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The Enzymatic Activity and Inhibition of DapE Encoded N-Succinyl-L,L-Diaminopimelic Acid Dessucinylase

Tahirah Heath

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THE ENZYMATIC ACTIVITY AND INHIBITION OF
DAPE ENCODED N-SUCCINYL-L,L-DIAMINOPIMELIC ACID DESUCCINYLASE

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN THE CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN CHEMISTRY AND BIOCHEMISTRY

BY
TAHIRAH K. I. HEATH

CHICAGO, IL
MAY 2018
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ACKNOWLEDGEMENTS

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For my parents, Monique and Myron Heath
TABLE OF CONTENTS

ACKNOWLEDGEMENTS iii

LIST OF TABLES viii

LIST OF FIGURES ix

LIST OF SCHEMES xi

LIST OF ABBREVIATIONS xiii

ABSTRACT xvi

CHAPTER ONE: AN INTRODUCTION TO BACTERIAL INFECTIONS AND ANTIBIOTIC RESISTANCE 1

CHAPTER TWO: N-SUCCINYL-L,L-DIAMINOPIMELIC ACID DESUCCINYLASE BACTERIAL ENZYME AS AN ANTIBIOTIC TARGET 4

  Introduction 4
  Results and Discussion 6
    DapE Crystal Structure and Important Residues 6
    In Silico Modeling for Enzyme Interactions 9
    Closed Conformation of DapE 10
    Substrate Requirements for DapE Activity 12
    Measuring Enzymatic Activity and Inhibition 16
    Identifying Appropriate Lead Inhibitors of DapE 17
  Conclusion 19

CHAPTER THREE: A SPECTROPHOTOMETRIC NINHYDRIN-BASED ENZYMATIC ASSAY 20

  Introduction 20
  Chemistry 21
  Results and Discussion 24
    Primary Amine Detection with Ninhydrin 24
    Quenching of DapE Hydrolytic Activity 28
    Circular Dichroism Denaturation Studies 29
    Pre-Heat Incubation Studies 30
    Enzyme Kinetics for N^6-methyl-SDAP 1b 31
    Reaction Rate/Velocity and Characterization of DapE Activity 32
    DapE Ninhydrin-based Enzymatic Assay and IC_{50} Determination 33
    Screening Primary Amine Inhibitors Using a Ninhydrin-Based Assay 33
  Conclusion 35
LIST OF TABLES

Table 1. Inhibitors of DapE identified by 225nm assay\textsuperscript{28} 17

Table 2. DapE inhibitor IC\textsubscript{50} values using ninhydrin-based enzymatic assay 34

Table 3. Molecular docking of 5-bromo-6-sulfonamide indoline 16 derivatives\textsuperscript{[a]} 43

Table 4. Ligand binding energies of 5-bromo-6-sulfonamide indoline 18,19 and 20\textsuperscript{[a]} 44

Table 5. Ligand binding energies of 5-chloro-6-sulfonamide indoline 22 derivatives\textsuperscript{[a]} 46

Table 6. Binding energies of N-substituted-5-bromo-6-sulfonamide Indolines 25, 27 and 29\textsuperscript{[a]} 47

Table 7. Inhibitory data (IC\textsubscript{50}) of N-acetyl-5-bromo-6-sulfonamide indolines 49

Table 8. Inhibitory data (IC\textsubscript{50}) of N-acetyl-5-chloro-6-sulfonamide indolines 22 50

Table 9. Binding energies of compound E with (R)- vs (S)-enantiomers with R\textsubscript{1} variation \textsuperscript{[a]} 54

Table 10. Binding energies of compound E serine derivatives 38\textsuperscript{[a]} 56

Table 11. Ligand binding energies of compound E phenylalanine derivatives 39\textsuperscript{[a]} 57

Table 12. Ligand binding energies of compound E tyrosine derivatives 40\textsuperscript{[a]} 59

Table 13. Inhibitory data of HTS compounds B 65

Table 14. Inhibitory data of miscellaneous compounds using ninhydrin-based assay 66

Table 15. Molecular docking energies of brominated 7- vs. 6-sulfonamide indolines\textsuperscript{[a]} 71

Table 16. Ligand binding energies for 1-acyl-7-N-acylsulfonamide indolines \textsuperscript{[a]} 86

Table 17. Inhibition data (IC\textsubscript{50}) of 7-sulfonamide indolines\textsuperscript{[a]} 90
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Side-by-side comparison of $[\text{ZnZn(HiDapE)}]^{a}$ open and closed conformations.</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>Active Site of $[\text{ZnZn(HiDapE)}]^{a}$</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>Active site of $[\text{ZnZn(NmDapE)}]$ 1.3Å crystal structure from PDB:5EUJ $^{[a]}$</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>Active site of $[\text{ZnZn(HiDapE)}]^{[a]}$ products-bound structure $^{[19]}$</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>$[\text{ZnZn(HiDapE)}]$ products-bound active site binding regions $^{[a]}$ $^{[19]}$</td>
<td>11</td>
</tr>
<tr>
<td>6</td>
<td>L,L-SDAP N$^{6}$-mono-methyl and N$^{6}$-di-methyl substrate analogs</td>
<td>13</td>
</tr>
<tr>
<td>7</td>
<td>Molecular docking of L,L-SDAP substrate analogs $^{[a]}$</td>
<td>14</td>
</tr>
<tr>
<td>8</td>
<td>N$^{6}$-acetylated and N$^{6}$-methylated L,L-SDAP substrate analogs $\textbf{1b}$ and $\textbf{1d}$</td>
<td>21</td>
</tr>
<tr>
<td>9</td>
<td>UV Absorption spectra of primary and secondary amines with ninhydrin $^{[a]}$</td>
<td>25</td>
</tr>
<tr>
<td>10</td>
<td>Absorption spectra of primary and secondary amines as a function of time $^{[a]}$</td>
<td>27</td>
</tr>
<tr>
<td>11</td>
<td>Circular Dichroism thermal denaturation study of DapE $^{[a]}$</td>
<td>29</td>
</tr>
<tr>
<td>12</td>
<td>Development of primary amine standard with ninhydrin $^{[a]}$</td>
<td>31</td>
</tr>
<tr>
<td>13</td>
<td>Enzyme saturation curve of DapE $^{[a]}$</td>
<td>32</td>
</tr>
<tr>
<td>14</td>
<td>HTS lead compounds identified with the DapE coupled assay</td>
<td>37</td>
</tr>
<tr>
<td>15</td>
<td>Indoline, indole, and natural indole-containing compounds</td>
<td>37</td>
</tr>
<tr>
<td>16</td>
<td>HTS lead N-acetyl-6-sulfonamide compound $\textbf{C}$ $^{[a]}$</td>
<td>38</td>
</tr>
<tr>
<td>17</td>
<td>Phenyltetrazole thioether HTS compound $\textbf{E}$ with plan for future analogs</td>
<td>51</td>
</tr>
<tr>
<td>18</td>
<td>Molecular docking of HTS compound $\textbf{E}$ $^{[a]}$</td>
<td>52</td>
</tr>
<tr>
<td>19</td>
<td>Phenyltetrazole (S-$\textbf{38b}$) docking interaction with the Zn(II)s at DapE active site $^{[a]}$</td>
<td>57</td>
</tr>
</tbody>
</table>
Figure 20. Phenyltetrazole inhibitor (R)-40b interaction with Zn(II)s at DapE active site[a]  58
Figure 21. Difluoromethyl sulfonamide HTS lead compound B with points of modification  51
Figure 22. Difluoromethyl sulfonamide B interaction with the Zn(II) ions at DapE[a]  63
Figure 23. Methyl sulfonamide analogs of compound B  64
Figure 24. Conformation of N-acetyl-5-bromoindoline rotamers  68
Figure 25. Molecular docking interaction of 7-sulfonmaide indolines with DapE[a]  70
Figure 26. 7-Sulfonamide indolines synthesized by improved Borror method  74
Figure 27. Ligand-enzyme interaction of N-acetyl-7-(N-acetyl)sulfonamide indolines 79a [a]  87
Figure 28. Synthesis of 1-Cbz-7-(N-acyl)sulfonamide indolines 76  88
Figure 29. Synthesis of indoline 7-sulfonylureas 78 from Cbz-7-sulfonamide indoline 64  88
**LIST OF SCHEMES**

<table>
<thead>
<tr>
<th>Scheme</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Enzymatic cleavage of L,L-SDAP (1a) by DapE to succinate (2) and DAP (3a)</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>Asymmetric synthesis of N^{6}-Methyl-L,L-SDAP substrate 1b&lt;sup&gt;[a]&lt;/sup&gt;</td>
<td>22</td>
</tr>
<tr>
<td>3</td>
<td>Synthesis of N-acetyl-5-bromo-6-sulfonamide indolines 16a and 16b&lt;sup&gt;[a]&lt;/sup&gt;</td>
<td>38</td>
</tr>
<tr>
<td>4</td>
<td>Synthesis of N-acetyl-5-bromo-6-sulfonamide indolines derivatives 16&lt;sup&gt;[a]&lt;/sup&gt;</td>
<td>39</td>
</tr>
<tr>
<td>5</td>
<td>Synthesis of 6-(N-acyl) sulfonamides, sulfonylureas and sulfonyl thioureas&lt;sup&gt;[a]&lt;/sup&gt;</td>
<td>39</td>
</tr>
<tr>
<td>6</td>
<td>Synthesis of N-acetyl-5-chloro-6-sulfonamide indolines derivatives 22&lt;sup&gt;[a]&lt;/sup&gt;</td>
<td>40</td>
</tr>
<tr>
<td>7</td>
<td>Synthesis of 6-sulfonamide indoline derivatives with R&lt;sub&gt;1&lt;/sub&gt; variation&lt;sup&gt;[a]&lt;/sup&gt;</td>
<td>41</td>
</tr>
<tr>
<td>8</td>
<td>Synthesis of the HTS lead compound E&lt;sup&gt;[a]&lt;/sup&gt;</td>
<td>53</td>
</tr>
<tr>
<td>9</td>
<td>Synthesis of HTS lead compound E amine analog&lt;sup&gt;[a]&lt;/sup&gt;</td>
<td>53</td>
</tr>
<tr>
<td>10</td>
<td>Diazotization and bromination of α-amino acids to α-bromo carboxylic acids&lt;sup&gt;[a]&lt;/sup&gt;</td>
<td>55</td>
</tr>
<tr>
<td>11</td>
<td>Synthesis of HTS lead difluoromethyl sulfonamide compound B&lt;sup&gt;[a]&lt;/sup&gt;</td>
<td>62</td>
</tr>
<tr>
<td>12</td>
<td>Synthesis of compound B analogs with modification at R&lt;sub&gt;3&lt;/sub&gt;</td>
<td>62</td>
</tr>
<tr>
<td>13</td>
<td>Borror’s synthesis of N-acetyl-7-sulfonamide indolines&lt;sup&gt;[a]&lt;/sup&gt;</td>
<td>73</td>
</tr>
<tr>
<td>14</td>
<td>Improved synthesis of N-acetyl-7-sulfonamide indolines&lt;sup&gt;[a]&lt;/sup&gt;</td>
<td>74</td>
</tr>
<tr>
<td>15</td>
<td>Proposed synthesis of 7-sulfonyl chloride indoline 60&lt;sup&gt;[a]&lt;/sup&gt;</td>
<td>76</td>
</tr>
<tr>
<td>16</td>
<td>Acetylation of primary sulfonamide 58 using acetyl chloride</td>
<td>76</td>
</tr>
<tr>
<td>17</td>
<td>Selective aniline protection using carboxybenzyl (Cbz) group.</td>
<td>77</td>
</tr>
<tr>
<td>18</td>
<td>Unsuccessful attempts at deaminative chlorination</td>
<td>77</td>
</tr>
<tr>
<td>19</td>
<td>Proposed synthesis of 7-sulfonic acid indoline 67 from the primary 7-sulfonamide indoline 64</td>
<td>78</td>
</tr>
</tbody>
</table>
Scheme 20. Use of Ph$_3$P to couple aryl sulfonic acids to form sulfonamides 80
Scheme 21. Use of polymer-bound Ph$_3$P for coupling aryl sulfonic acids 81
Scheme 22. Selective acetylation of 7-sulfonamide indoline 58. 82
Scheme 23. Modified synthesis of N-acetyl-7-sulfonamide indolines$^a$ 83
Scheme 24. Variation of N-Cbz-7-sulfonamide indoline 64 at R$_3$$^a$ 84
Scheme 25. Hydrogenolysis to produce N-acyl-7-(N-acetyl)sulfonamide indolines 79 85
Scheme 26. Sulfonamide rearrangement reactions 65,66 91
Scheme 27. Lithiation-mediated synthesis of 7-sulfonamide indolines 92
Scheme 28. Ortho directed lithiation using CO$_2$$^a$ 93
Scheme 29. Synthesis of indoline sulfonic acid$^a$ 94
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>angiotensin-converting-enzyme</td>
</tr>
<tr>
<td>acetyl-CoA</td>
<td>acetyl coenzyme A</td>
</tr>
<tr>
<td>AcOH</td>
<td>acetic acid</td>
</tr>
<tr>
<td>AlCl₃</td>
<td>aluminum trichloride</td>
</tr>
<tr>
<td>BOC</td>
<td>tert-butyloxycarbonyl</td>
</tr>
<tr>
<td>Cbz</td>
<td>carboxybenzyl</td>
</tr>
<tr>
<td>CDM</td>
<td>charge density mismatch</td>
</tr>
<tr>
<td>ClSO₃H</td>
<td>chlorosulfonic acid</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>DapE</td>
<td>diamino pimelic acid dessuccinylase enzyme</td>
</tr>
<tr>
<td>dapE</td>
<td>diamino pimelic acid dessuccinylase enzyme gene</td>
</tr>
<tr>
<td>DBU</td>
<td>1,8-diazabicyclo[5.4.0]undec-7-ene</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DMAc</td>
<td>N,N-dimethylacetamide</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>ESKAPE</td>
<td><em>Enterococcus faecium, Staphylococcus aureus, Klebsiella species, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species</em></td>
</tr>
</tbody>
</table>

xiii
Et$_3$N \hspace{1cm} \text{triethylamine}

EtOH \hspace{1cm} \text{ethanol}

H. influenzae \hspace{1cm} \textit{Haemophilus influenzae}

H. pylori \hspace{1cm} \text{helicobacter pylori}

HEPES \hspace{1cm} 2-[4-(2-hydroxyethyl)piperizin-1-yl]ethanesulfonic acid

HiDapE \hspace{1cm} \textit{Haemophilus influenzae} L,L-diaminopimelic acid desuccinylase enzyme

HPLC \hspace{1cm} \text{high pressure liquid chromatography}

HTS \hspace{1cm} \text{high-throughput screening}

K$_2$CO$_3$ \hspace{1cm} \text{potassium carbonate}

K$_{\text{cat}}$ \hspace{1cm} \text{turnover number}

KDa \hspace{1cm} \text{kilodalton}

K$_m$ \hspace{1cm} \text{Michaelis-Menten constant}

L,L-DAP \hspace{1cm} L,L-diaminopimelate

L,L-SDAP \hspace{1cm} N-succinyl-L,L-diaminopimelic acid

LogP \hspace{1cm} \text{partition coefficient}

LysA \hspace{1cm} \text{lysin enzyme A}

M. smegmatis \hspace{1cm} \textit{Mycobacterium smegmatis}

m-DAP \hspace{1cm} \text{meso-diaminopimelate}

MeCN \hspace{1cm} \text{acetonitrile}

MMFF94X \hspace{1cm} \text{Merck Molecular Force Field 94X}

MOE \hspace{1cm} \text{Molecular Operating Environment}

N. meningitidis \hspace{1cm} \textit{Neisseria meningitidis}
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$CO$_3$</td>
<td>sodium carbonate</td>
</tr>
<tr>
<td>NaBH$_4$</td>
<td>sodium borohydrate</td>
</tr>
<tr>
<td>n-BuLi</td>
<td>n-butyl lithium</td>
</tr>
<tr>
<td>NCS</td>
<td>N-chlorosuccinamid</td>
</tr>
<tr>
<td>NmDapE</td>
<td><em>Nesseria meningitidis</em> dianminopimelic acid dessucinylase enzyme</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>OTf</td>
<td>trifluoromethanesulfonate</td>
</tr>
<tr>
<td>PCC</td>
<td>pyridinium chlorochromate</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>PDC</td>
<td>pyridinium dichromate</td>
</tr>
<tr>
<td>pH</td>
<td>potential of hydrogen</td>
</tr>
<tr>
<td>Ph$_3$P</td>
<td>triphenyl phosphine</td>
</tr>
<tr>
<td>Ph$_3$PO</td>
<td>triphenyl phosphine oxide</td>
</tr>
<tr>
<td>POCl$_3$</td>
<td>phosphorus oxychloride</td>
</tr>
<tr>
<td>R&amp;D</td>
<td>research and development</td>
</tr>
<tr>
<td>SAR</td>
<td>structure-activity relationship</td>
</tr>
<tr>
<td>SDAP</td>
<td>N-succinyl-L,L-diaminopimelic acid</td>
</tr>
<tr>
<td>STTAC</td>
<td>sulfur trioxide trimethylamine complex</td>
</tr>
<tr>
<td>t-Bu</td>
<td>tert-butyl group</td>
</tr>
<tr>
<td>t-BuOH</td>
<td>tert-butyl alcohol</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoro acetic acid</td>
</tr>
<tr>
<td>TMEDA</td>
<td>tetramethylethylenediamine</td>
</tr>
</tbody>
</table>
ABSTRACT

The bacterial enzyme DapE is a hydrolase in the late stage of the mDAP/lysine biosynthetic pathway that catalyzes the hydrolysis of N-succinyl-L,L-diaminopimelic acid (L,L-SDAP) to succinic acid and L,L-diaminopimelate (DAP). The product DAP is ultimately converted to m-DAP and then to lysine which is used in the construction of proteins, whereas for humans, lysine is an essential amino acid obtained through diet. DapE has been shown to be conserved across many strains of bacteria. The m-DAP produced through the succinylase pathway is used by both gram negative and gram-positive bacteria in the construction of the essential peptidoglycan cell wall. Because of conservation across bacterial strains and its absence in humans, DapE has been identified as an ideal drug target for antibiotics with a new mechanism of action. Inhibitors of this enzyme may serve as broad-spectrum antibiotics with selective toxicity towards bacterial strains without mechanism-based toxicity in humans.

Previously, the enzymatic activity was determined by monitoring the cleavage of the substrate’s amide bond at 225 nm. The development of a new assay which can be observed at a wavelength of 570 nm through detection of Ruhemann’s purple complex produced by reaction of ninhydrin and the hydrolyzed primary amine product of a newly synthesized L,L-SDAP substrate analog enables identification of new inhibitors and establishing SAR. Inhibitory data of ligands identified through a HTS screen and their analogs have been obtained using the new ninhydrin-based enzymatic assay. A High-Throughput Screening (HTS) of over 33,000 compounds licensed from Chembridge Corporation utilizing the enzyme coupled assay identified five
lead ligands as inhibitors with inhibition of >20% at 12 μM of DapE. The results consist of four series of inhibitors: a simple amide with a β-sulfonyl moeity, a difluoromethyl sulfonamide ligand, two N-acyl-6-sulfonamide indolines and a chiral phenyltetrazole thioether. Inhibitory data of ligands identified through a HTS screen and their analogs have been obtained using the new ninhydrin-based enzymatic assay. Herein, we report the synthesis, inhibitory data and SAR of analogs from the last three series of inhibitors as well as a novel synthesis for the formation of 7-sulfonamide indolines as a new series of inhibitors of DapE.
CHAPTER ONE

AN INTRODUCTION TO BACTERIAL INFECTIONS AND ANTIBIOTIC RESISTANCE

Bacterial infections are a significant and growing medical problem in both the United States and around the world.\(^1\) It is estimated that at least 90 million new tuberculosis cases were reported worldwide in the 1990’s resulting in about 30 million deaths.\(^2\) These data reveal that tuberculosis is the leading cause of death in adults by an infectious disease, worldwide. \(^2\) The use of antibiotics has substantially decreased the number of deaths due to infectious diseases.

Antibiotics are used to treat both gram-negative and gram-positive bacterial infections. Both gram-negative and gram-positive bacteria include pathogenic as well as non-pathogenic bacteria. Most bacteria are harmless or even beneficial while only some are pathogenic, and pathogenic bacteria lead to infectious diseases. The term gram-negative and gram-positive comes from the crystal violet staining that is used to classify bacteria based on the structure of the peptidoglycan cell wall.\(^3\) Gram-positive bacteria, aptly named so due to the positive test result, have a thick cell wall that can absorb the crystal violet dye. Gram-negative bacteria do not absorb the violet staining and instead turn pink showing a negative result. This is due to the fact that gram-negative bacteria have a thinner peptidoglycan layer and an extra outer lipid membrane which makes cell wall penetration more difficult. Despite having a thicker cell wall than gram-negative bacteria, gram-positive bacteria are more susceptible to antibiotics because they lack that outer membrane.
As of 2004, more than 70% of pathogenic bacteria were resistant to at least one of the currently used antibiotics. Resistance stems from a spontaneous or induced genetic mutation or the acquisition of resistant genes from other bacteria by horizontal gene transfer. This enables bacteria to become immune to specific antibiotics and potentially to structurally-related variants as well. Unfortunately, the widespread distribution and over-administration of antibiotics by physicians has contributed to the development of antibiotic resistance. Antibiotic resistance has been recognized since the advent of penicillin-resistant *Staphylococcus aureus* infections rapidly appeared. Nevertheless, penicillin is still widely effective against many bacteria today. MRSA or methicillin-resistant *Staphylococcus aureus* is probably the most familiar drug-resistant bacteria. However, the emergence of ESKAPE pathogens is the biggest threat to the successful treatment of infectious diseases thus far. ESKAPE pathogens are six strains of bacteria that have become resistant to many antibiotics on the market, and include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species. At least two other strains of common bacterial species capable of causing life-threatening illnesses (*Mycobacterium tuberculosis* and *Escherichia coli* O157:H7) are already resistant to all drugs in clinicians’ current arsenal of more than 100 antibiotics.

Once easily treatable infections can render a person extremely sick and can be fatal. A bacterial strain such as *E. coli* developing resistance is especially dangerous because it is commonly found in cases of food poisoning and urinary tract infections in otherwise healthy people. This new phenomenon of drug-resistant bacteria passing on resistance to other bacterial
strains can create a nightmare scenario. Consequently, the risk that more pathogenic bacterial strains will evolve resistance against new antibiotics is very high.\textsuperscript{6,9}

Penicillin was among the first drugs to be effective against bacterial infections. It was discovered in 1928 by Scottish scientist Alexander Fleming from the penicillium fungi, but was not used in humans until 1942.\textsuperscript{10} Penicillin is a part of the β-lactam antibiotic family which is comprised of many classes of β-lactams. Penicillin resistance in bacteria developed rapidly within the first 4 years of use. Since the introduction of penicillin, many other antibiotics have been introduced including other β-lactam antibiotics such as carbapenems and cephalosporins which helped to combat antibiotic resistance in bacteria up until now. Most novel antibiotics that are developed are structural variants of a limited set of known bioactive compounds including those with β-lactam functionality. Many of these compounds target the same enzymatic pathways which include protein and cell wall synthesis.\textsuperscript{11}

There is a limited lifetime of usefulness of antibiotics from when they are first identified as effective against bacteria to when bacteria eventually develop resistance. There is an urgent call for the introduction of new antibiotics in the R&D pipeline to help combat this problem. There has been a decrease in the number of antibiotics that have been introduced on the market over the past few decades. It takes an average of 10 years between initial discovery of a drug compound and when it first enters the market. This means that antibiotics launched today are the products of drug discovery projects initiated at least a decade ago.\textsuperscript{12} The development of resistance to current antibiotics makes the discovery of novel compounds towards new target pathways a critically important challenge. To help overcome bacterial resistance to antibiotics, new enzyme targets must be identified and small molecule inhibitors developed.\textsuperscript{13}
CHAPTER TWO

N-SUCCINYL-L,L-2,6-DIAMINOPIMELIC ACID DESSUCINYLASE BACTERIAL ENZYME AS AN ANTIBIOTIC TARGET

Introduction

A previously unexplored target for the development of new antibiotics is the enzymatic pathway for the biosynthesis of lysine in bacteria. The m-DAP/lysine biosynthetic pathway in plants and bacteria converts L-aspartate to the amino acid lysine through its immediate precursor meso-diaminopimelate (m-DAP). In the late stage of the lysine biosynthetic pathway, m-DAP is synthesized by bacteria through three subset parallel pathways: the succinylase, acetylase, and the dehydrogenase pathways. The succinylase pathway is the most widely distributed in bacteria being utilized by all gram-positive and nearly all gram-negative bacteria. The dapE gene encoded N-succinyl-L,L-2,6-diaminopimelic acid desuccinylase (DapE) is a hydrolase enzyme in the late stage of the succinylase pathway that catalyzes the hydrolysis of N-succinyl-L,L-diaminopimelic acid (L,L-SDAP) 1a to succinic acid 2 and L,L-diaminopimelate (DAP) 3a (Scheme 1). DAP is then converted to meso-DAP and ultimately to lysine.

Scheme 1. Enzymatic cleavage of L,L-SDAP (1a) by DapE to succinate (2) and DAP (3a)
Lysine is used by gram-negative and gram-positive bacteria in the construction of the peptidoglycan cell wall. Once m-DAP is synthesized, it is either incorporated into the peptidoglycan cell wall of bacteria or decarboxylated to lysine by LysA. It has been shown that the deletion of the dapE gene is lethal to *H. pylori* and *M. smegmatis*. Bacteria present in lysine substituted media with the dapE gene deletion could not grow, verifying that lysine cannot be effectively imported. This proves that lysine is essential to the proliferation of bacteria and strongly suggests that the m-DAP/lysine biosynthetic pathway is the only source for lysine in bacteria and it cannot be provided by the environment. In contrast, lysine is an essential dietary amino acid that humans must obtain through their diet as the lysine biosynthetic pathway is not present in mammals. In humans, lysine plays a major role in the muscle building, calcium absorption, injury recovery, and in the production of hormones, enzymes and antibodies. Humans also metabolize lysine to give acetyl-CoA used in protein synthesis. Inhibition of DapE therefore suggests prospects of a broad spectrum antibiotic with selective toxicity towards bacterial strains as free of mechanism-based toxicity in humans.

The lysine biosynthetic pathway enzymes, specifically DapE, are an ideal drug target for antibiotics in that this pathway is not present in humans. Furthermore, DapE has been shown to be conserved across many strains of bacteria and essential to their peptidoglycan cell wall. DapE has been identified in many drug resistant bacteria. In summary, inhibition of the m-DAP/lysine biosynthetic pathway could provide a new class of broad-spectrum antibiotics with a new mechanism of action that inhibit synthesis of the bacteria cell wall and are then lethal to bacteria. The identification of new classes of inhibitors of DapE and the development of an enzymatic assay to determine inhibitory potency of these compounds will be described in this
dissertation. The two key steps to the structural insight of inhibitor binding is obtaining a crystal structure of the enzyme with key catalytic residues identified as well as elucidating substrate structural requirements for catalysis. We report new insights on the physical structure and the catalytic activity of DapE obtained through a combination of crystallography, molecular docking and enzyme inhibitory data. Understanding how the enzyme works is the most effective way of understanding how to inhibit its activity. The results herein advance our knowledge toward development of new antibiotics through inhibition of DapE.

