PROGRESS TOWARDS THE SYNTHESIS OF THE SUBSTRATE FOR THE
BACTERIAL MENAQUINONE BIOSYNTHETIC ENZYME, 1,4-DIHYDROXY-6-
NAPHTHOATE SYNTHASE

by

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Abstract

1,4-dihydroxy-6-naphthoate (DHN) synthase is one of several enzymes of the menaquinone biosynthetic pathway found in pathogenic bacteria including *Campylobacter jejuni* and *Helicobacter pylori*. It is responsible for catalyzing the formation of the 1,4-dihydroxy-6-naphthoate core of the electron transport system cofactor, menaquinone. Since humans lack the ability to synthesize menaquinone, enzymes of bacterial menaquinone biosynthesis have been targeted for inhibitor design.

In order to aid the design of potential inhibitors of DHN synthase, its mechanism of catalysis has to be conclusively proven. We have proposed two possible reaction mechanisms that can be distinguished from one another, as they release different 3-carbon byproducts in addition to DHN. In order to establish the identity of these byproducts, and thus establish DHN synthase’s mechanism of action, hundred milligram quantities of substrate, much higher than the natural quantity of enzymatically-produced substrate, have to be synthesized. Access to synthetic substrate will allow for kinetic testing of potential inhibitors.

In this thesis, progress towards the synthesis of two compounds will be presented. One is the natural substrate, CDHF, cyclic de-hypoxanthine futalosine and the other, de-carboxy CDHF, is a substrate analog devoid of a carboxylic acid functionality. We have demonstrated that an oxidative aromatic cyclization of a naphthol core successfully produces a key intermediate in the overall synthesis. This should ultimately allow for the completion of the synthesis of the substrate and the elucidation of the mechanism.
Preface

All of the data and findings in this dissertation have been independently collected by the author, Ursula Hrysio. None of the original research data that was collected by this author have been published.
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<td>3,5-dimethylpyrazole</td>
</tr>
<tr>
<td>[4Fe-4S]</td>
<td>4 iron-4 sulfur center</td>
</tr>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>atm</td>
<td>atmospheric pressure units</td>
</tr>
<tr>
<td>ATP</td>
<td>adenine triphosphate</td>
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<tr>
<td>B:</td>
<td>enzymatic base residue</td>
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<tr>
<td>BH</td>
<td>protonated enzymatic base residue</td>
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<tr>
<td>CDHF</td>
<td>cyclic de-hypoxanthine futalosine</td>
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<td>COBA</td>
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<td>EPSP</td>
<td>5-enolpyruvylshikimate-3-phosphate</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>electrospray ionization mass spectrometry</td>
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FAD  flavin adenine dinucleotide
FADH$_2$  reduced flavin adenine dinucleotide
GGCX  $\gamma$-glutamylcarboxylase
Gla  $\gamma$-carboxyglutamic acid
$K_i$  inhibition constant
KH$_2$  vitamin K hydroquinone
KIE  kinetic isotope effect
KO  vitamin K epoxide
LDA  lithium diisopropylamine
MIC$_{90}$  minimum inhibitory concentration required to inhibit the growth of 90% of organisms
mol %  mol percent
MTAN  methylthioadenosine nucleosidase
NAD$^+$  nicotinamide adenine dinucleotide
NADH  reduced nicotinamide adenine dinucleotide
NADPH  reduced nicotinamide adenine dinucleotide phosphate
NaHMDS  sodium hexamethyldisilazane
NMP  n-methyl-2-pyrrolidone
NMR  nuclear magnetic resonance
OSB  o-succinylbenzoic acid
P$_i$  phosphate
PCC  pyridinium chlorochromate
PEG  polyethylene glycol
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<tr>
<td>PIFA</td>
<td>phenyliodine (III) bis(trifluoroacetate)</td>
</tr>
<tr>
<td>pKa</td>
<td>acid dissociation constant</td>
</tr>
<tr>
<td>PP, i</td>
<td>pyrophosphate</td>
</tr>
<tr>
<td>PPTS</td>
<td>pyridinium p-toluene sulfonic acid</td>
</tr>
<tr>
<td>psi</td>
<td>pounds per square inch</td>
</tr>
<tr>
<td>p-TosOH</td>
<td>para-toluenesulfonic acid</td>
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<td>quant.</td>
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<td>ribonucleic acid</td>
</tr>
<tr>
<td>rt</td>
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</tr>
<tr>
<td>SAM</td>
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<td>SPhos</td>
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<td>TBHP</td>
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<td>tert-butyllithium</td>
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<td>tetrahydrofuran</td>
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<td>TIPSOTf</td>
<td>triisopropylsilyl trifluoromethanesulfonate</td>
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<tr>
<td>TPP</td>
<td>thiamine pyrophosphate</td>
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<tr>
<td>VKD</td>
<td>vitamin K dependant</td>
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Acknowledgements

It was an absolute privilege working in the lab of Dr. Martin Tanner. I feel that I have acquired much valuable knowledge from him about the field of Bioorganic Chemistry, both in theory and in synthesis. I was honoured to be given the opportunity to fulfill my goal of doing a graduate research project where chemical synthesis and biological theory were combined. Although I fell into several ruts along the synthetic pathway, I discovered how common this actually is in everyday research. It taught me how to effectively exercise patience, perseverance, and acceptance during those times, which is an absolute must in any work setting.

I would also like to thank my lab members who continue to work diligently every day, facing challenges and making accomplishments in their own research. I want to thank them for providing guidance on any technique or specific issue that they themselves had learned: either from themselves or from their former lab members. Their presence also was morally supportive, entertaining, and meaningful. I wish them all the best in their future endeavors.

I would finally like to thank my family for their love, humour, and support during the hard times and attempting to understand my research woes despite not having a background in Bioorganic Chemistry.
**Chapter 1: Introduction to Menaquinone and Its Biosynthesis**

Menaquinone, also known as vitamin K₂, is a methylated and variably prenylated napthoquinone with a diverse array of functions in bacteria and animals (Figure 1.1). It is synthesized *de novo* in pathogenic bacteria such as *Helicobacter pylori* and *Campylobacter jejuni* to act as a cofactor in the respiratory electron transport chain.¹ The focus of this thesis involves research on the enzymes of menaquinone biosynthesis in these organisms.

![Figure 1.1 Structure of a general menaquinone](image)

**1.1 General Properties of Naphthoquinones**

Naphthoquinones, fused six-membered rings conjugated with carbonyls, are electrophilic molecules that serve to shuttle electrons between organic molecules.² Naphthoquinones are found in the environment and can beneficial to medicine as therapeutic agents.²,³,⁴,⁵ In the environment, naphthoquinones arise from the combustion of fossil fuel, diesel fuel, and tobacco smoke.²,⁶ They are also produced on particle surfaces from the oxidation of aromatic hydrocarbons, such as naphthalene².

In the human body, naphthalene, a polyaromatic hydrocarbon, is metabolically converted into 1,2-naphthoquinone through the reactions of a series of enzymes: cytochrome P450, epoxide hydrolase, and dihydrodiol dehydrogenase (Figure 1.2).²,⁷,⁸,⁹
Naphthoquinones, as quinones, are diketone compounds, which may accept a single electron to become semiquinone radicals. Therefore, quinones have the ability to generate and propagate free radical chain reactions. A second electron transfer can then produce dihydroquinones. An example of a general quinone with these properties is shown below (Figure 1.3).  

Figure 1.2 The oxidation of naphthalene in the body

Figure 1.3 An example of a general quinone converted to a dihydroquinone via two single electron transfers
1.2 Quinones in the Environment and in Health

Reduced quinones that are present in the environment can transfer electrons to oxygen and generate reactive oxygen species which can oxidize amino acid functionalities.\textsuperscript{2,3} Furthermore, when radicals, such as hydroxyl radicals, are generated, they have the ability to react with unsaturated fatty acids of the cell membrane, causing lipid peroxidation, and ultimately the formation of malondialdehyde.\textsuperscript{3} Malondialdehyde is a carcinogenic aldehyde that can form covalent adducts with DNA and RNA bases.\textsuperscript{3,7}

Cells are protected from oxidative cellular damage by glutathione reductases that utilize glutathione as a repair molecule.\textsuperscript{11} The cysteine thiol groups of glutathione are oxidized to disulfides, and in turn, oxidized compounds are reduced back to their normal state.\textsuperscript{11} Nonetheless, the greater the concentration of semiquinones, the higher percentage of oxidized glutathione, and the less efficient the cellular repair system will be.

