirred for 14 h. The reaction mixture was cooled to 0 °C and quenched with the following: 0.16 mL H₂O,
0.16 mL 1N NaOH, and 0.5 mL H₂O. After 5 min, the mixture was allowed to warm to 25 °C. Then the mixture was filtered, and the filtrate was washed with brine and dried over anhyd Na₂SO₄. Excess solvent was removed under reduced pressure. The resulting oil was purified via flash chromatography (1:1 hexanes/ethyl acetate). This yielded 0.382 g (90%) of 4.22 as a light brown oil. ¹H NMR (CDCl₃) δ 7.95 (d, 1H, J = 8.8 Hz), 7.67 (d, 1H, J = 8.1 Hz), 7.39 (t, 1H, J = 8.2 Hz), 7.23 (m, 2H), 3.98 (t, 2H, J = 6.6 Hz), 3.94 (s, 3H), 3.32 (t, 2H, J = 6.6 Hz), 1.47 (s, 1H); (lit.¹⁰⁸¹H NMR)

![4.23*]

2-(6-Methoxynaphthalen-1-yl)ethyl methanesulfonate (4.23*)¹²⁸

Methanesulfonyl chloride (0.09 mL, 1.11 mmol) was added dropwise to a solution of 4.22 (0.150 g, 0.741 mmol) in pyridine (5 mL) at 0 °C under argon. This mixture was stirred for 6 h at 0 °C. Afterwards, the reaction mixture was poured into ice and extracted 3 times with toluene. The organic layer was washed with cold 5% aq HCl and water, twice with 5% NaHCO₃ and water again. Then the organic layer was dried over anhyd Na₂SO₄, and the excess solvent was removed under reduced pressure. The resulting oil was purified via flash chromatography (3:1 hexanes/ethyl acetate). This yielded 0.186 g (90%) of 4.23* as a beige solid. mp 44-46°C; ¹H NMR (300 MHz, CDCl₃), δ 7.92 (d, 1H, J = 9.2 Hz), 7.68 (d, 1H, J = 8.2 Hz), 7.38 (t, 1H, J = 7.2 Hz), 7.25 (d, 1H, J = 97 Hz), 7.23 (dd, 1H, J = 2.7, 6.5 Hz), 7.17 (d, 1H, J = 2.6 Hz), 4.52 (t, 2H, J = 7.2 Hz), 3.93 (s, 3H, -
OCH$_3$), 3.50 (t, 2H, $J$ = 7.2 Hz), 2.78 (s, 3H, -OSO$_2$CH$_3$); $^{13}$C NMR (125 MHz, CDCl$_3$), $\delta$ 157.44, 135.22, 132.11, 127.17, 126.83, 126.17, 125.26, 124.73, 119.14, 106.83, 69.71, 55.32, 37.34, 32.85; HRMS (FAB) calcd for C$_{14}$H$_{17}$O$_4$S (M+H)$^+$ 281.0842, found for 281.0817; IR (solid) 2357.4, 1626.2, 1342.1.

4.23

1-(2-Iodoethyl)-6-methoxynaphthalene (4.23)\textsuperscript{128} A solution of 4.23* (0.050 g, 0.178 mmol) in dry acetone (2 mL) was added to a mixture of 18-crown-6 ether (0.030 g, 0.357 mmol) and potassium iodide (0.059 g, 0.357 mmol) in acetone (3 mL) at 25 °C under argon. The mixture was heated to reflux for 14 h. Afterwards, the solvent was removed under reduced pressure, and diethyl ether was added to the resulting residue. The cloudy mixture was then, filtered, and the filtrate was concentrated under reduced pressure. The resulting residue was purified via flash chromatography (3:1 hexanes/ethyl acetate). This yielded 0.024 g (43%) of 4.23 as a yellow solid. $^1$H NMR (CDCl$_3$) $\delta$ 7.89 (d, 1H, $J$= 9 Hz), 7.69 (d, 1H, $J$= 8.3 Hz), 7.39 (t, 1H, $J$= 7 Hz), 7.21 (m, 3H), 3.94 (s, 3H), 3.62 (t, 2H, $J$= 7.2 Hz), 3.45 (td, 2H, $J$= 7.1, 2 Hz); (lit.\textsuperscript{128} $^1$H NMR)

\textit{Alternative Procedure}\textsuperscript{108}

A solution of 4.22 (0.641 g, 3.17 mmol) in carbon disulfide (14 mL) was added to a round bottom flask containing diphosphorous tetraiodide (0.488 g, 0.856 mmol) at 25
°C under an atmosphere of argon. The mixture was stirred for 48 h at constant
temperature. Next the mixture was concentrated under vacuum, and the residue was
diluted with diethyl ether. The organic layer was washed with water and aqueous
potassium carbonate and dried over anhyd Na₂SO₄. Once the excess solvent was
removed under reduced pressure, the resulting mixture was purified via flash
chromatography (3:1 hexanes/ethyl acetate). This yielded 4.23 (0.516 g, 52%) as a yellow
solid. The ¹H NMR matched the spectrum from the preceding procedure.