**Results and Discussion**

**DapE Crystal Structure and Important Catalytic Residues.**

DapE is a small, homodimeric enzyme (41.5 KDa/subunit) that contains one catalytic and one dimerization domain per monomer, and it has been shown that the dimerization domain is required for catalysis.\(^{18}\) DapE has two known conformations: an open conformation that resembles an old-school rotary telephone receiver, and a closed conformation.\(^{19}\)

Figure 1. Side-by-side comparison of [ZnZn(HiDapE)]\(^a\) open and closed conformations.\(^{19}\)

*[a] (A) The previously obtained apo “open” conformation of HiDapE (PDB:3IC1). (B) The “closed” conformation of HiDapE with products bound; succinic acid 2 in magenta and L,L-DAP 3a in yellow (PDB:5VO3). The two subunits of the homodimer are colored in green and orange.*
The DapE enzyme is conserved across various strains of bacteria. Structurally and experimentally we have a logical first step in inhibitor design that is understanding how the substrate binds to the active site.\textsuperscript{13} Two crystal structure of the open conformation of DapE with mono zinc center \([\text{Zn}(\text{HiDapE})]\) (PDB:3ISZ) and di-zinc center \([\text{ZnZn}(\text{HiDapE})]\) (PDB:3IC1) active sites with A 2.0 and 2.3\textdegree{} resolution, respectively, were obtained that demonstrated that two Zn(II) atoms per monomer are needed for full enzymatic activity while the mono Zn(II) enzyme retains only \(\sim 60\%\) activity.\textsuperscript{20} DapE can exist as different metallo-isozymes including physiologically relevant variants where one Zn(II) is substituted with other divalent metals retaining enzymatic activity. The Co(II)-substituted DapE enzyme was 25\% more active than the Zn(II)-loaded form of the enzyme.\textsuperscript{21} Interestingly, Mn(II) can activate DapE, but the activity is just to \(\sim 20\%\) of the Zn(II)-bound enzyme.\textsuperscript{21} The order of the observed \(k_{\text{cat}}\) values are Co(II) > Zn(II) > Cd(II) > Mn(II) > Ni(II) \(\sim\) Cu(II) \(\sim\) Mg(II).\textsuperscript{21} The Zn(II) atoms sit approximately 3.30 \textdegree{} apart with a distorted tetrahedral geometry involving one imidazole group, one carboxylate group and a bridged carboxylate group (Figure 2).\textsuperscript{20, 22} The Zn(II) atoms in the active site are coordinated to a central catalytic water molecule that is employed in the hydrolysis of the substrate N-succinyl-L,L-diaminopimelic acid (L,L-SDAP).\textsuperscript{20, 22}

Figure 2. Active Site of \([\text{ZnZn}(\text{HiDapE})]\)\textsuperscript{[a] 20}

\textsuperscript{[a]} (A) Crystal structure PDB:3IC1. (B) DapE active site with bond distances of residues to Zn 1 (blue) and Zn2 (red).
The PDB:3IC1 crystal structure revealed that residues Glu134 and Thr325 of the *H. influenzae* DapE were important residues for substrate catalysis. Glu134 serves as a general acid/base while Thr325 hovers over the active site and plays a role in L,L-SDAP substrate recognition and transition state stabilization.\textsuperscript{20} Positively-charged residues likely interact with the negatively charged carboxylic acid groups on the substrate. More recently, a co-crystallization attempt of [ZnZn(NmDapE)] (*N. meningitidis*) in the presence of inhibitors and 5\% DMSO produced crystals of DapE that diffracted at 1.30 Å. This structure was deposited in the RSCB Protein Data Bank with the entry 5UEJ (Figure 3).

**Figure 3.** Active site of [ZnZn(NmDapE)] 1.3Å crystal structure from PDB:5UEJ \[a\]

\[a\] Crystal structure obtained in the presence of 5\% DMSO with a refinement $R/R_{\text{free}}=13/16\%$.

Although the crystal structure obtained was unbound (no ligand), this is significant advance for two main reasons: 1) previous crystal structures of DapE were of much poorer resolution, the best being at 1.80 Å, and 2) this is the first time that DapE crystals were obtained in the presence of 5\% DMSO. This is significant because DMSO can aid in the solubility of inhibitors but can effect crystal growth. This new, near atomic resolution of DapE allows us to refine the structure with a more detailed and accurate position of atoms which can translate to better molecular docking of inhibitors involving the active site. To further elucidate the structural
requirements for DapE enzymes to recognize and bind L,L-SDAP, we have determined the X-ray crystal structure of [ZnZn(HiDapE)] bound by the products of hydrolysis: succinic acid 2 and L,L-DAP 3a (Figure 4). The atomic coordinates and structure have been deposited in the RCSB Protein Data Bank as entry 5VO3.

Figure 4. Active site of [ZnZn(HiDapE)]$^{[a]}$ products-bound structure$^{19}$

$^{[a]}$ Electron density map of DapE enzymatic products succinic acid (cyan) and L,L-DAP (yellow) bound in the active site with Zinc ions shown in black.

**In Silico Modeling for Enzyme Interactions**

*In silico* studies help predict the binding modes and energies of ligand-enzyme interactions. X-ray crystallography was used to guide our efforts in designing and synthesizing inhibitors of DapE. Structure-based ligand discovery uses crystal structures obtained from proteins and predicts the binding affinities of ligands to the enzyme through molecular docking. This approach identifies several ligands from a screening library that have a high affinity for the target. There are many different software programs available for molecular docking and virtual screening such as AutoDock, DOCK, SwissDOCK and FlexX.$^{23, 24}$ Virtual screening can suggest new potential lead compounds, and this approach can also be a key tool for mechanistic studies in enzyme-substrate interactions. The use of molecular docking has led to the development of protocol termed Products-Bound Transition-State Modeling (PBTSM) developed by Cory Reidl
which utilizes the product-bound crystal structure of an enzyme in combination with the unbound crystal structure to perform *in silico* studies for mapping out enzyme-substrate mechanisms and interactions. A step-by-step reconstruction of the product back to the via the transition state aids in the identification of key residues essential for substrate binding and catalysis. The PBTSM protocol was first seen published in Nocek and Reidl’s work for determination of the critical residues responsible for the conformational change observed in DapE PDB:5VO3.

**Closed Conformation of DapE.**

The products-bound structure of [ZnZn(HiDapE)] reveals previously unknown substrate-enzyme interactions and a catalytically significant, dramatic conformational change of the enzyme from the known open-conformation obtained through previous crystal structures to a previously unobserved closed-conformation of the enzyme. The closed-conformation crystal structure reveals that residues from the dimerization domain of the B subunit come in contact with the active site of the A subunit. An overlay of the open and closed conformation shows that the catalytic domain rotates ~50° and moves 29 Å at the exterior and 10 Å at the interior of the enzyme. In addition, the newly formed amine of L,L-DAP is only 2.9 Å from the newly formed succinic acid carbon. There is significant movement of the enzyme which results in closing of the Zn(II) active site and defining two distinct binding regions: the succinic acid binding region and the L,L-DAP binding region (Figure 5).
In addition, a previously unidentified residue, His194.B from subunit B, was shown to be critical in the formation of the closed-conformation of DapE. When there is no substrate in the active site, His194.B from the dimerization domain in the B subunit sits 10 Å away from the subunit A active site. Upon binding of the substrate, His194.B moves to a distance of 2.9 Å away from the succinic acid and forms an H-bond to the oxygen that binds to Zn2 forming an oxyanion hole. The closed crystal structure depicting the His194.B movement shows that this residue is critical in activity by activating the scissile bond for nucleophilic attack by the active site H2O. This stabilization possibly allows for catalysis in the absence of Zn2 by coordination of His194.B in this oxyanion hole. We proposed that the presence of the substrate induces a conformational change in the enzyme structure that facilitates catalytic activation within the active site, and without the substrate present the enzyme remains in the open, inactive conformation. In effect, the substrate acts as the glue that links the catalytic domain of subunit A to the dimerization/cap domain of subunit B. This is evidenced by the NmDapE mutant His195.B, which corresponds to His194.B in HiDapE, that provides a [ZnZn(NmDapE)] enzyme.
that exhibits only ~3% of its WT activity.\textsuperscript{19} These data provide critical structural insights on the catalytic activity of DapE and the structural residues that are important for hydrolytic activity.

**Substrate Requirements for DapE Activity.**

Now that the mechanism of catalytic activity of the enzyme and the important residues necessary for catalytic activity, determination of the substrate requirements for hydrolysis can aid in the development of inhibitors of DapE. Understanding which structural components are necessary for DapE to recognize and bind the L,L-SDAP substrate can guide the design of inhibitors by competing with or complying with substrate specificity. The endogenous substrate L,L-SDAP is hydrolyzed by DapE, and screening substrate analogs for hydrolysis can help validate important structural requirements. DapE is shown to only selectively hydrolyze N-succinyl-L,L-diaminopimelic acid (L,L-SDAP) and is inactive toward D,L-, L,D-, and D,D-SDAP.\textsuperscript{21} Further work on substrate analogs was taken up by Holz, in addition to investigating the differences in activities of the zinc and cobalt metal isozymes of DapE, and it was revealed that the free N\textsuperscript{6}-amino group of the substrate is crucial for recognition and cleavage by DapE.\textsuperscript{21} Gelb’s and Holz’s work demonstrated that there are indeed strict structural requirements for the substrate in order for a substrate to be cleaved by the enzyme: The free amino group of SDAP is important for substrate recognition, as SDAP lacking the basic 6-amino group (at the non-succinylated end) was hydrolyzed at only 0.036% of the rate of the natural SDAP substrate.\textsuperscript{17}

These data imply that the carboxylate of the succinyl moiety and the amine form important interactions with the active site of DapE as consistent with the newly obtained data from the closed-conformation crystal structure.\textsuperscript{19} Additional modified substrate analogs have been designed to explore the positive characteristic interaction of the free amine in the active
sight. These modifications include altering the substrate primary amine 1a to the N\(^6\)-mono-methyl secondary amine 1b and the N\(^6\)-dimethyl tertiary amine 1c (Figure 6). This approach was first explored in silico using molecular modeling to predict substrate-enzyme interactions involving recognition of the amine.

Figure 6. L,L-SDAP, N\(^6\)-methyl and N\(^6\)-dimethyl substrate analogs

DapE Molecular Modeling to Assess N\(^6\)-Mono- vs. N\(^6\),N\(^6\)-Dimethyl-SDAP Analogs.

Ligand models of L,L-SDAP 1a, N\(^6\)-methyl-L,L-SDAP 1b and N\(^6\),N\(^6\)-dimethyl-L,L-SDAP 1c were built using the Molecular Operating Environment (MOE) computational suite’s Builder utility followed by minimization in the gas phase using the force field MMFF94X. An X-ray crystal structure of the product-bound DapE crystal structure in the closed conformation was utilized (PDB: 5VO3). Following receptor preparation, molecular docking was performed using ligand conformation databases. Docking poses of the substrate L,L-SDAP (1a) and methylated analogs 1b and 1c were assessed as judged by their similarity to the product binding interactions seen in the original product-bound crystal structure. The three ligand-enzyme models were then solvated in a simple water box at pH of 7.4. The system atoms were then optimized with a short, localized molecular minimization process. Molecular Dynamics parameters were set to globally minimize the protein, ligand and solvent atoms with a typical heating and cooling protocol. Simulation results were then minimized once again before the
final binding poses were obtained for comparison. The final solvated and minimized substrate-bound DapE models are shown in Figure 7.

Figure 7. Molecular docking of L,L-SDAP substrate analogs\textsuperscript{[a]}

\textsuperscript{[a]} Energy-Minimized substrate/analogs docked and modeled in the DapE active site. The diaminopimelate moiety is depicted in yellow, and the succinate in turquoise. a) Native substrate L,L-SDAP, b) N\textsuperscript{6}-methyl-L,L-SDAP, c) N\textsuperscript{6},N\textsuperscript{6}-dimethyl-LL-SDAP. The catalytic domain of Subunit A is depicted in green, whereas the dimerization domain of Subunit B is shown in orange.

The docked and modeled endogenous substrate L,L-SDAP amide carbonyl is bound to one of the Zn(II) atoms while the amide N-H acts as an H-bond donor to the backbone carbonyl of Thr325:A. The proximal carboxylate participates in bifurcated H-bonds with the side chains of Arg258:A, Thr325:A, and Asn245:B. The free primary amino group acts as an H-bond donor to the backbone carbonyl of Ala136:A and a water molecule, which in turn participates in H-bond donation to the backbone carbonyl of Glu135:A and to the side chain carbonyl of Asn245:B. The terminal carboxylate of the pimelic acid moiety is H-bonded to the N-H of Asn244:B, the side chain hydroxyl of Ser181:A, and Ser290:A, and a water molecule, which in turn H-bond donates to the side chain hydroxyls of Thr183:A and Thr325:A. We observe by
comparing the enzyme-substrate interactions in the crystal-bound L,L-SDAP product(s) and the docked & modeled L,L-SDAP structures, that the substrate links the catalytic domain of subunit A to the dimerization/cap subunit of chain B. The interactions of the N⁶-methyl-L,L-SDAP analog 1b are identical to those of the endogenous substrate, except for the added methyl group on the primary amino group that eliminates the H-bond interaction to the backbone carbonyl of Ala136:A. The presence of the N⁶-methyl group also leads to perturbations of the water interaction at the ammonium N-H. However, H-bond bridging between the backbone carbonyl of Glu135:A and the side chain carbonyl of Asn245:B by the ammonium N-H(s) are maintained, while the N-methyl forms a new hydrophobic interaction with the adjacent Ala136:A residue. In contrast, the N⁶,N⁶-dimethyl-L,L-SDAP substrate 1c is quite distinct from the natural L,L-SDAP substrate’s binding due to the presence of the two additional methyl groups. Loss of the interfacial domain interaction between the backbone carbonyl of Glu135:A and the side chain carbonyl of Asn245:B by the ammonium N-H species, due to interference of the additional methyl groups, appear to be the key difference to the endogenous L,L-SDAP substrate binding mode. The additional bulk of the two methyl groups also leads to the migration of the N-H bond from the ammonium species of the potential substrate from Glu135:A to the backbone carbonyl of Glu134:A. Significantly, Glu134 (E134) is proposed to act as the general acid/base during the hydrolysis reaction catalyzed by DapE²⁶ and this residue is shifted further away from the active site. Thus, it is concluded that the N⁶-methyl-L,L-SDAP substrate analog 1b retains overall similar binding to the endogenous L,L-SDAP 1a, while the N⁶,N⁶-dimethyl-L,L-SDAP analog 1c suffers loss of significant binding interactions due to the presence of the second methyl group. These in silico studies provide additional evidence of the binding interaction of the substrate to
the active site and the induced conformational change that results in the closed conformation and ultimate hydrolysis of the substrate. Combination of these data will aid in the development of inhibitors for DapE by enabling design of inhibitors that bind to the active site in lieu of the substrate or alternatively disrupt the closing of the enzyme to inhibit hydrolytic activity. A competitive inhibitor should compete with the substrate by binding at the active site. The important catalytic and surrounding residues at the active site which help coordinate the substrate for catalysis can also be important to bind the inhibitor at the active site in an ideal orientation to allow for binding and inhibition.

**Measuring Enzymatic Activity and Inhibition of DapE.**

To produce an effective inhibitor of DapE, the enzymatic activity and inhibition thereof must be able to be monitored and quantified. The hydrolysis of the endogenous substrate L,L-SDAP produces the two products, succinic acid and L,L-diaminopimelate, by cleavage of the amide bond. This allows the enzymatic activity of DapE to be observed spectrophotometrically by monitoring the decrease in absorbance by the cleavage of the amide bond at 225 nm. Similarly, the inhibition of DapE can be determined by the absorbance at 225 nm. Inhibitors of DapE will effectively decrease the rate of hydrolysis and the amide bond should still be detected at 225 nm.

**Initial Screening for Inhibitors of DapE.** High-throughput screening (HTS) is the dominant technique for lead-based discovery in pharmaceuticals and more recently, virtual screening for inhibitors has become routine. To identify appropriate lead molecules for inhibition of DapE, initially over 30 molecules representing different structural classes and containing different zinc binding groups were screened against DapE using L,L-SDAP as the
substrate. These classes of compounds were selected initially because of the Zn(II) atoms at the active site of DapE for which at least one is essential for catalytic activity. Of these molecules, thiols, carboxylic acids, phosphates and boronic acid functional groups were demonstrated as inhibitors of DapE with low micromolar IC$_{50}$ values including the ACE inhibitor L-captopril shown in Table 1.

Table 1. Inhibitors of DapE identified by 225nm assay

<table>
<thead>
<tr>
<th>Zinc Binding Groups</th>
<th>Structure</th>
<th>Compound</th>
<th>[HiDapE] IC$_{50}$ (μM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiol/ Carboxylic Acid</td>
<td><img src="image1" alt="Structure" /></td>
<td>3- Mercaptobenzoic Acid</td>
<td>34</td>
</tr>
<tr>
<td>Thiol/ Carboxylic Acid</td>
<td><img src="image2" alt="Structure" /></td>
<td>L-Penicillamine</td>
<td>13.7</td>
</tr>
<tr>
<td>Thiol/ Carboxylic Acid</td>
<td><img src="image3" alt="Structure" /></td>
<td>L-Captopril</td>
<td>3.3</td>
</tr>
<tr>
<td>Boronic Acid</td>
<td><img src="image4" alt="Structure" /></td>
<td>Phenylboronic Acid</td>
<td>107</td>
</tr>
<tr>
<td>Boronic Acid</td>
<td><img src="image5" alt="Structure" /></td>
<td>2-Thiopheneboronic Acid</td>
<td>92</td>
</tr>
</tbody>
</table>

$^a$IC50 values were obtained using D,D- and L,L-SDAP for which DapE selectively cleaves the L,L isoform. Activity was monitored by absorbance of the amide bond cleavage at 225nm at 25°C.

This inhibitor screen provided two key pieces of information: first, that DapE binding is stereoselective in both the substrate and inhibitor preferring the L- over D-enantiomer of both captopril and penicillamine, and second, that L-captopril is a potent and competitive inhibitor of DapE with a measured IC$_{50}$ of 3.3 μM and a $K_i$ of 1.82 μM. Because the enzyme has high specificity for L,L-SDAP functional groups and for the stereochemistry of both the L,L-SDAP
isomer and L-inhibitors among those tested, DapE has the potential to demonstrate high specificity for drug target inhibitors with high potency and selectivity.\textsuperscript{20}

**Identifying Appropriate Lead Inhibitors of DapE.**

Although the clinical target use for L-captopril is not a bacterial enzyme inhibitor, it is the most potent DapE inhibitor identified in the literature to-date. L-captopril can thus serve as the control in an enzymatic assay for which synthesized inhibitors will be compared to. The next step in the design of inhibitors takes in account the inhibitor’s \textit{in vivo} efficacy which includes the incorporation of functional groups for increased water solubility and oral bioavailability.\textsuperscript{29} Oral bioavailability of a drug is the potential for the drug to be administered orally and enter the blood circulation and to have an active effect.\textsuperscript{29} The preditiction of oral bioavailability can be aided by Lipinski’s rule of five (RO5) which is used to determine drug likeness of a compound.\textsuperscript{29} Lipinski’s rule states that a substance should have no more than one violation of the five rules which include the following: 1) no more than 5 hydrogen bond donors, 2) no more than 10 hydrogen bond acceptors, 3) a molecular mass less than 500 Daltons and 4) an octanol-water partition coefficient logP less than or equal to 5. The Lipinski rule of five considerations along with molecular docking and inhibitory potency can be used for the assessment of structure-activity relationship (SAR) to guide the development of potent inhibitors of DapE.

**Conclusion**

We have demonstrated that DapE is a promising inhibitory target for the development of inhibitors as antibiotics. X-Ray crystallography combined with computational studies have identified key residues necessary for substrate recognition and hydrolysis by DapE. The activity of DapE can be monitored by the decrease in absorbance from the cleavage of the amide bond at
225 nm, and a screening of inhibitors using this method has identified several low micromolar inhibitors. The assay measuring cleavage of the simple amide bond of the substrate at 225 nm is simple and reliable, but precludes the testing of inhibitors that absorb strongly in the ultraviolet region thus ruling out testing of many preferred medicinal chemistry leads and analogs. The development of a more reliable method of measuring inhibition is needed to screen and identify any inhibitors of DapE.
CHAPTER THREE
A SPECTROPHOTOMETRIC NINHYDRIN-BASED
ENZYMATIC INHIBITION ASSAY FOR DAPE

Introduction

Several assays have been developed for evaluating inhibitors of DapE. The original assay for DapE took advantage of the fact that the product L,L-DAP reacts somewhat faster with ninhydrin than with the L,L-SDAP substrate, but this assay suffered from poor reproducibility. Gelb then developed two new assays for DapE. The first employed $^{14}$C-labeled SDAP followed by scintillation counting of liberated radioactive $^{14}$C-succinate. The second was a complex, coupled-assay utilizing porcine succinate thiokinase and inositol triphosphate to convert liberated succinate to succinyl-CoA and inositol diphosphate. The inositol diphosphate was then detected by its reaction with phosphoenolpyruvate to yield liberated pyruvate, itself being detected spectrophotometrically using lactate dehydrogenase. In our own earlier work, inhibitors of DapE were assayed against zinc-reconstituted DapE by monitoring amide bond cleavage of L,L-SDAP at 225 nm utilizing L,L-SDAP as a substrate ($\varepsilon_{225\text{nm}} = 304 \text{ M}^{-1} \text{ cm}^{-1}$)\textsuperscript{28}. All of these assays suffered significant limitations. The original ninhydrin assay employing native or racemic SDAP, as noted, had very poor reproducibility. The $^{14}$C-labeled substrate assay required working with radioactivity with extra safety protocols and waste disposal issues. We employed Gelb’s coupled assay with some success, but found it to be technically troublesome and therefore difficult to reproduce. The assay involved the use of more than one
enzyme which meant that the inhibition measured could not be attributed reliably as coming from one exclusive enzyme. Finally, the previously mentioned assay measuring cleavage of the simple amide bond of the substrate at 225 nm is simple and reliable, but precludes the testing of inhibitors that absorb strongly in the ultraviolet region, thus ruling out testing of many preferred medicinal chemistry leads and analogs. The desire for a robust and higher-throughput assay for supporting our medicinal chemistry efforts toward the discovery of DapE inhibitors as new antibiotics led us to consider developing a new assay.

Ninhydrin assays have been previously employed as a tool for screening inhibitors of enzymes. Recognizing the simplicity and reliability of a ninhydrin-based assay to detect the release of the primary amino group upon cleavage of the succinate amide, we explored whether we could block the reactivity of the free amino group of the substrate to prevent interference caused by its reaction with ninhydrin. We prepared both N6-acetylated (1d) and N6-methylated (1b) derivatives of L,L-SDAP, and deprioritized the contemplated N6,N6-dimethyl L,L-SDAP analog 1c based on previous in silico studies mentioned in Chapter Two (Figure 8).

Figure 8. N6-Acetylated and N6-methylated L,L-SDAP substrate analogs 1b and 1d.

Chemistry

The N6-acetyl-L,L-SDAP substrate analog (1d) was prepared by direct acylation of L,L-SDAP, but this analog was not cleaved by the enzyme, either due to the need for a charged
cationic species at that position suggestion from studies in Chapter Two, or due to unfavorable steric interactions. In contrast, considering the more conservative change of adding a single methyl group involving minimal steric bulk and still maintaining a positively-charged ammonium species at physiological pH suggested preparation of the N⁶-methyl-L,L-SDAP acid (1b) analog for study (scheme 2). Cleavage of N⁶-methyl-SDAP substrate 1b would yield succinate (2) and the N⁶-methyl L,L-diaminopimelic acid, a secondary amine which may be differentiated from primary amines in reactivity with ninhydrin. The N⁶,N⁶-dimethyl-SDAP substrate 1c is inert to reaction with ninhydrin, but is more sterically encumbered at the critical cationic ammonium moiety. We selected the more conservative N⁶-monomethyl derivative 1b, based on modeling described above that suggests that the tertiary N⁶,N⁶-dimethylamine substrate 1c would be a very poor substrate.

The N²-succinyl-N⁶-methyl-L,L-diaminopimelic acid substrate 1b was prepared enantioselectivity by Marlon Lutz as illustrated in Scheme 2 beginning with methylation of BOC-L-glutamic acid t-butyl ester 4, first with potassium carbonate and methyl iodide to form the methyl ester, then in the presence of silver oxide in the same pot to afford the α-N-methylated ester 5. Reduction of the methyl ester with sodium borohydride afforded the primary alcohol 6 which was oxidized back to the aldehyde 7 with PCC or PDC. Horner-Wadsworth-Emmons olefination with Cbz-α-phosphonoglycine ester gave the olefin 8a, which was enantioselectively hydrogenated in the presence of catalytic 1,2-bis[(2S,5S)-2,5-diethylphospholano]benzene(1,5-cyclooctadiene)rhodium(I) trifluoromethanesulfonate to afford the L,L-Cbz-protected amino acid 9a in 93% yield. Removal of the Cbz protecting group by hydrogenolysis followed by reaction with succinic anhydride afforded succinamide derivative...
10a in 99% yield, which was subjected to hydrolysis with aqueous HCl to yield N⁶-methyl SDAP as the hydrochloride salt (1b.HCl) in 97% yield.

The synthetic route via the methyl ester intermediate was improved by proceeding with the benzyl ester instead. Thus, the Wadsworth-Emmons reaction of aldehyde 7 with benzyl 2-\{([benzyloxy]carbonyl)amino\}-2-(dimethoxyphosphoryl)acetate (R = Bn) afforded olefin 8a, which was enantioselectively hydrogenated in the presence of catalytic 1,2-bis[(2S,5S)-2,5-diethylphospholano]benzene(1,5-cyclooctadiene)rhodium(I) trifluoromethanesulfonate to afford the L,L-Cbz-protected amino acid benzyl ester 9b in 94% yield. Hydrogenolytic removal of the Cbz group followed by reaction with succinic anhydride gave the succinate amide (99% for 10a, 92-97% for 10b). Removal of the benzyl ester with trifluoroacetic acid in methylene chloride gave the trifluoroacetate salt 1b.TFA in nearly quantitative yield.

Scheme 2. Asymmetric synthesis of N⁶-methyl-L,L-SDAP substrate 1b[^a]

[^a]: Preparation of N⁶-Me-L,L-SDAP 1b as either the hydrochloride salt (1b.HCl) via the methyl ester (8a-10a derivatives) or trifluoroacetate salt (1b.TFA) via the benzyl ester (8b-10b derivatives); a1) K₂CO₃, MeI, DMAC a2) Ag₂O, MeI, DMAc; b) NaBH₄, EtOH, 0°C to rt; c) PCC, SiO₂, DCM; d) DBU, DCM; e) [Rh(I)COD(S,S)-Et-DuPHOS][Otf], H₂ (50 psi), MeOH; f1) H₂ (1 atm) Pd/C, MeOH; f2) succinic anhydride, Et₃N, DCM; g) TFA, DCM, 25°C
Results and Discussion

Having synthesized the N$^6$-methyl L,L-SDAP substrate 1b, we then demonstrated that it functions as a substrate by observing hydrolysis of the amide bond at 225 nm in the presence of DapE, with a measured $k_{cat}/K_m$ of $3.87 	imes 10^4 \text{M}^{-1}\text{S}^{-1}$ compared to the endogenous L,L-SDAP substrate with a $k_{cat}/K_m$ of $1.92 	imes 10^5 \text{M}^{-1}\text{sec}^{-1}$.

Primary Amine Detection with Ninhydrin.

Since we demonstrated that 1b can function as a substrate we set out to design a robust and operationally straightforward enzymatic assay that would be amenable to inhibition studies for drug discovery. While ninhydrin reacts with both primary and secondary amines, only primary amino acids can form the Schiff base known as Ruhemann's purple, which has a longer wavelength absorption with $\lambda_{max}$ at 570 nm and 450 nm. In contrast, ninhydrin reacts with cyclic secondary amino acids such as proline to form an iminium salt that is yellow-orange in color, with $\lambda_{max}$ at 440 and 405 nm. Other amines are known to react with ninhydrin without creating the Ruhemann’s purple complex.$^{32}$ Initial control reactions were performed with ninhydrin in the absence of enzyme monitoring between 300 and 800 nm with ninhydrin to determine the optimal time of heating and color development. Glutamic acid was utilized as a model primary amino acid, while N-methylglycine (sarcosine) was selected as a model for our acyclic secondary amino acid substrate 1b rather than proline. Some secondary amines are known to react with ninhydrin in the presence of polar aprotic solvents such as DMSO, the solvent in which the ninhydrin reagent is provided.$^{33}$ Heating of these model primary and secondary amines with ninhydrin at 100°C for 15 min surprisingly provided very similar absorption spectra with
identical $\lambda_{\text{max}}$ values of 404 and 570 nm for the products of ninhydrin with glutamic acid and sarcosine, respectively (Figure 9).