Although radicals are generally detrimental to cells, radical mechanisms are employed by some biological enzymes. An example of a radical enzyme is nitric oxide synthase, which is responsible for generating nitric oxide to control vascular tone in blood vessels.\textsuperscript{2,12} A large concentration of extraneous radicals in the body will shuttle electrons away from the enzyme’s cytochrome P450 site, and reduce its nitric oxide-generating activity.\textsuperscript{2}

Naphthoquinones can also form covalent bonds with enzyme cysteine thiols, and thereby inactivate them (\textbf{Figure 1.4}).\textsuperscript{2} In some cases, this can be beneficial to human health; allowing them to serve as potential anticancer agents.\textsuperscript{5} For example, 1,2-napthoquinone derivatives, α- and β-lapachone, inhibit the DNA repair enzyme, DNA topoisomerase II (\textbf{Figure 1.5}).\textsuperscript{5} A study on nasopharyngeal tumor cells showed that the antitumor activity of α- and β-lapachone, evident in the formation of DNA fragments, was not the result of oxidative stress, but the result of the
compound binding to the enzyme-DNA complex and causing dissociation of the DNA before it was repaired by DNA topoisomerase II.\textsuperscript{5}

![Chemical structures](image)

Figure 1.4 The formation of a covalent bond between a quinone and an enzyme cysteine thiol\textsuperscript{2}

![Chemical structures](image)

Figure 1.5 Chemical structures of a) $\alpha$- and b) $\beta$-lapachone

1.3 **Cellular Respiration in Bacteria and the Role of Menaquinone as a Cofactor**

The single electron transferring activity of quinones is evident in menaquinone’s role in the electron-transport chain during cellular respiration.\textsuperscript{1} Cellular respiration is a mandatory process required for the survival of all living cells. In the process of cellular respiration, the energy harnessed inside organic nutrients becomes available to do work as these compounds are oxidized and broken down into smaller carbon skeletons.\textsuperscript{13} In prokaryotes, the process of cellular respiration takes place across the plasma membrane, while in eukaryotes, it takes place across the mitochondrial membrane.\textsuperscript{13,14} The membranes where cellular respiration occurs
contain several transmembrane proteins which shuttle the electrons released during the oxidation of organic substrates to a final electron acceptor. Also moving across the membrane, are the protons released as the organic compound is oxidized.\textsuperscript{13,14} A build up of these protons outside of the cell membrane generates a chemiosmotic gradient, which ultimately drives ATP biosynthesis by ATP synthases.\textsuperscript{13,14} ATP contains high energy phosphate anhydride bonds and is the energy source for all cellular energy-demanding processes.\textsuperscript{13,14} It is also the source of activated phosphate utilized by proteins such as actin and kinases.\textsuperscript{13}

The protons and electrons which travel across the membrane ultimately reduce either an organic or inorganic substrate.\textsuperscript{1} In the case of aerobic respiration, the inorganic substrate, oxygen, is reduced to water. Under anaerobic conditions, the substrate to be reduced can be an organic substrate such as fumarate.\textsuperscript{13}

Helping to shuttle electrons from the membrane-bound proteins are quinone cofactors, which are capable of being cyclically oxidized and reduced. The quinone head is the location of electron transport while the hydrophobic prenyl tail allows for membrane association.\textsuperscript{2} Many strains of bacteria, such as \textit{Escherichia coli}, utilize menaquinone when undergoing anaerobic respiration but utilize ubiquinone under aerobic conditions.\textsuperscript{15} Other strains, such as \textit{H. pylori} and \textit{C. jejuni}, utilize menaquinone as the sole quinone under all growth conditions.\textsuperscript{15} In humans, ubiquinone is the sole cofactor in respiration.\textsuperscript{15}
1.4 The Role of Menaquinone in Humans

Menaquinone has a plethora of roles in the human body, including the induction of blood coagulation\textsuperscript{16}, bone mineralization\textsuperscript{17}, and the posttranslational modification and activation of vitamin K-dependent proteins (VKD) proteins.\textsuperscript{18}

1.4.1 Menaquinone in the Vitamin K Cycle

Menaquinone is involved in the post-translational modification of VKD proteins by acting as a cofactor for $\gamma$-glutamylcarboxylase (GGCX), the enzyme responsible for catalyzing the carboxylation of glutamic acid residues in VKD proteins, to $\gamma$-carboxyglutamic acid (Gla) residues (Figure 1.6).\textsuperscript{18,19} This carboxylation is driven by the vitamin K cycle; a cycle in which vitamin K is first reduced to vitamin K hydroquinone (KH\textsubscript{2}) by vitamin K reductase, followed by an oxidization to vitamin K epoxide (KO) by vitamin K epoxidase, and finally a reduction back to vitamin K by vitamin K epoxide reductase (Figure 1.6).\textsuperscript{20}
The carboxylation of glutamic acid residues on VKD proteins makes it possible for calcium to bind to these proteins and subsequently expose a binding site required to activate processes related to bone mineralization. In addition, VKD proteins are responsible for generating the thrombin necessary for the blood clotting process.

Menaquinone supplements are commercially available for bone health in the form of menaquinone-2, -4 and -7, with menaquinone-7 having the longer half-life and greater potency due to its increased chain length. Nonetheless, a study found that although a dosage of
menaquinone-7 is beneficial for bone health, it was found to simultaneously reduce the effect of the anticoagulant, acenocoumarol.\textsuperscript{23}

1.4.2 The Bioconversion of Phylloquinone to Menaquinone

Humans do not synthesize menaquinone; rather, it is either synthesized by intestinal flora or enzymatically-derived from ingested plant vitamin K\textsubscript{1}, phylloquinone.\textsuperscript{24,25} Phylloquinone is similar in structure to menaquinone but contains only a single unsaturation in the lipophilic side chain. It is converted into menaquinone through the following steps (Figure 1.7): first, phylloquinone is reduced to a dihydroquinone via NADPH dehydrogenase or glutathione reductase; second, the side chain is cleaved by an intestinal enzyme to release menadione; third, menadione is prenylated in the tissues by geranylgeranyldiphosphate from the mevalonate pathway to yield menaquinone hydroquinone; and finally, the hydroquinone is oxidized to menaquinone-4.\textsuperscript{25}
There are several forms of menaquinone present in the human body other than vitamin K₁ (MK-4) which differ in the number of prenyl groups making up the hydrophobic side chain. The highest sources of vitamin K₁ are leafy green vegetables and vegetable oils. Vitamin K₂, which includes MK-8 and MK-9, is found in cheese and MK-7 is found in Japanese natto.

1.4.3 Menaquinone’s Role in Human Astrocyte Electron Transport

Despite the fact that humans use ubiquinone as the sole cofactor in cellular respiration, menaquinone may also have an electron transport function in brain astrocytes. Although the brain has low levels of γ-carboxylase activity, the brain regions have differing levels of vitamin K. High levels of menaquinone are found in the mid-brain to assist with glutamate metabolism. During aerobic glycolysis, the brain becomes an anoxic environment and with the
generation of lactate and pyruvate, a hypoxic response is stimulated. Under these anaerobic conditions, menaquinone is required for electron transfer during the reduction of the low potential electron acceptor, fumarate. In astrocytes, reduced menaquinone and menadione were proven to carry electrons from NADPH, bypass mitochondrial complex I, and directly reduce mitochondrial complex III to generate ATP.

1.5 The Biosynthesis of Menaquinone

One of the most interesting features of menaquinone biochemistry is the variety of pathways that lead to its biosynthesis (Figure 1.8). The biosynthesis of menaquinone begins with chorismate, derived from erythrose-4-phosphate and phosphoenolpyruvate in the shikimate pathway (Figure 1.9). Subsequently, the menaquinone pathway diverges to three separate pathways: the classical pathway, the futalosine pathway, and the adenofutalosine pathway.
1.5.1 The Formation of Menaquinone’s Aromatic Core: The Shikimate Pathway

Many of the aromatic compounds produced by microorganisms and plants, from alkaloids to the aromatic amino acids, tyrosine, tryptophan, and phenylalanine, originate from the shikimate pathway (Figure 1.9). The initial building blocks for this pathway are byproducts of
metabolism and a series of seven enzymes ultimately produce chorismate, the precursor of more complex aromatic compounds. Since humans lack a shikimate biosynthetic pathway, the enzymes of the pathway are targets for synthetic herbicides.\(^2\)

The shikimate pathway begins with the condensation of phosphoenol pyruvate (PEP) and erythrose-4-phosphate via 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase to form the initial ring scaffold compound, 3-deoxy-D-arabino-heptulosonate-7-phosphate.\(^2\) 3-Dehydroquinate synthase then produces the all-carbon ring skeleton of 3-dehydroquinate.\(^2\) The elimination of water from this intermediate by 4-dehydroquinate dehydratase generates 3-dehydroshikimate. 3-Dehydroshikimate is reduced by NADPH-dependant shikimate dehydrogenase to yield shikimate.\(^2\) Shikimate is then phosphorylated to shikimate-3-phosphate by shikimate kinase.\(^2\) An enol ether tail is added to shikimate via condensation with another phosphoenolpyruvate molecule to yield 5-enolpyruvylshikimate-3-phosphate (EPSP) by EPSP synthase.\(^2\) A final dephosphorylation via a \textit{trans}-1,4 elimination by chorismate synthase, yields chorismate.\(^2\)
1.5.2 The Classical Menaquinone Biosynthetic Pathway

Most bacteria employ the classical pathway that has been largely worked out in the model organism, *E. coli*. In this pathway, the MenA-G genes convert the chorismate from the shikimate pathway into the precursor 1,4-dihydroxy-2-naphthoate (Figure 1.8).

The classical pathway, shown in Figure 1.10, begins with the activity of MenF, which isomerizes the first intermediate, chorimate, to isochorismate.15,31
Isochorismate is then transformed into 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate via a 1,4 Stetter-like, thiamine-diphosphate-catalyzed addition of a
decarboxylated 2-ketoglutarate molecule to isochorismate by the enzyme MenD.\textsuperscript{15,32} A pyruvate molecule is then removed from the molecule by a serine protease-like enzyme, MenH to yield 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate.\textsuperscript{33} Before the work of Jiang \textit{et al.}\textsuperscript{33}, it was thought that both the 1,4 conjugate addition and the pyruvate elimination were catalyzed by MenD, since past studies found that pyruvate can spontaneously eliminate from the product of MenD.\textsuperscript{34,35}

A subsequent dehydration of 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate produces \textit{o}-succinylbenzoate via MenC, \textit{o}-succinylbenzoate synthase.\textsuperscript{15} Next, MenE, an \textit{o}-succinylbenzoate-CoA ligase, adds a CoA moiety to \textit{o}-succinylbenzoate to yield \textit{o}-succinylbenzoate-CoA.\textsuperscript{36} MenB then catalyzes an intramolecular Claisen condensation of \textit{o}-succinylbenzoate-CoA, to give 1,4-dihydroxy-2-naphthoyl-CoA.\textsuperscript{37,38} A thioesterase, encoded by yfbB, hydrolyzes the 1,4-dihydroxy-2-naphthoyl-CoA to 1,4-dihydroxy-2-naphthoate.\textsuperscript{15} Next, MenA, a 1,4-dihydroxy-2-naphthoate prenyltransferase, catalyzes both the attachment of the prenyl tail of varying length and decarboxylation.\textsuperscript{15,39} Finally, MenG, a SAM-methyltransferase, methylates the molecule to yield menaquinone.\textsuperscript{15,40}