(2E,4E)-methyl 4-(6-methoxy-3,4-dihyronaphthalen-1(2H)-ylidene)but-2-enoate 4.25\textsuperscript{127} Methyl 4-bromocrotonate (2.20 mL, 18.73 mmol) was added to a mixture
of 6-methoxy-1-tetralone (3 g, 17.03 mmol), zinc (1.34 g, 20.43 mmol) and iodine (1
crystal) in benzene (40 mL) at 25 °C. Then, the mixture was heated at reflux for 2.5 h.
Afterwards, the mixture was cooled to 0 °C, and 10% aq H₂SO₄ was added. Next, the
mixture was extracted with ethyl acetate, washed with brine and dried over anhyd
Na₂SO₄. Excess solvents were removed under reduced pressure to yield the alcohol as an
orange oil, which was dehydrated without any further purification. The oil was dissolved
THF (20 mL), and a solution of 6M HCl (20 mL) was added to this solution. The
mixture was stirred for 14 h at 25 °C . Afterwards, the mixture was extracted with ethyl
acetate, and the organic layer was washed with sat aq NaHCO₃, water and brine and dried over anhyd Na₂SO₄. Once the excess solvent was removed under reduced pressure, the resulting mixture was purified via Biotage system (5% to 50% ethyl acetate in hexanes). This yielded 4.25 (2.10 g, 48%) as an orange solid. mp 61-63°C; ¹H NMR (CDCl₃) δ 7.80 (dd, 1H, J = 15, 3.2 Hz), 7.65 (d, 1H, J = 8.9 Hz), 6.77 (dd, 1H, J = 2.7, 6.1 Hz), 6.67 (d, 2H, J = 11 Hz), 5.96 (d, 1H, J = 15 Hz), 3.82 (s, 3H), 3.77 (s, 3H), 2.80 (dt, 4H, J = 6.1, 4.7 Hz), 1.88 (quintet, 2H, J = 6.4); ¹³C NMR (125 MHz, CDCl₃), δ 168.08, 159.85, 145.04, 140.98, 140.79, 127.53, 125.98, 118.95, 118.59, 113.32, 112.89, 55.25, 51.44, 30.78, 27.32, 22.91; HRMS (FAB) calcd for C₁₆H₁₉O₃ (M+H)+ 259.1329, found for 259.1345; IR (solid) 2929, 1705, 1597

Methyl 4-(6-methoxynaphthalen-1-yl)butanoate (4.26)⁹⁴ 10% Palladium on carbon (0.115 g, 0.44 mmol) was added to a degassed solution of 4.25 (1.15 g, 4.45 mmol) in triglyme (20 mL). The mixture was heated to reflux under argon for 2 h before it was allowed to cool to 25 °C. Next, the mixture was filtered through a pad of celite, and the filtrate was concentrated. The resulting oil was purified via Biotage system (2% to 20% ethyl acetate in hexanes). This yielded 1.15 g (quant. yield) of 4.26 as a yellow solid. mp 27-29°C; ¹H NMR (CDCl₃) δ 7.99 (d, 1H, J = 9.1 Hz), 7.63 (d, 1H, J = 8.3 Hz),
7.36 (t, 1H, J = 7 Hz), 7.18 (m, 3H), 3.94 (s, 3H), 3.69 (s, 3H), 3.08 (t, 2H, J = 7.6 Hz),
2.42 (t, 2H, J = 7.3 Hz), 2.08 (quintet, 2H, J = 7.6 Hz); \(^{13}\)C NMR (125 MHz, CDCl\(_3\)),
\(\delta\) 174.04, 157.35, 137.68, 135.31, 127.36, 126.27, 125.85, 125.52, 124.19, 118.58,
106.73, 55.36, 51.64, 33.75, 32.49, 25.99; HRMS (FAB) calcd for C\(_{16}\)H\(_{19}\)O\(_3\) (M+H)\(^{+}\)
259.1329, found for 259.1347; IR (solid) 2955, 1726, 1714, 1622, 1433

4.27

4-(6-Methoxynaphthalen-1-yl)butanoic acid (4.27) Potassium hydroxide (0.365 g) was added to a solution of 4.26 (1.15 g, 4.43 mmol) in 1:1 ethanol-water (20 mL). The mixture was heated to reflux for 12 h. Afterwards, the mixture was diluted with water and extracted with diethyl ether. The aq layer was acidified with 1M HCl, which resulted in the formation of a white precipitate. The precipitate was filtered, washed twice with water and dried under vacuum to yield 0.875 g (80%) of 4.27 as a white solid. \(^{1}\)H NMR (CDCl\(_3\)) \(\delta\) 7.98 (d, 1H, J = 9.1 Hz), 7.64 (d, 1H, J = 8.3 Hz), 7.37 (t, 1H, J = 7 Hz), 7.18 (m, 3H), 3.94 (s, 3H), 3.12 (t, 2H, J = 7.5 Hz), 2.47 (t, 2H, J = 7.3 Hz), 2.10 (quintet, 2H, J = 7.5 Hz) (lit. \(^{94}\) \(^{1}\)H NMR)
7-Methoxy-3,4-dihydrophenanthren-1(2H)-one (4.28) 4.27 (0.150 g, 0.614 mmol) was added to polyphosphoric acid (~ 0.75 g) which was heated in a beaker to 90 °C. The mixture was stirred for 15 min before additional polyphosphoric acid (~ 0.75 g) was added. This mixture was heated to 100 °C and immediately allowed to cool to 60 °C. Then ice water was added to the mixture, which caused a yellow precipitate to form. The precipitate was filtered, washed with water, and dried under vacuum. This resulted in 0.103 g (74%) of 4.28 as a gray solid. ¹H NMR (CDCl₃) δ 8.09 (dd, 2H, J = 10.3, 8.7 Hz), 7.66 (d, 1H, J = 8.6 Hz), 7.24 (dd, 1H, J = 2.6, 6.6 Hz), 7.17 (d, 1H, 2.6 Hz), 3.96 (s, 3H), 3.36 (t, 2H, J = 6.1 Hz), 2.73 (t, 2H, J = 5.9 Hz), 2.29 (quintet, 2H, J = 6.6 Hz) (lit.⁹⁴ ¹H NMR)
APPENDIX A:

NMR SPECTRA
2.19
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