Figure 9. UV Absorption spectra of primary and secondary amines with ninhydrin$^[a]$}

![Graph showing UV absorption spectra of primary and secondary amines with ninhydrin](image)

$^[a]$ Sarcosine (Sarc) is used as a model secondary amine depicted in blue and glutamic acid (Glu) is used as a model primary amine depicted in orange.

It was apparent that absorption from the existing secondary amine of N$^6$-methyl-L,L-SDAP substrate 1b, which is a primary $\alpha$-amino acid, would interfere with accurate determination of the primary amine from enzymatic hydrolysis of 1b, but the solution to this problem lay in the slower rate of reaction of the secondary amine with ninhydrin relative to the primary amine. Reducing the time of incubation of the mock substrates glutamic acid and sarcosine with ninhydrin at 100°C to only 2 minutes (data in appendix B) provided significantly greater absorption at 570 nm for the primary amine than for the secondary amine, a difference which diminished upon continued heating of the samples to 15 minutes. These data suggested that a very short incubation time of ca. 2 minutes should enable a clear distinction between the primary and secondary amines of the N$^6$-methyl-L,L-SDAP 3b product. However, at two minutes, even the primary amine had not completely reacted, and furthermore, the practical
challenge of a short incubation time, combined with associated increased error precluded this option, but encouraged us to examine lower incubation temperatures to enable the reaction of the primary amine with ninhydrin while minimizing the reaction of the secondary amine. The first directed approach was to monitor product absorption development over 15 minutes at varying temperatures in 20-degree increments. Time course plots of the reaction with glutamic acid or sarcosine (1.0 mM) and ninhydrin (Figure 10) show positive reactions with the primary amine at 80°C and 60°C, whereas little or no detectable reaction is observed with the secondary amine at these same temperatures. The absorbance from the primary and secondary amines at these lower temperatures reaches a maxima and plateaus around 15 min of development. This demonstrates that adding ninhydrin and heating the amine reaction mixture at 80°C for 15 minutes provides easily detectable absorbance of ~1 AU for the primary amine product without reacting to any significant extent with secondary amines.
Figure 10. Absorption spectra of primary and secondary amines as a function of time

[A] Glutamic acid (Glu) serves as a standard from primary amines depicted in orange and sarcosine (Sar) serves as a standard for acyclic secondary amines depicted in blue. (A) The time course plot of ninhydrin with primary and secondary amines at 100°C. (B) Development of ninhydrin with primary and secondary amines at 80°C. (C) Development of ninhydrin with primary and secondary amines at 60°C.
Quenching of DapE Hydrolytic Activity.

Given the somewhat lower temperature (80°C) selected for the N⁶-methyl-L,L-SDAP (1b) hydrolyzed product development with ninhydrin upon completion of the enzymatic reaction, it was also essential to confirm that the enzyme activity is effectively halted at the time of quench. When quenching the enzymatic reaction, ninhydrin is added as a commercially-available 2% solution in DMSO, so both the addition of DMSO as well as the elevated reaction quench temperature needed to be examined independently to ensure that the enzymatic reaction was halted. The total final assay volume was 300 µL after the addition of 100 µL of ninhydrin reagent giving a final concentration of 33 vol% of DMSO. The solvent DMSO is known to decrease or halt enzyme activity due to denaturation in the presence of as little as 10% by volume. Therefore, control reactions of standard enzyme activity were carried out in triplicate as follows: to a buffered DapE solution at 30°C was added the N⁶-methyl-L,L-SDAP TFA salt 1b. The reaction proceeded for 10 minutes after which a 2% ninhydrin reagent in 100% DMSO was added and subsequently heated to 80°C for 15 minutes. The ninhydrin reaction was quenched by placing the mixture in ice for 2 minutes after which the absorbance was read at 570 nm. This control reaction was set as 100% standard enzymatic activity of DapE.

DapE was incubated with DMSO prior to addition of N⁶-methyl-L,L-SDAP substrate under various times to show the effects of DMSO on the hydrolytic activity of DapE. The DMSO incubation reactions were carried out with the exchange of buffered solution with DMSO. The enzyme was incubated for the desired time between 0 and 10 minutes. After the allotted incubation time, N⁶-methyl-SDAP TFA salt 1b was added and the reaction proceeded for 10 minutes. The enzymatic reaction was quenched by addition of 2% ninhydrin solution and was
subsequently heated to 80°C for 15 minutes. The ninhydrin reaction was quenched by placing in an ice bath for 2 minutes and the absorbance was measured 570 nm. Plots of normalized 100% enzymatic activity versus reaction rates obtained in the presence of DMSO indicate a ca. 50% decrease in enzymatic activity over a 10-minute time frame in the presence of ninhydrin/DMSO, but enzymatic activity is not quenched even after 10 minutes. This demonstrates that the addition of the ninhydrin/DMSO solution does not fully quench the catalytic activity of DapE.

**Circular Dichroism Denaturation Studies.**

The effect of heating the DapE reaction mixture at 80°C was examined to determine if all DapE hydrolytic activity is halted as when heating to 100°C. Denaturation upon heating was examined by Circular Dichroism (CD) spectroscopy as a function of temperatures up to 80°C (Figure 11).

Figure 11. Circular Dichroism thermal denaturation study of DapE$^{[a]}$

![Graph A](image1.png) ![Graph B](image2.png)

$^{[a]}$ CD $\alpha$-helical structure represented in red and $\beta$-sheets represented in blue. (A) percent secondary structure observed over the course of heating from 20-80°C; and (B) percent secondary structure remaining with continued heating at 80°C.

A loss of ca. ~5% in the $\alpha$-helical structure was used as an indication that DapE was unfolding, while $\leq$10% total remaining $\alpha$-helical structure suggested completely denatured
protein. DapE begins to denature at ~60 °C as evidenced by a ~15% reduction in α-helical secondary structure. Upon heating to 80 °C, the percent observed α-helical structure decreased to ~10%. Thus, complete denaturation of DapE after 2 minutes of heating at 80°C was confirmed.

**Pre-Heat Incubation Studies.**

To complement the CD studies, DapE was pre-heated to 80°C and to 100°C independently to determine the effect on enzymatic activity. The DapE enzyme was incubated at 80°C or 100°C in increments from 0 to 10 minutes and was then cooled to room temperature before addition of N6-methyl-L,L-SDAP substrate 1b. Incubating DapE at 80°C for 0 to 5 minutes before adding substrate shows the dramatic decrease of enzymatic activity over one to two minutes with enzyme activity completely disrupted after 2 minutes. Incubating DapE at 100°C shows that enzymatic activity is eliminated within one minute of heating (Plot given in Appendix B). However, as noted, heating to 100°C also causes both primary and acyclic secondary amino acids to react at an appreciable rate with ninhydrin within this time. It was therefore concluded that heating the enzyme at 80°C both stops enzyme activity within 1-2 minutes and enables enzyme activity to be measured accurately by allowing only the primary amine from the cleaved substrate to react with the ninhydrin. To minimize the associated error, DapE enzymatic activity is initially quenched by heating at 100°C before addition of ninhydrin/DMSO and subsequent development to 80°C for detection of N6-methyl-SDAP hydrolyzed product.
**Enzyme Kinetics for N⁶-methyl-SDAP 1b.**

We determined the $k_{cat}/K_m$ for the modified N⁶-methyl-L,L-SDAP substrate 1b to compare with the endogenous substrate SDAP. The optimized experimental assay conditions enable the detection of the formation of products as well as providing proper conditions for quenching DapE hydrolytic activity. The saturation of DapE concentration versus product formation was examined by reacting the glutamic acid standard to obtain a DapE concentration that would produce hydrolyzed product with a normalized absorbance less than 1 AU, so as to not saturate the spectrophotometer. Based on the glutamic acid standards, the ideal DapE concentration will hydrolyze 2 mM of N⁶-methyl-SDAP to produce around 0.4 mM (80 nmol) of product in ten minutes (Figure 12).

Figure 12. Development of primary amine standard with ninhydrin.$^[a]$ 

\[ [\text{Glutamic Acid mM}] \]

\[ 0 \quad 0.1 \quad 0.2 \quad 0.3 \quad 0.4 \quad 0.5 \]

\[ 0 \quad 0.2 \quad 0.4 \quad 0.6 \quad 0.8 \quad 1.0 \quad 1.2 \]

$^[a]$ Glutamic acid is used as a primary amine control in the development with ninhydrin at 80°C for 15 minutes.

The N⁶-methyl SDAP (1b) concentration was increased to 2 mM to mimic the original L,L- SDAP concentration employed in the 225 nm enzymatic assay. The plot of Abs vs DapE
concentration reveals the need for a final adjusted enzyme concentration of around 8 nM to keep
the product absorbance under 1 AU (Figure 13).

Figure 13. Enzyme saturation curve of DapE\(^\text{[a]}\)

\[\text{[a]}\] Enzyme saturation curve of DapE using 2 mM of N\(^6\)-Me-SDAP substrate. Optimal enzyme concentration
selected for absorbance of primary amine product at or around 1 AU as to not saturate the spectrophotometer.

**Reaction Rate/Velocity and Characterization of DapE Activity.**

The rate of the enzymatic reaction was observed to check the linearity of the hydrolysis.

Using an N\(^6\)-methyl SDAP (1b) concentration of 2 mM with a DapE concentration of 8 nM, the
hydrolysis was plotted with reaction time in minutes versus product formed to check for linearity
followed by saturation along the whole curve. A reaction time of 10 minutes should lie along the
linear line of the curve and ideally should be followed by some additional time to allow for some
variation in quenching enzyme activity after the 10-minute reaction time period. The next step
was to determine the velocity of the reaction to properly characterize the activity of the enzyme.

Varying the concentration of N\(^6\)-methyl SDAP and plotting product versus time enabled
determination of the velocity of the reaction. This was performed using smaller concentrations of
substrate with the ninhydrin-based assay to avoid associated error with increased absorbance
well above 1 AU as observed with higher concentrations. This analysis was also performed on the original 225 nm assay for comparison and in attempt to define $K_m$ through an experimentally obtained $V_{\text{max}}$. It is important to note that the use of the $N^6$-methyl-SDAP TFA salt in a 50 mM HEPES buffered solution of pH 7.5 at concentrations above 9 mM lowers the pH of the solution making the DapE enzyme hydrolytically inactive. The actual $V_{\text{max}}$ of $N^6$-methyl-SDAP could not be experimentally determined under these conditions by use of the TFA salt. $N^6$-Methyl-SDAP TFA salt 1b was determined to have a $k_{\text{cat}}/K_m$ of $3.87 \times 10^4$ M$^{-1}$S$^{-1}$ using the 225 nm assay vs $3.3 \times 10^4$ M$^{-1}$S$^{-1}$ using the ninhydrin-based 570 nm assay.

**DapE Ninhydrin-based Enzymatic Assay and IC$_{50}$ Determination.**

Combination of these data indicate that the optimal DapE assay conditions using the trifluoroacetate (TFA) salt of $N^6$-methyl-$L,L$-SDAP 1b as the substrate and with ninhydrin as the colorimetric probe are 8 nm DapE in 50 mM HEPES buffer at pH 7.5 at 30°C followed by the addition of 2 mM $N^6$-methyl-SDAP. After 10 min. the reaction is quenched by heating at 100°C for 1 minute followed by cooling on ice. A 2% ninhydrin solution in 100% DMSO (100 μL) is added and the solution heated at 80°C for 15 minutes followed by cooling on ice. Once the sample is cooled to 30°C the absorbance at 570 nm is determined.

To test $N^6$-methyl SDAP as a substrate for screening potential inhibitors of DapE, the potencies of several previously-identified inhibitors were examined and found to be comparable with results from the previous assay monitoring amide bond cleavage at 225 nm. Specifically, captopril was found to have an IC$_{50}$ value of $3.4 \pm 0.2$ μM which is almost identical to that reported using SDAP in the 225 nm assay (IC$_{50}$ = 3.3 μM). Likewise, the IC$_{50}$ values for 3-mercaptopbenzoic acid (IC$_{50}$ = 21.8 ± 2.2 μM), phenylboronic acid (IC$_{50}$ = 316 ± 23.6 μM), and
thiophene boronic acid (IC$_{50} = 111 \pm 16 \, \mu$M) were found to be in good agreement with the literature values of 35 μM, 107 μM, and 92 μM, respectively.$^{28}$

Table 2. DapE inhibitor IC$_{50}$ values using ninhydrin-based enzymatic assay

<table>
<thead>
<tr>
<th>Structure</th>
<th>Compound</th>
<th>[HiDapE] IC$_{50}$ (μM)$^{[a]}$</th>
<th>[HiDapE] IC$_{50}$ (μM)$^{[b]}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image.png" alt="Structure" /></td>
<td>3- Mercaptobenzoic Acid</td>
<td>35</td>
<td>21.8 ± 2.2</td>
</tr>
<tr>
<td><img src="image.png" alt="Structure" /></td>
<td>L-Captopril</td>
<td>3.3</td>
<td>3.4 ± 0.2</td>
</tr>
<tr>
<td><img src="image.png" alt="Structure" /></td>
<td>Phenylboronic Acid</td>
<td>107</td>
<td>316 ± 23.6</td>
</tr>
<tr>
<td><img src="image.png" alt="Structure" /></td>
<td>2-Thiopheneboronic Acid</td>
<td>92</td>
<td>111 ± 16</td>
</tr>
</tbody>
</table>

[a] Inhibitor IC$_{50}$ values obtained from original 225 nm assay using 4 mM racemic SDAP. [b] Inhibitor IC$_{50}$ values obtained from ninhydrin-based enzymatic assay using 2 mM N-Me-L,L-SDAP substrate and ~10nM DapE.

Screening Primary Amine Inhibitors Using a Ninhydrin-Based Assay

Amines are critically important functional groups in drugs and in compound screening libraries. We were therefore very concerned that primary amines might appear as false positives in an inhibitor library screen due to their reactivity with ninhydrin, so we screened benzylamine, cyclohexylamine, and aniline as potential inhibitors in the assay. At a concentration of 200 μM none of these amines inhibit or give the appearance of inhibiting DapE using this assay. A blank control of these primary amines from 100 μM up to 500 μM demonstrates that these primary amines are not detected at 570 nm, consistent with the fact that their reaction with ninhydrin does not produce the Ruhemann’s purple complex that absorbs at 570 nm, and instead produces an iminium salt complex which is not detected at this wavelength. In addition, we screened
glutamic acid as a potential inhibitor of DapE at 200 μM, and glutamic acid does not appear to inhibit the DapE enzyme at this concentration, even though it is an alpha-amino acid that can produce Ruhemann’s purple, due to subtraction of the blank.

**Conclusion**

In summary, a new DapE spectrophotometric method is described for identification and optimization of DapE inhibitors as potential antimicrobial agents. Captopril displayed modest antibiotic activity against *Escherichia coli* and *Salmonella enterica* confirming our own observation of antibiotic activity, but Udo found that captopril does not inhibit the Mn²⁺ enzyme, and that, surprisingly, the antibiotic activity of captopril was independent of DapE inhibition in bacteria. Ultimately, it will be imperative to screen different metalloisozymes of DapE, in particular the Mn²⁺ enzyme in addition to the Zn²⁺ form, as captopril was found to be a moderately potent inhibitor of the Zn²⁺ enzyme but not of the Mn²⁺ enzyme. Even within the divalent zinc enzyme, mono- and di-zinc enzymes were determined to show different activities, and their crystal structures have been determined with structural insights that impact rational design of inhibitors. In summary, utilizing N⁶-methyl L,L-SDAP 1b as the modified substrate enables a new ninhydrin-based assay for measuring inhibition of the bacterial enzyme DapE that should support both lead discovery as well as lead optimization toward the discovery of new antibiotics with a new mechanism of action to treat bacterial infections.
CHAPTER FOUR
DESIGN, SYNTHESIS, MOLECULAR DOCKING, AND INHIBITORY POTENCY OF INHIBITORS OF DAPE

Now that we have new reliable enzymatic assay available for the identification of inhibitors of DapE and an understanding of the active site of the enzyme with molecular docking to guide the development of antibiotics, our efforts are focused on synthesizing new inhibitors of DapE. We took another approach at identifying lead inhibitors of DapE with more novel structures that were not biased for Zn(II) binding groups as the enzyme has many possible metallo-isozymes. A High-Throughput Screening (HTS) of over 33,000 compounds licensed from by Chembridge Corporation utilizing the enzyme-coupled assay identified five inhibitors with inhibition of >20% at 12 μM of DapE. The results consist of four series of inhibitors: a simple amide with a β-sulfonyl compound A, the difluoromethyl sulfonamide compound B, two N-acyl-sulfonamide indolines compounds C and D and a chiral phenyltetrazole thioether compound E (Figure 14). Interestingly, all five ligands contain two potential zinc binding groups within several atoms of each other which could suggest strong interactions with both zins at the active site. Compound A has not been pursued as a possible inhibitor due to the simplicity of the ligand and the lack of novelty in the structure. Herein, we report the synthesis, inhibitory data and SAR of analogs from the other three series of inhibitors as well as a screening of new inhibitors of DapE.
Figure 14. HTS lead compounds identified with the DapE coupled assay

![Image of HTS lead compounds](image)

**Part I. 6-Sulfonamide Indolines as Inhibitors of DapE**

**Introduction.**

The N-acetyl-5-bromo-6-sulfonamide indoline HTS leads C and D were of greater initial interest because these two compounds were identified independently of each other which was confirmation that this series of inhibitors was the most promising of the leads identified. Many substances, both natural and synthetic, with medicinal properties contain an indole structure such as serotonin, indole alkaloids and tryptophan (Figure 15).\(^35\) Tryptophan, an essential amino acid, also contains the indole moiety and is the precursor to the synthesis of serotonin and other indole alkaloids. Indoline is a saturated indole and derivatives of indoline are known to have inhibitory properties against enzymes as well as therapeutic use in the treatment of cancer.\(^36,37\)

Figure 15. Indoline, indole, and natural indole-containing compounds

![Image of indoline, indole, tryptophan, and serotonin](image)

In addition, sulfonamides are also a familiar moiety in medicinal chemistry. Molecular docking using the open crystal structure of DapE (PDB:3IC1) was used to determine relevant possible binding poses of inhibitors to the enzyme. *In silico* studies containing the two lead indoline compounds suggest interaction at the zinc active site which is indicative of possible
competitive inhibition. Optimization of the initial indoline lead C with systematic SAR modifications at the 1-, 5-, and 6-positions were carried out corresponding to derivatives varying $R_1$, $R_2$, and $R_3$, respectively, in Figure 16.

Figure 16. HTS lead N-acetyl-6-sulfonamide indoline C $^{[a]}$

![Compound C](image)

$^{[a]}$ Indoline C and points of modification through synthesis.

**Chemistry.**

The N-acetyl-6-sulfonamide indoline HTS lead compounds have been produced according to the literature which makes neither the synthetic route nor the structures themselves novel. N-acetyl-5-bromo-6-sulfonamide indoline C was synthesized first, through acetylation of indoline to produce 13, followed by bromination of 13 using elemental bromine to produce the 5-brominated indoline 14. Next, sulfonation of indoline 14 in a neat reaction with chlorosulfonic acid produced the 6-sulfonamide indoline 15. Finally, reacting the sulfonyl chloride 15 with isopentyl amine produced 16a (HTS compound C) shown in Scheme 3.

Scheme 3. Synthesis of N-acetyl-5-bromo-6-sulfonamide indolines 16a and 16b$^{[a]}$

![Scheme 3](image)

$^{[a]}$ Synthesis of the HTS lead compound C. a) Acetyl chloride, Et$_3$N, DCM, rt to 0°C; b) Br$_2$, AcOH, rt; c) CISO$_3$H, 65°C, 3 hrs; d) isopentylamine amine for 16a or piperidine for 16b, DCM, Et$_3$N, DMAP.

The piperidine analog 16b was synthesized instead of the dimethyl piperidine HTS compound D because first, the stereocenters were not specified in the screening result and
second, to avoid preparing a mixture of stereoisomers during synthesis. Initial SAR modification was pursued with the sulfonamide at the R3-position because that is the only structural difference between the two leads identified. Finally, diversification by the reaction of chlorosulfonyl indoline 15 with simple alkyl, cyclic and aromatic primary and secondary amines in a traditional fashion afforded the desired R3 modified sulfonamides 16 (Scheme 4).

Scheme 4. Synthesis of N-acetyl-5-bromo-6-sulfonamide indoline 16 derivatives\(^{[a]}\)

Further diversification of the R3 sulfonamide substituent was achieved by conversion of the indoline 6-sulfonylchloride 15 to the primary sulfonamide 17 by ammonia in methanol. This enabled the synthesis of 7-N-acylsulfonamides 18 by reaction with acyl chlorides or the production of sulfonylureas 19 and sulfonyl thioureas 20 by reaction with isocyanates and Isothiocyanates, respectively (Scheme 5). These derivatives are in the process of being synthesized.

Scheme 5. Synthesis of 6-(N-acyl) sulfonamides, sulfonylureas and sulfonyl thioureas\(^{[a]}\)

\(^{[a]}\) X=primary, secondary and aromatic amines and amino acids

\(^{[a]}\) Synthesis of N-acyl sulfonamides, sulfonylureas and sulfonyl thioureas. a) NH\(_3\)/MeOH; b) an acyl chloride; c) an isocyanate or a carbamoyl chloride; d) an isothiocyanate.
Modification of the $R_2$ group to develop structure activity relationships resulted in the conservative switch from the larger and heavier bromine atom to the smaller and lighter chlorine to aid in water solubility. The 5-chloro analogs were originally synthesized by published methods using NCS in acetonitrile and sodium acetate as a catalyst to produce 21 (Scheme 6). Since then, chlorination using NCS and DMF as a solvent in a new flow chemistry synthetic route employed by our lab. To date, no other substitutions at the 5-position have been tested in this synthesis but will be explored in time.

Scheme 6. Synthesis of N-acetyl-5-chloro-6-sulfonamide indoline derivatives 22$^{[a]}$

![Chemical Structure](image)

[a] Synthesis of indoline 22 derivatives. a) NCS, MeCN, NaOAc for batch synthesis and NCS, DMF, 70°C for flow synthesis; b) ClSO$_3$H, 65°C, 3 hrs; c) desired amine, Et$_3$N, DCM, DMAP.

Variation at the $R_1$-position was accomplished by the hydrolysis of the acetyl group of 16 to produce 23 with the unprotected aniline nitrogen. Subsequently, reaction of 23 with carbamoyl chlorides, isocyanates, or other acyl chlorides produced amides (25) and carbamate indoline derivatives (27 and 29) (Scheme 7). This synthetic route was chosen for two reasons: first to avoid chlorosulfonation of indoline with possible labile groups present at $R_1$ and to produce a single point variation at only the $R_1$ position while keeping the $R_3$ substituent constant.
Scheme 7. Synthesis of 6-sulfonamide indole derivatives with $R_1$ variation\(^{[a]}\)

\[ \text{Variation at } R_1: \text{alkyl, cyclic, or aryl derivatives.}\]
\[ \text{a) } H_3O^+, \text{ heat; b) acyl chloride; c) isothiocyanate; d) carbamoyl chlorides or isocyanates}\]

**Results and Discussion.**

The HTS leads were first synthesized and tested for inhibitory potency to validate that they were identified as inhibitors of DapE. The isopentyl indole compound 16a showed an IC\(_{50}\) of over 200 \(\mu M\) from the ninhydrin-based assay but did confirm modest inhibition. A measured IC\(_{50}\) of 130 \(\mu M\) for 16b showed that this compound’s inhibition is more potent than the isopentyl ligand 16a. Indoline 16b was very insoluble at high concentrations and could therefore exhibit greater potency if modified. Considering this data, SAR was first explored by continued variation of the sulfonamide group $R_3$ producing alkyl, cyclic, aromatic and amino acid derivatives.

**Computational Studies.** Preliminary molecular docking data suggests that incorporation of amino acids with the free carboxylate end (16l(-)-16o(-)) would be more tightly bound to the active site Zn(II) atoms leading to more potent inhibitors than the various other sulfonamide derivatives shown in Table 3. This is evidenced by the docking pose produced in silico and the lower, more stable binding energies of 16l(-)-16o(-) calculated using SwissDock.\(^{40}\) The methyl
ester analogs 16l-16o of the amino acids were synthesized and tested for inhibition initially instead of the carboxylate counterparts. Introduction of aromatic heterocycles 16p-16s provided possible allosteric inhibitors that bind to the dimerization domain of DapE rather than the active site based on docking studies. These data suggest that these compounds will be less likely to act as competitive inhibitors of DapE. Considering the closed conformation of DapE and the role that His194 plays in the conformational change, it would be beneficial to explore this proposed binding at the dimerization domain of compounds 16p-16s in the near future.
Table 3. Molecular docking of 5-bromo-6-sulfonamide indoline 16 derivatives[^a]

<table>
<thead>
<tr>
<th>Entry</th>
<th>R₃</th>
<th>Δ G[^b]</th>
<th>Entry</th>
<th>R₃</th>
<th>Δ G[^b]</th>
</tr>
</thead>
<tbody>
<tr>
<td>16a</td>
<td></td>
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<td>16m</td>
<td></td>
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<td>16f</td>
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<td>-8.3</td>
<td>16r</td>
<td></td>
<td>-8.5</td>
</tr>
<tr>
<td>16g</td>
<td></td>
<td>-8.5</td>
<td>16s</td>
<td></td>
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</tr>
<tr>
<td>16h</td>
<td></td>
<td>-7.4</td>
<td>16l(-)</td>
<td></td>
<td>-13.2</td>
</tr>
<tr>
<td>16i</td>
<td></td>
<td>-8.3</td>
<td>16m(-)</td>
<td></td>
<td>-12.5</td>
</tr>
<tr>
<td>16j</td>
<td></td>
<td>-8.6</td>
<td>16n(-)</td>
<td></td>
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<tr>
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<td>-8.7</td>
<td>16o(-)</td>
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</tr>
<tr>
<td>16l</td>
<td></td>
<td>-9.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[^a] Molecular docking was performed using SwissDock with the [ZnZn(HiDapE)] open crystal structure PDB:3IC1.
[^b] ΔG ligand binding energies are calculated in kcal·mol⁻¹.

N-Acyl sulfonamides are a biocompatible scaffold in medicinal chemistry and are often employed in in pharmacological uses.[^41] The N-acyl sulfonamide is very acidic and can resist hydrolysis under many conditions in biological systems. Molecular docking was performed on
some N-acyl sulfonamides with DapE to observe the potential binding energy and use of this functional group as an element of potential inhibitors given these considerations. The results of the *in-silico* studies suggest that these moieties are more tightly bound than the original HTS compounds containing simple alkyl, cyclic and aryl sulfonamides (Table 4). Aromatic substituents 18b, 19c and 20c (R₃) also shows lower binding energies, however, there is no uniform pattern in binding energies compared to other substituents apparent from the data obtained.

Table 4. Ligand binding energies of 5-bromo-6-sulfonyamide indoline 18, 19 and 20[^a]

<table>
<thead>
<tr>
<th>Entry</th>
<th>R₃</th>
<th>ΔG[^b]</th>
<th>Entry</th>
<th>R₃</th>
<th>ΔG[^b]</th>
</tr>
</thead>
<tbody>
<tr>
<td>18a</td>
<td></td>
<td>-8.9</td>
<td>19e</td>
<td></td>
<td>-8.1</td>
</tr>
<tr>
<td>18b</td>
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<td>19f</td>
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<td>-10.4</td>
</tr>
<tr>
<td>18c</td>
<td></td>
<td>-10.1</td>
<td>19g</td>
<td></td>
<td>-10.6</td>
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<tr>
<td>19a</td>
<td></td>
<td>-9.7</td>
<td>19h</td>
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<td>20a</td>
<td></td>
<td>-8.1</td>
</tr>
<tr>
<td>19c</td>
<td></td>
<td>-10.1</td>
<td>20b</td>
<td></td>
<td>-9.9</td>
</tr>
<tr>
<td>19d</td>
<td></td>
<td>-10.3</td>
<td>20c</td>
<td></td>
<td>-9.3</td>
</tr>
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</table>

[^a]: Molecular docking of 6-sulfonyamide derivatives N-acyl sulfonamide 18, sulfonylurea 19 and sulfonyl thiourea 20 performed with SwissDock using [ZnZn(HiDapE)] open crystal structure PDB:3IC1. [^b]: ΔG ligand binding energies calculated in kcal·mol⁻¹.
As previously mentioned, while attempting crystallization of DapE in the presence of using 6-sulfonamide indolines, it was discovered that these compounds were not very water-soluble despite having cLogP values of 3.5 to as low as 0.17. During the co-crystallization attempt, the addition of 5 % DMSO was used to aid in the solubility of the inhibitors, although water solubility is essential for oral bioavailability of a drug candidate in vivo.