\textbf{1.5.3 The Futalosine Pathway}

In 2008, Hiratsuka \textit{et al.} discovered a non-classical futalosine pathway in \textit{Streptomyces} after observing that the MenA-G genes were absent in these organisms (\textbf{Figure 1.8}).\textsuperscript{41} This was an intriguing finding since the intermediates in the futalosine pathway were unrelated in structure to those of the classical pathway and the isomeric 1,4-dihydroxy-6-naphthoate served as the precursor to menaquinone.\textsuperscript{41}
1.5.3.1 Discovery and Identification of the Futalosine Pathway

Using bioinformatics studies, Hiratsuka was able to identify potential genes encoding the enzymes of the alternate menaquinone biosynthetic pathway. He targeted genes that were present in bacterial species that employ the alternate pathway but absent in those that employ the classical pathway.\textsuperscript{41} Candidate enzymes were correctly identified by producing mutants. Mutant enzymes were produced by a process called gene knockout, in which the genes that normally expressed the enzyme, were made inactive.\textsuperscript{13} When each gene encoding a specific pathway enzyme was knocked out, the next step in the enzymatic pathway could not occur, thus the intermediate accumulated in the organism.\textsuperscript{13,41} Incubating each of the enzyme-deficient bacterial mutants with menaquinone (to allow for growth) and observing the products that accumulated, allowed the Dairi group to correctly order the alternate pathway enzymes.\textsuperscript{41}

1.5.3.2 The Futalosine Pathway Enzymes

The enzymes of the futalosine pathway are seen in Figure 1.8. The first futalosine pathway enzyme that acts after the formation of chorismate is MqnA. When the pathway was initially proposed, it was believed that MqnA participates in the formation of futalosine from chorismate and inosine, even though the Dairi group was unable to show activity with the enzyme in 2008.\textsuperscript{41} Following the formation of futalosine, futalosine hydrolase then cleaves off the hypoxanthine base to give de-hypoxanthine futalosine (DHF).\textsuperscript{42} DHF is then cyclized to CDHF via DHF cyclase, or MqnC, through a radical SAM mechanism.\textsuperscript{43} Next, DHN synthase
generates 1,4-dihydroxy-6-naphthoate (DHN). Finally, DHN is prenylated and methylated via enzymes with activities reminiscent of MenA and MenG respectively. Hiratsuka was able to observe activity with futalosine hydrolase and DHN synthase, however, he was unable to show activity for either MqnA or DHF cyclase. It was not until 2013 that the activities of MqnA and DHF cyclase were observed by the Begley group.

1.6 The Adenofutalosine Pathway

Both the Tanner and Dairi groups identified a modified alternate menaquinone pathway, the adenofutalosine pathway that operates in the human pathogens, H. pylori and C. jejuni (Figure 1.8). In this pathway, the nucleoside formed from chorismate bears an adenine base instead of a hypoxanthine base. In addition, a devoted hydrolase is not required and the ubiquitous nucleosidase, methylthioadenosine nucleosidase (MTAN), is used to remove the adenine base and produce DHF. The subsequent steps in the pathway are the same as in Streptomyces.

1.6.1 Recent Updates to the Adenofutalosine Pathway: Demonstration of Enzyme Activity with MqnC in the Formation of CDHF

In 2013, the Begley group was able to show activity with MqnC, which catalyzes the formation of CDHF. CDHF is produced from de-hypoxanthine futalosine (DHF) through a radical S-adenosyl methionine (SAM)-dependant mechanism (Figure 1.11). Radical SAM enzymes contain a cysteine-rich motif in their active site which bears a [4Fe-4S] cluster. The
iron sulfur cluster is responsible for initiating single electron transfers that cleave SAM into a 5’-deoxyadenosyl radical and methione. The 5’-deoxyadenosyl radical is highly reactive and can in turn, generate high energy intermediates during catalysis. In the case of CDHF synthase (MqnC), the 5’-deoxyadenosyl radical generated from SAM abstracts a hydrogen atom from DHF to generate a radical intermediate. This radical undergoes an intramolecular cyclization, and a final electron transfer back to the [4Fe-4S]^{2+} cluster, generates CDHF. Unfortunately, the activity of this enzyme was low and only trace amounts of CDHF could be produced in this fashion.

![Diagram of CDHF formation](image)

Figure 1.1 The formation of CDHF from DHF via MqnC
1.6.2 Recent Updates to the Adenofutalosine Pathway: Demonstration of Enzyme Activity with MqnA and MqnE

New research has also uncovered more information on the futalosine and adenofutalosine pathways and modifications have been made to the previously proposed routes. Initially, the production of either futalosine or adenofutalosine from chorismate via MqnA had not been clearly demonstrated. However, in 2013, the Begley group provided the answer after envisioning that the reaction may take place through either a TPP-dependent ketoacid decarboxylase-catalyzed reaction or a radical SAM enzyme mechanism. After consulting the gene database and finding a candidate gene in the neighborhood of other menaquinone biosynthetic genes, they tested a putative radical SAM enzyme found in both S. coelicolor and T. thermophilus. They were able to successfully prove that adenofutalosine synthesis is catalyzed by a unique radical SAM enzyme, adenofutalosine synthase, MqnE, which generates adenofutalosine by the addition of an adenosyl radical to the enol ether of 3-[(1-carboxyvinyl)oxy]benzoic acid (COBA) (Figure 1.12). In addition, the group also conclusively proved that MqnA catalyzes the dehydration-promoted cyclization of chorimate to give the intermediate, COBA. Thus it would appear that both pathways must involve adenofutalosine as the first formed nucleoside intermediate.
1.6.3 Recent Updates to the Adenofutalosine Pathway: Identification of 6-
Adenodeoxyfutalosine Deaminase

Another modification to the initially proposed futalosine pathway was reported by the
Raushel group that further indicates futalosine is not produced directly from chorismate. Instead, futalosine is synthesized via the deamination of 6-adenodeoxyfutalosine via 6-
adenodeoxyfutalosine deaminase, an amidohydrolase which was originally thought to deaminate adenosine.\textsuperscript{45} Thus, both of the new pathways appear to involve 6-adenodeoxyfutalosine production and a revised biosynthetic scheme is shown in Figure 1.13.

![Chemical Reaction Diagram](image_url)

**Figure 1.13** The revised menaquinone biosynthetic pathways in bacteria

### 1.7 The Design of Menaquinone Inhibitors

The appearance of different enzymes operating in plants, bacteria, and humans, allows for the design of enzyme inhibitors that may serve as pesticides and antibiotics. As mentioned
previously, since humans lack the shikimate pathway, herbicides and antibiotics can be made that have no effect on human enzymes. While human flora such as Lactobacilli and E. coli utilize the classical pathway to synthesize menaquinone, pathogens such as H. pylori, the causative agent of gastric ulcers and C. jejuni, the causative agent of inflammatory enteritis and Guillian Barre Syndrome, utilize the adenofutalosine pathway. This allows for research into the design of inhibitors that specifically inhibit the pathways of pathogens while sparing the pathway of commensal bacteria. Nonetheless, a complete separation of menaquinone biosynthesis between pathogens and non-pathogens does not exist, since Mycobacterium tuberculosis and Lactobacilli both utilize the classical pathway.

In the past, inhibitors have been synthesized to act against several of the enzymes in the menaquinone pathway. An inhibitor works by binding to the enzyme active site either reversibly or irreversibly. In reversible inhibition, the inhibitor can be competitive, uncompetitive or non-competitive, depending on whether the inhibitor binds to the same site or an alternate site on the enzyme as the normal substrate. Meanwhile, an irreversible inhibitor binds to the enzyme active site irreversibly by formation of a covalent bond. A potent inhibitor typically acts as a mechanism-based inhibitor. This means that the inhibitor contains functionalities that resemble the intermediate or the transition state of the normal reaction. Meanwhile, a substrate analog inhibitor contains functionalities similar to the normal substrate which to allow it to bind to the enzyme’s active site. Since the inhibitor is not the normal substrate, it is not consumed, and the enzyme’s activity is brought to a halt by preventing the normal substrates from binding.

Several menaquinone classical pathway inhibitors have been designed to target Mycobacterium tuberculosis and include inhibitors of MenD: acylphosphonate esters, MenE: vinylsulfonamide analogues, and MenA: aurachin RE and allylaminomethanone-A. There
is one reported example of an adenofutalosine pathway inhibitor that targets MTAN: BuTDADMe-ImmA.\textsuperscript{53}

\subsection*{1.7.1 MenD Inhibitors}

Examples of competitive inhibitors that target MenD include acylphosphonate esters (Figure \ref{fig:1.14}), substrate analogs of \(\alpha\)-ketoglutarate with \(K_i\) values as low as 700 nM.\textsuperscript{49} Although these inhibitors provided information about MenD’s catalytic mechanism, these inhibitors were found to not significantly inhibit the growth of \textit{M. tuberculosis} under aerobic or anaerobic conditions.\textsuperscript{49}

![Acylphosphonate ester inhibitor of MenD](image1.png)

\textbf{Figure 1.14} Acylphosphonate ester inhibitor of MenD

\subsection*{1.7.2 MenE Inhibitors}

Literature examples of MenE inhibitors are mechanism-based inhibitors. As previously mentioned, MenE is an acyl-CoA synthetase which catalyzes the adenylation and thioesterification of \(\alpha\)-succinylbenzoic acid (OSB) during menaquinone biosynthesis (Figure \ref{fig:1.15}).\textsuperscript{15} The most potent inhibitor of the enzyme is a vinyl sulfonamide, MeOSB-AVSN, which inhibits MenE by reacting with CoA-SH in the active site of the enzyme.\textsuperscript{50} The inhibitor contains an alkene to act as the electrophile for the nucleophilic CoA thiol to attack, yet is devoid of a leaving group (Figure \ref{fig:1.16}).\textsuperscript{50}
1.7.3 MenA Inhibitors

