Functionalization of Novel PP5 Inhibitors

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Functionalization of Novel PP5 Inhibitors

By
Hope P. Hill

A thesis submitted in partial fulfillment of the requirements of the Honors College at University of South Alabama and the Bachelor of Sciences in the Department of Chemistry

University of South Alabama
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Dedication:

To my grandparents: Nina, Papa, Pammie, and Doc
Acknowledgements:

It is not an exaggeration to say that Dr. Forbes is the reason I successfully completed the undergraduate research experience. There were many days I became quite frustrated with our results, but Dr. Forbes was always there to offer a word of encouragement and highlight the positive outcomes of our work. I had a lot to learn coming into his research lab in the summer of 2019. Yet, through it all he was patient and continued to remind me that not only was I capable but that organic chemistry was worth learning. I enjoyed our almost daily conversations about the progress of the research and my own personal progress as a student and person. Dr. Forbes has been a fantastic mentor and is a fantastic human being. I would also like thank Dr. Richard Rogers for also motivating me to learn organic chemistry to the best of my ability and Dr. Yet for proving through every lecture that chemistry is something to get excited about. To all of my other instructors in the chemistry, Dr. Davis, Dr. Stenson, Dr. Coym, Dr. Duranty, and Dr. Migaud, I am so grateful for all you taught me throughout these past four years.

The work of my amazing research team should not be forgotten. Noah Baker and Ivy Nguyen have been a phenomenal duo to work with and worked tirelessly to make this thesis possible. I appreciate their skills and diligence every day that resulted in a final product we could all be proud of.

Finally, I would like to thank my family for supporting me throughout these past four years. It was through their guidance that I learned resilience and hard work are foundational to success. Although two of my grandparents are no longer here to witness the completion of my undergraduate degree, I would like to specifically thank them as well for teaching me that knowledge is always worth pursing but a kind heart should be pursued most of all.
Abstract:

The overexpression of protein phosphatase 5 has been correlated to tumor cell reproduction making it a candidate for small molecule drug therapy. Selective and potent inhibition of protein phosphatase 2A (PP2A) enzyme has been previously achieved through the development of the molecule fostriecin with key functionality being a lactone and unsaturated linear chain. The large synthetic overhead of fostriecin has led to exploration of other small molecule inhibitors that could mirror fostriecin’s interaction with the active site in the catalytic domain of PP5. The naturally occurring inhibitor, cantharidin, is functionalized with an epoxy containing eleven carbon chain to optimize binding with the active site of PP5 and to promote selectivity and potency through unique interactions with the amino acid residues. The stereoisomers formed in the Diels-Alder reaction between the robust dienophile N-phenylmaleimide and furfuryl alcohol are isolated and identified. The biologically active exo confirmation is selectively recrystallized and employed in further synthetic steps with the final product tested for inhibitory action against the PP5 enzyme.
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LIST OF ABBREVIATIONS

Ac₂O: Acetic Anhydride
CDCl₃: Deuterated Chloroform
COSY: Correlation Spectroscopy
DMF: Dimethylformamide
EtOAc: Ethyl Acetate
KOH: Potassium hydroxide
mCPBA: meta-Chloroperoxybenzoic acid
MgSO₄: Magnesium Sulfate
NaH: Sodium hydride
NMR: Nuclear Magnetic Resonance
PPP: Phosphoprotein Phosphatase
PP: Protein Phosphatase
PP5: Serine/Threonine Protein Phosphatase 5
RBF: Round-Bottomed Flask
THF: Tetrahydrofuran
TLC: Thin-Layer Chromatography
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Introduction:

Cancer is among the leading causes of death in America. The rapid reproduction of cells is detrimental to the human body presenting a painful death for many individuals.\(^1\) Treatments such as chemotherapy and radiation are familiar to most Americans; however, these techniques are not completely effective in eliminating cancerous cells nor are they without dangerous side effects such as lung damage and congestive heart failure.\(^2\) These therapies are also expensive and time-consuming, yet still lack the specificity needed to prevent widespread side effects.

Research in cancer drug development has sought to understand what enables long term cancer cells continued survival. For this reason, the division of biological enzymes known as protein phosphatases have been taken into consideration due to their vital role in cell regulation.\(^3\) For instance, protein phosphatase 5 aids in the regulation of cell proliferation and apoptosis. However, the overexpression of protein phosphatase 5, PP5, displayed a positive correlation to tumor cell development in breast cancer, prostate cancer, and pancreatic cancer.\(^4\) It is hypothesized that PP5 is aiding cancer cell proliferation by negatively regulating physiological processes such as programmed cell death.\(^5\) Because of this possible link, protein phosphatase 5 has become an area of interest for anti-cancer drug research.

Various natural products have been shown to inhibit the activity of ser/thr protein phosphatases. The derived natural product fostriecin (Figure 1) has successfully been used as an antitumor agent in clinical trials.\(^6\) However, fostriecin requires more than 20 steps to synthesize and is therefore not ideal for mass drug production.\(^7\) Current research aims to develop alternative inhibitors that are similar in function to fostriecin. The naturally occurring inhibitor, cantharidin (Figure 2), is a much smaller molecule than fostriecin that has been shown to successfully
suppress the activity of the PP5. Unfortunately, dangerous side effects have resulted from cantharidin’s lack of selectivity. Various derivatives of cantharidin have been able to increase selectivity through new interactions between the inhibitor and active site. This study seeks to use these previous results further increase selectivity. The effectiveness of the lactone functionality of fostriecin can be mimicked through a suitable equivalent such as an epoxy functionality strategically tethered to a cantharidin scaffold. It is hypothesized that the epoxide functionality will promote unique interactions with the polar amino acid residues, such as Cysteine, found in and near the catalytic pocket which in turn increases potency while maintaining specificity for PP5.