The introduction of a 5-chloro substituent at R₂, compared to the 5-bromo counterpart, should aid in water solubility as predicted by lower cLogP values. This halogen substitution with an atom of lower atomic weight also decreases the total molecular weight of the inhibitor to allow for additional variations at other positions while keeping the molecular weight under 500 Daltons as per Lipinski’s RO5. Further, this bioisosteric replacement could potentially increase binding in the active site. *In silico* studies suggest that the chloro-substituent may increases and other times decreases the binding energies of the inhibitor-enzyme interactions as shown in Table 5. The chlorine analogs of the aromatic sulfonamides with heteroatoms in analogs 22p-s have slightly lower more favorable, binding energies than the bromine counterparts 18p-s. Most of the aromatic indolines have lower binding energies as well. This is indicative of more tightly bound ligands and potentially better inhibitors. In the cases of cyclic and acyclic sulfonamide indolines, the energies vary and no drastic energy difference is observed. Inhibitory data of the 5-chloro analogs of the original HTS ligands ultimately showed increased potency with a measured IC₅₀ values of 44 μM for 22b and 54 μM 22a.
Table 5. Ligand binding energies of 5-chloro-6-sulfonamide indoline 22 derivatives [a]

<table>
<thead>
<tr>
<th>Entry</th>
<th>R₃</th>
<th>ΔG [b]</th>
<th>Entry</th>
<th>R₃</th>
<th>ΔG [b]</th>
</tr>
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<tbody>
<tr>
<td>22a</td>
<td>H</td>
<td>-8.8</td>
<td>22j</td>
<td></td>
<td>-8.8</td>
</tr>
<tr>
<td>22b</td>
<td>N</td>
<td>-8.4</td>
<td>22k</td>
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<td>-8.1</td>
</tr>
<tr>
<td>22c</td>
<td>N</td>
<td>-8.6</td>
<td>22m</td>
<td></td>
<td>-8.8</td>
</tr>
<tr>
<td>22d</td>
<td>N</td>
<td>-7.4</td>
<td>22n</td>
<td></td>
<td>-8.4</td>
</tr>
<tr>
<td>22e</td>
<td>N</td>
<td>-8.4</td>
<td>22p</td>
<td></td>
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<tr>
<td>22h</td>
<td>N</td>
<td>-7.9</td>
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<td>-8.4</td>
</tr>
<tr>
<td>22i</td>
<td>N</td>
<td>-9.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[a] Molecular docking of 5-chloro-6-sulfonamide indolines by SwissDock using the [ZnZn(HiDapE)] open crystal structure PDB:3IC1. [b] ΔG ligand binding energies are calculated in kcal·mol⁻¹

In these cases, analogs benefit from making the conservative change from the bromo to the chloro analogs for the overall decrease in molecular weight. Additionally, the aromatic heterocycles 22p-22s were also predicted to bind at an allosteric site at the dimerization domain rather than the active site of DapE similar to the 5-bromo counterparts.

For further SAR evaluation for R₁ modifications, the N-carbamate-6-sulfonamide indoline analogs of the N-acetyl indolines were docked with DapE to examine possible binding configurations at the active site. Ureas and thioureas are very good zinc binding groups and the
formation of these functional groups at \( R_1 \) could potentially create increased binding at the active site. *In silico* studies show that the incorporation of these moieties notably improves the binding energies of these ligands (Table 6).

Table 6. Binding energies of N-substituted-5-bromo-6-sulfonamide indolines 25, 27 and 29 \[^a\]

<table>
<thead>
<tr>
<th>Entry</th>
<th>( R_1 )</th>
<th>( \Delta G^{[b]} )</th>
<th>Entry</th>
<th>( R_1 )</th>
<th>( \Delta G^{[b]} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>25a</td>
<td>( \text{---} )</td>
<td>-8.9</td>
<td>27e</td>
<td>( \text{---} )</td>
<td>-8.1</td>
</tr>
<tr>
<td>25b</td>
<td>( \text{---} )</td>
<td>-10.4</td>
<td>27f</td>
<td>( \text{---} )</td>
<td>-10.4</td>
</tr>
<tr>
<td>25c</td>
<td>( \text{---} )</td>
<td>-10.1</td>
<td>27g</td>
<td>( \text{---} )</td>
<td>-10.6</td>
</tr>
<tr>
<td>27a</td>
<td>( \text{---} )</td>
<td>-9.7</td>
<td>27h</td>
<td>( \text{---} )</td>
<td>-8.7</td>
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<td>27b</td>
<td>( \text{---} )</td>
<td>-10.1</td>
<td>29a</td>
<td>( \text{---} )</td>
<td>-8.1</td>
</tr>
<tr>
<td>27c</td>
<td>( \text{---} )</td>
<td>-10.1</td>
<td>29b</td>
<td>( \text{---} )</td>
<td>-9.9</td>
</tr>
<tr>
<td>27d</td>
<td>( \text{---} )</td>
<td>-10.3</td>
<td>29c</td>
<td>( \text{---} )</td>
<td>-9.3</td>
</tr>
</tbody>
</table>

\[^a\] Molecular docking of N-substituted-5-bromo-6-sulfonamide indolines by SwissDock using the [ZnZn(HiDapE)] open crystal structure PDB:3IC1. \[^b\] \( \Delta G \) ligand binding energies are calculated in kcal mol\(^{-1}\)

The least favorable addition at the \( R_1 \) N-position is by the isothiocyanate to produce the thioureas 29a-c where the ligand binding energy is distinctly weaker. Additionally, longer alkyl substituents such as a propyl group as opposed to a methyl group are more favorable as the methyl derivatives 25a, 27a and 29a all have higher and therefore weaker binding energies than the propyl derivatives 25c, 27b and 29b. Similarly, bulkier functional groups such as 5-
membered and 6-membered cyclic substituents or aryl groups have lower calculated binding energies. The production of these analogs will be pursued in time and tested for inhibition against DapE using the ninhydrin-based enzymatic assay.

**Inhibitory Data.** Inhibitory activity of N-acetyl-6 sulfonamide indolines against [ZnZn(HiDapE)] was measured using the new ninhydrin-based enzymatic assay. The ninhydrin-based assay protocol was similar to the protocol reported in Chapter Two, except for the inhibitor dilution and incubation method. All inhibitors were diluted to 5 mM or 10 mM stock solutions in 100 % DMSO to aid in inhibitor solubility. Serial dilutions were then made from this stock solution with final stock solutions containing 50 % DMSO. The addition of 20 μL of 50 % DMSO to 200 μL reactions provided a final concentration of 5% DMSO. Because of the addition of DMSO, the incubation time was cut from 10 minutes to 0-2 minutes. Inhibition was tested at 20, 50, 100 and 200 μM and the IC$_{50}$ was determined for these inhibitors where applicable and reported in Table 7.

These data show that inclusion of the methyl ester amino acid derivatives did not increase inhibition of DapE compared to the alkyl, cyclic and aromatic sulfonamides, in fact inhibition was comparable to some of the other inhibitors tested, as expected.
Table 7. Inhibitory data (IC\textsubscript{50}) of N-acetyl-5-bromo-6-sulfonamide indoles

<table>
<thead>
<tr>
<th>Entry</th>
<th>R\textsubscript{2}</th>
<th>R\textsubscript{3}</th>
<th>IC\textsubscript{50} (μM) \textsuperscript{[a]}</th>
<th>Entry</th>
<th>R\textsubscript{2}</th>
<th>R\textsubscript{3}</th>
<th>IC\textsubscript{50} (μM) \textsuperscript{[a]}</th>
</tr>
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<tbody>
<tr>
<td>16a</td>
<td>Br</td>
<td></td>
<td>&gt;200</td>
<td>16j</td>
<td>Br</td>
<td></td>
<td>86</td>
</tr>
<tr>
<td>16b</td>
<td>Br</td>
<td></td>
<td>130\textsuperscript{[b]}, 134\textsuperscript{[b]}</td>
<td>16k</td>
<td>Br</td>
<td></td>
<td>ND\textsuperscript{[c]} 56% at 200</td>
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<tr>
<td>16e</td>
<td>Br</td>
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<td>99</td>
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<td>16f</td>
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<td></td>
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<td>16o</td>
<td>Br</td>
<td></td>
<td>82</td>
</tr>
<tr>
<td>16g</td>
<td>Br</td>
<td></td>
<td>97</td>
<td>16t</td>
<td>Br</td>
<td></td>
<td>ND\textsuperscript{[c]} 39% at 100</td>
</tr>
<tr>
<td>16h</td>
<td>Br</td>
<td></td>
<td>172</td>
<td>16u</td>
<td>Br</td>
<td></td>
<td>ND\textsuperscript{[c]} 26% at 100</td>
</tr>
</tbody>
</table>

\textsuperscript{[a]} Data obtained from a set of triplicates and IC\textsubscript{50} values were determined from a set of three or four % inhibition points. \textsuperscript{[b]} A different batch tested separately for inhibition. \textsuperscript{[c]} Not determined; solubility of the compound did not allow for determination of IC\textsubscript{50} experimentally, inhibition determined for a single concentration.

Direct comparison of inhibitory data of 5-chloro and 5-bromo analogs shows that the inclusion of a 5-chloro substitute increased inhibition of DapE relative to 5-bromo with the 5-chloro isopentyl sulfonamide 22a showing an IC\textsubscript{50} of 44 μM and the piperidine derivative showing and IC\textsubscript{50} of 54 μM. Additionally, the water solubility increased for the chloro derivatives as expected as some 5-chloro analogs were sufficiently soluble for identification while the 5-bromo counterparts were insoluble at the higher concentrations (Table 8).
Table 8. Inhibitory data (IC₅₀) of N-acetyl-5-chloro-6-sulfonamide indolines 22

<table>
<thead>
<tr>
<th>Entry</th>
<th>R₂</th>
<th>R₃</th>
<th>IC₅₀ (µM) [a]</th>
<th>Entry</th>
<th>R₂</th>
<th>R₃</th>
<th>IC₅₀ (µM) [a]</th>
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<tbody>
<tr>
<td>22a</td>
<td>Cl</td>
<td></td>
<td>54</td>
<td>22h</td>
<td>Cl</td>
<td></td>
<td>ND[ᵇ] &lt;200</td>
</tr>
<tr>
<td>22b</td>
<td>Cl</td>
<td></td>
<td>44</td>
<td>22l</td>
<td>Cl</td>
<td></td>
<td>&gt;200</td>
</tr>
<tr>
<td>22c</td>
<td>Cl</td>
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<td>88</td>
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<td>Cl</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>22e</td>
<td>Cl</td>
<td></td>
<td>ND[ᵇ] 43% at 100</td>
<td>22u</td>
<td>Cl</td>
<td></td>
<td>ND[ᵇ] &gt;200</td>
</tr>
<tr>
<td>22g</td>
<td>Cl</td>
<td></td>
<td>172</td>
<td>22v</td>
<td>Cl</td>
<td></td>
<td>ND[ᵇ]</td>
</tr>
</tbody>
</table>

[a] Data obtained from a set of triplicates and IC₅₀ values were determined from a set of three or four % inhibition points. [ᵇ] Not determined; solubility of the compound did not allow for determination of IC₅₀ experimentally, inhibition reported for a single concentration.

Conclusion.

Several N-acetyl-5-bromo-6-sulfonamide indolines have been designed, synthesized and tested for inhibition of DapE. The original HTS lead inhibitors exhibited an IC₅₀ of over 200 µM and 130 µM for the isopentyl sulfonamide 16a and piperidine sulfonamide 16b, respectively, which were modest. However, the change from 5-bromo to 5-chloro substituents increased the inhibition noticeably. In-silico studies suggest favorable modifications of these compounds to produce more tightly-bound ligands and ultimately increased potency. The 5-chloro analogs of all 5-bromo ligands will be synthesized for continued SAR comparisons as well as the corresponding N-acylsulfonamides and sulfonylureas, in addition to this also includes the synthesis of N-carbamates and other acyl groups at the R₁ position of indoline. Overall, inclusion of more water-soluble groups such as carboxylate groups, which also can bind tightly to Zn(II)
atoms, and tertiary amines, which will allow production of HCl salts, will be included in the
design of future analogs. The ninhydrin-based enzymatic assay will provide inhibitory data and
further SAR analysis to proceed with future modification of these compounds to produce more
potent inhibitors of DapE.

**Part II. Phenyltetrazole Thioethers as Inhibitors of DapE**

**Introduction.**

The phenyltetrazole thioether class of inhibitors has a more complex structure than the
other HTS ligands, including a chiral center. There several potential Zn(II) binding groups
located on the ligand. The pharmacophore of the HTS ligand compound E is unknown therefore
any one of these moieties or a combination thereof could be essential to DapE binding. This
exact ligand was synthesized in-house first to confirm inhibition and ultimately to enable
variation at various positions. We will first perform optimization of compound E with SAR
assessment by variations at the X, R1 and R2 positions (Figure 17).

Figure 17. Phenyltetrazole thioether HTS compound E with plan for future analogs

Molecular docking of the lead compound E with DapE performed using SwissDock
showed that there is a significant binding energy difference between the (R)- and (S)-
enantiomers. The (R)-enantiomer was predicted to bind at an allosteric site with weaker and
poorer binding energy than the (S)-enantiomer (Figure 18). One could assume this is indicative
of a noncompetitive or uncompetitive inhibitor with less potency.
Figure 18. Molecular docking of HTS compound E\[^{[a]}\]

![Molecular docking images](image)

\[^{[a]}\] Molecular docking performed using SwissDock and DapE open crystal structure (PDB:3IC1) and output is visualized using UCSF Chimera. The (S)-enantiomer is shown interacting with the Zn(II) atoms at the active site (picture left) with the Zn(II) atoms shown as grey spheres. The (R)-enantiomer is shown binding to an allosteric site (pictured right) with the Zn(II) atoms shown as purple spheres. The secondary structure is shown in grey.

DapE has shown preference for both L-inhibitors and L-substrates. This chiral phenyl tetrazole compound could potentially show selective inhibition for DapE by the (S)-enantiomer in a similar fashion. Our goal was to confirm this series as inhibitors of DapE using the ninhydrin-based enzymatic assay and obtain SAR from molecular docking studies to guide the synthesis of novel and more potent inhibitors of DapE.

**Chemistry.**

The lead compound E was successfully synthesized with two synthetic steps: first, alkylation of the 5-thiol(phenyltetrazole) 30 with racemic bromopropionic acid 31 to produce the thioether 32.\[^{42}\] Next, compound 33 was produced by amide coupling of the carboxylic acid 32 with 2-amino thiazole\[^{43}\] (Scheme 8).
Scheme 8. Synthesis of the HTS lead compound \( E^{[a]} \)

\[
\begin{align*}
\text{30} & \quad \text{N-S} \quad + \quad \text{Br-COOH} & \quad \xrightarrow{a} & \quad \text{32} & \quad + \quad \text{R}_1 \\
\text{31} & \quad \text{N-S} \quad + \quad \text{Br-COOH} & \quad \xrightarrow{a} & \quad \text{32} & \quad + \quad \text{R}_1
\end{align*}
\]

\( \text{R}_1 = \quad \)

[\( [a] \) Synthesis of HTS lead compound \( E \). (a) EtOH, NaOH, reflux; b) EDCI, DMF, DMAP, 0°C-rt.]

Modification of \( \text{R}_1 \) can be achieved by amide coupling of 32 with the desired primary or secondary amine in lieu of 2-aminothiazole. Variation of \( \text{R}_2 \) comes by the same synthetic route with the use of the appropriate \( \alpha \)-bromo carboxylic acid 34 for alkylation of 30.

Variation at \( \text{X} \) where \( \text{X} = \text{N} \) is achieved by the proposed synthesis of a thiourea 35 by reaction of a racemic \( \alpha \)-amino acid 34 with phenyl isothiocyanate.\(^{44} \) Next, the tetrazole 36 is formed by reaction with Mukaiyama’s reagent (N-Methyl-2-chloro-pyridinium iodide) and sodium azide with 35.\(^ {45} \) Finally, coupling of 36 with 2-aminothiazole using EDCI produces 37 (Scheme 9).

Scheme 9. Synthesis of HTS lead compound \( E \) amine analog\([a]\)

\[
\begin{align*}
\text{34} & \quad \xrightarrow{a} \quad \text{35} & \quad \xrightarrow{b} \quad \text{36} & \quad \xrightarrow{c} \quad \text{37}
\end{align*}
\]

[\( [a] \) Synthesis of the amine derivative of compound \( E \) where \( \text{X} = \text{N} \). a) Phenyl isothiocyanate, acetone; b) Mukaiyama’s reagent, NaN\(_3\); c) EDCI HCl, 2-aminothiazole.]

Results and Discussion.

Computational Studies. Compound \( E \) (33a) was synthesized as a racemic mixture and tested for inhibition against DapE and exhibited an IC\(_{50}\) of 89 \( \mu \text{M} \). This is validation that this HTS ligand was correctly identified as a new class of inhibitors of DapE. Variation at \( \text{R}_1 \) using
other aromatic heterocycles with both (R)- and (S)-enantiomers in molecular docking showed that there is no explicit preference for either enantiomer by DapE in this case. Both bind to DapE with the (R)-enantiomers having a carbonyl oxygen bridging between both Zn(II) atoms at the active site, like L-captopril, while the (S)-enantiomers are bis-ligated to the active site with the carbonyl oxygen bridging between both Zn(II)s and either a nitrogen or sulfur coordinating to one Zn(II). Bis-ligated compounds could be more tightly bound ligands but in these cases, it is not indicative of such trends with this variation of R₁ (Table 9). This was evidenced by an increase in energy of the bridging inhibitors analogs (R)-33b and (R)-33c compared to the bis-ligated inhibitors (S)-33b and (S)-33c.

Table 9. Binding energies of compound E with (R)- vs (S)-enantiomers with R₁ variation [a]

<table>
<thead>
<tr>
<th>R₁</th>
<th>Entry</th>
<th>R/S</th>
<th>ΔG [b]</th>
<th>Binding Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(S)-33a</td>
<td>S</td>
<td>-8.2</td>
<td>C=O to Zn1; NH to Zn2</td>
</tr>
<tr>
<td></td>
<td>(R)-33a</td>
<td>R</td>
<td>-8.1</td>
<td>Allosteric</td>
</tr>
<tr>
<td></td>
<td>(S)-33b</td>
<td>S</td>
<td>-9.8</td>
<td>C=O bridging; C=N close by</td>
</tr>
<tr>
<td></td>
<td>(R)-33b</td>
<td>R</td>
<td>-10.1</td>
<td>C=O bridging</td>
</tr>
<tr>
<td></td>
<td>(S)-33c</td>
<td>S</td>
<td>-9.1</td>
<td>C=O bridging; C=N close by</td>
</tr>
<tr>
<td></td>
<td>(R)-33c</td>
<td>R</td>
<td>-9.4</td>
<td>C=O bridging</td>
</tr>
<tr>
<td></td>
<td>(S)-33d</td>
<td>S</td>
<td>-9.4</td>
<td>C=O bridging; S is close by</td>
</tr>
<tr>
<td></td>
<td>(R)-33d</td>
<td>R</td>
<td>-8.8</td>
<td>C=O bridging</td>
</tr>
</tbody>
</table>

[a] Molecular docking performed by SwissDock using ZnZn(HiDapE) open structure PDB:3IC1. [b] Ligand binding energies calculated in kcal·mol⁻¹.
Structure-activity relationships are in progress exploring variations of $R_2$. The synthesis of compound $E$ employs the use of chiral $\alpha$-bromo-carboxylic acids for alkylation of the thiol tetrazole. Taking advantage of the fact that 2-bromopropionic acid is a direct analog of alanine, amino acid analogs can be synthesized by diazotization and substitution with bromine$^{46}$ which would provide a wider variety of analogs of compound $E$ to be tested for inhibition (Scheme 10).

Scheme 10. Diazotization and bromination of $\alpha$-amino acids to $\alpha$-bromo carboxylic acids$^{[a]}$

This prompted molecular docking studies of $\alpha$-bromo-carboxylic acid derivatives of natural $\alpha$-amino acids. This design first included the change of methyl to the primary alcohol -CH$_2$OH (38) which corresponds to a change from alanine to serine shown in Table 10 below. Similarly, the incorporation of an aromatic motif along with a hydroxyl group that can serve as a hydrogen bond donor or acceptor prompted the docking of the phenyl alanine and tyrosine analogs (39 and 40). A substructure search via Scifinder Scholar shows that serine and tyrosine derivatives 38 and 40, respectively, are more novel than the alanine derivative 33. Combination of these derivatives with a variety of different heterocyclic amines at $R_1$ can provide for a novel series of inhibitor. Both (R)- and (S)-enantiomers were docked with a variety of proposed analogs with $R_1$ and $R_2$, variations.

$^{[a]}$ Diazotization and bromination of non-functionalized amino acids (top) and functionalized amino acids (bottom).
In silico studies were promising with the indication of the novel tyrosine-derived analogs 40 as well as serine-derived analogs 38. The R₂ variation with -CH₂OH showed decreased binding energies for each analog docked compared to their methyl counterparts (Table 10).

Table 10. Binding energies of compound E serine derivative 38

<table>
<thead>
<tr>
<th>R₁</th>
<th>Entry</th>
<th>R/S</th>
<th>ΔG[^a]</th>
<th>Binding Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Structure" /></td>
<td>(S)-38a</td>
<td>S</td>
<td>-8.9</td>
<td>C=O bridging</td>
</tr>
<tr>
<td><img src="image2.png" alt="Structure" /></td>
<td>(R)-38a</td>
<td>R</td>
<td>-8.2</td>
<td>Allosteric</td>
</tr>
<tr>
<td><img src="image3.png" alt="Structure" /></td>
<td>(S)-38b</td>
<td>S</td>
<td>-10.9</td>
<td>C=O bridging; OH-Zn₁</td>
</tr>
<tr>
<td><img src="image4.png" alt="Structure" /></td>
<td>(R)-38b</td>
<td>R</td>
<td>-10.7</td>
<td>C=O bridging; OH-Zn₁</td>
</tr>
<tr>
<td><img src="image5.png" alt="Structure" /></td>
<td>(S)-38c</td>
<td>S</td>
<td>-10.5</td>
<td>C=O bridging; OH-Zn₁</td>
</tr>
<tr>
<td><img src="image6.png" alt="Structure" /></td>
<td>(R)-38c</td>
<td>R</td>
<td>-9.4</td>
<td>C=O bridging; OH-Zn₁</td>
</tr>
<tr>
<td><img src="image7.png" alt="Structure" /></td>
<td>(S)-38d</td>
<td>S</td>
<td>-9.5</td>
<td>C=O bridging; S-Zn₁</td>
</tr>
<tr>
<td><img src="image8.png" alt="Structure" /></td>
<td>(R)-38d</td>
<td>R</td>
<td>-10.2</td>
<td>C=O bridging; tetrazole-Zn₁</td>
</tr>
</tbody>
</table>

[^a]: Molecular Docking performed using SwissDock with the [ZnZn(HiDapE)] structure PDB:3IC1.[^b]: Ligand binding energies calculated in kcal·mol⁻¹.

We did note that there was no distinct preference for one enantiomer over the other except for aminothiazole (R)-38a similar to the original HTS ligand. The active-site binding of these analogs was presented as bridged inhibitors with interaction of both Zn(II) atoms in a mono-ligated fashion. In most cases, the -OH group is positioned to coordinate with Zn₁ allowing for a bis-ligated coordination with both Zn(II) ions (Figure 19).
Figure 19. Phenyltetrazole (S)-38b interaction with the Zn(II)s at DapE active site

[a] Molecular docking of (S)-38b serine derivative was performed using SwissDock and visualized with UCSF Chimera. The Zn(II) atoms are shown as grey spheres with the secondary structure in grey.

The incorporation of the benzyl group similar in structure to phenylalanine, also showed decreased and more potent binding energies in most cases compared to their methyl counterparts (Table 11).

Table 11. Ligand binding energies of compound E phenylalanine derivatives 39[a]

<table>
<thead>
<tr>
<th>R1</th>
<th>Entry</th>
<th>R/S</th>
<th>ΔG[b]</th>
<th>Binding Orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(S)-39a</td>
<td>S</td>
<td>-8.9</td>
<td>C=O bridging; S-Zn1</td>
</tr>
<tr>
<td></td>
<td>(R)-39a</td>
<td>R</td>
<td>-9.5</td>
<td>C=O bridging; S-Zn1</td>
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<tr>
<td></td>
<td>(S)-39b</td>
<td>S</td>
<td>-9.7</td>
<td>C=O bridging; S-Zn1</td>
</tr>
<tr>
<td></td>
<td>(R)-39b</td>
<td>R</td>
<td>-10.2</td>
<td>C=O bridging; S-Zn1</td>
</tr>
<tr>
<td></td>
<td>(S)-39c</td>
<td>S</td>
<td>-10.3</td>
<td>C=O bridging</td>
</tr>
<tr>
<td></td>
<td>(R)-39c</td>
<td>R</td>
<td>-9.4</td>
<td>C=O bridging</td>
</tr>
<tr>
<td></td>
<td>(S)-39d</td>
<td>S</td>
<td>-10.4</td>
<td>C=O bridging; S-Zn1</td>
</tr>
<tr>
<td></td>
<td>(R)-39d</td>
<td>R</td>
<td>-9.4</td>
<td>C=O bridging; S-Zn1</td>
</tr>
</tbody>
</table>

[a] Molecular Docking performed using SwissDock with the [ZnZn(HiDapE)] structure PDB:3IC1. [b] Ligand binding energies calculated in kcal-mol⁻¹.
Thus, docking provided evidence of potentially more tightly-bound ligands. In the phenylalanine derivatives, no preference for either the (R)- or (S)-enantiomer was uniformly observed. However, variation of $R_1$ with other aromatic heterocycles showed that the original 2-aminothiazole was not as favorable for binding to DapE with both (S)-39a and (R)-39a having higher binding energies than the other substituents tested. Inhibitor binding was observed with a bridging mode between both Zn(II) ions and the sulfur coordinating to Zn1. More insight on the interactions with the binding pocket of DapE will be explored in future studies.

The biggest decrease in energy representing more tightly-bound ligands was observed with the tyrosine derivatives 40 of which most docked analogs showed binding in a bridging mode between the two Zn(II) ions by the amide carbonyl oxygen (Table 12, Figure 20).

![Figure 20. Phenyltetrazole inhibitor (R)-40b interaction with Zn(II)s at DapE active site](image)

[a] Molecular docking of (R)-40b tyrosine derivative was performed using SwissDock and visualized with UCSF rChimera. The Zn(II) atoms are shown as grey spheres with the secondary structure of DapE in grey.