MenA, 1,4-dihydroxy-2-naphthoate prenyltransferase, acts to prenylate 1,4-dihydroxy-2-naphthoate (DHNA) as it is decarboxylated (Figure 1.10). MenA is inhibited by the natural product, aurachin RE, which has a C9’ chiral centre in the alkyl side chain of the molecule that is required for binding to MenA (Figure 1.17). However, synthetic inhibitors of MenA were shown to be more bactericidal than aurachin RE and were able to kill non-replicating *M. tuberculosis* without inhibiting the growth of other Gram-positive bacteria. A synthetic inhibitor, allylaminomethanone-A, was found to inhibit MenA, by structurally mimicking the enzymatic product, demethylmenaquinone (DMMK) (Figure 1.18). Allylaminomethanone-A contains a tertiary amine which is believed to either interact with Asp residues in the MenA active site or interact with divalent cations in MenA’s active site. The inhibitor also contains a stable
hydrophobic benzophenone, unlike the unstable α,β-unsaturated functionality of aurachin RE.\textsuperscript{51,52} Kurosu et al. found that successful inhibitors of *Mycobacteria* have a tertiary or secondary amine near the centre of the molecule, while inhibitors of *Staphylococcus aureus* have a secondary or tertiary amine located at the end of the molecule.\textsuperscript{51,52}

![Figure 1.17 The natural product inhibitor of MenA, aurachin RE\textsuperscript{51}](image)

![Figure 1.18 Allylaminomethanone-A, a synthetic inhibitor of MenA\textsuperscript{52}](image)

**1.7.4 MTAN Inhibitor: BuT-DADMe-ImmA**

The previous examples all describe inhibitors that target the enzymes of the classical menaquinone biosynthetic pathway. As the adenofutalosine pathway was only recently discovered, there is only one example of an inhibitor that targets this pathway by acting on the enzyme MTAN. In the normal MTAN reaction, adenofutalosine is cleaved at the N-ribosidic
bond to release adenine and de-hypoxanthine futasline (Figure 1.13).\textsuperscript{30, 53} This reaction, therefore, has a transition state that includes a ribocarbocation, a water nucleophile and an N7-protonated neutral leaving group (Figure 1.19).\textsuperscript{53} A transition state-based inhibitor of MTAN is BuT-DADMe-ImmA (Figure 1.20).\textsuperscript{53} The structural features of this inhibitor that mimic the transition state of the reaction are: a hydroxyl-pyrollidine moiety that has a pKa of 9 to mimic the ribocarbocation, a methylene bridge between the base and the sugar to mimic the 3Å distance between the sugar and the purine base, and a 9-deaza adenine which alters the conjugation of the ring and causes N-7 to be protonated as in the N7-protonated adenine leaving group.\textsuperscript{53} The inhibitor was found to be more effective at inhibiting the growth of \textit{H. pylori} than amoxicillin, metronidazole, or tetracycline and had an MIC\textsubscript{90} of < 8 ng/mL.\textsuperscript{53}

Figure 1.19 Transition state of the MTAN-catalyzed reaction B = enzymatic base\textsuperscript{53}

Figure 1.20 BuT-DADMe-ImmA, an inhibitor of MTAN\textsuperscript{53}
1.7.5 Potential Design of a DHN Synthase Inhibitor

With studies beginning to be conducted on inhibitors of adenofutalosine pathway enzymes, the long term goal of this research will look into the design of a DHN synthase inhibitor. However, before design of an effective inhibitor can be determined, more insight must first be gained about the mechanistic characteristics of the enzyme.

1.8 Proposed Reaction Mechanism of DHN Synthase

We have proposed two mechanisms for this enzymatic reaction. In the isomerization mechanism (Figure 1.21), the aldehyde is first isomerized to a ketone. The acidic proton adjacent to the open chain form of the aldehyde will be removed by an active site base. A proton transfer between the oxygens of the ene-diolate and protonation on carbon will generate the isomeric ketone. The presence of this ketone at C-6 will allow a retro-aldol cleavage to occur to release the enol(ate) of dihydroxyacetone. The product quinone will spontaneously tautomerize and form dihydroxynaphthoate (DHN).

In the dehydration mechanism (Figure 1.22), the open chain form of the hemiacetal will undergo an E1cB dehydration that results in the formation of a 1,2-dicarboxyl. A retro-aldol cleavage will then release methylglyoxal as a byproduct.
Figure 1.21 Isomerization mechanism in which dihydroxyactone is a byproduct B: (BH) is an enzymatic base (acid)

Figure 1.22 Dehydration mechanism in which methylglyoxal is a byproduct
1.9 Research Goals

The goal of this research project was to probe the mechanism and inhibition of 1,4-dihydroxy-6-naphthoate (DHN) synthase, the enzyme which catalyzes the formation of the 1,4-dihydroxy-6-naphthoate intermediate in the futalsine / adenofutalsine pathway (Figure 1.13). The first step in this process would require the chemical synthesis of the substrate, CDHF. A main goal of this project was to determine the three carbon byproduct of the DHN synthase reaction and thereby eliminate one of the two possible mechanisms. Additional goals included probing the mechanism by isotopic labelling, kinetic characterization, and inhibitor design.
Chapter 2: Progress Towards the Synthesis of a Substrate for DHN Synthase

The first step *en route* to probing the mechanism of 1,4-dihydroxy-6-napthoate (DHN) synthase is to synthesize the substrate, cyclic de-hypoxanthine futalosine (CDHF) (Figure 2.1), in hundred milligram quantities. Attempts to prepare the substrate enzymatically using recombinant MqnC have been unsuccessful in both the Tanner and Dairi groups.\textsuperscript{54} This is perhaps not surprising as radical SAM enzymes require strictly anaerobic conditions and are notoriously difficult to work with. As discussed in Chapter 1, the Begley group was able to detect activity of CDHF synthase, however, turnover was very low and only minute amounts of product could be obtained.\textsuperscript{43}

![Figure 2.1 Structure of CDHF](image)

2.1 The Synthesis of CDHF by the Yajima Group

During the initial stages of my research, Yajima *et al.* reported the synthesis of (+/-) CDHF (Figure 2.2).\textsuperscript{55} However, the yields of CDHF obtained from this synthesis are extremely low.\textsuperscript{55} The main problem was the sensitivity of the final product to either acidic or basic deprotection conditions.\textsuperscript{55} Their synthesis of (+/-) CDHF began with the introduction of two allyl groups to (1) to generate a diene (2). This diene was used as the substrate for Grubbs 2\textsuperscript{nd} generation ring closing metathesis to generate (3). Compound (3) was then oxidized at the
allylic position using CrO₃ and 3,5-DMP to yield (4). OsO₄-promoted syn dihydroxylation of (4) along with the protection of the diol as an acetonide afforded (5). LiAlH₄ reduction of the lactone carbonyl yielded (6). Subsequent protection of the alcohol with a triisopropylsilyl group, and hydrogenolysis of the benzyl ether afforded (7). The phenol was then triflated and a methoxycarbonyl was introduced with a palladium catalyst to yield (8). A carboxyl group was introduced into the cyclohexanone ring via PhI(OAc)₂/TBHP-promoted oxidation in the presence of K₂CO₃ base and the methyl ester was saponified to give (9). The group finally obtained product, (+/-) CDHF, with BiCl₃-promoted hydrolysis. However, the acidic conditions required to remove the acetonide protecting groups in the final step, also resulted in the formation of the intramolecular aldol product, compound (10).
While the Yajima group has synthesized (+/-) CDHF and established that the recombinant DHN synthase is active and will convert CDHF into DHN, the small amounts of substrate available made it impossible for them to determine the nature of the 3-carbon byproduct.55
2.2 Planned Route to the Synthesis of CDHF

Since the yield of CDHF by the Yajima group was prohibitively low, they have not conducted further mechanistic studies on DHN synthase.\textsuperscript{55} We planned to develop a higher yielding synthesis that would have allowed us to identify the byproduct and to introduce isotopic labels into the substrate.

The retrosynthetic scheme of the substrate originally suggested by Dr. Ciufoli is shown in the figure below (Figure 2.3). The key synthetic steps are a stereoselective dihydroxylation followed by lactone reduction (A$\rightarrow$B), introduction of unsaturation into the lactone (B$\rightarrow$C), oxidative aromatic cyclization and alkene reduction (C$\rightarrow$D), and Heck coupling followed by alkene reduction and saponification (D$\rightarrow$E).

Figure 2.3 Retrosynthetic scheme originally suggested by Dr. Ciufoloni
The stereoselective dihydroxylation (A→B) could involve either osmium tetroxide or ruthenium tetroxide as the oxidant, and should selectively produce the desired stereochemistry because of steric control imparted by the bulky phenyl group that blocks one face of the alkene (Figure 2.4).\textsuperscript{56, 57}

![Figure 2.4 Stereoselective dihydroxylation of the lactone double bond using osmium tetroxide](image)

The double bond in structure (B) could be introduced by the trapping of a lithiated lactone enolate with phenyl selenyl chloride, and subsequently oxidizing and eliminating phenyl selenyl oxide.\textsuperscript{58}

The oxidative aromatic cyclization has literature precedence; Hata et al. have reported that this reaction is successful on compound (D) where R = H.\textsuperscript{59} They have also shown that a variety of lactones can be produced from different phenyl ether derivatives.\textsuperscript{59} Oxidative cyclizations have been successfully performed by different research groups to synthesize lactones, spiropyans, and spirolactams.\textsuperscript{59,60,61,62,64} Many of these cyclizations require the use of a hypervalent iodine reagent to activate a phenol or anisole and produce an activated electrophilic intermediate which can then be intramolecularly trapped by a nucleophilic side chain to generate the cyclic product.\textsuperscript{59,60,62,64} Meanwhile, the Taylor group used the metal reagent, thallium (III) trifluoroacetate to promote the generation of an aromatic radical cation
that would be trapped by nucleophilic intramolecular cyclization. In addition, the Hata group used the metal reagent, silicotungstostillic acid, to promote an acid-catalyzed intramolecular cyclization.