In this project, in addition to the introduction of new functionality, previous synthetic pathways are refined by introducing a more robust starting material, N-phenylmaleimide, to the Diels-Alder reaction. Diels-Alder adduct stereoisomers are isolated then identified through the use of the two-dimensional NMR technique COSY.

![Figure 1. Fostriecin](image1.png)  
![Figure 2. Cantharidin](image2.png)

**Protein Phosphatases**

Protein phosphorylation is a common cellular mechanism used to control intracellular transduction pathways. This process is the attachment a phosphate group to an amino acid residue such as serine or threonine by a protein kinase that can be subsequently dephosphorylated by protein phosphatases. Reversible phosphorylation directly affects the
interaction between proteins and thus regulates important cellular processes. Research has focused largely on the two classes of enzymes that participate in these reactions: protein kinases and protein phosphatases which contribute to cell growth and programmed cell death. Deregulation of these enzymes, more specifically, abnormal protein serine/threonine phosphatase activity, has been linked to several diseases including cancer.\textsuperscript{3-5}

**PP5 and Cancer Development**

The protein phosphatase 5, PP5, has been found in many protein complexes that contribute to signaling networks that regulate cellular proliferation and apoptosis. In a 2008 study, an analysis of human breast cancer cells revealed a correlation between the overexpression of PP5 and cancer cell development.\textsuperscript{4} Researchers performed a follow-up study in order to determine if overexpression of PP5 had a direct and positive effect on tumor growth. The experiment compared tumor size development in mice that were injected with differing amounts of PP5. It was concluded that PP5 overexpression was directly proportional to tumor size. There exist several possible explanations to this relationship.

Prior studies have implied that the PP5 responds to cellular stress in altering its cell growth regulatory mechanisms. Oxidative stress is known to be directly linked to gene mutation and subsequently cancer cell proliferation. Substantial data has suggested that, during periods of oxidative stress, PP5 protein levels increase.\textsuperscript{5} Such overexpression leads to an association with apoptosis signal regulating kinase (ASK1). This correlation suggests the possible prevention of apoptosis through inactivation of ASK1, therefore, allowing cells to survive in oxygen depleted environments. PP5 is also reported to act as a negative regulator of the DNA damage repair kinase, DNA-PKcs.\textsuperscript{5} Without repair, DNA damage is responsible for tumor cell development.
The relationships between PP5 and ASK1 and DNA-Pkcs demonstrate how the role of PP5 under genomic stress can lead to negative effects including cancer progression. For these reasons PP5 has become an attractive target for anti-cancer drug development.

**PP5: Target for Drug Development**

An understanding of the enzyme’s structure and function is necessary for drug design considerations. Protein phosphatases, PP1, PP2B, PP5, and PP2A, have very comparable catalytic active sites. The amino acids of the catalytic core coordinate with two metal ions, Mn, to provide the necessary interaction with the substrate and to allow dephosphorylation. The substrate will also coordinate with the active site water molecule and metal ions. A competitive inhibitor able to exploit these interactions would greatly limit enzyme activity.

Protein phosphatase 5 is slightly unique in several attributes of its catalytic domain. First, the tripartite tetratricopeptide-repeat (TPR) located at the N-terminus and the C-terminal interact with one another blocking access to the catalytic site. Access to the catalytic domain is gained after heat shock protein 90, HSP90, binds to the TPR. PP5 is activated only through the chaperone HSP90; this step is essential to the function of PP5. PP5 is therefore involved in the function of many HSP90 clients including the DNA-damage activated protein kinase and apoptosis signaling kinase. Negative regulation of these kinases is directly fostered by the sustained interaction between HSP90 and PP5; this relationship suggests the possible mechanism behind sustained cancer cell proliferation and over expression of PP5. It has been hypothesized that mutual inhibition may be necessary for optimal results. Therefore, these biological relationships are considered when determining a small molecule inhibitor.
Fostriecein: Antitumor Antibiotic

The molecule fostriecein (Figure 1) is a potent and selective antitumor agent that provides a novel mechanism for therapeutic implementation and has been tested in Phase I clinical trials at the National Cancer Institute. Antitumor activity originates in the prevention of reversible the phosphorylation of proteins that are necessary for the cell cycle. Dephosphorylation performed by protein phosphatases is interrupted through the inhibitory action of fostriecein. However, the effectiveness of fostriecein is in part due to its selective inhibition of PP2A (IC\textsubscript{50}=4.7 μM) versus PP1 (IC\textsubscript{50} > 1000 μM). Contact with the catalytic grooves in PP2A, leads to covalent interaction between the lactone of fostriecein and the nucleophilic amino acid residue Cys269. The absence of the Cys269 amino acid residue resulted in deselection of other protein phosphatases.

**Figure 3:** Fostriecein within the active site of PP2A. Contacts within active site enable selective inhibition.
Fostriecin was originally isolated through the fermentation of beer. Throughout the clinical trials, concerns were presented about the purity and storage stability of the naturally isolated product.\textsuperscript{15} In order to confirm structural and stereochemical assignments, as well as chemical purity, the synthetic assembly of fostriecin was necessary with total synthesis requiring twenty steps.\textsuperscript{7} When looking towards future medicinal large-scale production, large numerous-step synthesis processes are not cost or time efficient. A smaller molecule requiring fewer synthetic steps yet equal in effectiveness would be transformative for this field of study. Research has turned to focus on naturally occurring small inhibitors for a potential model.