As seen with the alanine 33, serine 38, and phenylalanine derivatives 39, there was no preference for one enantiomer over the other for tyrosine derivatives 40. There is a further decrease in energy with the incorporation of a pyridine or pyrimidine modification at $R_2$ (Table 12) suggesting favorable ligand design and binding.
Table 12. Ligand binding energies of compound E tyrosine derivatives 40\textsuperscript{[a]}

<table>
<thead>
<tr>
<th>R\textsubscript{1}</th>
<th>Entry</th>
<th>R/S</th>
<th>ΔG\textsuperscript{[b]}</th>
<th>Binding Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td>(S)-40a</td>
<td>S</td>
<td>-10.2</td>
<td>C=O-Zn1</td>
</tr>
<tr>
<td><img src="image2.png" alt="Image" /></td>
<td>(R)-40a</td>
<td>R</td>
<td>-9.0</td>
<td>OH- bridging</td>
</tr>
<tr>
<td><img src="image3.png" alt="Image" /></td>
<td>(S)-40b</td>
<td>S</td>
<td>-11.2</td>
<td>C=O bridging</td>
</tr>
<tr>
<td><img src="image4.png" alt="Image" /></td>
<td>(R)-40b</td>
<td>R</td>
<td>-11.0</td>
<td>C=O bridging</td>
</tr>
<tr>
<td><img src="image5.png" alt="Image" /></td>
<td>(S)-40c</td>
<td>S</td>
<td>-10.5</td>
<td>C=O bridging</td>
</tr>
<tr>
<td><img src="image6.png" alt="Image" /></td>
<td>(R)-40c</td>
<td>R</td>
<td>-10.99</td>
<td>C=O bridging</td>
</tr>
</tbody>
</table>

\textsuperscript{[a]} Molecular Docking performed using SwissDock with the [ZnZn(HiDapE)] structure PDB:3IC1. \textsuperscript{[b]} Ligand binding energies calculated in kcal mol\textsuperscript{-1}.

One clear indication for the variation of the R\textsubscript{1} and R\textsubscript{2} groups is that incorporation of a the -OH group decreases the ligand binding energies corresponding with tighter binding as evidenced by the serine 38 and tyrosine derivatives 40. Also, the introduction of a 6-membered aromatic heterocycle also decreases the ligand binding energies as seen in analogs b and c of each derivative. The in-silico studies using SwissDock provide a key starting point to the design of future analogs. The binding modes of these inhibitors may play a key role in the inhibition of DapE.

The aza analog 37 is an alternative bioisostere to the thioether 33. The secondary amine is less likely to by oxidized than the sulfur making the shelf life potentially longer than the thioether, which is preferred in medicinal chemistry applications. Most importantly, there is an ease of analog synthesis as single enantiomers from natural or unnatural amino acids. Also, the modification to the amine adds for another point of derivation compared to the thioether. A
Scifinder Scholar substructure search shows that this simple modification in itself is not novel but the incorporation of a simple methyl addition to the amine or the use of serine or tyrosine derivatives at R₂ provides a novel structure and perhaps may enhance the pharmacophore. The amine analog 37 is currently being synthesized as an alternative to be compared to the inhibitory data by the original HTS ligand compound E.

**Conclusion.**

The phenyltetrazole HTS ligand E was validated as a lead toward a series of potent inhibitors of DapE demonstrated by use of the ninhydrin-based enzymatic assay with an IC₅₀ of 89 μM for 33a. This is promising since this was a racemic mixture and if one enantiomer is completely inactive then the IC₅₀ is cut essentially in half. If one enantiomer shows slight inhibition while the other is more potent, the apparent IC₅₀ for a single enantiomer would still be lower than reported for the racemic. Molecular docking has guided the design of more potent inhibitors of this series. The novelty space on this series is easily achieved by slight variations such as the incorporation of serine or tyrosine derivatives and the change from a thioether to a secondary or tertiary amine, as shown by SciFinder Scholar. The use of MOE for more extensive molecular docking will be more beneficial because docking of the ligands is not computed with a rigid receptor as in SwissDock and will allow for the active site of the enzyme to more accurately accommodate the inhibitors. This will aid in the design of new inhibitors with increased interactions with the residues in proximity to the active site. Key interactions can be assessed that are not explicitly observed using SwissDock. Additionally, the MOE software suite can generate a library of potential inhibitors that fit in the active site that should improve the novelty and potency of the synthesized inhibitors. Results from the molecular docking
experiments using MOE are currently in progress. Analogs will be synthesized and inhibitory data using the newly-developed ninhydrin assay will be obtained in due course.

**Part III. Difluoromethyl Sulfonamides as Inhibitors of DapE**

Introduction.

The difluoromethyl sulfonamide compound B identified through HTS is a simplified class of inhibitors with much room for variation. Difluoromethyl (-CF$_2$H) groups are important structural motifs in medicinal chemistry as the group acts as a lipophilic hydrogen donor. The difluoromethyl group can also act as a bioisostere of thiols, anilines, amines and thiophenes but not of alcohols.$^{47}$ Possible inhibition of DapE could come from interaction with a negative charge on the enzyme or between F and the Zn(II) ion itself. Take in account the proximity of the methoxy oxygen and the sulfonyl oxygen, these both could act as Zn(II) ion binding groups through bis-ligation. With these possible modes of interaction, a HTS ligand expansion was performed with a goal of establishing SAR and obtaining proposed binding configurations of Compound B through *in silico* studies. The original HTS compound B was resynthesized along with analogs and tested for inhibition against DapE using the ninhydrin-based enzymatic assay.

Figure 21. Difluoromethyl sulfonamide HTS lead compound B with points of modification
Chemistry.

The lead inhibitor compound B was synthesized through the condensation of toluene sulfonyl chloride and 2-methoxy aniline to yield 41, followed by the difluoromethylation using potassium carbonate and chlorodifluoroacetate sodium salt (Scheme 11).

Scheme 11. Synthesis of HTS lead difluoromethyl sulfonamide compound B

\[
\text{Scheme 11. Synthesis of HTS lead difluoromethyl sulfonamide compound B}^{[a]}
\]

[a] a) DCM, Et3N, rt; b) MeCN, chlorodifluoroacetate sodium salt, K2CO3

[b] Synthesis of compound B, the synthesized ligand will be referred to synonymously as 42a.

Variation of the R1 and R2 groups is obtained through use of substituted aniline derivatives in a reaction with substituted benzene sulfonyl chloride. Further variation at position R2 meta and para to the amine can be achieved by use of appropriate-substituted anilines.

Modification of R3 is performed by reaction of 41 through alkylation, acylation, etc.

Scheme 12. Synthesis of compound B analogs with modification of R3

Results and Discussion.

For HTS compound B (42a), molecular docking simulations were performed in SwissDock using the PDB:3IC1 crystal structure to obtain predicted binding modes. The ligand scaffold of compound B from the HTS is not as complex as the other inhibitors identified and the
presence of potential zinc binding groups guided the design of analogs. The sulfonyl oxygens were observed coordinating to the zinc atoms at the active site of DapE with one oxygen bridging between both Zn(II)s in the docking results (Figure 22).

Figure 22. Difluoromethyl sulfonamide B interaction with the Zn(II) ions at DapE

[4] Molecular docking performed using SwissDock and DapE open crystal structure (PDB:3IC1) with outputs visualized using UCSF Chimera. Zn(II) atoms shown in purple with the inhibitor in light blue and the enzyme secondary structure in grey.

Interestingly, one zinc atom appeared to coordinate to the methoxy oxygen, the difluoromethyl, and to both sulfonyl oxygens. The proximity of these zinc-binding groups allows for multiple interactions at the active site where all three groups play a distinct role in coordination. To initiate the SAR studies, we utilized the synthesis of compound B to vary the most easily altered positions pertaining to these functional moieties. This included testing the location of the methoxy group to the sulfonamide by synthesizing both the ortho and para methoxy derivatives. Finally, the difluoromethyl group on the sulfonamide was replaced with isosteres including a traditional methyl group.

**Computational Studies.** The o-methoxy difluoromethyl sulfonamide 42a and the p-methoxy analog 42b were docked to DapE along with the methyl derivatives 43a and 43b (Figure 23). The methylated derivatives of o-methoxy and p-methoxy sulfonamides serve two
purposes: first, to assess SAR of the difluoromethyl group acting as a methyl isostere versus its potential to act as a more complex moiety; and second, to determine if ortho substitution aids in the binding of the inhibitor as demonstrated in the molecular docking of compound B.

Figure 23. Methyl sulfonamide analogs of compound B

![Methyl sulfonamide analogs of compound B](image)

Initial docking studies indicated that the difluoromethyl group decreased binding to the active site as the methyl derivatives showed lower binding energies of -8.29 kcal·mol\(^{-1}\) and -8.79 kcal·mol\(^{-1}\) for 43a and 43b, respectively, indicating tighter binding than the lead compound. No further computational studies have been performed to date on compound B, but initial results confirm the HTS ligand suggests active-site binding.

**Inhibitory data.** The methyl analogs 43a and 43b have also been synthesized alongside the original compound 42a. Inhibitory data showed that both methylated structures 43a and 43b have increased potency compared to the difluoro 42a which only inhibited 31 % at 100 μM. The methylated derivative 43a showed 60 % inhibition at 100 μM and 43b showed an IC\(_{50}\) of 151 μM. Interestingly, the non-functionalized sulfonamide 43c showed no measurable inhibition at 100 μM (Table 13).
Table 13. Inhibitory data of HTS compounds

<table>
<thead>
<tr>
<th>Entry</th>
<th>Structure</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; μM&lt;sup&gt;[a]&lt;/sup&gt;</th>
<th>Entry</th>
<th>Structure</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; μM&lt;sup&gt;[a]&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>42a</td>
<td><img src="image" alt="Structure" /></td>
<td>ND&lt;sup&gt;[b]&lt;/sup&gt;, 31% at 100</td>
<td>43b</td>
<td><img src="image" alt="Structure" /></td>
<td>151</td>
</tr>
<tr>
<td>43a</td>
<td><img src="image" alt="Structure" /></td>
<td>ND, 60% at 100</td>
<td>43c</td>
<td><img src="image" alt="Structure" /></td>
<td>ND, 0% at 100</td>
</tr>
</tbody>
</table>

[a] Data obtained from a set of triplicates and IC<sub>50</sub> values were determined from a set of three or four % inhibition points. [b] Not determined, low solubility at higher concentrations, inhibition reported by a single concentration.

**Conclusion.**

The difluoromethyl sulfonamide series was confirmed as inhibitors of DapE with compound E showing a 31% inhibition at 100 μM using the ninhydrin-based enzymatic assay. Although the inhibition of DapE by 42a was less than that of the methylated analogs 43a and 43b, expansion of the series by has already been proven to be beneficial. Further, molecular dockings studies are needed to further guide the design of future analogs of this series. *In silico* inhibitory screening of additional analogs to increase potency of this motif should prove advantageous. We will continue to focus on building a library of compounds to be synthesized in due course.

**Part IV. Inhibitory Data of Miscellaneous Compounds**

The spectrophotometric ninhydrin-based enzymatic assay was used to determine the inhibitory activity of the HTS compounds and analogs. Inhibition was screened at 20 and 200 uM and the IC<sub>50</sub> values were not yet determined for these inhibitors. The ninhydrin-based assay protocol was similar to the protocol for screening described in Chapter Two. The exception is that the inhibitor dilution and incubation methods were modified, in that all inhibitors were diluted to 5 mM or 10 mM stock solutions in 100% DMSO to aid in inhibitor solubility. Serial
dilutions were then made from this stock solution with final stock solutions containing 50 % DMSO. The addition of 20 μL of 50 % DMSO to 200 μL reactions provide a final concentration of 5 % DMSO. Because of the addition of DMSO, the incubation time was cut in half from 10 minutes to 5 minutes and reactions were incubated on ice. Inhibitory data is reported as percent inhibition.

**Inhibitory Data.**

Table 14 shows the inhibition of DapE by compounds prepared by our research group outside those identified in HTS.

Table 14. Inhibitory data of miscellaneous compounds using ninhydrin-based assay

<table>
<thead>
<tr>
<th>Entry</th>
<th>Structure</th>
<th>% Inhibition at 200 μM[^a]</th>
<th>% Inhibition at 20 μM[^a]</th>
</tr>
</thead>
<tbody>
<tr>
<td>44</td>
<td><img src="image1" alt="Structure" /></td>
<td>43</td>
<td>21</td>
</tr>
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<td>37</td>
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<td>47a</td>
<td><img src="image4" alt="Structure" /></td>
<td>86</td>
<td>32</td>
</tr>
<tr>
<td>47b</td>
<td><img src="image5" alt="Structure" /></td>
<td>25</td>
<td>12</td>
</tr>
<tr>
<td>48a</td>
<td><img src="image6" alt="Structure" /></td>
<td>79</td>
<td>22</td>
</tr>
<tr>
<td>48b</td>
<td><img src="image7" alt="Structure" /></td>
<td>27</td>
<td>25</td>
</tr>
</tbody>
</table>

[^a]: Data obtained from a set of triplicates; Inhibitors were allowed to incubate with HiDapE at 30°C for 10 minutes.
CHAPTER FIVE

7-SULFONAMIDE INDOLINES AS INHIBITORS OF DAP E

Introduction

The identification of 7-sulfonamide indolines as a class of possible inhibitors of DapE arose curiously from the mislabeling of the structures of the 6-sulfonamide indolines identified through the HTS performed on compounds licensed from Chembridge Corp. The actual 6-sulfonamide indoline structure was incorrectly labeled by Dorogov and others as the 7-sulfonamide indoline regioisomer produced from the sulfonylation of N-acetyl-5-bromo-indoline using chlorosulfonic acid in an electrophilic aromatic substitution reaction.\(^{50}\) Subsequently, Borror and colleagues confirmed through X-ray analysis and NMR spectral analysis that this chlorosulfonic reaction actually produces the sole 6-chlorosulfonated regioisomer.\(^{51}\) This may be due to the rotamer of the amide bond of N-acetyl indoline favoring an endo vs an exo position. The endo conformer is favored when the carbonyl oxygen is in close proximity to the 7-H on the aromatic ring due to the partial positive and partial negative charge the two atoms carry from hydrogen bonding (Figure 24). This endo conformation may block substitution at the 7-position on indoline leaving the 6-position open for substitution. This conformation also explains why halogenation of N-acetyl indoline occurs only para to the amide bond at the 5-position with exclusive formation of the 5-halo regioisomer.\(^{52}\)
The 7-sulfonamide indolines rose in interest due to molecular docking that was initially performed based on the incorrect HTS structure docking suggested that the proximity of the amide oxygen and a sulfonamide oxygen in these 7-sulfonamide indoline regioisomers could allow for a bis-ligated interaction to the Zn(II) ions at the active site of DapE. Furthermore, lower binding energies were observed for the 7-sulfonamide indolines compared to the 6-sulfonamide indolines which could be a direct correlation of suggested interaction at the active site. More tightly bound ligands should be more potent inhibitors of DapE, which led us to explore these structures. Correspondingly, we hypothesized that 7-sulfonamide indolines would function as potent inhibitors of DapE and herein report molecular docking data that predicts the increased potency over the 6-sulfonamide regioisomers. To date, the only published methods on a synthetic route to 7-sulfonamide indolines is by Borror et al. using a tricyclic indoline system containing a cyclic sulfonyl urea moiety as an intermediate. Our attempts to repeat this method revealed severe limitations on both the yield and the variety of analogs that could be produced. Herein, we report an improved method for the formation of 7-sulfonamide indolines via the tricyclic sulfonyleurea indoline, in addition to a novel synthetic approach to the direct 7-sulfonation of indolines. The inhibitory data of the 7-sulfonamide indolines have validated our hypothesis that 7-sulfonamide indolines should be more potent inhibitors of DapE than the 6-sulfonamide indolines.
Computational Studies

Based on the presence of two zinc binding groups on the indoline compounds it is suggestive of bis-ligation involving both the N-acetyl and the sulfonamide groups on the indoline with both Zn(II) ions at the DapE active site. The 7-sulfonamide indolines provide just the right distance for this ligand-zinc interaction to take place where the 6-sulfonamide groups are positioned farther apart in indoline making bis-ligation less possible. Molecular docking of the 7-sulfonamide indolines with [Zn2(HiDapE)] crystal structure 3IC1 performed with SwissDock showed six different possible modes of interaction with DapE. Upon completion of the calculations, the ligand conformations satisfying the di-zinc metal ion coordination with the lowest binding energies around the target active site were used to identify potential inhibitors and are represented in Figure 25.

In the docked structure, the 7-sulfonamide indolines were observed interacting with both zincs at the active site in a bis-ligated mode as hypothesized. Additionally, the 7-sulfonamide indolines were also observed binding in a bridging manner with either the acetyl or a sulfonyl oxygen coordinated to both Zn(II) ions. In some cases, one oxygen was shown coordinating to just one zinc while the other oxygen was bridged between both. And, like the 6-sulfonamide indolines, some inhibitors were observed bound to DapE at an allosteric site.
Figure 25. Molecular docking interaction of 7-sulfonamide indolines with DapE\textsuperscript{[a]}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure25.png}
\caption{Molecular docking of 7-sulfonamide indolines bound to DapE performed by SwissDock and visualized with UCSF Chimera with the Zn(II) ions shown as purple spheres, the inhibitors are shown in blue with the sulfonyl group in yellow and the secondary structure of DapE shown in grey. A) Bis-ligated pose. B) Sulfone oxygen bridging Zn(II) ions. C) Acetyl oxygen bridging the Zn(II)s with the sulfonyl oxygen coordinating to only one Zn(II) ion. D) Both sulfonyl and acetyl oxygens coordinating to one Zn(II). E) Allosteric binding. F) carboxylate coordinating to active site.}
\end{figure}

\textsuperscript{[a]} Molecular docking of 7-sulfonamide indolines bound to DapE performed by SwissDock and visualized with UCSF Chimera with the Zn(II) ions shown as purple spheres, the inhibitors are shown in blue with the sulfonyl group in yellow and the secondary structure of DapE shown in grey. A) Bis-ligated pose. B) Sulfone oxygen bridging Zn(II) ions. C) Acetyl oxygen bridging the Zn(II)s with the sulfonyl oxygen coordinating to only one Zn(II) ion. D) Both sulfonyl and acetyl oxygens coordinating to one Zn(II). E) Allosteric binding. F) carboxylate coordinating to active site.

The overall binding energies of the 5-brominated-7-sulfonamide indolines versus the 6-sulfonamide indoline regioisomers were markedly lower representing superior predicted binding (Table 15). The arithmetic differences in the 7-sulfonamides compared to the 6-sulfonamides that result in a negative number indicate more tightly-bound ligands favoring the 7-sulfonamide regioisomer. The 7-sulfonamide indolines could act as more tightly-bound ligands to the active sites based on lower calculated energies and the suggestive binding configurations obtained from the molecular docking. Some of the trends between the direct analogs of the regioisomers were similar such as for the nitrogen-containing aromatic sulfonamide derivatives which are predicted to act as allosteric inhibitors.
Table 15. Molecular docking energies of brominated 7- vs. 6-sulfonamide indolines $^{[a]}$

<table>
<thead>
<tr>
<th>Entry</th>
<th>Entry</th>
<th>R$_2$</th>
<th>R$_3$</th>
<th>ΔG for 49</th>
<th>ΔG for 16</th>
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<tbody>
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<td>16a</td>
<td>-Br</td>
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<td>-8.9</td>
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<tr>
<td>49b</td>
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[a] Molecular docking performed with SwissDock using PDB:3IC1. [b] Only four of the twenty indoline 6-sulfonamides were predicted to be better than the corresponding 7-sulfonamides.

**Part I. N-Acetyl-7-Sulfonamide Indoline Syntheses**

**Borror Synthetic Route to 7-Sulfonamide Indolines.**

The 7-sulfonamide indolines were synthesized using a unique tricyclic indoline system containing a cyclic sulfonyl urea moiety as an intermediate. The key step in this synthesis is using the indoline nitrogen as an anchor to deliver the sulfonyl group for formation of the 7-sulfonamide indoline. The 7-sulfonamide indolines are synthesized by the condensation of indoline and chlorosulfonyl isocyanate (51) followed by thermally-induced intramolecular cyclization. This produces the unique tricyclic indoline system with a cyclic sulfonyl urea 52. Alkylation of this cyclic sulfonylurea 52 using a haloalkane and K₂CO₃ produced 53 and was followed by a base-catalyzed hydrolysis using KOH afforded 7-sulfonamide indoline 54. Finally, acetylation using acetyl chloride produces the desired N-acetyl-5-bromo-7-sulfonamide indoline 49 (Scheme 13). ⁵¹
Scheme 13. Borror’s synthesis of N-acetyl-7-sulfonamide indolines\[^{[a]}\]

\[
\begin{align*}
\text{Br} & \xrightarrow{\text{a}} \text{Br} & \xrightarrow{\text{b}} \text{Br} \\
50 & & 71\% & & 51 & & 34\% \\
\text{Br} & \xrightarrow{\text{c}} \text{Br} & \xrightarrow{\text{d}} \text{Br} & \xrightarrow{\text{e}} \text{Br} \\
49 & & 52 & & 53 & & 54
\end{align*}
\]

\[^{[a]}\text{Only published synthesis of 7-sulfonamide indolines.}^{51} \text{ R= alkyl halide. (a) ClSO_2NCO, MeCN, 0 °C; b) toluene, 116 °C, 4 hrs; (c) K_2CO_3, DMF, R-X, rt, 72 hrs; (d) KOH, MeOH, rt, 24 hrs; (e) acetyl chloride, DCM, Et_3N, 1 hr.}\]

The literature procedure provided the desired indoline 51, however, the isolated product was not stable over time. Additionally, the heat-mediated cyclization produced an even lower yield of 52 over two steps. By the time we synthesized derivatives of 53, the overall yield was barely enough to characterize the products, let alone enough to take into the next step.

**Improved Borror Synthetic Route.**

Synthesis of 7-sulfonamide indolines proved difficult through this method of intramolecular cyclization, alkylation, and hydrolysis, which led us to pursue modifications of the synthesis described by Borror. The first modification came by performing an intramolecular cyclization under Friedel-Crafts conditions\[^{53}\] in a one-pot synthesis to create the tricyclic sulfonymurea indoline 56. After alkylation and halogenation of the tricyclic indoline 56 to produce 53, hydrolysis was performed with 50% sulfuric acid heated to 135 °C rather than using KOH. We observed that the viscous nature of the sulfuric acid aided in solubility (Scheme 14).\[^{53}\]
Scheme 14. Improved synthesis of N-acetyl-7-sulfonamide indolines\textsuperscript{[a]}

\begin{center}
\begin{tikzpicture}
  \node (12) at (0,0) {12};
  \node (1a) at (1.5,0) {1a};
  \node (55) at (3,0) {55};
  \node (2a) at (4.5,0) {2a};
  \node (56) at (6,0) {56};
  \node (57) at (7.5,0) {57};
  \node (53) at (1.5,-3) {53};
  \node (54) at (3,-3) {54};
  \node (49) at (1.5,-6) {49};

  \draw[->] (12) -- (1a);
  \draw[->] (1a) -- (55);
  \draw[->] (55) -- (2a);
  \draw[->] (2a) -- (56);
  \draw[->] (56) -- (57);
  \draw[->] (53) -- (54);
  \draw[->] (54) -- (49);

  \node (Br) at (1.5,-4.5) {Br};
  \node (R') at (3,-4.5) {R'};
  \node (H) at (5.5,-4.5) {H};
  \node (N) at (3,-5) {N};
  \node (O) at (3,-5.5) {O};
  \node (C) at (3,-6) {C};
  \node (CH3) at (3,-6.5) {CH3};

  \node (O=SO2) at (1.5,-7) {O=SO2};
  \node (HN) at (1.5,-7.5) {HN};

  \node (O=SO2) at (3,-7) {O=SO2};
  \node (HN) at (3,-7.5) {HN};

  \node (O=SO2) at (5.5,-7) {O=SO2};
  \node (HN) at (5.5,-7.5) {HN};

  \node (Br) at (1.5,-8) {Br};
  \node (R') at (3,-8) {R'};
  \node (H) at (5.5,-8) {H};
  \node (N) at (3,-8.5) {N};
  \node (O) at (3,-9) {O};
  \node (C) at (3,-9.5) {C};
  \node (CH3) at (3,-10) {CH3};

  \node (O=SO2) at (1.5,-10.5) {O=SO2};
  \node (HN) at (1.5,-11) {HN};

  \node (O=SO2) at (3,-10.5) {O=SO2};
  \node (HN) at (3,-11) {HN};

  \node (O=SO2) at (5.5,-10.5) {O=SO2};
  \node (HN) at (5.5,-11) {HN};

  \node (Br) at (1.5,-11.5) {Br};
  \node (R') at (3,-11.5) {R'};
  \node (H) at (5.5,-11.5) {H};
  \node (N) at (3,-12) {N};
  \node (O) at (3,-12.5) {O};
  \node (C) at (3,-13) {C};
  \node (CH3) at (3,-13.5) {CH3};
\end{tikzpicture}
\end{center}

\textsuperscript{[a]} Improved Borror synthesis. R= alkyl (1a) One-pot synthesis employing ClSO\textsubscript{2}NCO, nitroethane, -42\degree C; followed by (2a) AlCl\textsubscript{3}, rt-110\degree C, 2 hrs; (b) alkyl halide, NaH, DMF, 60\degree C; (c) Br\textsubscript{2}, AcOH or NBS in MeCN; (d) 1) 50\% H\textsubscript{2}SO\textsubscript{4}, 135\degree C, 2 hrs; 1) 50\% H\textsubscript{2}SO\textsubscript{4}, 135\degree C, 2 hrs (e) acetyl chloride, Et\textsubscript{3}N, DCM, DMAP.

The improved Borror synthetic route allowed for the synthesis of three N-acetyl-5-bromo-7-sulfonamide indolines: the isopentyl sulfonamide 49\textsuperscript{a}, the propyl sulfonamide 49\textsuperscript{b} and the benzyl sulfonamide 49\textsuperscript{c} shown in Figure 26.

Figure 26. 7-Sulfonamide indolines synthesized by the improved Borror method

Although three N-acetyl-7-sulfonamide indolines were successfully synthesized through these methods, this synthetic route has severe synthetic limitations. First the alkylation step is limited to the synthesis of secondary sulfonamides from the S\textsubscript{N}2 of available primary and unhindered haloalkanes. This precludes the synthesis of any cyclic sulfonamides through this method including the reproduction of the piperidine sulfonamide HTS inhibitor compound D.
Second, the harsh hydrolysis conditions brought about by the use of 50 % sulfuric acid heated to 135°C for two hours narrows the use of many substituents unable to tolerate these severe conditions including alcohols, alkenes, and other easily-hydrolyzed and other labile functional groups. Therefore, a more practical method for synthesizing 7-sulfonamide indoline analogs for evaluation as inhibitors of DapE, in addition to other potential application of indoline-7-sulfonamides is needed.

**Modified Synthesis of 7-Sulfonamide Indolines.**

To reliably evaluate inhibition against DapE and to develop structure-activity relationships between the 6-sulfonamide indolines versus the 7-sulfonamide indoline regioisomers, is to synthesize direct analogs of each one though a similar method. Ideally, it would be conducive to the efficient synthesis of both in parallel with the production of the indoline 7-sulfonyl chloride 60 could react with primary and secondary amines with varying functional groups should be analogous to the synthesis of the 6-sulfonamide indolines. This could be achieved by a practical alternative synthesis of 7-sulfonamide indolines using the existing tricyclic sulfonyl urea indoline 56 from the previously described modified method (Scheme 15)\(^{51}\). Upon hydrolysis of the non-alkylated cyclic sulfonylurea 56 the primary 7-sulfonamide indoline 58 is produced. Acylation on the aniline nitrogen to produce 59 allows the primary sulfonamide to be free for conversion to the sulfonyl chloride 60 which is ultimately reacted with amines to provide the desired 7-sulfonamide indoline.
Scheme 15. Proposed synthesis of 7-sulfonyl chloride indoline 60\textsuperscript{[a]}

\[ \text{Scheme 15. Proposed synthesis of 7-sulfonyl chloride indoline 60} \]

[\text{[a] Proposed synthetic route of sulfonyl chloride 61 (a) 1) 50\% H}_2\text{SO}_4 \text{at 135 °C, 2 hrs; 2) 10N NaOH to pH 7; (b) acetylation; (c) deaminative chlorination}\]

Initially, acetylation of sulfonamide 58 using both acetyl chloride and the milder reagent acetic anhydride to obtain the N-acetyl-7-sulfonamide indoline 59 produced a mixture of N-acylated products. This included the di-acetylated product 62 as the major product with the desired N-acetyl 7-sulfonamide indoline 59 as well as the 7-(N-acetyl)sulfonamide indoline 63 and unreacted starting material 58 as shown in Scheme 16, demonstrating that, both the aniline nitrogen of indoline and the primary sulfonamide had similar reactivity towards acetylation.