### 2.2.1 Progress Towards the Synthesis of Natural Substrate, CDHF

Our initial approach towards the synthesis of CDHF (Figure 2.5) began with 4-(4-methylphenyl) butyric acid as the starting material (11) based on the idea that the C-4 methyl can ultimately be oxidized to give the carboxylic acid of CDHF. Compound (11) was first converted into an acyl chloride (12) to act as substrate for a Friedel Crafts acylation to form the tetralone (13) in 58% yield. Purification at this step required column chromatography to give a red syrup followed by Kugelrohr distillation to afford the tetralone. The tetralone was then brominated at the alpha position to give compound (14) in 68% yield. An issue with the bromination reaction was that di-brominated byproducts were also inevitably produced. Bromination of the tetralone was used to prime the substrate for base-promoted elimination of HBr and aromatization to form the naphthol (15) in 96% yield.
The original plan to affix a propanoic acid moiety to the para position of the naphthol involved an irregular Hoesch electrophilic addition reaction with an $\alpha,\beta$-unsaturated nitrile, acrylonitrile, followed by acid hydrolysis.\(^6^5\) However, as discovered by the Das Gupta group, only 17% of the desired carboxylic acid was formed with a similar substrate (Figure 2.6). Even though the Das Gupta group had discovered that para addition of a nitrile group was produced in higher yield when methoxynaphthalene was used as substrate, the reported yield was only 40-60%, along with 20-30% of a dimerized byproduct (Figure 2.7).\(^6^5\) Dr. Ciufolini also pointed out that the crude product is a black tar which is difficult to purify and he suggested a new approach. This approach involved a Heck coupling of para-iodinated methoxynaphthalene to ethyl acrylate.\(^6^6\)

Figure 2.5 Initial synthetic pathway towards the production of CDHF
The aryl halide substrate required for the Heck reaction required the para iodination of (15). Iodination was initially attempted on (15) using \( n \)-iodo succinamide as the electrophilic iodine source and \( p \)-toluene sulfonic acid monohydrate as the catalyst. Unfortunately, a major impurity was di-iodinated product. The solution to this problem was to protect the napthol hydroxyl as a methoxy group. Therefore, compound (15) was converted into compound (16) in 80% yield. At this point in the synthesis, only low amounts of product (16) (approximately 278 mg) remained from the total of \( \sim 15 \) g of starting material (11). Therefore, a more cost effective starting material, or new synthetic target, had to be found.
2.2.2 Progress Towards the Synthesis of De-Carboxy CDHF

Given the structural complexity of the product, CDHF, we began to think of simpler structures that would be easier to synthesize, yet may still serve as substrates for MqnC. We focussed on de-carboxy CHDF, as it should be markedly easier to synthesize (Figure 2.8). The missing carboxylate may result in weaker binding to the enzyme, but may still allow for reaction to occur and permit the characterization of the three-carbon byproduct.

Since methoxynaphthalene (17) was cheap and readily available in large quantities, it was chosen as a reasonable starting material. Iodination of methoxynaphthalene (17) selectively yielded the para iodinated methoxynaphthalene (18). Via Heck coupling, this iodinated substrate

![Pathway diagram](image-url)
was coupled to ethyl acrylate to produce (19), a pure *trans*-acrylate, with an alkene proton coupling constant of 15 Hz, in high yield. The specific procedure followed was that of Hwang *et al.* in which inexpensive N-phenyl urea served as the ligand in the coupling, rather than the costly SPhos ligand.\(^{66}\)

The acrylate double bond could not be reduced at atmospheric pressure and required 50 psi of H\(_2\) gas with Pd/C as the catalyst to yield the ester (20). This ester side chain was then saponified to the propanoic acid (21), a necessary precursor required for an intramolecular cyclization to form the five-membered ring lactone (22).

The cyclization was accomplished via an oxidative de-aromatization of the electron-rich aryl propanoic acid (21). The resulting lactone (22) was produced by a procedure requiring a hypervalent iodine agent, phenyliodine(III) bis(trifluoroacetate) (PIFA), and a heteropoly acid, silicotungstostilic acid.\(^{59}\) Hypervalent iodine reagents have the advantage that they have a low toxicity, are readily available, and are easy to handle.\(^{59}\) While either phenols or phenol ethers can undergo the oxidative cyclization, methoxynaphthalene was chosen as the starting material because it was reported that phenyl ethers are more stable and are easier to handle under the given oxidation reaction conditions.\(^{59}\) Nonetheless, a cyclization reaction that involves a phenyl ether derivative, requires a heteropoly acid such as silicotungstostilic acid for activation. This acid is readily available, less expensive, and has a low toxicity compared to heavy metal thallium reagents.\(^{59}\)

Oxidative de-aromatization is believed to take place through radical cation formation and subsequent cyclization by the carboxylic acid side chain (Figure 2.9).\(^{59}\) In the first step of the reaction, one electron is removed from the electron-rich benzyl ring to generate a radical cation.\(^{59}\) This cation is then intercepted by the carboxylic acid side chain.\(^{59}\) A second one-
electron transfer and deprotonation produces the methyloxonium that is subsequently demethylated to give product.$^{59}$

![Diagram showing the single electron transfer mechanism for the formation of lactone (22)](image)

**Figure 2.9** Single electron transfer mechanism for the formation of lactone (22)$^{59}$

The next steps involved the introduction of a double bond into the lactone ring, followed by dihydroxylation (A→C in **Figure 2.3**). This would only be possible if the alkene of (22) was reduced first. The direct reduction of this double bond using H$_2$ gas proved to be a difficult endeavor no matter which catalyst or pressure conditions were used: Pt/O$_2$, Rh/Al$_2$O$_3$, Pd/C; 1 atm or 50 psi (**Figure 2.10**). In each case; instead of reducing the double bond, re-aromatization
to the naphthol ring occurred with the opening of the lactone. Even running the reaction at 0 °C for less than 20 minutes, showed the presence of the re-aromatized naphthol (23).

![Chemical structure](image)

**Figure 2.10** The unsuccessful reduction of alkene 22

Dr. Ciufolini suggested a new method to reduce the α,β unsaturated carbonyl that involved a 1,4 conjugate addition of mercaptoethanol followed by Raney nickel reduction of the C-S bond. Although the 1,4 addition afforded the thiol (24) in 68% yield, unfortunately, Raney nickel reduction of the thiol was shown to generate a large quantity of the re-aromatized naphthol (Figure 2.11).

![Chemical structure](image)

**Figure 2.11** The unsuccessful reduction of the double bond using Raney nickel
Successful reduction of the double bond was finally achieved after borohydride reduction followed by PCC oxidation (Figure 2.12). The borohydride reduction reduced both the double bond and the ketone to generate the clear, crystalline alcohol as a 1:1 mixture of diastereomers in 62% yield (25). PCC oxidation of the alcohols afforded the ketone (26) in 97% yield.

![Chemical diagram showing the synthesis of de-carboxy CDHF]

Figure 2.12 Successful steps towards the synthesis of de-carboxy CDHF

The next step was protection of the ketone in order to carry out selective oxidation adjacent to the lactone carbonyl. The protecting group chosen was (R,R)-hydrobenzoin. The purpose of using this chiral protecting group is that deprotection requires simple hydrogenolysis and thus, acidic or basic deprotection conditions are avoided. This is important because product decomposition was a major issue for the Yajima group for their acid-catalyzed
deprotection of the carbonyl. Furthermore, with this protecting group, additional chirality is introduced into the molecule creating diastereomers. The formation of diastereomers could allow for the separation of the two original lactone enantiomers.

Although the two diastereomers (27a) and (27b) were formed during the protection reaction, the reaction went no further than 50% completion even with several different procedural modifications. The initial acid catalyst used was the mild acid catalyst, pyridinium p-toluene sulfonic acid, prepared from pyridine and p-toluene sulfonic acid monohydrate. However, this procedure gave a yield of less than 50% presumably because water is a byproduct of the reaction which must be continually removed to drive the reaction to completion. Therefore, a non-hydrated catalyst, camphorsulfonic acid, was then tested. However, using this acid as the catalyst only slightly increased the product yield. The yield was also slightly increased by attaching a drying tube filled with magnesium sulfate below the condenser to trap the water byproduct rather than using a Dean-Stark trap.

Due to the incomplete reaction and a complex NMR spectrum with the chiral protecting group, an alternate achiral protecting group, which could also be removed by hydrogenolysis, 1,2 dihydroxymethylbenzene, was tested. Unfortunately, once the reactive orthoester, prepared from 1,2 dihydroxymethylbenzene and trimethyl orthoformate, was combined with (26), protection of the carbonyl did not occur.

With the available R,R-hydrobenzoin product (27a/b) that formed, unsaturation of the lactone was attempted by first forming an enolate, trapping the enolate as a selenide, and subsequently oxidizing the selenide to promote elimination. Unfortunately, just as with the protection reaction, the yield of selenide could not be further increased to an acceptable value despite modifying reaction parameters.
The formation of a lithium enolate was first attempted using either prepared LDA from n-BuLi and diisopropyl amine or using a commercially available solution of LDA. In both trials, an excess of selenide trapping agent, phenyl selenyl chloride, was used. With both procedures, the yield of the selenide was no more than 10%. Fortunately, with the low quantity of isolated product, oxidation of the selenide (28) to the unsaturated lactone (29) was successful with an acetic acid-catalyzed hydrogen peroxide oxidation. The oxidation was easy to monitor by TLC as a non-polar, yellow-staining spot, believed to be the phenyl selenyl alcohol, appeared in addition to the product spot.

Producing the selenide was also attempted by forming a sodium enolate using NaHMDS as the base and PhSeBr, prepared from diphenyl diselenide and bromine, as the trapping electrophile. Nonetheless, the yield of selenide was not significantly increased.