**Overview of Cantharidin**

Cantharidin is a naturally occurring inhibitor found in blister beetles. It has been used in Chinese medicine for centuries for a wide range of ailments most commonly warts and molluscum.\textsuperscript{8} Cantharidin consists of a six-membered ring where carbons 1 (C1) and 4 (C4) are bridged by oxygen and carbon 2 (C2) and carbon 3 (C3) are fused with anhydride functionality (Figure 2). Collectively, this core scaffold is 7-oxabicyclo[2.2.1]heptane. This novel tricyclic system which consists of four oxygen atoms has been shown to be active as is the case in its ability of this inhibitor to induce cell death and DNA breakage which explains its effectiveness in dermatological removals. While activity is important, the potentially dangerous side effects of cantharidin have led to its use being less attractive.\textsuperscript{8}
Cantharidin and its Analogs

Cantharidin inhibits members of the serine/threonine-family protein phosphatases: PP1CA, PP2CA, PP5C, and PP6C while PP4C was insensitive to cantharidin inhibition. The catalytic pocket of these protein phosphatases displays strong correlation between the carboxylate oxygens and the bridgehead oxygen of the inhibitor and the manganese ions. Specifically, studies on selectivity revealed close proximity between the oxygen bridgehead of the inhibitor and both the manganese catalytic ions and the amino acid side chain (Phe446) of PP5. Steric hindrance provided by the bulkier side chain (Trp257) in PP4C provided a plausible explanation to minimal inhibitory action against PP4C as observed in the assay. Experiments were further conducted to increase the selectivity for PP5 by testing various derivatives of the 7-oxabicyclo [2.2.1] heptane scaffold.

The demethylated analog of cantharidin, norcantharidin (Figure 4), is easily synthesized as well and has been shown to also be an effective inhibitor with potential anticancer properties. The elimination of various negative side effects through the use of norcantharidin over cantharidin has increased its use in drug testing. Further modifications to the structure display potentially useful trends. Reduction of the C8-C9 double bond (Figure 5) when substituted increased potency but did not increase selectivity for PP5. Addition of heteroatomic functionality such as found in the propoxymethyl group at the C8 position (Figure 6) also increased the inhibition against PP5 while largely deselecting for PP1C. The propoxymethyl group allowed for favorable interaction through a supplementary hydrogen bond with a neighboring amino acid residue while still avoiding steric clashes. These results are exploited in further development of selective and potent inhibitors of PP5.
**Figure 4.** Norcantharidin. Displaying demethylated analog of cantharidin with carbon numbering.

**Figure 5.** Reduction of C8/C9 bond. Displaying addition of substituent at C8 and reduction of C8/C9 bond.
Figure 6. **Propoxymethyl substitution.** Addition of substituent at position C8 promotes selective inhibition.

Figure 7a

**Co-crystal Structure of PP5C with Norcantharidin.** Norcantharidin, in the bis-acid form within the catalytic site of PP5C (7a). Catalytic manganese ions are shown as purple spheres. A close-up view of the co-crystal structure of PP5C with norcantharidin displaying contact between the bridgehead oxygen of norcantharidin with both catalytic metal ions and a non-catalytic phenylalanine residue of PP5C (7b).
It is also observed that the inhibitory activity of norcantharidin correlates to whether or not the starting structure is in anhydride or bis-carboxylic acid form. Furthermore, research results have suggested that the more active form of cantharidin and its derivatives is in fact the acidic, ring-opened, form. In mirroring this form, the molecule endothall (Figure 8) is the hydrolyzed derivative of cantharidin sharing the same 7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid scaffold. Endothall exhibits competitive inhibition as both the carboxylic acid oxygens and oxygen bridgehead coordinate with the metal ions to block water molecules from participating in hydrolysis. However, the electrophilic carbon present in the anhydride is vulnerable to a nucleophilic attack by a water molecule. Consequently, when working with the carboxylic acid derivative, such as esters and amides, these moieties are liable to be removed through hydrolysis reactions. Modifications at C8/C9 and C2/C3 positions diminish susceptibility to hydrolysis and were found to be tolerated by PP5.

![Figure 8. Endothall. The Bis acid and active form of norcantharidin.](image)
Functionalization:

The catalytic mechanism of PPP-family ser/thr is very similar for all members.\textsuperscript{11} Subtle differences in the catalytic pockets can be utilized to promote inhibitor specificity. Derivatives containing additional functional groups are shown to increase potency and selectivity as illustrated by propoxymethyl moiety at position C8.\textsuperscript{9} Increasing the carbon chain length from 3 to ten carbons also displayed an increase in selectivity as shown by the differing IC\textsubscript{50} values of PP5 and PP1.

Table 1: Comparison of differing IC\textsubscript{50} values of cantharidin derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>R-group</th>
<th>PP5 IC\textsubscript{50}</th>
<th>PP1 IC\textsubscript{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td>R=H</td>
<td>1.0 μM</td>
<td>8.9 μM</td>
</tr>
<tr>
<td><img src="image2.png" alt="Image" /></td>
<td>R = CH\textsubscript{2}OCH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{3}</td>
<td>0.7 μM</td>
<td>3.7 μM</td>
</tr>
<tr>
<td><img src="image3.png" alt="Image" /></td>
<td>R = CH\textsubscript{2}O(CH\textsubscript{2})\textsubscript{9}CH\textsubscript{3}</td>
<td>14.3 μM</td>
<td>21.1 μM</td>
</tr>
</tbody>
</table>
Using fostriecin as a model system, next generation candidates can be prepared and tested by employing specific functionality that potentially promotes covalent bonding to polar amino acid residues. It is hypothesized that an epoxide could mirror the covalent interactions between the lactone of fostriecin and the nucleophilic amino acid residue of the catalytic groove.9,10