Scheme 16. Acetylation of primary sulfonamide 58 using acetyl chloride

Instead, a selective carboxybenzyl (Cbz) protection of the aniline nitrogen of the indoline ring was achieved by use of a Cbz protecting group (64) as supported by NMR. This selective amine protection allows for the primary sulfonamide to be converted to the sulfonyl chloride 65 (Scheme 17).
Scheme 17. Selective aniline protection using carboxybenzyl (Cbz) group.

\[
\text{Scheme 18. Unsuccessful attempts at deaminative chlorination}
\]

**Deaminative chlorination of 7-sulfonamide indolines.** The deaminative chlorination of the primary sulfonamide 64 to produce the 7-chlorosulfonyl indoline 65 was explored. An attempt to change the primary sulfonamide directly to a sulfonyle chloride using thionyl chloride and dioxane at reflux as per Park’s procedure yielded <20% desired product with unreacted starting material. 1,4-Dioxane and thionyl chloride proved troublesome to remove and the low product yield compared to the expected yield of 55% proved this route may not be practical. The use of chlorosulfonic acid followed by thionyl chloride would not be applicable due to reaction of the strong electrophilic with the more sensitive Cbz group (Scheme 18).
Due to a lack of other literature procedures for the direct deaminative chlorination of aryl sulfonamides, a more productive synthetic route to synthesize the sulfonyl chloride through a sulfonic acid intermediate was pursued since there are many known literature procedures for the synthesis of sulfonyl chlorides from sulfonic acids. Conversion of the N-Cbz-7-sulfonamide indoline 64 has been achieved using isoamyl nitrite suing to produce\textsuperscript{56} the 7-sulfonic acid indoline 67, which can then be more efficiently converted to the sulfonyl chloride 65 (Scheme 19).

Scheme 19. Proposed synthesis of 7-sulfonic acid 67 from the primary 7-sulfonamide indoline 64

A neat reaction with thionyl chloride (SOCl\textsubscript{2}) and the N-Cbz-7-sulfonic acid indoline 67 produced a complex mixture of products. This could be due to the proximity of the Cbz group to the 7-sulfonyl chloride. The proximity of these groups could allow for intramolecular reactions between these two groups due to a 6-membered ring that could be formed. In addition, this could have been the result of thionyl chloride reacting directly with the Cbz group despite reports that the Cbz protecting group is stable to thionyl chloride. This is hypothesized because of the presence of 7-sulfonamide indoline 58 was detected as one of the products produced. The two variables of this reaction are the reagents used and the Cbz protecting group and the most logical remedy to this problem is to use a milder reagent. We decided to preserve starting materials by substitution of toluene sulfonic acid (TsOH) as a model aryl sulfonic acid to optimize the
reaction conditions for converting an aryl sulfonic acid to a sulfonyl chloride and ultimately to a sulfonamide.

**Toluene sulfonic acid as a model aryl sulfonic acid.** We decided to address the two concerns of the Cbz group and the thionyl chloride as two separate problems. First, we looked at the use of milder reagents and/or reaction conditions for the chlorination of sulfonic acid. Cyanuric trichloride (CTC) is often employed for the conversion of alcohols and carboxylic acids to alkyl chlorides and acyl chlorides, respectively, CTC can convert aryl sulfonic acids to sulfonyl chlorides in different applications. A solvent-free reaction of TsOH, CTC and KCl ground in a mortar and pestle for 5 minutes with the addition of a drop water followed by a workup with EtOAc showed mainly unreacted CTC in the organic layer by HPLC and the toluene sulfonic acid remained in the aqueous layer unreacted. A more direct approach employing TsOH, CTC, Et₃N in acetone at reflux showed full and clean conversion to toluene sulfonyl chloride (TsCl). The HPLC retention times and the NMR reference peaks matched those of commercially available TsCl.

Second, we addressed the possibility of the sulfonyl chloride 65 being successfully synthesized and soon after reacting intramolecularly or intermolecularly with the Cbz group. To avoid this potential problem, we considered synthesizing 65 as an intermediate without isolation, or technically in a one-pot reaction to produce the desired sulfonamide. When reacted with DMF, CTC forms a complex known as Gold’s reagent, where it subsequently reacts with sulfonic acid and “alkylates” the added amine to form a sulfonamide. Although we could form Gold’s reagent, we were not able to get the TsOH to react and therefore no product was observed. We are currently pursuing a straightforward *in situ* attempt by synthesizing the TsCl using CTC and
acetone, as described above, while closely monitoring the reaction followed by addition of the
desired amine to form the sulfonamide in a one-pot reaction. In addition, there are a few other
one-pot reactions involving CTC and the addition of an amine to form the sulfonamide.\textsuperscript{60}

**Aryl sulfonamide formation using triphenylphosphine.** More recently, we have
focused on coupling the indoline 7-sulfonic acid 67 with primary and secondary amines using
triphenylphosphine (Ph\textsubscript{3}P). In this case, we used benzene sodium sulfonate 69 as a model aryl
sulfonic acid. The use of a halogenating agent, Br\textsubscript{2} \textsuperscript{61} or trichloroacetonitrile,\textsuperscript{62} converts the Ph\textsubscript{3}P
to Ph\textsubscript{3}PX\textsubscript{2} (X=Br or Cl). From there, reaction with the sulfonic acid generates the sulfonyl halide
which can react with the amine in situ to yield the desired 7-sulfonamide indoline. Ph\textsubscript{3}PBr\textsubscript{2} 68
was generated by the addition of Br\textsubscript{2} to Ph\textsubscript{3}P in acetonitrile. Next, benzene sodium sulfonate 69
was added to generate the sulfonyl bromide 70 in situ which then reacted with aniline to afford
the desired sulfonamide 71 (Scheme 20).

Scheme 20. Use of Ph\textsubscript{3}P to couple aryl sulfonic acids to form sulfonamides

\[
\begin{align*}
\text{Ph}_3\text{P:} & \xrightarrow{\text{Br}_2} \text{Ph}_3\text{PBr}_2 + \left\{ \begin{array}{c}
\text{PhO}_2\text{SO}_2\text{Na}^+ \quad 69 \\
\text{PhO}_2\text{SO}_2\text{Br}^- \quad 68 \\
\end{array} \right\} \\
\text{Ph}_3\text{PBr}_2 & \xrightarrow{\text{TEA}} \text{Ph}_3\text{PO} + \text{Ph}_3\text{PBr}_2 \quad 70 \\
\end{align*}
\]

The reaction was complete as determined by HPLC with full conversion of the starting
material. However, the separation of the triphenylphosphine oxide byproduct (Ph\textsubscript{3}PO) 72 proved
difficult based on the similar properties of benzene sodium sulfonate and the Ph\textsubscript{3}PO byproduct.
Also, use of aniline produced a product containing only aromatic hydrogens and the presence of
the unreacted Ph\textsubscript{3}PBr\textsubscript{2} and Ph\textsubscript{3}PO makes confirmation of the product more difficult. By using a
polymer-bound Ph₃P reagent, the removal of the Ph₃PO was possible by filtration. Also, the switch from aniline to cyclohexyl amine should allow the detection of protons outside of the aromatic region via NMR (Scheme 21).

Scheme 21. Use of polymer-bound Ph₃P for coupling aryl sulfonic acids

While monitoring the reaction by TLC and HPLC, no aromatic products were detected which led us to conclude that the TsOH was attached to the polymer-bound Ph₃P. We decided to return our focus to the unbound Ph₃P for these reactions as we wanted to confirm the actual products of the reaction as a priority in determining how to isolate the product if possible. The reaction was scaled up 10-fold from 20 mg to 200 mg of TsOH and the product formed was detected as well as the observed disappearance of most of the starting material. The sulfonamide 71b was synthesized in a separate reaction by a known procedure using cyclohexyl amine and commercially available TsCl for use as a standard. Unfortunately, neither the HPLC retention times nor the TLC rf values matched as they were distinctly different. Co-chromatography of the Ph₃P reaction and the sulfonamide 71b showed two different retention times instead of the increase in the concentration of the product as expected. Lastly, we attempted to synthesize the sulfonyl chloride in situ by use of trichloroacetonitrile as described by Chantarasriwong but this proved unsuccessful as very little TsOH was converted to the TsCl.
A Successful Selective Acetylation of 7-Sulfonamide Indoline 58.

We have concluded that the Cbz group is unsuitable for further use in the synthesis of N-acyl-7-sulfonamide indolines through the formation of the sulfonyl chloride 65 due to the labile nature of this protecting group in proximity to the electrophilic sulfonyl species on the 7-position of the indoline. We decided to revisit the selective amine protection of 7-sulfonamide indoline 58 by acetylation. The use of Et$_3$N led to the di-acetylation of 7-sulfonamide indoline 58. Borror used K$_2$CO$_3$ as a base for the alkylation of the 5-bromo cyclic sulfonamide indoline 51, however this was not successful when attempted with the cyclic indoline 56. We attempted acetylation of 58 using either K$_2$CO$_3$ or the weaker base NaHCO$_3$. We observed the formation of one main product and two smaller byproducts with K$_2$CO$_3$ and one main product and a small byproduct with the use of NaHCO$_3$ as a base. Acetylation of 58 in THF with 3 equivalents of solid NaHCO$_3$ cooled to 0 °C and 1.05 equivalents of acetyl chloride dissolved in THF was added slowly. The temperature was kept below room temperature (15-18°C), and the formation of the desired mono-acetylated indoline 59 product (Scheme 22) was suggested by the disappearance of the aniline nitrogen and the retention of the correctly integrated sulfonamide hydrogens on $^1$H NMR.

Scheme 22. Selective acetylation of 7-sulfonamide indoline 58.
The successful synthesis of the N-acetyl-7-sulfonamide indoline 59, demonstrates that we have designed a new modified synthetic route of N-acetyl-7-sulfonamide indolines through a sulfonyl chloride. With the robust acetyl functional group in contrast to Cbz, the synthesis of the sulfonyl chloride is synthesized with greater ease. The hydrolysis of the cyclic sulfonyl urea indoline 56 to produce the 7-sulfonamide indoline 58 allows for the acetylation of the indoline nitrogen to produce 59. The primary sulfonamide 59 is available for conversion to the sulfonic acid 73 by isoamyl nitrite. From here, the sulfonamide can be synthesized by formation of the sulfonyl chloride 74 and either isolated or generated in situ and reacted with the desired amine to produce the sulfonamide 75 (Scheme 23).

Scheme 23. Modified Synthesis of N-acetyl-7-sulfonamide indolines

[a] R= alkyl, cyclic, aromatic amines. (a) One-pot synthesis of cyclic sulfonurea 1) ClSO2NCO, nitroethane, -42°C; 2) AlCl3, rt-110°C, 2 hrs; (b) 1) 50% H2SO4, 135°C, 2 hrs; 2) 10°C, 10 N NaOH, pH 7; (c) 1.05 eq of acetyl chloride, 3 eq NaHCO3, THF 0°C to 15°C; (d) isoamyl nitrite, dioxane, reflux; (e) Cyanuric trichloride, acetone; (f) amine; (g) optional one-pot reaction of e) and f) without isolation of indoline 74.
Substituted N-Cbz-7-Sulfonamide Indoline Derivatives.

We used the N-Cbz-7-sulfonamide indoline 64 intermediate to synthesize derivatives of the primary sulfonamide at R₃. Similar to the derivatives designed for the 6- sulfonamide indolines, we aimed to synthesize N-acyl sulfonamides 76 by reaction of acyl chlorides or carboxylic acids. In addition, thioureas 77 can be synthesized by the reaction of the primary sulfonamide with isothiocyanates, and sulfonyleureas 78 can be synthesized by reaction with isocyanates or carbamoyl chlorides (Scheme 24).

Scheme 24. Variation of N-Cbz-7-sulfonamide indoline 64 at R₃

The N-acyl sulfonamides were explored in Chapter Four as precursors to the compounds in the 6-sulfonamide indoline series. As previously mentioned, our aim is to have direct head-to-head comparisons of 6- and 7-sulfonamide regioisomers. The Cbz at the R₁-position of the indoline is intended as a protecting group that can be replaced with various acyl groups. To assess SAR of the N-acylsulfonamides, molecular docking was performed on N-acyl-7-(N-acyl)sulfonamides and [ZnZn(HiDapE)] utilizing SwissDock. N-Acyl sulfonamides are
moderately acidic with a pKa of 5-6 which is similar to a carboxylic acid. In aqueous solutions, this functional group exists predominantly as an anion. Both the neutral acylsulfonamides and their anions were docked, along with 5-bromo and 5-chloro analogs.

**Computational Studies of 7-(N-acyl)sulfonamides and DapE Binding.**

Initial molecular docking showed that 1-N-acetyl-5-bromo-7-(N-acetyl)sulfonamide indoline 79a (X=Br) as the neutral compound has a ΔG of -8.5 kcal·mol⁻¹ which is not significantly different from the original piperidine sulfonamide 16b (-8.93 kcal·mol⁻¹) but is predicted to be significant more stable than the isopentyl sulfonamide 16a (-9.67 kcal·mol⁻¹). Docking was then performed on the 5-chloro analog 79a (X=Cl) for SAR comparison showing a very significant decrease in energy to -10.24 kcal·mol⁻¹ (Table 16). Using this proposed synthetic route, the halogenation can be performed early in the synthesis directly after hydrolysis. Because of the favorable difference in binding energies, the non-halogenated 79a was docked for comparison. The data showed that the non-halogenated 79a (X=H) ligand has a binding energy comparable to the 5-chloro analog (-10.13 kcal·mol⁻¹). Docking with a longer acyl group and a bulkier acyl group with 5-bromo, 5-chloro and 5-hydro substituents was performed (Table 16) as comparison. The butanamide derivative 79b and benzamide derivative 79c were used as reference. Docking results show the opposite effect for butanamide 79b with 5-bromo (X=Br) and 5-hydro (X=H) substituents having comparable energies and the 5-chloro (X=Cl) analog has a significantly higher energy and weaker binding, whereas the benzamide 79c substituent showed the same trend as the acetyl groups with less of a numerical difference between either substituent but with 5-chloro analog 79c (X=Cl) having the lowest binding energy. The acyl
sulfonamide amino anions were later docked as well for a more physiological relevant comparison of the potential binding for the ligands shown in Table 16.

Table 16. Ligand binding energies of the 1-acyl-7-N-acylsulfonamide indolines \([a]\)

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>(\Delta G) kcal·mol(^{-1}) (X=H)</th>
<th>(\Delta G) kcal·mol(^{-1}) (X=Br)</th>
<th>(\Delta G) kcal·mol(^{-1}) (X=Cl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>79a</td>
<td>(-CH_3)</td>
<td>-10.1</td>
<td>-8.7</td>
<td>-10.3</td>
</tr>
<tr>
<td>79b</td>
<td>(-CH_2CH_2CH_3)</td>
<td>-11.1</td>
<td>-11.1</td>
<td>-9.3</td>
</tr>
<tr>
<td>79c</td>
<td>(-)</td>
<td>-10.3</td>
<td>-10.3</td>
<td>-10.8</td>
</tr>
<tr>
<td>79a(-)</td>
<td>(-CH_3)</td>
<td>-12.7</td>
<td>-12.3</td>
<td>-12.1</td>
</tr>
<tr>
<td>79b(-)</td>
<td>(-CH_2CH_2CH_3)</td>
<td>-13.7</td>
<td>-13.9</td>
<td>-12.9</td>
</tr>
<tr>
<td>79c(-)</td>
<td>(-)</td>
<td>-12.5</td>
<td>-13.4</td>
<td>-13.6</td>
</tr>
</tbody>
</table>

\([a]\) Molecular docking performed with SwissDock and visualized with UCSF Chimera.

The deprotonated form of the acylsulfonamide anions 79a(-), 79b(-), and 79c(-) showed much lower binding energies, as low as \(-13.88\) kcal·mol\(^{-1}\) as expected. These very low binding energies were similar to the carboxylate anions for both the 6- and 7-sulfonamide indolines which is important because those energies were reflective of the carboxylate moiety interacting with the Zn(II) ions. Another important take-away from the \textit{in-silico} data is that substitution at the 5-position with chlorine supports our decision to abandon the 5-bromo substituent. Although
initial inhibitory data and inhibitor development with SAR can be performed without 5-substitution, this position maybe prone to metabolism in vivo application so the 5-chloro analogs should continue to be analyzed. Figure 27 shows the binding of the 5-substituted-7-N-acylsulfonamide indoline inhibitors to the active site with the anions in A-C and the protonated counterparts in D-F. Docking shows the weakest interactions with the neutral 5-bromo-7-acylsulfonamide 79a. The orientation of the possible interactions with zinc the binding groups is different in the representations, in addition, the anions show a distinct interaction with the dimerization domain of DapE which will be further explored at a later date.

Figure 27. Ligand-enzyme interaction of N-acetyl-7-(N-acetyl)sulfonamide indolines 79a

[a] Molecular docking of 79a performed with SwissDock and visualized with UCSF Chimera with the ligand shown in blue, the Zn(II)s shown as purple spheres and the secondary structure of the enzyme shown in grey. Yellow lines from the ligand-enzyme interactions are visualization outputs that show direct coordination between atoms. Top row is 79a(-) in its ionic form where (A) X=H; (B) X=Br; (C) X=Cl; and the bottom row is the 79a in its neutral form where (D) X=H; (E) X=Br; (F) X=Cl
Three 1-Cbz-7-(N-acyl)sulfonamide indoline derivatives were successfully synthesized by reaction of the primary sulfonamide 64 with acetyl chloride to produce 76a, reaction with benzoic anhydride produced 76b, and reaction with p-anisoyl chloride produced 76c shown in Figure 28. Two heterocyclic acylsulfonamides 76d and 76e were formed by the reaction of thiazole carbonyl chloride and isoxazole carbonyl chloride respectively.

Figure 28. Synthesis of 1-Cbz-7-&N-acyl)sulfonamide indolines 76

Three cyclic 7-sulfonylurea indolines have been synthesized from 67 and are shown below (Figure 29). Ideally, inhibitors tested should be at least 95% in purity, however an initial screening at slightly lower purity is practical.

Figure 29. Synthesis of indoline 7-sulfonylureas 78 from Cbz-7-sulfonamide indoline 64
Next, modification of \( \textbf{R}_1 \) was achieved by removal of the Cbz group from \( \textbf{76} \) through hydrogenolysis with \( \text{H}_2 \) and Pd/carbon. From here, several acyl groups can be added on the aniline nitrogen to produce analogs of \( \textbf{75} \) for SAR comparison. Several derivatives of \( \textbf{75} \) are currently in progress (Scheme 25).

Scheme 25. Hydrogenolysis to produce N-acyl-7-(N-acetyl)sulfonamide indolines \( \textbf{79} \)

Synthesis of 7-sulfonylureas \( \textbf{78} \) and thioureas \( \textbf{77} \) using isocyanates and isothiocyanates as reagents proved unsuccessful as the degradation of material was observed under a variety of conditions. Analysis by HPLC showed many products while \( ^1\text{H} \) NMR confirmed the disappearance of the methylene hydrogens from the Cbz group. This was further evidence that the Cbz group is indeed too labile and therefore unsuitable for further use in the synthetic route as it limited most of the chemistry necessary for functionalization of the compounds we designed.

**Inhibitory Data of 7-Sulfonamide Indolines.**

N-Cbz-7-(N-acyl)sulfonamide indolines \( \textbf{74a} \) and \( \textbf{74c} \) have been submitted and tested for inhibition of [ZnZn(\( \text{HiDapE} \))]. These indolines showed more potent inhibition of DapE having \( \text{IC}_{50} \) of 32 \( \mu \text{M} \) for the N-acetyl sulfonamide \( \textbf{74a} \) and 57 \( \mu \text{M} \) for the anisoyl sulfonamide derivative \( \textbf{74c} \). This is very promising considering that the Cbz was intended only as a protecting group that was essential for the functionalization of the primary 7-sulfonamide group (Table 17).
Table 17. Inhibition data of 7-sulfonamide indolines\textsuperscript{[a]}

<table>
<thead>
<tr>
<th>Entry</th>
<th>Structure</th>
<th>IC\textsubscript{50} μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>76a</td>
<td><img src="image" alt="Structure" /></td>
<td>32</td>
</tr>
<tr>
<td>76c</td>
<td><img src="image" alt="Structure" /></td>
<td>57</td>
</tr>
</tbody>
</table>

\textsuperscript{[a]} Data obtained from a set of triplicates and IC\textsubscript{50} values were determined from a set of three or four % inhibition points

**Conclusion.**

The N-Cbz-7-N-acetysulfonamide indolone 76a is the most potent inhibitor synthesized and tested from any series of the HTS inhibitors identified. The anisole analog 76c also showed increased potency as well which is further confirmation that this new series should be prioritized for the development of a ligand library for DapE inhibition. The focus on further development of the synthetic route to produce 7-sulfonamide indolines is supported by both \textit{in silico} data and the inhibitory data obtained.

**Part III. Ortho-Directed Synthesis of 7-Sulfonamide Indolines**

**Synthesis of 7-Substituted Indolines in Literature.**

Synthesis of 7-substituted indolines in general has proven to be especially troubling. Our goal is to develop a novel synthesis of N-protected 7-sulfonamide indolines with direct introduction of the sulfonaryl group. A number of reactions in which Rh, Ru and Ir catalysts were used to introduce alkenes, amines and other functionalities directly onto the 7-position of indole were identified.\textsuperscript{63, 64} However, no catalytic introduction of a sulfonaryl group to the 7-
position of indoline or a position ortho to an aniline by metellation was found. We then focused on identifying procedures with the direct addition of any SO₂ to the 7-position of indoline by any means. A literature procedure where PPA (polyphosphoric acid) activates the rearrangement of an N-sulfonamide moiety (80) to the 7-position⁶⁵ as a sulfone (81) in addition to a Photo-Fries rearrangement were identified but these reactions gave a low yield with a mixture of products (Scheme 26).⁶⁶

Scheme 26. Sulfonamide rearrangement reactions⁶⁵,⁶⁶

Ortho-Directed Lithiation Towards Synthesis of 7-Sulfonyl Indolines.

The Iwao group, which is well known for developing indole, indene and indoline substitution chemistry, has used ortho-directed lithiation of Boc-protected indoline to produce 7-substituted indolines.⁶⁷ These include reactions with aldehydes and a range of electrophiles (I, Cl, Me, Me₃Si).⁶⁷ Smith synthesized aryl and alky sulfonic acids by insertion of sulfur trioxide via STTAC into a carbon-lithium bond of alkyl and aryl organolithium reagents.⁶⁸ With the combination of these two organic processes, we have designed a novel and promising synthesis of 7-sulfonamide indolines. Boc-protected indoline 85 undergoes ortho-directed lithiation using
n-BuLi. The carbamate acts as a stabilizer for lithium at the 7-position by coordination to the carbonyl oxygen (86). The 7-sulfonic acid indoline 87 is then obtained by mild insertion of sulfur trioxide by STTAC (sulfur trioxide trimethyl amine complex) into the carbon-lithium bond followed by acidification. The sulfonic acid 87 should then react with CTC in acetone to produce the sulfonyl chloride 88. The Boc group should be robust enough to remain intact under these conditions, unlike the Cbz from the modified synthesis in the previous section. The sulfonyl chloride 88 can then be reacted with a variety of amines to produce the desired 7-sulfonamide indolines 89. The Boc deprotection and replacement with similar moieties at the 1-position for SAR can be achieved (75) (Scheme 27).

Scheme 27. Lithiation-mediated synthesis of 7-sulfonamide indolines

Reaction of Boc-indoline 85 produced the sulfonic acid 86 with 45% conversion of starting material as determined by HPLC, however, after workup only 35% of product was detected via NMR. The humidity present in the lab and/or the degradation of both n-BuLi and STTAC may have been contributing factors. The lithiation step is performed at -78 °C and then warmed to room temperature before cooling back to -78 °C for the duration of the reaction before
addition of STTAC. Variation in number of equivalents of n-BuLi, the reaction temperature, and variation in reaction time could result in a higher conversion of starting material. There is enough evidence to support pursuing this synthesis as an applicable route for 7-sulfonic acid indolines 87. Optimization of reaction methods for the synthesis of 7-sulfonic acid indoline 87 through ortho-directed lithiation is currently in progress.

**Ortho-Directed Lithiation by CO₂.**

The use of carbon dioxide as an ortho-directing group for sulfonylation has also been pursued. Following a similar procedure, n-BuLi was introduced to a solution of indoline 12 in hexane and the lithium carbamate 90 was generated by introduction of CO₂ from the sublimation of dry ice.⁶⁹ The lithium carbamate 90 was exposed to another equivalent of n-BuLi followed by TMEDA and STTAC to produce 7-sulfonic acid indoline 91 (Scheme 28).

Scheme 28. Ortho-directed lithiation using CO₂

\[ \text{[a]} \]

Ortho-directed sulfonylation of indoline (a) 1) n-BuLi, hexane, rt; 2) CO₂, n-BuLi, -78°C; b) STTAC, TMEDA, THF, -78 °C

HPLC showed full conversion of starting material before isolation. During an aqueous workup, there was an observed effervescence, probably CO₂, and a final HPLC showed a different retention time, but the product that was isolated appeared to resemble starting material. A proton NMR confirmed the isolated product was indeed starting material. A second attempt of ortho-directed lithiation by CO₂ involved the use of CO₂ generated from the addition of 12M HCl to Na₂CO₃ which was pumped through a drying tube and delivered to the reaction flask just
below the surface of the solvent. The HPLC showed full conversion of starting material with retention times that did not match the previous attempt. A proton NMR of the product was noticeably different from the indoline starting material. The aromatic region did however show 4 unique aromatic protons suggesting that the 7-sulfonic acid indoline 91 was not produced but instead indoline-1-sulfonic acid 92. This suggests that the 7-position of indoline had not been lithiated under these conditions (Scheme 29).

Scheme 29. Synthesis of indoline sulfonic acid$^{[a]}$

[a] Ortho-directed sulfonylation of indoline (a) 1) n-BuLi, hexane, rt; 2) CO$_2$, n-BuLi, -78°C; b) STTAC, TMEDA, THF, -78 °C

Conclusion.

A new practical synthetic route for the production of 7-sulfonamide indolines has been developed by formation of a tricyclic sulfonylurea indoline extended from a published synthesis by Borror and colleagues.$^{51}$ By hydrolysis of the cyclic sulfonylurea to produce the primary sulfonamide, the formation of the sulfonyl chloride by way of the sulfonic acid allows for production of a wider variety of analogs compared to the current literature procedure. Inhibitory data supports the use of N-acyl-7-sulfonamide indolines as inhibitors of DapE with data from two inhibitors, with IC$_{50}$s of 32 μM for the N-acetylsulfonamide 76a and 57 μM for the anisoyl sulfonamide derivative 76c. Molecular docking studies also supports the application of 7-(N-
acyl)sulfonamide indolines as inhibitors of DapE as evidenced by the favorable low binding energies and observed positive interactions at the active site with both Zn(II) atoms. Progress toward novel synthesis of lithium-mediated sulfonylation of N-Boc indoline as a route to produce 7-sulfonamide indolines is very promising. The optimization of the method will be addressed in due course. Some 7-sulfonamide indolines are awaiting inhibitory data and the development of new analogs from the improved methods are currently in progress.
APPENDIX A
SUPPLEMENTAL DATA FOR CHAPTER TWO
DapE molecular modeling protocol.

Models of L,L-SDAP (1a), N6-methyl-L,L-SDAP (1b), and N6-acetyl-L,L-SDAP (1c) bound to HiDapE were developed using the Molecular Operating Environment (MOE)3 computational suite’s Builder utility followed by minimization in the gas phase using the force field MMFF94X. The minimized ligands were then subjected to the Conformational Search protocol to generate structural-conformation data bases populated with as many as 10,000 individual conformations. Conformational data bases were generated for all three ligands of interest for use in the following docking experiments.