Thinking that perhaps low product yield was the result of selenide decomposition, hydrogen peroxide oxidation was also attempted on crude selenide. However, this was unsuccessful as the oxidation of the crude generated an impurity with an identical polarity to that of the product, making purification difficult. Another future approach to introduce unsaturation into the lactone is to brominate next to the carbonyl and de-brominate with triethylamine.

With 17 mg of the unsaturated lactone (29a/b), Upjohn dihydroxylation of the double bond was attempted in hopes of yielding a syn-dihydroxylated product using osmium tetroxide as the oxidant (Figure 2.13). Osmium tetroxide is a highly toxic and expensive reagent which sublimes at room temperature. Fortunately, catalytic quantities of the reagent can be used if n-methyl morpholine oxide is added to the reaction mixture to re-oxidize osmium back to its +8 oxidation state. Unfortunately, the reaction was unsuccessful after three attempts. Perhaps with more substrate available, ruthenium tetroxide oxidation should be tested. Paquette et al.
found that ruthenium tetroxide worked efficiently to dihydroxylate a 5-membered lactone ring with a 81% yield in less than two minutes.\textsuperscript{57}

![Figure 2.13 Stereoselective attack by OsO\textsubscript{4} to form the dihydroxylated lactone](image)

2.2.3 Future Steps in the Synthesis of De-Carboxy CDHF

Once the lactone double bond is dihydroxylated, the next step of the synthesis would be to protect the diol as two benzyl ethers (31) (\textbf{Figure 2.14}). DIBAL reduction of the lactone ester will give the hemiacetal (32). Finally, both the hydroxyls and ketone can be deprotected by hydrogenolysis to give de-carboxy CDHF.
2.2.4 Synthesis of CDHF Using a Similar Synthetic Route as that Used for De-Carboxy CDHF

The synthesis of de-carboxy CDHF would allow one to determine whether in the absence of the 7-carboxylic acid functionality, it would still bind to *C. jejuni* DHN synthase and serve as substrate. It may also be possible to improve the activity with this compound by a “chemical rescue” experiment involving the addition of large amounts of bicarbonate to the enzymatic reaction. If the model substrate is ineffectual, the natural substrate will be synthesized from the 7-bromo-3,4-dihydronaphalen-1-(2H)-one (33) using a similar synthetic route (Figure 2.15).
The previous studies demonstrated that it was possible to prepare a key unsaturated spirolactone *en route* to the synthesis of de-carboxy CDHF. Given that the ultimate goal would be to synthesize the actual substrate, CDHF, a study was made into looking at a similar synthetic route which would allow for the incorporation of a carboxylic acid moiety (Figure 2.15). Given that the bromotetralone 33 is commercially available, we chose to start with this compound and ultimately convert the bromo functionality into a carboxylic acid ester (Figure 2.15).

![Figure 2.15 Steps towards the synthesis of CDHF](image)

2.2.4.1 Aromatic Esterification of Bromotetralone With Organozinc Reagents

Aromatic esterification can be accomplished via a halide-metal exchange between an aryl halide and a metal reagent followed by trapping of the metalized species with a carbonyl electrophile. In halide-metal exchange reactions, organozinc reagents have been used as they tolerate a variety of functional groups. Furthermore, addition of inexpensive Mg, LiCl and
ZnCl$_2$, forms stoichiometric amounts of MgCl$_2$, a Lewis acid, to further increase the reactivity of the organozinc reactant.$^{75}$ A typical reaction with bromo-anisole is shown in Figure 2.16:

![Figure 2.16 Organozinc reagents used in a halide-metal exchange reaction$^{75}$](image)

2.2.4.2 Aromatic Esterification of Bromotetralone With Copper Cyanide

Another organometallic reagent used in literature is copper cyanide.$^{76}$ With this reagent, a cyanide group will replace the aromatic ring bromine.$^{76}$ A subsequent acid hydrolysis of the cyano group will yield a carboxylic acid.$^{76}$ However, the addition of the cyano group requires harsh reaction conditions in which the mixture is refluxed in a volume of concentrated HCl.$^{76}$ Sonawane used the following procedure with 3-bromopropiophenone (Figure 2.17).$^{76}$

![Figure 2.17 Esterification reaction using copper cyanide$^{76}$](image)
2.2.4.3 The Chosen Aromatic Esterification Procedure

The procedure chosen was a halide-metal exchange reaction between the bromo tetralone 33 and n-BuLi (Figure 2.15). Prior to the halide-metal exchange, the cyclohexanone ketone was protected as an acetonide in order to prevent lithiation adjacent to the carbonyl. Compound 33 was treated with ethylene glycol and p-toluene sulfonic acid monohydrate, while refluxing in benzene. The procedure followed was identical to that followed by the Chen group, however, the acid catalyst chosen was p-toluene sulfonic acid monohydrate rather than vanadyl triflate and the byproduct water was captured in a magnesium sulfate-filled drying tube attached below the condenser rather than using a Dean-Stark trap. The reaction yield of compound 34 was 73%.

With the carbonyl protected, the halogen-metal exchange reaction could then be performed. t-BuLi is a common reagent used to provide the lithiated intermediate which will subsequently be quenched with dry carbon dioxide gas. Although it would appear that a way to avoid using gaseous carbon dioxide as the trapping agent would be to use ethyl chloroformate, it has been found in literature that this reagent would lead to a number of unfavourable byproducts including diaryl ketones and diaryl methanol (Figure 2.18).

![Chemical Reaction](image)

Figure 2.18 The formation of unfavourable byproducts with the use of ethyl chloroformate as the trapping reagent.
The procedure chosen was that in which the less reactive butyllithium, \(n\)-BuLi, reacts with the bromo tetralone to form an aryllithium which can then form an adduct with DMF (Figure 2.19).\(^7\) The adduct will then be transformed into a hemiacetal with methanol.\(^7\) In the presence of molecular iodine, this hemiacetal will become a hemiacetal-hypiodite.\(^7\) Finally, in the presence of base, the desired aryl ester will be formed following the elimination of HI.\(^7\)

![Figure 2.19: Mechanism for the formation of the product methyl ester by halogen-metal exchange](image)

In the first attempt, a small quantity of pure ester 35 was obtained in low yield. The cyclohexanone function was successfully deprotected to give compound 36 without hydrolysis of the methyl ester.

Attempting the lithiation again, the product yield was also low, however, this time, some of the product also contained compound 36 in addition to the major product, 33. As shown in Figure 2.19, hydrogen iodide is a byproduct of the halogen-metal exchange reaction, thus making deprotection of the acetal group possible to give compound 36 as one of the products.
2.2.4.4 Future Method of Aromatic Esterification

In future studies, an alternate method of installing an ester onto the tetralone without the use of gaseous carbon dioxide will be tested using a palladium(II)acetate-catalyzed cross coupling with potassium ethyl oxalate. This reaction, if successful, will allow the installation of an ester without the need to protect and subsequently deprotect the cyclohexanone (Figure 2.20).

![Chemical Reaction](image)

Figure 2.20 The installation of an ester onto the tetralone using a palladium(II) acetate catalyzed cross coupling with potassium ethyl oxalate

2.3 Future Goals for DHN Synthase Research: Analysis of the Enzymatic Reaction Mechanism

2.3.1 Coupled Enzyme Assay

Once a viable synthesis is in hand, the three-carbon byproduct will be identified using $^1$H NMR spectroscopy and spiking with authentic standards. Depending on the identity of the three-carbon byproduct, a particular enzymatic assay can be performed to monitor the kinetics of the enzyme. If the product is identified as methylglyoxal, the addition of glyoxylase I and
glutathione will generate (R)-S-lactoylglutathione which absorbs at 240 nm (Figure 2.21). However, if the product is dihydroxyacetone, a coupled enzyme assay employing two enzymes will be used (Figure 2.22). Dihydroxyacetone is first phosphorylated to give dihydroxyacetone phosphate and is then reduced by glycerol-3-phosphate dehydrogenase and NADH to generate glycerol-3-phosphate. The rate will be determined by the disappearance of NADH absorption at 340 nm. A complementary approach to obtaining kinetic data would be to directly monitor the appearance of the DHN chromophore using UV spectroscopy.

Figure 2.21 Coupled enzymatic assay for methylglyoxal produced from the isomerization mechanism

Figure 2.22 Coupled enzymatic assay for dihydroxyacetone produced from the dehydration mechanism
2.3.2 Isotopic Labelling of CDHF Substrate

Isotopic labelling will be used to probe the reaction mechanism in two ways. In the first method, CDHF is incubated with DHN synthase in D$_2$O solvent and the reaction is rapidly monitored by NMR to avoid results skewed by non-enzymatic wash-in of isotopic labels. The presence of deuterium in specific regions of the byproduct would confirm proposed reaction steps where deuteration can occur by solvent exchange. The products that would be expected from pathway A dehydration and pathway B isomerisation are shown in Figure 2.23.

![Figure 2.23 Expected byproducts formed by the enzymatic reaction in D$_2$O](image)

The second method of isotopic labelling involves the synthetic incorporation of deuterium into CDHF. We will monitor for the observance of “internal return” in which a deuterium atom in the substrate is transferred to a different carbon in the product (Figure 2.24). This would allow one to determine if a single enzymatic base is responsible for both the deprotonation of the substrate and the reprotonation at a different carbon. The observation of “internal return” requires that the proton cannot exchange with bulk solvent during the lifetime
of the enolate intermediate and has been observed with triosephosphate isomerase (Figure 2.25). The presence of deuterium in either product would indicate a one base mechanism. Of course, the absence of deuterium in the product would be consistent with either a “two-base” mechanism or with rapid solvent exchange.