Preliminary hypotheses support the endothall derivative containing tethered oxirane functionalization would utilize close contact and interaction with the catalytic groove, leading to an increase in potency and selectivity. Initial testing revealed enzyme inhibition of 4 nM and therefore considerably more potent than previously tested molecules. Given the possibility of hydrolysis of carboxylic acid derivatives, placements of the tethered epoxide at positions 1, 2, and 5 of the norcantharidin scaffold will avoid hydrolysis while still demonstrating comparable levels of inhibition. While the ideal length for the chain can be experimentally tested, the eleven-carbon chain is an entry point exploiting contacts as shown in previously tested systems only to be confirmed upon a proper study of tethered epoxy functionality. The results from the PP5 assays will guide future alterations to the chain length.
Scheme 1. Synthetic Overview:

1) KOH, heat, 48h
2) Ac₂O

mCPBA
Experimental methods:

1. General Considerations
2. Preparation of Undecenyl Tosylate
3. Preparation of 3-(Hydroxymethyl)furan
4. Diels-Alder Cycloaddition using N-Phenylmaleimide and Furan
5. Diels-Alder Cycloaddition using N-Phenylmaleimide and 2-(Hydroxymethyl)furan
6. Diels-Alder Cycloaddition using N-Phenylmaleimide and 3-(Hydroxymethyl)furan
7. Etherification of Hydroxymethyl Diels-Alder Adduct using Undecenyl Tosylate
8. Etherification of Hydroxymethyl Diels-Alder Adduct using Allyl Bromide
9. Hydrolysis and Ring Closure using N-Phenylmaleimide Diels-Alder Adduct
1. General Considerations

The NMR which generated all the spectra as part of this submission was a JEOL ECA-500 spectrometer. The software used to process all the corresponding data was JEOL Delta™ Version 5.0.4.4 (PC) or 5.2.1 (MAC). $^1$H NMR (500 MHz) spectra were obtained as solutions in CDCl$_3$. Chemical shifts were reported in parts per million (ppm) and referenced to $\delta$ 7.27 ($^1$H NMR). For the synthetic procedures performed, additional considerations consisted of the following: TLC analyses were performed on flexible aluminum backed TLC plates with a fluorescent indicator. Detection was conducted by UV absorption (254 nm) followed by charring with 10% KMnO$_4$ in water. Solutions were concentrated in vacuo using a rotary evaporator. The resulting residue was purified using a silica gel column (70-230 mesh, 60 Å). All chemicals used for synthetic procedures were reagent grade or better.
2. Preparation of Undecenyl Tosylate

10-Undecen-1-ol (3.62 mL, 18 mmol, 1.5 equiv) was added using a syringe to a round bottomed flask (RBF) equipped with a magnetic stir bar, septum, and placed under a blanket of argon. The setup was secured with clamp and placed on stir plate. Approximately 45 mL of dichloromethane was added to the RBF while stirring. Next, 0.2 mL of 1-methylimidazole (0.2 mL, 2.52 mmol, 0.2 equiv.) and triethylamine (2.60 mL, 18 mmol, 1.5 equiv.) were added by syringe. The reaction mixture was then externally cooled using an ice-bath. In a separate container, tosyl chloride (2.3 g, 12 mmol, 1 equiv.) was dissolved using 20 mL of dichloromethane. That suspension was then transferred to the reaction mixture using a disposable glass Pasteur pipet. The reaction mixture was allowed to warm to room temperature and was stirred for approximately 72 h. The organic layer was washed with deionized water and once isolated, the organic layer was dried using anhydrous MgSO₄ and concentrated in vacuum. The crude tosylate was purified using column chromatography (SiO₂; gradient system of EtOAc/hexanes (hexanes, 1:16, 1:8, 1:4)) and yields averaged between 90-95%. Final product was a viscous yellow oil. (SiO₂, 1:16 EtOAc: hexanes, Rf = 0.3). δ 7.79 (d, 2H, CH, J = 8.6 Hz), 7.35 (d, 2H, CH, J = 7.5 Hz), 5.86-5.76 (m, 1H, CH), 5.01-4.92 (m, 2H, CH₂), 4.02 (t, 2H, CH₂, J = 6.3 Hz), 2.45 (s, 3H, CH₃), 2.03 (q, 2H, CH₂, J = 8.0 Hz) 1.68-1.60 (m, 2H, CH₂), 1.37-1.21 (m, 14H, CH₂).
3  Preparation of 3-Hydroxymethylfuran

3-Furfural (1.94 g, 20 mmol, 1.75 mL) was added via syringe to a 250 mL RBF equipped with a small magnetic stir bar. Approximately 100 mL of THF was next added to the RBF. The solution was externally cooled using an ice bath while stirring under a blanket of argon. Sodium borohydride (0.769 g, 20 mmol) was transferred to the reaction mixture portion wise. An additional 1 mL of THF was used to completely transfer reductant to RBF. Reaction mixture was kept externally cooled using an ice bath and allowed to stir under a blanket of argon for 48 h. The crude reaction mixture was diluted using tBuOMe (100 mL) and washed first with a saturated aqueous solution of ammonium chloride (20 mL) followed by a saturated aqueous solution of sodium chloride (20 mL). The organic layer was dried over anhydrous MgSO₄ and concentrated in vacuo while keeping the water bath at room temperature in order to minimize loss of the volatile hydroxymethylfuran. The crude material was used as is once complete conversion using TLC analysis was confirmed (SiO₂, EtOAc:hexanes (1:1), \( R_f = 0.51 \)).
**Diels-Alder Cycloaddition using N-Phenylmaleimide and Furan**