The previously-reported X-ray crystal structure of the product-bound form of HiDapE in a closed conformation (PDB: 5VO3)4 and the molecular system were uploaded into MOE. Following receptor preparation, molecular docking was performed using the previously-generated ligand conformation data bases. Substrate analog docking was carried out in the prepared HiDapE enzyme model with the products and solvent atoms inactivated and the docking site specified at the catalytic Zn(II) atoms. Substrate analog placement employed the Proxy Triangle method with London dG scoring generating 50 data points that were further refined using the induced fit method with GBVI/WSA dG scoring to obtain the top 30 docking results. The docking protocol was repeated for all three substrates and analyzed for selection of best docking pose. Docking poses of the substrate 1a and analogs 1b and 1c were assessed as judged by their similarity to the product binding interactions seen in the original product-bound X-ray crystal structure. A single substrate analog docking pose was selected for each of the three substrates providing initial substrate-bound enzyme models.

The three substrate/enzyme models were then solvated in a simple water box at pH of 7.4 that was treated with NaCl counterions to balance the charge. Periodic boundary conditions were enabled, and the hydrogen bonding network of the model was optimized by automatically sampling different tautomer/protomer states using Protonate3D,1 which calculates optimal protonation states, including titration, rotamer, and “flips” using a large-scale combinatorial search.2 The system atoms were then optimized with a short, localized molecular minimization process with atoms further than 8 Å from the substrate fixed. System refinement continued until an RMS Gradient of 0.1 kcal/mol/Å was attained. Molecular Dynamics parameters were set to globally minimize the protein, substrate, and solvent atoms in the system using an NPA algorithm with an Amber12:EHT force field, with a typical heating and cooling protocol. Simulation results were then minimized once again before the final binding poses were obtained for comparison.

Modified L.L-SDAP as potential substrates.
The hydrolytic activity of HiDapE towards 1b and 1d was monitored by the decrease in absorbance at 225 nm using a Shimadzu UV-2450 UV/Visible Spectrophotometer. All reaction volumes were 1 mL with 8 nM HiDapE, 5 mM 1b or 5 mM 1d in 50 mM HEPES buffer, pH 7.5. Spectra were recorded over 35 min with one scan per minute. Enzyme dilutions were made directly before each trial from a concentrated stock solution that was stored at -80°C.
APPENDIX B

EXPERIMENTAL DATA FOR CHAPTER THREE
General Experimental Methods
All solvents were distilled prior to use, and all reagents were used without further purification unless otherwise noted. Ninhydrin solution was purchased as a 2% solution in 100% DMSO with a lithium acetate buffer at pH 5.2 from Santa Cruz. All synthetic reactions were conducted under a nitrogen atmosphere. Silica gel 60A, 40–75 μm (200 × 400 mesh), was used for column chromatography. Aluminum-backed silica gel 200 μm plates were used for TLC. $^1$H NMR spectra were obtained using either a 300 MHz spectrometer or a 500 MHz spectrometer with trimethylsilyl (TMS) as the internal standard. $^{13}$C NMR spectra were obtained using a 75 MHz spectrometer or a 125 MHz spectrometer. The purity of all compounds was determined to be $\geq$95% unless otherwise noted by high performance liquid chromatography (HPLC) employing Mobile phase A = 0.1% TFA in water, Mobile phase B= 0.1% TFA in acetonitrile, with a gradient of 60% B increasing to 95% over 10 min, holding at 95% B for 5 min, then returning to 60% B and holding for 5 min. HRMS spectra were measured on a TOF instrument by electrospray ionization (ESI). HRMS spectra were collected using a Waters Acquity I class UPLC and Xevo G2-XS QTof mass spectrometer with Waters Acquity BEH C18 column (1.7 μm, 2.1x50 mm). Mobile phase A was 0.05% formic acid in water and mobile phase B was 0.05% formic acid in acetonitrile. A gradient of 5% to 90% B in 5 minutes was applied. Enzyme activity curves were fitted with a program that uses a non-linear least squares regression of the Gauss-Newton algorithm with optional damping using an ad hoc program. $^5,6$

Synthesized Compounds from Chapter Three
All methods can be found in the published article referenced.

Protein Purification and Expression
Recombinant DapE from H. influenzae was expressed and purified according to a published protocol. $^{10}$ Briefly, several grams of cell paste were thawed at room temperature. Cells were lysed by sonication in 2 second pulses with 5 seconds rest, repeatedly, and the cell debris was removed by centrifugation at 4 °C for 40 min at 12,000 g. The supernatant was applied to a column packed with 10 mL of HisTrap HP resin (GE Healthcare), connected to VacMan (Promega) and the chromatographic process was accelerated with a vacuum pump. The column was washed with 20 bed volumes of lysis buffer and the His$_6$-tagged DapE were eluted with 25 mL of elution buffer (50 mM HEPES pH 8.0, 500 mM NaCl; 500 mM imidazole; 2 mM DTT). The His$_6$-tag was cleaved with TEV protease (2 mg of a His$_6$-tagged form) overnight at 4°C and dialysis to remove the excess of imidazole was carried out simultaneously. The resulting solution was mixed with His-Trap HP resin to capture the cleaved His$_6$-tag and the His$_6$-tagged TEV protease and the flow through containing DapE was collected and concentrated. A SEC column was run and the eluent was concentrated using an Amicon YM-10 membrane to a concentration 20mg x ml$^{-1}$ then the reducing agent TCEP (1mM) was added. Purified DapE from H. influenzae exhibited a single band on SDS-PAGE indicating a molecular weight of 41,500 Da. Protein concentrations were determined from the absorbance at 280 nm using molar absorptivity calculated using the method developed by Gill and Hipple. $^{11}$ The protein concentration determined using this molar absorptivity was in close agreement to that obtained using a Bradford assay. Individual aliquots of purified DapE were stored in liquid nitrogen until needed.

Enzyme Zinc Metal Installation.
Apo-DapE was prepared by extensive dialysis for 3 to 4 days against 10 mM EDTA in 50 mM HEPES buffer, pH 7.5. The enzyme was then exhaustively dialyzed against metal-free (chelexed) 50 mM HEPES buffer, pH 7.5. Apo-DapE samples were incubated with 1.7 equivalents of ZnCl₂ (97%; Sigma-Aldrich) in 50 mM HEPES buffer for 30 min.

**Modified SDAP as Potential Substrates**

The hydrolytic activity of DapE with modified SDAP analogs was monitored by the decrease in absorbance at 225 nm via Shimadzu UV-2450 UV/Visible Spectrophotometer. All reaction volumes were 1mL with 8 nM DapE and 5 mM of N-Methyl-SDAP **1b** or 5 mM N-Ac-SDAP **1d** in 50 mM HEPES buffer pH 7.5. Spectra were recorded over 35 minutes with one scan per minute. Enzyme dilutions were made directly before each trial from the concentrated stock solution at -80°C.

**DapE Assay Protocol**

All reaction volumes are 200 μL with 25 nM DapE and 1 mM of N-methyl-SDAP **1b** unless otherwise stated. Enzyme assay absorbance values were read on a BioTek Syngen microplate reader. Initial assay conditions to measure the activity of DapE were carried out in triplicate as follows: to a 50 mM HEPES buffered DapE solution at pH 7.5 at room temperature (18-22°C) was added N-methyl SDAP **1b** TFA salt. The reaction proceed for 10 minutes after which 100 μL of the 2% ninhydrin solution was added, the reaction vortexed and heated to 100°C for 15 minutes to quench enzymatic activity and to promote the primary amine with ninhydrin. The ninhydrin reaction was then halted by placing the tube in ice for 2 minutes, and the absorbance of 80 μL was then recorded at 570 nm.

**Control Ninhydrin Reactions with Glutamic Acid and Sarcosine.**

Control reactions were carried out using glutamic acid as a primary amine model and sarcosine as a model secondary amine. UV/Vis absorbances between 300 and 800 nm were recorded on Shimadzu UV-2450 UV/Visible Spectrophotometer, and the assay was followed by reading the absorbance at 570 nm using a BioTek Syngen microplate reader. Control reactions for the detection of primary amine were carried out in triplicate as follows: to a 50 mM HEPES pH 7.5 buffered solution at 30 °C was added glutamic acid (0-1.2 mM) and 100 μL of the 2% ninhydrin solution. The reaction mixture was heated to 100 °C (2 min vs 15 min), cooled on ice and the absorbance spectra was examined via UV/Vis spectroscopy. Control reactions for the detection of secondary amine were carried out in triplicate in the same manner using the secondary amine sarcosine. The final concentration of the UV-Vis spectroscopy samples were 1 mL 0.04 mM dilutions made from 0.2 mM samples.

Entire time course plots of glutamic acid or sarcosine and ninhydrin development were carried out in triplicate as follows: To a 50 mM HEPES buffered solution pH 7.5 was added 1 mM amine at 30 °C. After a 5-minute incubation time the 2% ninhydrin solution was added, the reaction vortexed and heated at 100 °C with time intervals of 0, 2, 3, 5, 10, 15 and 20 minutes independently. The mixtures were cooled on ice for 2 minutes and 80μL of each reaction was examined via microplate reader at 570 nm. These experiments were repeated at 80 °C and 60 °C.
Incubation of DapE by DMSO.

All reaction volumes were 200 μL with 25 nM of DapE and 1 mM N⁶-methyl-SDAP TFA salt substrate 1b, and the standard ninhydrin solution in DMSO. UV/Vis spectra were recorded on 80 μL aliquots of ninhydrin-developed reactions unless otherwise stated, and spectra were measure on a BioTek Syngen microplate reader. Control reactions of enzymatic activity were carried out in triplicate as follows: to a 50 mM HEPES pH 7.5 buffered DapE at 30°C was added N⁶-methyl SDAP 1b. The reaction proceeded for 10 minutes after which 100 μL of the 2% ninhydrin solution was added and subsequently heated to 80°C for 15 minutes. This reaction was quenched by placing in ice, cooled to 30 °C and the absorbance was recorded at 570 nm. These control reactions were set as 100% standard enzymatic activity of DapE.

The DMSO incubation reactions were carried out in triplicate as follows: to a 50 mM HEPES pH 7.5 buffered DapE solution at 30°C was added DMSO (75% v/v). DapE was allowed to incubate between 0 and 10 min after which time N⁶-methyl-SDAP 1b was added and the reaction was allowed to proceed for 10 minutes. The enzymatic reaction was then quenched by addition of the 2% ninhydrin solution, the reaction vortexed and heated to 80°C for 15 minutes. The ninhydrin reaction was the quenched by placing the tube in ice and cooling to 30°C, and the absorbance was then read at 570nm.

Monitoring the Thermal Denaturation of DapE using Circular Dichroism (CD).

Denaturation of DapE was measured using an Olis DSM 20 circular dichroism spectrophotometer. Samples were measured in a cylindrical quartz cuvette with a 1 mm path length, and contained 10 mM phosphate, pH 8.0, and 0.4 μM of DapE. Data was collected every 1 nm with a wavelength range of 190 – 260 nm. The temperature was increased from 20°C to 80°C using 10°C increments and incubation time of 2 min at each temperature (available in Appendix C). Once 80°C was attained, this temperature was held constant until complete denaturation was observed. OlisGlobalworks software v1.3 was used to deconvolute the spectra and calculate the percent of secondary structure. The secondary structure was discerned by measuring at the percent alpha helices and beta sheets.

Pre-Heat Incubation Control Reactions.

All reaction volumes were 200 μL with 25 nM of DapE and 1 mM N⁶-methyl SDAP TFA salt substrate, and the standard ninhydrin solution. UV/Vis spectra were examined on 80 μL of ninhydrin-developed reactions unless otherwise stated. UV/Vis Spectroscopy was observed via BioTek Syngen microplate reader. The control reactions were carried out in triplicate under the previous for 100% standard enzymatic activity of DapE. A second set of control reactions were used to show no enzymatic activity. These were carried out using denatured DapE with heating to 100°C for 3 min prior to use and with all other standard conditions stated above.

The measurement of the decrease in activity of DapE due to incubation at 100°C was carried out as follows: a 50 mM HEPES pH 7.5 buffered DapE solution was heated at 100°C with an incubation time from one to 5 min independently followed by cooling in ice to 30°C. To this solution was added N⁶-methyl SDAP 1b and the reaction was allowed to proceed for 10 minutes after which the 2% ninhydrin solution was added, the reaction vortexed and heated at 80
°C for 15 minutes. The ninhydrin reaction was quenched by placing in ice and the absorbance was examined at 570 nm. This experiment was carried out in triplicate under the same conditions for pre-heating at 80°C.

Enzyme concentration reactions were carried out as follows: To a solution of 50 mM HEPES pH 7.5 buffer was added the desired concentration of enzyme at 30°C followed by the addition of 20 µL of N⁶-methyl- L,L-SDAP 1b TFA salt (final concentration of 1 mM SDAP, 200 µL total volume). The reaction proceeded for 10 minutes after which it was heated at 100°C for 1 minute then cooled on ice for an additional 1 minute. Next, 100 µL of 2% ninhydrin reagent was added to a final concentration of 300 µL. The reactions were then heated to 80°C for 15 minutes then cooled on ice for an additional 2 minutes and to which absorbance of 80 µL was read via a microplate reader at 570 nm.

**Ninhydrin-based Assay for DapE Enzymatic Activity**

The enzymatic activity of DapE is measured in triplicate as follows: To 50 mM HEPES at pH 7.5 buffered solution with 8 nM DapE at 30°C was added 2 mM N⁶-methyl SDAP 1b (final volume 200 µL). The reaction was allowed to proceed for 10 minutes and quenched by heating at 100°C for 1 minute and subsequent cooling on ice for 1 minute. To the cooled reaction was added the 2% ninhydrin solution, and the reaction vortexed and subsequently heated at 80°C for 15 minutes for the ninhydrin reaction. This reaction was quenched by placing in ice 2 minutes and the absorbance of 80 µL was read at 570 nm. These reactions were set as 100% standard enzymatic activity of DapE. Glutamic acid concentrations of 0 mM, 0.1 mM, 0.2 mM, 0.3 mM, 0.4 M, and 0.5 mM were used as a standard control of the measurement of primary amine.

**IC₅₀ Determinations.**

IC₅₀ values and enzyme activity curves were fitted with a program that uses a non-linear least squares regression of the Gauss-Newton algorithm with optional damping using an ad hoc program.⁵,⁶ All inhibition assays were conducted with a reaction volume of 200 µL, 2 mM N⁶-methyl SDAP 1b and 8 nM DapE unless otherwise stated. Glutamic acid standards of 0 to 0.5 mM were used in every trial. To a 50 mM HEPES pH 7.5 buffered solution was added desired inhibitor concentration at 30°C followed by DapE and allowed to incubate for 10 min. N⁶-methyl SDAP 1b was added and allowed to react for an additional 10 min and quenched by heating at 100°C for 1 minute and cooled on ice to 30°C. The 2% ninhydrin solution (100 µL) was added, the reaction vortexed and heated at 80°C for 15 min. after which the reaction was quenched by cooling on ice to 30°C and the absorbance was examined at 570 nm.
APPENDIX C
SUPPLEMENTAL FIGURES FOR CHAPTER THREE
Circular Dichroism UV/Vis spectra of thermal denaturation of DapE observing the α-helical secondary structure.
DapE enzymatic activity after pre-heat incubation reactions at 0°C, 80°C and 100°C.
UV-Vis spectral overlay of primary amine standard (glutamic acid) development with ninhydrin from 2- 20 minutes at 100°C.
UV-Vis spectral overlay of secondary amine standard (sarcosine) ninhydrin development from 2-20 minutes at 100°C.
Plot of DapE enzymatic activity after DMSO incubation reactions:

- Buffer Avg
- DMSO Avg
- No Substrate
Inhibition plot for 3- Mercaptobenzoic Acid against DapE.
Inhibition plot of phenylboronic acid against DapE.
Inhibition plot of 2-thiopheneboronic acid against DapE.
Inhibition plot of L-captopril against DapE.
APPENDIX D

EXPERIMENTAL INFORMATION FOR CHAPTER FOUR
General Experimental Details

All solvents were distilled prior to use unless purchased as anhydrous or as 98%+ purity. All reagents were used without further purification unless otherwise specified. $^1$H $^{13}$C NMR spectra were obtained on 500 MHz spectrometer or 300 MHz spectrometer and $^{13}$C NMR spectra were obtained at 125 MHz or 75 mHz, respectively on these instruments and processed manually or using MestreNova software. Percent yields were reported as single yields for final inhibitors and average of yields for intermediate materials. Reactions were run in round bottom flasks for large scale reactions and in vials for final small-scale reactions. Purities were obtained on Agilent 1050 and 1100 UV-Vis HPLC with a Mobile phase A: 5% Acetonitrile in water with 0.1% TFA and Mobile phase B: Acetonitrile with 0.1% TFA.

Synthesis of Compounds from Chapter Four

$^1$-Acetyl-indoline (13): To a solution of indoline 12 (20.0 g, 0.168 mol) in triethylamine (81.6 mL) was added dichloromethane (193 mL) via pouring with stirring in air for 10 min at room temperature. Another solution of acetyl chloride (17.4 g, 0.222 mol) in dichloromethane (129 mL) was added to the first solution via pressure equalizing funnel slowly and the temperature maintained at 0-5$^\circ$C for 15 minutes. The resulting solution was stirred at room temperature under air overnight and checked for completion by TLC with a mobile phase of 50% EA/Pet ether. The reaction was quenched with water and extracted with DCM three times. The organic portion was washed once with deionized water, once with 1N HCl, and once with brine and dried over Na$_2$SO$_4$. After drying, the solution was concentrated by rotary evaporation to yield the desired N-acetyl indoline 13 (25.2 g, 93.2%). $^1$H NMR (500 MHz CDCl$_3$): δ 8.22 (d, 1 H), 7.19 (t, 2 H), 7.02 (d, 1 H), 4.06 (t, 2 H), 3.20 (t, 2 H), 2.23 (s, 1H).

$^1$-Acetyl-5-bromoindoline (14): To a solution of 13 (2.00 g, 12.4 mmol) in glacial acetic acid (13 mL) at room temperature was added Br$_2$ (0.61 mL) dropwise with stirring. The solution turned from dark brown to orange/yellow upon stirring. Upon completion, the reaction was quenched by pouring into water with rapid stirring and a gold solid remained. Sodium thiosulfate solution was added to quench excess bromine. The heterogeneous mixture was filtered through a Buchner funnel and dried under high vacuum to obtain the desired 5-bromo-substituted N-acetyl indoline 14 (94% yield). $^1$H NMR (500 MHz CDCl$_3$): δ 8.09 (d, 1 H), 7.30 (s, 1 H). 7.29 (d, 1 H), 4.07 (t, 2 H), 3.19 (t, 2 H), 2.22 (s, 3 H).
1-Acetyl 5-bromoindoline -6-sulfonyl chloride (15): Chlorosulfonic acid (18.75 mL) was slowly added to dried round bottom flask with indoline 14 (2.50 g, 10.41 mmol) drop wise by addition funnel. The neat reaction was stirred and heated between 60°-70°C for 3hr. The black solution was cooled to room temperature and then quenched by pouring slowly over ice with stirring. An off-white precipitate was isolated via vacuum filtration, washed with deionized water and dried in vacuo to produce 15 (52% yield). 1H NMR (500 MHz CDCl3): δ 9.00 (s, 1 H), 7.60 (s, 1 H), 4.18 (t, 2 H), 3.31 (t, 2 H), 1.60 (s, 3 H).

General procedure for the synthesis of N-acetyl 5-bromo-6-sulfonamide indolines (16).

To a solution of sulfonyl chloride 15 (1eq, 0.148 mmol) and triethylamine (1.25 eq, 31.0 µL) in dichloromethane was added the desired amine (1.25 eq, 0.185 mmol) and the reaction stirred at room temperature until complete as determined by TLC. Upon completion the reaction was diluted with dichloromethane, washed once with water, twice with 1M HCl and once with brine. The reaction was then dried over Na2SO4, filtered and concentrated on the rotovap to obtain indoline derivatives 16. Secondary amines and nitrogen heterocycles were reacted with the addition of DMAP as a catalyst (10 mol%) and reaction times were longer than primary amine derivatives.

1-Acetyl-5-bromo-N-isopentylindoline-6-sulfonamide (16a): The synthesis of 16a was achieved by following the general procedure for 16 using isopentyl amine to produce a light yellow solid (82%) mp: 184-186°C. 1H NMR (500 MHz, Chloroform-d) δ 8.90 (s, 1H), 7.48 (s, 1H), 4.98 (bs, 1H), 4.14 (t, J = 8.7 Hz, 2H), 3.25 (t, J = 8.6 Hz, 2H), 3.14 (s, 1H), 2.92 (q, J = 6.9 Hz, 2H), 2.47 (s, 1H), 1.67 – 1.56 (m, 2H), 1.37 (q, J = 7.2 Hz, 1H), 0.85 (dd, J = 6.7, 2.3 Hz, 6H).

1-Acetyl-5-bromo-6-(piperidin-1-sulfonyl) indoline (16b): The synthesis of 16b was achieved by following the general procedure for 16 using piperidine to produce an off-white solid. (79% yield) MP: 197-201°C. 1H NMR (500 MHz, Chloroform-d) δ 8.76 (s, J = 6.1 Hz, 1H), 7.49 (s, 1H), 4.13 (t, J = 8.6 Hz, 2H), 3.30 (q, J = 4.9 Hz, 4H), 3.24 (t, J = 8.7 Hz, 2H), 2.23 (s, J = 2.7 Hz, 3H), 1.67 – 1.60 (m, 2H), 1.56 (t, J = 7.3 Hz, 2H).
1-Acetyl-5-bromo-N,N-dipropylindoline-6-sulfonamide (16c): The synthesis of 16c was achieved by following the general procedure for 16 using dipropyl amine to produce a brown waxy substance (78%). $^1$H NMR (500 MHz, Chloroform-$d$) $\delta$ 8.73 (s, 1H), 7.51 (s, 1H), 4.15 (t, $J$ = 8.6 Hz, 2H), 3.33 (s, 1H), 3.34 – 3.22 (m, 4H), 2.26 (s, 3H), 1.70 – 1.53 (m, 4H), 1.28 (s, 1H), 0.88 (t, $J$ = 7.4 Hz, 6H).

1-Acetyl-5-bromo-6-(pyrrolidin-1-sulfonyl) indoline (16g): The synthesis of 16g was achieved by following the general procedure for 16 using pyrrolidine to produce an off-white solid (62%) mp: 190-198°C. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 8.58 (d, $J$ = 3.3 Hz, 1H), 7.69 (s, 1H), 4.13 (q, $J$ = 10.4, 9.7 Hz, 2H), 3.31 (d, $J$ = 3.4 Hz, 4H), 3.20 (t, $J$ = 8.8 Hz, 2H), 2.16 (s, 3H), 1.86 (q, $J$ = 4.8, 4.1 Hz, 4H)

1-Acetyl-5-bromo-N-cyclohexylindoline-6-sulfonamide (16h): The synthesis of 16h was achieved by following the general procedure for 16 using cyclohexylamine (77%) mp: 217-221°C. $^1$H NMR (500 MHz, Chloroform-$d$) $\delta$ 8.93 (s, 1H), 7.48 (s, 1H), 5.01 (d, $J$ = 7.6 Hz, 1H), 4.14 (t, $J$ = 8.6 Hz, 2H), 3.25 (t, $J$ = 8.7 Hz, 3H), 2.48 (m, 1H), 2.24 (s, 3H), 1.81 – 1.75 (m, 2H), 1.50 (d, $J$ = 12.7 Hz, 3H), 1.27 – 1.19 (m, 5H), 1.14 (t, $J$ = 12.3 Hz, 1H)

1-Acetyl-5-bromo-N-phenylindoline-6-sulfonamide (16i): The synthesis of 16i was achieved by following the general procedure for 16 using aniline to produce an off-white solid (82%). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 10.50 (s, 1H), 8.76 (s, 1H), 7.61 (s, 1H), 7.20 (t, $J$ = 7.9 Hz, 2H), 7.08 (d, $J$ = 8.0 Hz, 2H), 6.97 (t, $J$ = 7.7 Hz, 1H), 4.10 (t, $J$ = 8.0, 7.3 Hz, 2H), 3.19 – 3.06 (t, 2H), 2.14 (s, 3H).
1-Acetyl-5-bromo-6-(indolin-1-sulfonyl) indoline (16j): The synthesis of 16j was achieved by following the general procedure for 16 using indoline to give a dark brown solid (83.4% yield) MP: 211-218°C. $^1$H NMR (500 MHz CDCl$_3$) δ 8.86 (s, 1 H), 7.47 (s, 1 H), 7.31 (d, 1 H), 7.10 (d, 1 H), 7.07 (t, 1 H), 6.95 (t, 1 H), 4.26 (t, 2 H), 4.10 (t, 2 H), 3.19 (m, 4 H), 1.25 (s, 3 H). $^{13}$C NMR (126 MHz, DMSO-d$_6$) δ 169.1, 138.5, 131.6, 127.6, 125.5, 123.4, 118.9, 113.9, 113.6, 50.9, 49.3, 29.9, 28.1, 27.9, 24.4.

1-Acetyl-N-benzyl-5-bromoindoline-6-sulfonamide (16k): The synthesis of 16k was achieved by following the general procedure for 16 using benzylamine to produce a yellow solid (81%) mp: 202-208°C. $^1$H NMR (500 MHz, Chloroform-d) δ 8.96 (s, 1H), 7.50 (s, 1H), 7.31 – 7.22 (m, 5H), 5.34 (s, 2H), 4.21 – 4.10 (m, 4H), 3.28 (t, $J = 8.7$ Hz, 2H), 2.27 (s, 3H).

Methyl 3-(1-acetyl-5-bromoindoline)-6-sulfonamido)propanoate (16l): The synthesis of 16l was achieved by following the general procedure for 16 using β-alanine methyl ester to give a light brown solid (60%) mp: 180-184°C. $^1$H NMR (500 MHz, Chloroform-d) δ 8.91 (s, 1H), 7.50 (s, 1H), 5.78 (s, 1H), 4.14 (t, $J = 8.6$ Hz, 2H), 3.70 (s, 2H), 3.69 (s, 1H), 3.26 (t, $J = 8.7$ Hz, 2H), 3.24 – 3.16 (m, 2H), 2.53 (t, $J = 6.0$ Hz, 2H), 2.24 (s, 3H), 1.26 (s, 3H). $^{13}$C NMR (500 DMSO-d6) δ 171.6, 169.9, 143.3, 139.3, 138.4, 131.8, 117.7, 112.8, 52.17, 49.4, 46.6, 39.2, 34.7, 27.8, 24.7.
1-Acetyl-5-bromoindolin-6-(sulfonyl glycin methyl ester) (16m): The synthesis of 16m was achieved by following the general procedure for 16 using glycine methyl ester to produce a light yellow solid (72.5%) mp: 163-167°C. ¹H NMR (500 MHz, Chloroform-d) δ 8.87 (s, 1H), 7.50 (s, 1H), 5.69 (s, 2H), 4.13 (t, J = 8.6 Hz, 2H), 3.82 (d, J = 5.4 Hz, 2H), 3.68 (s, 3H), 3.15 – 3.06 (m, 2H), 2.24 (s, 3H).

[Chemical Structure Image]

Methyl ((1-acetyl-5-bromoindolin-6-yl)sulfonyl)valinate (16n): The synthesis of 16n was achieved by following the general procedure for 16 using valine methyl ester to produce an off white solid (55%) mp: 165-176°C. ¹H NMR (500 MHz, Chloroform-d) δ 8.85 (s, 1H), 7.48 (s, 1H), 5.68 (d, J = 9.4 Hz, 1H), 4.13 (t, J = 8.5 Hz, 2H), 3.85 (d, J = 8.0 Hz, 1H), 3.54 (d, J = 1.8 Hz, 3H), 3.24 (s, 2H), 3.11 (s, 3H), 2.23 (s, 3H), 2.12 – 2.04 (m, 1H), 0.97 – 0.88 (m, 6H). ¹³C NMR (126 MHz, DMSO-d₆) δ 171.6, 169.9, 143.3, 139.3, 138.4, 131.8, 117.7, 112.8, 52.2, 49.4, 46.6, 39.2, 34.7, 27.9, 24.7.