Figure 2.24 Expected byproducts formed by enzymatic reaction of a deuterated substrate in H₂O

![Diagram showing CDHF (open chain form) and reaction pathways](image)

Figure 2.25 Internal return with triosephosphate isomerase

In addition, the synthesis of deuterated substrate will allow us to measure a kinetic isotope effect (KIE) and to determine whether the proposed α-proton removal is a rate
determining step (RDS) in the mechanism. If this is the case, we would expect that the deuterated substrate would react slower by a factor of 2-4. The deuterium could be incorporated into the substrate by the treatment of compound (27a/b) in basic D₂O (Figure 2.26).

![Figure 2.26 The incorporation of deuterium into the substrate](image)

2.3.3 Design of DHN Synthase Inhibitors

Future aims of this project include the synthesis and testing of potential DHN synthase inhibitor, compound 37 (Figure 2.27). Compound 37 has anionic forms that mimic the proposed enolate intermediates produced in either of the proposed DHN synthase mechanisms. If inhibition is observed with this compound, more complex versions that more closely resemble the true substrate can be prepared.
2.4 Concluding Rationale for Future DHN Synthase Research

Further studies include site-directed mutagenesis of active site residues and crystallographic studies on enzyme-inhibitor complexes. This research is important since there is no conclusive data on the mechanism of DHN synthase. Further knowledge about the DHN synthase mechanism could lead to the development of active site inhibitors which would serve as potential antibiotics against an enzyme that is specifically found in the pathogens, *H. pylori* and *C. jejuni*. The design of novel inhibitors is an essential process in keeping ahead of the evolution of newly resistant bacterial strains.
Chapter 3: Experimental Procedures for the Synthesis of CDHF and De-Carboxy CDHF

3.1 Materials and General Methods

Chemicals were purchased from Alfa Aesar, Sigma Aldrich, and Ontario Chemicals and used without further purification. Silica gel chromatography was performed using Silica Gel SiliaFlash F60 (40-63 μm, Silicycle). Triethylamine, methylene chloride, and methanol solvents were distilled over CaH$_2$ under an atmosphere of N$_2$.

$^1$H NMR spectra were obtained on either a Bruker AV300 (300 MHz field strength) or a AV400 (400 MHz field strength) NMR spectrophotometer at the UBC NMR Facility.

Mass spectra were obtained using an Esquire LC mass spectrometer at the UBC Mass Spectrometry Facility. Electrospray ionization mass spectrometry (ESI-MS) was performed to detect neutral compounds as positive ions and negatively charged compounds as negative ions.

3.2 Experimental Procedures for CDHF and De-Carboxy CDHF Synthesis

3.2.1 4-(4-Methylphenyl) butanoyl chloride (12)

The acid chloride (12) was synthesized using an identical procedure to that of Sorensen et al. $^{63}$ The product (12) was obtained in quantitative yield as a yellow oil. $^1$H NMR data was identical to that reported in the literature.$^{63}$
3.2.2 3,4-Dihydro-7-methyl-(2H)-naphthalenone (13)

Friedel Crafts acylation of (12) was performed to afford the cyclohexanone (13) using a procedure identical to Sorensen et al.\textsuperscript{63} The product was further purified via Kugelrohr distillation to afford (13) as a white solid in 58% yield. \textsuperscript{1}H NMR data was identical to that reported in the literature.\textsuperscript{63}

3.2.3 2-Bromo-3,4-dihydro-7-methyl-(2H)-naphthalenone (14)

Compound 13 (1.83 g, 11.42 mmol) was dissolved in 3 mL of diethyl ether. Bromine (0.62 mL, 24.13 mmol) was added dropwise to the reaction flask while stirring at 0 °C under argon for 0.5 h.\textsuperscript{84} The reaction mixture was diluted with 20 mL diethyl ether and washed with a 5% sodium thiosulfate solution, followed by a water wash. Solvent was removed under vacuum to yield a yellow solid. The crude product was purified by silica gel chromatography using an eluent of 1:3 ethyl acetate:petroleum ether to yield (14) as a yellow solid (1.75 g, 68%). \textsuperscript{1}H NMR data was identical to that reported in the literature.\textsuperscript{85}

3.2.4 7-Methyl-1-naphthol (15)

To aromatize the cyclohexanone ring, a modified procedure of Grimshaw et al. was followed.\textsuperscript{86} Compound 14 (1.18g, 2.51 mmol) was dissolved in 25 mL of DMF and the resulting solution was charged with LiBr (987 mg, 11.4 mmol) and Li\textsubscript{2}CO\textsubscript{3} (739 mg, 10.0 mmol). The reaction was refluxed for 3.5 h. Solids were removed by gravity filtration and the residual DMF solvent was removed by short path distillation. The crude product was treated with ice cold
water and extracted with diethyl ether. The combined organic extracts were washed with 1N HCl, dried over sodium sulfate, and concentrated to yield an orange-red syrup. The crude product was purified by silica gel chromatography with an eluent of 1:7 ethyl acetate: petroleum ether to yield (15) as an orange brown solid (0.639g, 96%). $^1$H NMR data was identical to that reported in the literature.$^{87}$ ESI-MS m/z 157.3 [M - 1]$^-$. 

3.2.5 1-Methoxy-7-methyl-naphthalene (16)

1-Methoxy-7-methyl-naphthalene was prepared by dissolving compound 15 (332 mg, 2.1 mmol) in 62 mL of acetone distilled over molecular sieves. Three equivalents of K$_2$CO$_3$ (871 mg, 6.30 mmol) were added along with 2 equivalents of MeI (0.28 mL, 4.47 mmol). The reaction was stopped after 6 h. Solids were removed by filtration and the resulting solution was purified by silica gel chromatography using an eluent of 1:15 ethyl acetate: petroleum ether. The product (16) was a clear oil (0.278 g, 78%). $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ ppm 8.08 (s, 1H), 7.73 (d, 1H, $J = 9$ Hz), 7.28-7.44 (m, 3H), 6.83 (d, 1H, $J = 6$ Hz), 4.03 (s, 3H), 2.56 (s, 3H). ESI-MS m/z 173.4 [M + 1]$^+$, 195.4 [M + Na]$^+$. 

3.2.6 4-Iodo-1-methoxynaphthalene (18)

Mono-iodination of compound 17 was achieved following the procedure of Das et al.$^{68}$ The product (18) was obtained as a light yellow solid with a yield of 80%. $^1$H NMR data was identical to that reported in the literature.$^{88}$
3.2.7 Ethyl 3-(4′-methoxy-1′-naphthyl)-2-propenoate (19)

A Heck reaction on compound 18 was achieved following the procedure from Liang et al.\textsuperscript{66} Compound 20 (40.2 mg, 0.149 mmol) was dissolved in 3 mL of DMF and charged with Pd(OAc)\textsubscript{2} (1.61 mg, 0.007 mmol), ethyl acrylate (12.0 mg, 0.120 mmol), K\textsubscript{2}CO\textsubscript{3} (19.9 mg, 0.144 mmol), and N-phenyl urea (1.96 mg, 0.144 mmol) while stirring under argon. The mixture was heated to 100 °C for 2 h, cooled to rt, neutralized with a solution of saturated ammonium chloride, and extracted twice with ethyl acetate. The combined organic extracts were washed three times with a saturated ammonium chloride solution and twice with brine. The extracts were then dried over sodium sulfate and concentrated to give (21) as an orange syrup (40.2 mg, 98%). The product appeared to be pure by \textsuperscript{1}H NMR, thus, no further purification was required. \textsuperscript{1}H NMR data was identical to that reported in the literature.\textsuperscript{89} ESI-MS m/z 279.4 [M + Na]\textsuperscript{+}.

3.2.8 Ethyl 3-(4′-methoxy-1′-naphthyl)-2-propanoate (20)

To hydrogenate the acrylate double bond, compound 19 (14.9 g, 58.2 mmol) was dissolved in 80 mL of ethanol and Pd/C (1.14 g) was added. The reaction mixture was hydrogenated under 50 psi H\textsubscript{2} (g) for 3 h. The Pd/C catalyst was removed by filtering the reaction mixture through a celite bed. The crude product was purified by silica gel chromatography with an eluent of 1:15 ethyl acetate: petroleum ether to afford (20) as a yellow syrup (10.1 g, 67%). \textsuperscript{1}H NMR data was identical to that reported in the literature.\textsuperscript{89} ESI-MS m/z 281.5 [M + Na]\textsuperscript{+}. 
3.2.9 (4-Methoxy-1-naphthyl) propionic acid (21)

Saponification of the ester was performed following the procedure of Khosla\textsuperscript{90} by dissolving compound 20 (22.5 mg, 0.087 mmol) in 2.5 mL of ethanol and adding 4 mL of 4N NaOH. The reaction was complete after 14 h. The product was diluted with ethyl acetate and acidified to pH 2 using 1 N HCl. The aqueous layer was extracted with ethyl acetate and the combined organic extracts were washed three times with brine. Compound 21 was obtained as an opaque white solid (14.26 mg, 71.3 %). This product was used in the next step without further purification.

3.2.10 3,4-Dihydro-spiro[furan-2(5H),1′(4′H)-naphthalene]-4′,5-dione (22)

Lactonization was performed following the procedure by Hata \textit{et al.}\textsuperscript{59} The product was purified by silica gel chromatography using an eluent of 1:5 acetone: petroleum ether to yield (22) in 89% yield. \textsuperscript{1}H NMR data was identical to that reported in the literature.\textsuperscript{64}

3.2.11 Compound 25

Reduction of both the alkene and ketone of Compound 22 was accomplished using a procedure reported by Couche \textit{et al.}\textsuperscript{91} Lactone (22) (757 mg, 3.54 mmol) was dissolved in 138 mL of ethanol. After cooling to 0 °C, NaBH\textsubscript{4} (80.2 mg, 2.12 mmol) was added to the solution, producing a slightly grey mixture. After 1 h, an additional equivalent of NaBH\textsubscript{4} was added. No further reaction progress was observed by TLC after 4 h and the reaction was quenched with distilled water. The aqueous layer was extracted with ethyl acetate and dried over sodium
sulfate. This material was purified by silica gel chromatography with an eluent of 1:3 acetone:petroleum ether. Compound 25 (mixture of diastereomers) was produced as a clear syrup (481 mg, 62%). In addition, starting material (155 mg) was recovered. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ ppm 7.20-7.58 (m, 4H), 4.70-4.80 (m, 1H), 2.70-2.80 (m, 2H), 1.80-2.50 (m, 6H). ESI-MS m/z 241.3 [M + Na]$^+$. 