\[
\begin{align*}
&\text{ Cycloaddition using } N\text{-Phenylmaleimide and Furan} \\
&
\text{N-phenylmaleimide (400 mg, 2.31 mmol, 1.0 equiv) was added to a sealed pressure flask} \\
&\text{equipped with a stir bar and dissolved in 1.6 mL of hot ethyl acetate forming a 1.4 M solution.} \\
&\text{Furan (252 µL, 3.5 mmol, 1.5 equiv) was next added via syringe once the reaction mixture was} \\
&\text{allowed to cool back to room temperature. The pressure flask was sealed, placed in a sand bath} \\
&\text{and externally heated to 100 °C. The reaction was allowed to stir for 16 h. The crude reaction} \\
mixture was immediately hydrogenated 25 mg of 10% Pd/C. after transferring the material to a \\
Parr flask and diluted using as a ratio 20 mL of THF per gram of material. The hydrogenation \\
was set at 55 psi and allowed to mix for 1 h after purging the initial blanket of molecular \\
hydrogen three times. After removing the catalyst via filtration using a Celite pad, the product \\
was concentrated under reduced pressure and chromatographed (SiO}_2; \text{EtOAc/hexanes (2:1)).} \\
\text{Reaction was replicated on several occasions with yields averaging between quantitative and} \\
95\%.^1\text{H NMR (CDCl}_3) \ \delta 7.46 (t, 3H, CH, }J = 7.5 \text{ Hz), 7.26 (d, 2H, CH, }J = 5.7 \text{ Hz) 5.00-5.99} \\
(m, 2H, CH), 3.05 (s, 2H, CH) 1.96-1.86 (m, 2H, CH}_2) 1.69-1.65 (m, 2H, CH}_2).
5 Diels-Alder Cycloaddition using N-Phenylmaleimide and 2-Hydroxymethylfuran

\[
\begin{align*}
\text{ HO} & \quad + \quad \begin{array}{c}
\text{HO} \\
\text{N} \\
\text{O}
\end{array}
\end{align*}
\text{EtOAc} \quad \text{sealed tube} \quad \begin{align*}
\text{HO} & \quad + \quad \begin{array}{c}
\text{HO} \\
\text{N} \\
\text{O}
\end{array}
\end{align*}
\text{H}_2 \quad 10\% \text{ Pd/C}
\]

2-Furfuryl alcohol (303 µL, 3 mmol, 1.5 equiv) and N-phenylmaleimide (400 mg, 2.3 mmol, 1.0 equiv) were added to a sealed pressure flask equipped with a magnetic stir bar and dissolved in ethyl acetate (1.6 mL) to form a 1.4 M solution. The reaction was placed in a sand bath and externally heated to 100 °C. Reaction was allowed to stir for 16 h. The crude reaction mixture was immediately hydrogenated using 25 mg of 10% Pd/C after transferring the material to a Parr flask and diluted using as a ratio 20 mL of THF per gram of material. The hydrogenation was set at 55 psi and allowed to mix for 1 h after purging the initial blanket of molecular hydrogen three times. After removing the catalyst via filtration using a Celite pad, the product was concentrated under reduced pressure and chromatographed (SiO₂; EtOAc/hexanes (2:1)). For purposes of determining the isomeric composition, a small portion of chromatographed material was recrystallized (EtOAc with hexanes) resulting in isomerically pure material. Reaction was replicated on several occasions with yields of both the exo and endo adducts averaging between quantitative and 95%. Recrystallized product was white powder. (SiO₂, 6:1, \(R_f = 0.41\)) \(^1\)H NMR (CDCl₃) \(\delta\) 7.50-7.46 (m, 2H, CH), 7.43-7.39 (m, 1H, CH), 7.28-7.25 (m, 2H + CDCl₃, CH), 5.01 (d, 1H, CH, \(J = 5.2\) Hz), 4.07 (dd, 2H, CH₂), 3.21-3.17 (m, 2H, CH₂), 2.10-2.02 (m, 2H, CH₂), 1.92-1.87 (m, 2H, CH₂), 1.81-1.76 (m, 2H, CH₂).
Using the identical protocol (setup, purification, and isolation) as described above using not 2-(hydroxymethyl)furan but 3-(hydroxymethyl)furan (3 mmol, 1.5 equiv.), an average isolated yield between quantitative and 95% yield was observed. Recrystallized product was white powder. (SiO₂, EtOAc:hexanes (5:1), Rf = 0.34) ¹H NMR (CDCl₃) δ 7.48 (t, 2H, CH, J = 6.3 Hz), 7.39 (t, 2H, CH, J = 9.2 Hz), 7.27-6.24 (m, 2H, CH+CDCl₃), 4.97 (q, 2H, CH, J = 5.15 Hz), 3.7 (t, CH, J =10.3 Hz), 3.48 (d, 1H, CH, J = 7.4 Hz), 3.02 (d, 1H, CH, J = 7.5 Hz), 2.58-2.51(m, 1H, CH), 2.09 (m, 1H, CH₂), 1.32 (q, 1H, CH₂, J = 6.8 Hz).
Etherification of Hydroxymethyl Diels-Alder Adduct using Undecenyl tosylate

The Diels-Alder adduct (680 mg, 2.5 mmol, 1 equiv) was dissolved in 6 mL of THF and placed in a separate RBF. Sodium hydride (120 mg, 5.0 mmol, 2.0 equiv) was dissolved in 5 mL of THF and externally cooled using an ice bath while under a blanket of argon. To this RBF was transferred via syringe to the solution of Diels-Alder adduct. The reaction mixture was allowed to gradually warm to room temperature and allowed to stir for 30 min at which time the reaction mixture was externally cool again using an ice bath. The previously formed tosylate (973 mg, 3.0 mmol, 1.2 equiv) was dissolved in 2 mL of THF and added dropwise to reaction flask via syringe. The reaction mixture was allowed to stir overnight. Upon quenching the reaction mixture with aqueous HCl (0.1M), the reaction mixture was extracted with ethyl acetate, dried with MgSO₄, and filtered to remove the drying agent. Product was then concentrated under vacuum and purified by column chromatography. (SiO₂; gradient system of EtOAc/hexanes (1:16, 1:8, 1:4, 6:1)). While successful in conducting the reaction, percent yield and spectrum are not reported as the desired material was not isolated due to, what we suspect to be, multiple competing side reactions.
Using the identical protocol (setup, purification, and isolation) and replacing undecenyl tosylate with allyl bromide (260 µL, 3 mmol, 1.2 equiv), a small amount of product as a yellow viscous oil was isolated ~100 mg having the following spectral data: $^1$H NMR (CDCl$_3$) $\delta$ 7.49 (t, 2H, CH, $J$ = 7.5 Hz), 7.42 (t, 2H, CH, $J$ = 7.5 Hz), 7.26-7.23 (m, 1H, CH+CDCl$_3$), 5.99-5.91 (m, 1H, CH), 5.33 (dq, 1H, CH$_2$, $J$ = 7.6, 1.2 Hz) 5.23 (dd, 1H, CH$_2$, $J$ = 9.2, 1.2 Hz), 4.97 (t, 1H, CH, $J$ = 5.2 Hz), 4.15 (d, 2H, CH$_2$, $J$ = 5.7 Hz), 3.90 (dd, 2H, CH$_2$, $J$ = 11.5, 5.0 Hz), 3.73-3.67 (m, 2H, CH), 2.06-1.99 (m, 1H, CH$_2$), 1.87-1.81 (m, 2H, CH$_2$), 1.77-1.71 (m, 1H, CH$_2$).
9 Hydrolysis and Ring Closure using \(N\)-Phenylmaleimide Diels-Alder Adduct

\[
\begin{align*}
\text{1) KOH, heat} \\
\text{2) Ac}_2\text{O}
\end{align*}
\]

A 25 mL RBF was placed in a sand bath and externally warmed to 100 °C after the addition of the following materials: 2.5 mL of DI water, 100 mg KOH (1.8 mmol), and 217 mg of the Diels-Alder adduct (0.1 mmol). An additional 2.5 mL of water was added to RBF solution to assure formation of a solution. A reflux condenser was attached to reaction set-up and the reaction mixture was allowed to stir for 48 h. For this analysis, 2.5 mL of the reaction mixture was transferred, concentrated in vacuo, and then dissolved in using 4 mL of DMF prior to the addition of acetic anhydride (127 \(\mu\)L, 1.35 mmol, 3 equiv). Once added, the reaction mixture was allowed to stir at room temperature for an additional 48 h. Analysis of the crude reaction mixture upon multiple washes using EtOAc, drying over anhydrous MgSO\(_4\), and concentrating in vacuo revealed the presence of the desired material confirming proof of principle. While successful with conducting the reaction, percent yield and spectral data are not reported as the desired product has not been properly isolated using optimized reaction conditions.
**Results and Discussion:**

Forming the norcantharidin scaffold has been commonly achieved through reacting furan and maleic anhydride. Addition of functional groups was consistently added to furfuryl alcohol and the subsequent product reacted with maleic anhydride. However, subsequent reaction steps, specifically Williamson ether synthesis and epoxidation of alkene, consistently resulted in low yields due to competitive side reactions when specifically working with furan derivatives. Average yields of over two dozen systems tested were between 20-40%. In seeking to minimize the manipulation furan, altering the order of the synthetic pathway became necessary.

The likelihood of anhydride hydrolysis had previously not allowed for the Diels-Alder reaction to be used as the first synthetic step. In contrast to the labile maleic anhydride knowing that downstream synthetic steps may compromise the anhydride functionality, \(N\)-phenyl maleimide was seen as a stable alternative. The strength of the imide bond alone sharply decreased the possibility of a hydrolysis reaction from occurring. An addition, reaction monitoring through thin-layer chromatography was also a challenge when using maleic anhydride as the formed product was not UV active and, unfortunately, not readily charred using as stain potassium permanganate. In contrast and by design de novo, \(N\)-phenylmaleimide addressed both prior obstacles. Product formation and starting material consumption could now be easily monitored throughout the reaction sequence. Therefore, \(N\)-phenylmaleimide is considered a great candidate for Diels-Alder reactions in which the product can be further manipulated in subsequent reactions. The imide can be transformed in a final synthetic step restoring the anhydride that is has been shown key in inhibition of PP5.\(^{11}\)

Diels-Alder reactions are known to produce asymmetric racemic mixtures favoring the endo-adduct. However, co-crystal structures of PP5 and cantharidin reveal the \textit{exo} confirmation
allows for optimal interaction within the active site particularly with the manganese ions\textsuperscript{18}. Prior efforts were directed at developing a stereoselective synthesis protocol. Based on the knowledge that \textit{exo}-adduct is more thermodynamically favorable and that the cycloaddition is reversible, reaction temperatures were increased to favor formation of the desired \textit{exo} adduct. The performed Diels-Alder reaction between furan, parent compound, and \textit{N}-phenylmaleimide resulted in the stereoselective product formation. With the reaction performed at elevated temperatures, it was predicted that the thermodynamically favorable product, \textit{exo}, would become favored increasingly as the reaction proceeded.