### 3.2.12 Compound 26

For the PCC oxidation of the alcohol, a procedure by Schmid et al. was followed. The alcohol (25) (427 mg, 1.96 mmol) was dissolved in 125 mL of DCM and charged with PCC (720 mg, 3.34 mmol). The reaction was stirred at rt and was complete after 45 min. The mixture was diluted with DCM and filtered through a bed of celite. The filtrate was then washed with distilled water and the organic extract was dried over sodium sulfate and purified by silica gel chromatography using an eluent of 1:3 acetone:petroleum ether to yield (26) as a clear crystalline solid (404 mg, 97%). $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ ppm 8.08 (d, 1H, $J = 6$ Hz), 7.67 (t, 1H, $J = 6$ Hz), 7.50 (m, 2H), 2.80-3.15 (m, 4H), 2.63 (m, 2H), 2.40 (m, 2H). ESI-MS m/z 239.4 [M + Na]$^+$. 

### 3.2.13 Compound 27

The procedure used to protect the ketone with the $R,R$-hydrobenzoin group was that followed by Cossy et al. with the modification of using 10% camphorsulfonic acid and a drying tube to maintain anhydrous conditions. Compound 26 (4.16 g, 19.3 mmol) was dissolved in 600
mL of benzene and charged with an excess of $R,R$-hydrobenzoin (8.24 g, 38.5 mmol) and 10 mol % of camphorsulfonic acid (447 mg). The reaction was stirred under argon and anhydrous conditions were maintained with the installation of a MgSO$_4$-filled addition funnel below the condenser. After 96 h, the reaction was diluted with diethyl ether and then washed with a saturated sodium bicarbonate solution, followed by a saturated ammonium chloride solution. The organic layer was dried over sodium sulfate and concentrated. Purification using silica gel chromatography with an eluent of 1.0: 2.9: 5.7 ethyl acetate: diethyl ether: petroleum ether separated the clear crystalline product (27a/b) (2.94 g, 37% yield with approximately 5% diethyl ether solvent contamination, as a mixture of diastereomers) and 1.83 g of unreacted starting material. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ ppm 7.92 (d, $\frac{1}{2}$ H, $J = 8$ Hz), 7.84 (d, $\frac{1}{2}$ H, $J = 8$ Hz), 7.46-7.51 (m, 3H), 7.40-7.46 (m, 10H), 5.13 (m, 1H), 4.99 (m, $\frac{1}{2}$ H), 4.92 (m, $\frac{1}{2}$ H), 2.80-2.90 (m, 2H), 2.49-2.66 (m, 2H), 2.35-2.47 (m, 3H), 2.18-2.26 (m, 1H). ESI-MS m/z 435.3 [M + Na]$^+$. 

3.2.14 Compound 28

The procedure used to obtain the selenide was that of Gu et al.$^{58}$ All glassware was dried and flushed with argon. LDA was first prepared under argon at -78 °C in a 10 mL RBF by adding 0.21 mL of n-BuLi dropwise to a solution of 0.05 mL of distilled diisopropylamine dissolved in 2 mL of dry THF. The reaction was allowed to stir for 45 min. Compound 27a/b (120 mg, 0.292 mmol) dissolved in 1 mL of THF, was added dropwise to the prepared LDA solution. 1 mL of THF was used to rinse the RBF with a syringe while under argon. The reaction was stirred for 45 min at -78 °C. PhSeCl (62.8 mg, 0.328 mmol) dissolved in 2 mL of
THF was added dropwise to the enolate. After 3 h, the reaction was quenched with 2 mL of distilled water at rt. The solution was extracted three times with ether and the organic extracts were washed with brine, dried over sodium sulfate, and concentrated. Product was purified using silica gel chromatography with 1:2 diethyl ether: petroleum ether as eluent. Compound 28 was obtained as a yellow syrup (14.2 mg), along with 65.2 mg of unreacted starting material. The resulting \( ^1\)H NMR spectrum indicated the compound was present, but impure. This material was carried on without further purification. ESI-MS m/z (selenium isotopes) 589.2, 591.2, 592.3 [M + Na]⁺.

### 3.2.15 Compound 29

The oxidation of the resulting selenide was performed with 30% \( \text{H}_2\text{O}_2 \) using the procedure followed of Chavan et al.\textsuperscript{73} Acetic acid (0.25 mL) was added to crude compound 28 (14.2 mg, 0.025 mmol) dissolved in THF. \( \text{H}_2\text{O}_2 \) (30%, 2 mL) was added dropwise at 0 °C. The reaction was complete after 1 h and was easy to monitor by TLC, as the alcohol byproduct became visible as a very non-polar yellow spot on the TLC. The reaction was quenched with saturated sodium bicarbonate, extracted three times with diethyl ether, dried over sodium sulfate, and concentrated. The reaction was purified by silica gel chromatography using an eluent of 1:3 ethyl acetate: petroleum ether, to give (29) as a clear syrup (9.3 mg, 7.8% over two steps). This material was not completely pure as evident by downfield signals in the \( ^1\)H NMR spectrum. \( ^1\)H NMR  (400 MHz, CDCl\textsubscript{3}) δ ppm 8.42 (s, 1H), 7.98 (m, ½ H), 7.90 (m, ½ H), 7.60 (m, 1H), 7.50 (m, 1H), 7.20-7.39 (m, 10H), 7.07-7.10 (m, ½ H), 6.24-6.29 (m, 1H), 5.60 (m, ½ H), 4.92-5.60 (m, 2H), 2.46-2.79 (m, 4sH). ESI-MS m/z  433.3 [M + Na]⁺.
3.2.16 Compound 34

The bromo tetralone was protected as an acetal with ethylene glycol following the procedure of Chen et al. with the modification of using $p$-toluene sulfonic acid monohydrate as the acid catalyst and using a drying tube to maintain anhydrous conditions. The product was purified by using silica gel chromatography with an eluent of 1:20 ethyl acetate: petroleum ether to yield (34) as a yellow syrup (73%). $^1$H NMR and ESI-MS data were identical to those reported in the literature.

3.2.17 Compound 35

The esterification reaction was performed following the procedure of Ushijima et al. $n$-BuLi (1.2 mol equivalents, 1.52 mmol) was added dropwise to compound 34 (342 mg, 1.27 mmol) dissolved in 3 mL of dry THF while under argon at -78 °C. The reaction was stirred at -78 °C for 30 min, then DMF (0.12 mL, 1.52 mmol) was added and the mixture was allowed to stir for 15 min at -78 °C, then for 1.5 h at rt. THF was then removed under vacuum and 4 mL of MeOH was added. The reaction was allowed to stir for 30 min, at which point, I$_2$ (968 mg, 3.81 mmol) and K$_2$CO$_3$ (527 mg, 3.81 mmol) were added at 0 °C. The mixture was stirred at rt for 20 h. The reaction was quenched with sodium thiosulfate, extracted with chloroform, and dried over sodium sulfate. The crude product was purified by silica gel chromatography using an eluent of 1:10 ethyl acetate: petroleum ether to give (35) as a yellow syrup (42.5 mg, 10% with approximately 5% diethyl ether solvent contamination). $^1$H NMR (400 MHz, CDCl$_3$) δ ppm 8.17 (s, 1H), 7.89 (d, 1H, $J$ = 8 Hz), 7.17 (m, 1H, $J$ = 8 Hz), 4.25-4.29 (m, 2H), 4.13-4.15 (m, 2H), 3.95 (s, 3H), 2.80 (m, 2H), 2.00 (m, 4H). ESI-MS m/z 249.4 [M + 1]$^+$, 271.4 [M + Na]$^+$. 
3.2.18 7-(Carbomethoxy)-1-tetralone (36)

The carbonyl was deprotected following the procedure of Grieco et al.\textsuperscript{93} Compound 35 (14.5 mg, 0.067 mmol) in 3 mL of THF containing 0.13 mL of 5% HCl, was stirred at rt for 16 h. The reaction was extracted with diethyl ether and dried over sodium sulfate. The crude product was purified by silica gel chromatography using an eluent of 1:6 diethyl ether: petroleum ether to give a quantitative yield of a clear, crystalline product (36) (8 mg with approximately 5% diethyl ether and water solvent contamination). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ ppm 8.69 (s, 1H), 8.14 (d, 1H, $J = 8$ Hz), 7.35 (d, 1H, $J = 8$ Hz), 3.93 (s, 3H), 3.03 (t, 2H, $J = 4$ Hz), 2.70 (t, 2H, $J = 4$ Hz), 2.14-2.21 (m, 2H). ESI-MS m/z 205.3 [M + 1]$^+$, 227.3 [M + Na]$^+$.
References


54. Li, X.; Tanner, M.E. unpublished results


Appendix: $^1$H NMR Spectra of Selected Compounds

Figure A. $^1$H NMR (300 MHz, CDCl$_3$) spectrum of compound 16
Figure A. 2 $^1$H NMR (400 MHz, CDCl$_3$) spectrum of compound 25 (mixture of diastereomers)
Figure A. 3 $^1$H NMR (300 MHz, CDCl$_3$) spectrum of compound 26
Figure A. 4 $^1$H NMR (400 MHz, CDCl$_3$) spectrum of compound 27
Figure A. 5 $^1$H NMR (400 MHz, CDCl$_3$) spectrum of compound 28
Figure A. 6 $^1$H NMR (400 MHz, CDCl$_3$) spectrum of compound 29
Figure A. $^1$H NMR (400 MHz, CDCl$_3$) spectrum of compound 35
Figure A. $^1$H NMR (400 MHz, CDCl$_3$) spectrum of compound 36