The resulting NMR confirmed the formation of one isomer with only two peaks in the diagnostic region due to the symmetry of the molecule.
**Spectrum 1.** NMR spectrum of parent compound of Diels-Alder adduct following hydrogenation. Note the limited peaks in the diagnostic region at δ5.0 (H7), and δ3.0 (H6,H2).

However, under replicated reaction conditions both isomers were present when replacing furan with either 2-(hydroxymethyl)furan or 3-(hydroxymethyl)furan.
**Spectrum 2.** NMR spectrum of exo/endo mixture of Diels-Alder adduct following hydrogenation. In comparison to parent compound, the diagnostic region contains significantly more peaks between $\delta$5.0-3.0.
Enantioselective recrystallization of the \textit{exo} product with a similar system, a methyl substituent instead of hydroxymethyl at \( C_1 \), was referred to when interpreting NMR spectrum.\textsuperscript{19} Chemical shifts of the literature values could not be used to confirm which stereoisomer was generated. The isomers were separated using chromatographic techniques and further analyzed to determine which chemical shifts correspond to each.

Initially, thin layer chromatography was used to determine a solvent system that allowed for isomer separation. Separation was achieved using 5:1 EtOAc:hexanes. Translating this information to normal phase chromatography, the separation of Diels-Alder products produced stereochemically enriched samples of both the \textit{endo} and \textit{exo} isomers.

When interpreting the NMR spectra of the enriched samples, protons at positions 6 and 7 were the primary diagnostic peaks. Hydrogens at positions 6 and 2 are in the \textit{exo} position when overall product formed is \textit{endo} (Figure 9). Conversely, the \textit{exo} product results in hydrogens 6 and 2 being in the \textit{endo} position (Figure 10).

The change in position of H\(_6\) and H\(_2\) produces changes in coupling with the “neighboring” protons as it relates to position 7. The higher \( R_f \) valued product displayed a triplet splitting pattern for the proton at position 7 (Spectrum 3, Table 2). This splitting pattern is in contrast to the doublet displayed at the same position in the lower \( R_f \) isomer; that is, the proton at position 7 is coupled to an additional hydrogen in the first isomer than the second (Spectrum 4, Table 3).
Figure 9. *Endo* Stereoisomer. Identified as higher $R_f$ isomer through COSY NMR correlations.

Figure 10. *Exo* Stereoisomer. Identified as lower $R_f$ isomer through COSY NMR correlations.
**Spectrum 3**: NMR spectrum displaying the diagnostic region of isomer of higher \( R_f \) (enriched). Peaks of interest shown at \( \delta 5.0 \) (\( H_7 \)), and \( \delta 3.7-3.6 \) (\( H_6 \)).
Spectrum 4: NMR spectrum displaying the diagnostic region of isomer of higher $R_f$ (enriched).

Peaks of interest shown at $\delta$ 5.0 (H$_7$), and $\delta$ 3.2-3.1 (H$_6$).
**Table 2.** Isomer of higher $R_f$ (enriched):

<table>
<thead>
<tr>
<th>Entry</th>
<th>Peak assignment</th>
<th>Splitting Pattern</th>
<th>Peak (ppm)</th>
<th>Coupling Hz</th>
<th>Image of spectrum reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$H_7$</td>
<td>1H, CH</td>
<td>t</td>
<td>4.97</td>
<td>5.7</td>
<td></td>
</tr>
<tr>
<td>$H_2, H_6$</td>
<td>2H, CH$_2$</td>
<td>m</td>
<td>3.70-3.63</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Isomer of lower $R_f$ (enriched):

<table>
<thead>
<tr>
<th>Entry</th>
<th>Peak assignment</th>
<th>Splitting Pattern</th>
<th>Peaks (ppm)</th>
<th>Coupling Hz</th>
<th>Image of spectrum reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{H}_7$</td>
<td>1H, CH</td>
<td>d</td>
<td>4.99</td>
<td>5.15</td>
<td>![Image of spectrum reference]</td>
</tr>
<tr>
<td>$\text{H}_2, \text{H}_6$</td>
<td>2H, CH$_2$</td>
<td>m</td>
<td>3.19-3.16</td>
<td></td>
<td>![Image of spectrum reference]</td>
</tr>
</tbody>
</table>
However, the isomers could not be identified by the signal splitting patterns and as detailed above, the model system used to confirm either stereoisomers, exclusive nor enriched, did not align with the spectra generated using both 2-(hydroxymethyl)furan and 3-(hydroxymethyl)furan as diene. Structural information provided by the NMR could however, provide connectivity information given that the spin-spin coupling denoted by splitting patterns alone was not able to reveal which protons were giving rise to the coupling displayed in individual peaks. To garner more information on positioning of the diagnostic protons, two-dimensional NMR became necessary. The popular 2D NMR technique, homonuclear correlation spectroscopy or COSY, reveals homonuclear through bond correlations. If the nuclei of protons have interaction and therefore display coupling, it is denoted by cross peak correlations. The resulting COSY graph displays the one-dimensional NMR on both the x and y axis. Plot points appear where a correlation exists; each peak will be correlated to itself resulting in a series of linear points whereas nonlinear points display correlations between protons of differing chemical shifts. These nonlinear points reveal which protons are connected to one another through bond. Therefore, combining the data of one-dimensional and two-dimensional NMR gives both structural information and connectivity information. Relative stereochemical positioning of the protons is directly revealed through the correlations or perhaps more importantly, lack of correlations displayed by the two-dimensional NMR.

Furthermore, literature sources highlighted the effects of bond angle on the coupling constant $J$ between the protons $H_a$-$C$-$C$-$H_b$. The Newman projection displays that as the dihedral angle between $H_a$ and $H_b$ approaches $90^\circ$ the coupling constant approaches $0$ Hz. Conversely, as the bond angle approaches $0^\circ$ or $180^\circ$, the larger the coupling constant becomes.
In the model system, bicyclo[2.2.1]heptan-2-one, this correlation between the dihedral bond angle and coupling values is clearly reflected.

![Figure 11](image.png)

**Figure 11.** Bicyclo[2.2.1]heptan-2-one. Used as model system in establishing relationship between dihedral bond angle and coupling.

H\textsubscript{6n}-C-C-H\textsubscript{1}, where the dihedral bond angle is 44.91° coupling is 4.7 Hz. H\textsubscript{6x}-C-C-H\textsubscript{1}, where the bond angle is 77.26°, coupling is 0.1 Hz.\textsuperscript{20} Dihedral bond angles were derived using Spartan software. Given the similarity between the two systems highlighting the proton at the bridgehead carbon and the adjacent methylene, the synthesized Diels-Alder stereoisomers using the decorated furan derivatives are predicted to follow this pattern. In the synthesized endo-adduct, the dihedral bond angle, as determined by Spartan, of H\textsubscript{7}-C-C-H\textsubscript{6x} is 36.23° while the dihedral bond angle of H\textsubscript{7}-C-C-H\textsubscript{6n} of the exo-adduct is 81.91°. Therefore, based on the literature values, positioned hydrogen (H\textsubscript{6x}) of the endo-Diels-Alder adduct will display far greater coupling to the (H\textsubscript{7}) than the (H\textsubscript{6n}) of the exo-adduct.\textsuperscript{21} This coupling will be reflected in the given correlations of the COSY NMR.
**Figure 12.** Diagnostic correlations of *Endo* Diels-Alder adduct. Dihedral bond angle of H7-C-C-H6x is 36.23°. Coupling is expected to be significant.

**Figure 13.** Diagnostic correlations of *Exo* Diels-Alder adduct. Dihedral bond angle of H7-C-C-H6n is 81.91°. Coupling is expected to be greatly reduced.
The higher $R_f$ isomer displayed nonlinear spots between the H$_7$ and H$_6$ peaks as illustrated in the COSY spectrum below.

**Spectrum 5.** COSY correlations of higher $R_f$ product. Note the highlighted areas which confirm correlations between protons of positions 6 and 7.
In contrast, the isomer of lower $R_f$ value, did not show non-linear correlation between the respective peaks.

**Spectrum 6.** COSY correlations of lower $R_f$ product. Note the highlighted areas which confirm the absence of correlations between protons of positions 6 and 7.
From these correlations it was deduced that the triplet displayed by the first isomer included coupling with H\textsubscript{6} and therefore H\textsubscript{6} is in the \textit{exo} position (Figure 12, Spectrum 5). Furthermore, it is also deduced that the doublet displayed by the H\textsubscript{7} of the second stereoisomer did not include coupling with H\textsubscript{6} leading to the conclusion that H\textsubscript{6} is representative of the \textit{endo} isomer (Figure 13, Spectrum 6). Combining this data leads to the conclusion that the first isomer is the \textit{endo} Diels-Alder adduct and the second isomer is the \textit{exo} Diels-Alder adduct.

Addition of functional groups to furan made isolation of a singular isomer through stereoselective synthesis unviable. Column chromatography and identification of the isomers was necessary to confirm the isolation of the desired \textit{exo} isomer through recrystallization techniques. Only by having both isomers either separated or enriched and conducting this type of analysis is it possible to offer stereochemical confirmation. The results given by COSY spectral analysis confirmed the literature predictions of effect of dihedral bond angle on coupling. Isolation of the \textit{exo} stereoisomer allowed for advancement of this system to be pursued.
Conclusion:

Initial synthetic attempts consistently resulted in low yields as a result of competitive side reactions. After the testing of various pathways, it was revealed that the reactivity of furan made it unsuitable for continuous manipulation. Furan derivatization was limited by performing the Diels-Alder reaction as the first synthetic step. Replacing the reactive maleic anhydride with N-phenylmaleimide drastically improved the stability resulting in higher yields and enabling breadth when considering the addition of functionality. Original reaction preps sought to isolate the exo stereoisomer exclusively. However, the addition of the hydroxymethyl substituent resulted in a mixture of both stereoisomers. Preliminary NMR results were unable to definitely identify the isomers. The two-dimensional NMR technique COSY gave diagnostic correlations between the protons bound to positions 6 and 7 that when compared to the known effect of dihedral angle on coupling gave the affirmative stereoisomer determination. The exo isomer, with key peaks at 4.99 ppm and 3.19-3.16 ppm, was isolated through chromatography followed by recrystallization. This final product will be carried on in further synthetic steps by other members of the research lab. Work will focus on modifying synthesis protocol for both etherification using undecenyl-tosylate and anhydride formation. Epoxidation will be the final step before biological testing. Modification to the reaction protocol seek to develop a unified, high-yielding synthetic approach toward the assembly of decorated norcantharidin scaffolds that can be easily subjected to biological testing.
REFERENCES


APPENDICES

APPENDIX A: NMR SPECTRA

APPENDIX B: IMAGES FOR REACTION SETUP
APPENDIX B: IMAGES FOR REACTION SETUP
Image 1: Use of round-bottom bottle with wire bail closure for the cycloaddition of 2-hydroxymethylfuran and $N$-phenylmaleimide. Note: Hot plate setting was 100 °C.
Image 2. Parr shaker (medium pressure hydrogenation apparatus)