[Chemical Structure Image]

Methyl 6-((1-acetyl-5-bromoindolino)-6-sulfonamido)6-hexanoate (16o): The synthesis of 16o was achieved by following the general procedure for 16 using amino valeric acid methyl ester to produce an off white solid (65.1% yield) MP: 119-123°C. ¹H NMR (500 MHz Chloroform-d) δ 8.90 (s, 1H), 7.49 (s, 1 H), 5.10 (bs, 1 H), 4.17 (t, 2 H), 3.67 (s, 3 H), 3.27 (t, 2 H), 2.94 (t, 2 H), 2.27 (t, 2 H), 2.25 (s, 3 H), 1.56 (m 2 H), 1.48 (m, 2 H), 1.32 (m, 2 H). ¹³C NMR (126 MHz, Chloroform-d) δ 138.3, 130.8, 119., 113.1, 51.8, 49.2, 46.2, 43.5, 34.1, 30.0, 29.6, 27.9, 26.4, 24.6, 24.4.

[Chemical Structure Image]

1-Acetyl-5-bromo-N-(5-methylpyridin-2-yl)indoline-6-sulfonamide (16p): The synthesis of 16p was achieved by following the general procedure for 16 using 2amino-5-methyl pyridine, ¹H NMR (500 MHz, DMSO-d₆) δ 8.73 (s, 1H), 7.69 (s, 1H), 7.41 (s, 1H), 7.20 (s, 1H), 6.67 (d, J = 8.8 Hz, 1H), 4.09 (t, J = 8.7 Hz, 2H), 3.11 (t, J = 8.4 Hz, 2H), 2.14 (s, 3H), 2.04 (s, 3H).
1-Acetyl-5-bromo-N-(pyridin-2-yl)indoline-6-sulfonamide (16q): The synthesis of 16q was achieved by following the general procedure for 16 using 2-aminopyridine to produce a brown solid (60%). mp: 197-200°C.

1-Acetyl-5-bromo-N-(pyrimidin-2-yl)indoline-6-sulfonamide (16r): The synthesis of 16r was achieved by following the general procedure for 16 using 2-aminopyrimidine to yield a brown sticky solid (19%).

1-Acetyl-5-bromo-N-(pyrazin-2-yl)indoline-6-sulfonamide (16s): The synthesis of 16s was achieved by following the general procedure for 16 using 2-amino pyrazine (22%).

1-Acetyl-5-chloroindoline (21a): The 5-chloro indoline compound 21 was synthesized by adding NCS (1.74 g, 13.03 mmol) to a solution of 13 (2.00 g, 12.4 mmol) and NH₄OAc (96.1 mg, 10 mol%) in acetonitrile (65.1 mL) slowly and stirred at room temperature under air and monitored by TLC. NCS was purified through recrystallization in glacial acetic acid (10.0 grams in 50.0 mL), filtered and washed with hexane and then dried under vacuum. ¹H NMR (500 MHz Chloroform-d) δ 8.16 (d, 1 H), 7.18 (m, 2 H), 4.09 (t, 2 H), 3.20 (t, 2 H), 2.79 (s, 1 H), 2.24 (s, 3 H).
1-Acetyl-5-chloroindoline-6-sulfonyl chloride (21b): Chlorosulfonic acid (3.75 mL) was slowly added to chloroindoline 21 (500 mg), drop-wise by pressure equalizing funnel with stirring and heated between 60°-70°C for 3 hr. The black solution was cooled to room temperature and quenched by pouring slowly over ice with stirring. A light yellow/gold precipitate was formed filtered via vacuum filtration. The precipitate was further washed with water and dried in vacuo to produce the desired product 21b (49%). ¹H NMR (500 MHz CDCl₃) δ 8.98 (s, 1 H), 7.42 (s, 1 H), 4.20 (t, 2 H), 3.33 (t, 2 H), 2.28 (s, 3 H).

General procedure for the synthesis of N-acetyl-5-chloroindoline-6-sulfonamides (22).

The reaction of 21b (50 mg, 0.168 mmol) was added to a round bottom flask with the desired primary amine (0.211 mmol) and triethylamine (35.0 uL) in dichloromethane (2 mL) and allowed to stir until completion. The reaction was diluted with dichloromethane and the organic layer was washed one time with deionized water, twice with 1M HCl, once with 1M NaCl and dried over sodium sulfate, filtered and concentrated on the rotovap to produce analogs represented by 22. The secondary amines and nitrogen heterocycles followed the same synthesis with addition of 4-dimethylamino pyridine (DMAP) (10 mol %) catalyst. The 5-chloro indoline reaction times ran longer than their 5-bromo indoline counterparts.

Methyl 3-((1-acetyl-5-chloroindoline)-6-sulfonamido)propanoate (221): The synthesis of 221 was achieved by following the general procedure for 22 using β-alanine methyl ester to produce a waxy solid (48%). ¹H NMR (500 MHz Chloroform-d) δ 8.89 (s, 1 H), 7.32 (s, 1 H), 4.42 (t, 2 H), 3.70 (m, 3 H), 3.29 (t, 2 H), 2.54 (t, 2 H), 2.28 (d, 2 H), 1.45 (t, 2 H).
*1-Acetyl-5-chloro-N-(5-methylpyridin-2-yl)indoline-6-sulfonamide (22p)*: The synthesis of 22p was achieved by following the general procedure for 22 using 2-amino-5-methyl pyridine to produce a brown solid (51%).

![Chemical Structure](attachment:image.png)

*1-Acetyl-5-chloro-N-(pyrimidin-2-yl)indoline-6-sulfonamide (22r)*: The synthesis of 22r was achieved by following the general procedure for 22 using 2-amino-pyrimidine to yield a brown waxy solid (34%).

![Chemical Structure](attachment:image.png)

*2-((1-Phenyl-1H-tetrazol-5-yl)thio)propanoic acid (32a)* To a solution of phenyl-1H-tetrazole thiol 30 (1 eq) in anhydrous EtOH was added KOH (1.1 eq) and refluxed for one hour. The mixture was then allowed to cool to room temperature and 2-bromopropionic acid (1.1 eq) was added and the reaction was refluxed for 20 hours and solvent was removed to produce a yellow oil. The reaction was diluted with water and extracted with diethyl ether and organic portion washed with brine and dried over Na$_2$SO$_4$ and solvent was removed to give 32a as a light-yellow oil. 69%. $^1$H NMR (500 MHz, Chloroform-d) $\delta$ 7.97 – 7.90 (m, 2H), 7.63 – 7.53 (m, 2H), 7.57 – 7.50 (m, 1H), 4.44 (q, $J = 6.9$ Hz, 1H), 1.91 – 1.73 (d, 3H).

![Chemical Structure](attachment:image.png)

*2-((1-Phenyl-1H-tetrazol-5-yl)thio)-N-(thiazol-2-yl)propenamide (33a)*. To a solution of carboxylic acid 33a (1 eq) dissolved in DMF (0.4 M) was added CDI (1.16 eq) and stirred for 2.5 hours at 80°C. The reaction was cooled and the amine (1.08 eq) was added and stirred for 4 hrs at 80°C in a sealed vial and effervescence was observed upon addition. The reaction was determined complete via HPLC. The mixtures were poured into 3% aqueous NaHCO$_3$ where a ppt formed. Aqueous portions were extracted with EA, dried over Na$_2$SO$_4$ and concentrated to give 33a as a light-yellow oil.
**N-(2-Methoxyphenyl)-4-methylbenzenesulfonamide (41a):** To a solution of p-toluene sulfonyl chloride (2.62 mmol) in DCM (0.524M) was added trimethylamine (1.2 eq). The vials were fitted with stir bars and the anisidine analogs (1.1 eq) were added in one portion, vials were sealed and purged with N₂ and allowed to stir to completion. The reaction was quenched by addition of water and the organic portions were further diluted with DCM, washed with 1 M HCl and brine and dried over Na₂SO₄ and concentrated on rotovap. Reaction gave near quantitative yield for 41a α-anisidine analog 87% purity HPLC. Products were recrystallized in methanol and taken into next step. ¹H NMR matched those found in the literature.

![N-(2-Methoxyphenyl)-4-methylbenzenesulfonamide](image)

**N-(4-Methoxyphenyl)-4-methylbenzenesulfonamide (41b):** Sulfonamide 41b was synthesized following the general procedure for 41a using p-anisidine. Reaction gave a light purple crystalline solid with near quantitative yield for 30b with 92% purity HPLC. ¹H NMR matched those in literature.

![N-(4-Methoxyphenyl)-4-methylbenzenesulfonamide](image)

**N-(Difluoromethyl)-N-(2-methoxyphenyl)-4-methylbenzenesulfonamide (42a):** To a vial of sulfonamide 30 (1eq) and K₂CO₃ (3 eq) was added anhydrous acetonitrile (0.3M) and flushed with N₂ and the slurry stirred for 10 minutes at rt and was then heated to 70 °C for 1 hour. The reaction was cooled to 0°C and sodium chlorodifluoroacetate (3 eq) was added slowly to the mixture. After complete addition the reaction was warmed to rt and then heated to 70°C until complete. The solvent was removed and the reaction was diluted with DCM, washed with water, and dried over MgSO₄ and concentrated on rotovap. Product was isolated by trituration of residue. 93% purity.

![N-(Difluoromethyl)-N-(2-methoxyphenyl)-4-methylbenzenesulfonamide](image)

**N-(2-Methoxyphenyl)-N,4-dimethylbenzenesulfonamide (43a):** To a solution of sulfonamide 41a dissolved in anhydrous acetonitrile (0.3 M) was added K₂CO₃ (1.05 eq) and sealed under N₂. After five minutes of stirring methyl iodide was added and heated to 70°C and allowed to stir until complete as determined by TLC. The solvent was removed on rotovap and organic residue
was dissolved in DCM and washed with 1M HCl and brine and dried over MgSO₄ and concentrated. **43a** was produced as a light brown solid (86%). ¹H NMR (300 MHz, Chloroform-d) δ 7.60 (d, J = 8.2 Hz, 2H), 7.35 – 7.23 (m, 4H), 7.01 – 6.89 (t, 1H), 6.87 – 6.77 (d, 1H), 3.45 (s, 3H), 3.22 (s, J = 0.5 Hz, 3H), 2.44 (s, 3H).

**N-(4-Methoxyphenyl)-N,4-dimethylbenzenesulfonamide (43b):** The methylated derivative **41b** was synthesized following the same procedure for **43a** to produce a light brown solid (82%). ¹H NMR (300 MHz, Chloroform-d) δ 7.48 (d, J = 8.3 Hz, 2H), 7.29 (d, J = 2.1 Hz, 2H), 7.07 – 6.96 (m, 2H), 6.88 – 6.79 (m, 2H), 3.83 (s, 3H), 3.16 (s, 3H), 2.46 (s, 3H).

### DapE Assay Protocol

All reaction volumes are 200 μL with 10 nM HiDapE and 2 mM of N-methyl SDAP TFA unless otherwise stated. Enzyme assay absorbances were read on BioTek Syngen microplate reader.

**Ninhydrin-based enzymatic assay.**

To 50mM HEPES at pH 7.5 buffered solution with 8nM DapE at 30°C was added 2mM N-methyl SDAP (final volume 200 μL). The reaction proceeded for 10 minutes and quenched by heating at 100°C for 1 minute and subsequent cooling on ice for 1 minute. To the cooled reaction was added the 2% ninhydrin, the reaction vortexed and subsequently heated at 80 ºC for 15 minutes for the ninhydrin reaction. This reaction was quenched by placing cooling on ice minutes and the absorbance of 80µL was read at 570nm.

**IC₅₀ Determinations.**

All inhibition assays were conducted with a reaction volume of 200 μL, 2 mM N-methyl SDAP and 8 nM DapE unless otherwise stated. Glutamic acid standards of 0 to 0.5 mM were used in every trial. All inhibitor stock solutions were made in 100% DMSO and serial dilutions to desired concentration with 50% DMSO. To a 50 mM HEPES pH 7.5 buffered solution was added desired inhibitor concentration at 0 ºC followed by DapE and allowed to incubate for 5 min. The reactions were warmed to 30°C and N-methyl SDAP TFA was added and allowed to react for an additional 10 min. The enzymatic reaction was quenched by heating at 100 ºC for 1 minute and cooled on ice to 30 ºC. The 2% ninhydrin solution (100 µL) was added, the reaction vortexed and heated at 80 ºC for 15 min. after which the reaction was quenched by cooling on ice to 30 ºC and
the absorbance of 80 μL was examined at 570 nm via microplate reader. IC₅₀ values were fitted to a curve through Excel.
APPENDIX E

NMR SPECTRA FOR CHAPTER FOUR
$^1$H NMR for 1-Acetyl-5-bromo-N-isopentylindoline-6-sulfonamide (16a)
$^{13}$C NMR for 1-Acetyl-5-bromo-N-isopentylindoline-6-sulfonamide (16a)
\( ^1 \text{H NMR for 1-Acetyl-5-bromo-6-(piperidin-1-sulfonyl) indoline (16b)} \)
$^1$H NMR for 1-Acetyl-5-bromo-N,N-dipropylindoline-6-sulfonamide (16c)
\(^1\)H NMR for 1-Acetyl-5-bromo-6-(pyrrolidin-1-sulfonyl) indoline (16g):
$^1$H NMR for 1-Acetyl-5-bromo-N-cyclohexylindoline-6-sulfonamide (16h)
$^1$H NMR for 1-Acetyl-5-bromo-N-phenylindoline-6-sulfonamide (16i)
$^1$H NMR for 1-Acetyl-5-bromo-6-(indolin-1-sulfonyl) indoline (16j)
$^{13}$C NMR for 1-Acetyl-5-bromo-6-(indolin-1-sulfonyl) indoline (16j)
$^1$H NMR for 1-Acetyl-N-benzyl-5-bromoindoline-6-sulfonamide (16k)
$^1$H NMR for methyl 3-((1-acetyl-5-bromoindoline)-6-sulfonamido)propanoate (161)
$^{13}$C NMR for methyl 3-((1-acetyl-5-bromoindoline)-6-sulfonamido)propanoate (16l)
$^1$H NMR for 1-Acetyl-5-bromoindolin-6-(sulfonyl glycine methyl ester) (16m)
$^1$H NMR for methyl ((1-acetyl-5-bromoindolin-6-yl)sulfonyl)valinate (16n)
$^{13}$C NMR for methyl ((1-acetyl-5-bromoindolin-6-yl)sulfonyl)valinate (16n)
13C NMR spectra of methyl 6-((1-acetyl-5-bromoindoline)-6-sulfonamido)6-hexanoate (160)
$^1$H NMR spectra of 1-Acetyl-5-bromo-N-(5-methylpyridin-2-yl)indoline-6-sulfonamide (16p)

![NMR spectrum](image)

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<th>B (δ)</th>
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<th>E (δ)</th>
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\(^1\)H NMR for 1-Acetyl-5-chloroindoline-6-sulfonyl chloride (21b)
1-Acetyl-5-chloro-N-(5-methylpyridin-2-yl)indoline-6-sulfonamide 22p
$^1$H NMR for 2-((1-phenyl-1H-tetrazol-5-yl)thio)propanoic acid (32a)
\[^1\text{H}\text{ NMR for 2-}((1\text{-phenyl-1H-tetrazol-5-yl})\text{thio})-\text{N-(thiazol-2-yl)propenamide (33a)}}\]
$^1$H NMR for $N$-(2-methoxyphenyl)-$N$,4-dimethylbenzenesulfonamide (43a)

![NMR spectrum image]

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$^1$H NMR for $N$-(4-methoxyphenyl)-$N$,4-dimethylbenzenesulfonamide (43b)
APPENDIX F

EXPERIMENTAL DATA FOR CHAPTER FIVE
1-Acetyl-5-bromo-N-isopentylindoline-7-sulfonamide (49a): To a solution of 5-bromo 7-sulfonamide indolines 54a dissolved in dichloromethane was added triethylamine and the reaction was stirred at room temperature for 15 minutes. The mixture was cooled to 0°C via ice bath where acetyl chloride was added slowly drop wise making sure the temperature remained between 0-10°C. After complete addition the reaction was warmed to room temperature and stirred until complete as determined by TLC. The reaction was diluted with dichloromethane and washed one time with dilute HCl, one time with water and then once with brine and the organic layer was dried over Na2SO4 and concentrated in vacuum producing 49a. 1H NMR (500 MHz, Chloroform-d) δ 7.68 (dd, J = 1.7, 0.9 Hz, 1H), 7.59 – 7.50 (m, 1H), 7.29 (d, J = 2.1 Hz, 1H), 4.26 (td, J = 8.4, 4.6 Hz, 1H), 3.88 (s, 1H), 3.32 (t, J = 8.5 Hz, 1H), 2.17 (s, 7H), 1.67 – 1.61 (m, 2H), 1.23 (s, 2H), 0.94 (p, J = 2.4 Hz, 3H).

1-Acetyl-5-bromo-N-propylindoline-7-sulfonamide (49b): Indoline 49b was synthesized using the general procedure for 49a to produce a light brown solid. 1H NMR (500 MHz DMSO-D6) δ 7.67 (s, 1H), 7.52 (s, 1H), 4.26 (t, 2H, J= 4.8), 3.81 (t, 2H, J=4.8), 3.31 (t, 2H, J=4.8), 1.75 (m, 2H, J=4.5) 1.21 (s, 3H), 0.92 (t, 3H).

1-Acetyl-5-bromo-N-benzylindoline-7-sulfonamide (49c): Indoline 49c was synthesized using the general procedure for 49a to produce a white solid. 1H NMR (300 MHz, DMSO D6) 7.70 (s, 1H), 7.58 (d, 2H), 7.51 (s, 1H), 7.46-7.40(dd, 1H), 7.15 (t, 2H), 5.01 (s, 2H), 4.23 (t, 2H), 3.29 (t, 2H), 1.21 (s, 2H).
The alkylated cyclic sulfonyl urea indolines 57a was dissolved in a minimum amount of glacial acetic acid and stirred vigorously. This solution was cooled in an ice bath and Br₂ was added slowly drop wise with stirring. The reaction was warmed to room temperature and left to stir for 75 minutes and then heated to 50 °C for 1 hour. Reaction was quenched by pouring into ice water and isolating the white solid via vacuum filtration and dried via high vacuum to give produce a light brown solid. (75 % yield.) ¹H NMR (300 MHz, DMSO D₆)

8-Bromo-2-propyl-5,6-dihydro-[1,2,4]thiadiazino[6,5,4-hi]indol-3(2H)-one 1,1-dioxide (53b):
Indoline 53b was synthesized using the general procedure for 53a. (71%) ¹H NMR (500 MHz DMSO D₆) δ 7.91 (s, 1 H), 7.83 (s, 1 H), 4.20 (t, 2 H), 3.75 (t, 2 H), 3.31 (t, 2 H), 1.67 (m, 2 H), 0.89 (t, 3 H).

2-Benzyl-8-bromo-5,6-dihydro-[1,2,4]thiadiazino[6,5,4-hi]indol-3(2H)-one 1,1-dioxide (53c):
Indoline 53c was synthesized using the general procedure for 53a to produce a light brown solid. (76.3 % yield.) ¹H NMR (500 MHz, DMSO-D₆) δ 7.96 (s, 1 H), 7.84 (s, 1 H), 7.38-7.25 (m, 5 H), 4.99 (s, 2 H), 4.21 (t, 2 H), 3.34 (t, 2 H).
5-Bromo-N-isopentylindoline-7-sulfonamide (54a): A solution of the 5-bromo cyclic sulfonylurea 54a in 50% sulfuric acid was heated to 135°C for two hours. The reaction was cooled to room temperature and then neutralized carefully with a 10 N NaOH solution while over ice. The aqueous layer was extracted with ethyl acetate three times and the organic fractions were combined and dried over magnesium sulfate and concentrated in vacuum yielding a light-yellow solid (50%).

5-Bromo-N-propylindoline-7-sulfonamide (54b): Indoline 54b was synthesized using the general procedure for 54a to produce a light brown solid. (50% yield.) ¹H NMR (500 MHz, DMSO-D₆) δ 7.90 (s, 1 H), 7.82 (s, 1 H), 4.20 (t, 2 H, J=8), 3.75 (t, 2 H, J=8), 3.31 (t, 2 H), 1.67 (m, 2 H), 0.89 (t, 3 H). ¹³C NMR (126 MHz, DMSO-d₆) δ 148.7, 137.6, 136.3, 133.8, 133.2, 130.8, 124.7, 121.54, 120.0, 119.4, 115.5, 49.0, 48.6, 43.6, 43.3, 32.1, 31.9, 30.6, 30.1, 29.8, 29.5, 27.6, 23.20, 23.2, 22.9, 14.8, 11.8, 11.7.

5-Bromo-N-benzylindoline-7-sulfonamide (54c): Indoline 54c was synthesized using the general procedure for 54a to produce a light yellow solid. (50%) ¹H NMR (500 MHz, DMSO-d₆) δ 7.95 (d, J = 1.6 Hz, 1H), 7.84 (t, J = 1.5 Hz, 1H), 7.70 (dt, J = 8.0, 1.1 Hz, 1H), 7.40 – 7.23 (m, 5H), 4.99 (s, 2H), 4.25 – 4.17 (t, 2H), 3.32 (d, J = 7.8 Hz, 3H).
5,6-Dihydro-[1,2,4]thiadiazino[6,5,4-hi]indol-3(2H)-one 1,1-dioxide (56): To a solution of chlorosulfonyl isocyanate (1.80 mL, 18.4 mmol) in nitroethane (32 mL) cooled to -42°C was added indoline 12 slowly drop wise with stirring. The intermediate precipitated and turned cloudy and white and the mixture was slowly warmed to room temperature over one hour with continued stirring. Aluminum chloride (2.45 g, 18.4 mmol) was added in one portion and allowed to stir for 15 minutes at room temperature. The solution went from cloudy and white to a clear, pale yellow solution to a light purple to a dark purple over time. The reaction was then heated to 110°C for one hour. The mixture was cooled to room temperature and quenched by pouring into ice water slowly where a black precipitate formed. The solid was isolated by filtering via vacuum filtration. A dark gray clay-like substance was obtained and dried overnight via vacuum yielding the cyclic sulfonyl urea indoline 56 (58-83%) as a dark gray solid compound. The solid was not purified and taken into the next reaction. ¹H NMR (300 MHz, DMSO D₆) δ 7.55 (2H, t), 7.22 (1H, t), 4.18 (2H, t), 3.35 (2H, t). ²³¹H NMR (500 MHz, DMSO-d₆) δ 7.57 (ddq, J = 13.3, 7.4, 1.1 Hz, 2H), 7.21 (t, J = 7.7 Hz, 1H), 4.12 (dd, J = 9.1, 7.9 Hz, 2H), 3.34 – 3.26 (m, 2H). ¹³C NMR (126 MHz, DMSO-d₆) δ 148.9, 138.8, 133.0, 130.3, 124.5, 120.6, 119.3, 47.6, 27.8.

2-Isopentyl-5,6-dihydro-[1,2,4]thiadiazino[6,5,4-hi]indol-3(2H)-one 1,1-dioxide (57a): To a solution of cyclic sulfonyl urea indoline 56 (150 mg, 0.67 mmol) in DMF (2 mL) was added NaN₃ in 60% mineral oil (30 mg) and allowed to stir under nitrogen. N-propyliodide (0.78 mmol) was added drop wise and left stirring until completion as determined by TLC. The reaction was diluted with ethyl acetate and washed with 0.1 M HCl followed by a wash with saturated NaHCO₃ and then brine. The organic portion was concentrated in vacuum to yield the solid alkylation product (43%). ¹H NMR (500 MHz, DMSO-D₆) δ 7.67 (d, 1 H), 7.63 (d, 1 H), 7.26 (t, 1 H), 4.21 (t, 2 H), 3.76 (t, 2 H), 3.29 (t, 2 H), 1.67 (m, 2 H), 0.97 (t, 3 H).
2-Propyl-5,6-dihydro-[1,2,4]thiadiazino[6,5,4-hi]indol-3(2H)-one 1,1-dioxide (57b): Indoline 57b was synthesized using the general procedure for 57a using isoamyl bromide to produce brown solid. (45 % yield.) $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.60 (d, 1 H), 7.44 (d, 1 H), 7.18 (t, 1 H), 4.29 (t, 2 H), 3.94 (t, 2 H), 3.34 (t, 2 H), 1.69 (m, 2 H), 0.99 (d, 6 H).

2-Benzyl-5,6-dihydro-[1,2,4]thiadiazino[6,5,4-hi]indol-3(2H)-one 1,1-dioxide (57c): Indoline 57c was synthesized using the general procedure for 57a using benzyl bromide to produce a solid. (69 %) $^1$H NMR (500 MHz, DMSO-D$_6$) $\delta$ 7.71 (d, 1 H), 7.65 (d, 1 H), 7.38 (d, 2 H), 7.34 (t, 1 H) 7.31-7.25 (m, 3 H), 4.99 (s, 2 H), 4.22 (t, 2 H), 3.33 (t, 2 H).

Indoline-7-sulfonylamide (58): Indoline 58 was synthesized by hydrolysis of cyclic indoline 56 using the general procedure for 54. $^1$H NMR (500 MHz, DMSO-d$_6$) $\delta$ 7.25 (d, $J = 8.1$ Hz, 1H), 7.16 (d, $J = 7.1$ Hz, 1H), 7.11 (s, 2H), 6.55 (t, $J = 8.0$ Hz, 1H), 5.79 (s, 1H), 3.57 (t, $J = 8.7$ Hz, 2H), 2.97 (t, $J = 8.7$ Hz, 2H). $^{13}$C NMR (126 MHz, DMSO-d$_6$) $\delta$ 149.1, 149.1, 132.2, 132.1, 128.1, 128.1, 128.0, 125.6, 125.5, 125.5, 122.0, 116.3, 116.3, 47.3, 47.3, 47.3, 29.0, 28.9, 28.9.

1-Acetylindoline-7-sulfonyamide (59): $^1$H NMR (500 MHz, DMSO-d$_6$) $\delta$ 7.66 (d, $J = 8.0$ Hz, 1H), 7.50 (d, $J = 7.3$ Hz, 1H), 7.26 (t, $J = 7.7$ Hz, 1H), 7.05 (s, 2H), 4.12 (t, $J = 7.6$ Hz, 2H), 3.08 (t, $J = 7.6$ Hz, 2H), 2.24 (s, 3H). $^{13}$C NMR (126 MHz, DMSO-d$_6$) $\delta$ 171.4, 138.9, 137.9, 133.8, 129.1, 127.6, 51.8, 29.85, 24.39.
N-(Indolin-7-ylsulfonyl)acetamide (63): $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 7.66 (d, $J = 8.0$ Hz, 1H), 7.50 (d, $J = 7.3$ Hz, 1H), 7.26 (t, $J = 7.7$ Hz, 1H), 7.05 (s, 2H), 4.12 (t, $J = 7.6$ Hz, 2H), 3.08 (t, $J = 7.6$ Hz, 2H), 2.24 (s, 3H).

1-((Benzzyloxy)carbonyl)indoline-7-sulfonamide (64): To a round-bottom flask was added 7-sulfonamide indoline 58 (1 eq) in THF (0.17M) followed by NaHCO$_3$ (11 eq) and water (1.76M). The round bottom flask was sealed and flushed with N$_2$ gas. To this sealed mixture was added benzyl chloroformate (3.8 eq) slowly over a few minutes by syringe. After complete addition the reaction was allowed to stir at room temperature overnight. The reaction was diluted with EA and filtered to remove undissolved excess NaHCO$_3$. The organic portion was acidified with 6M HCl. The aqueous portions were further extracted with EA two times. The organic fractions were combined and washed with brine and dried over NaSO$_4$ and concentrated giving a greasy off white solid. 4 mL of Et$_2$O was added along with 2 mL of pet ether and the white solid was collected via vacuum filtration and further washed with pet ether and placed on high vac to fully dry. This reaction yields 62% white, light fluffy and pure white solid. $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.87 (d, 1 H), 7.21 (t, 1 H), 7.44–7.29 (m, 6 H), 5.90 (bs, 2 H), 5.28 (s, 2 H), 4.24 (t, 2 H), 3.02 (t, 2 H). $^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta$ 155.5, 139.3, 137.3, 136.7, 133.1, 129.1, 129.0, 128.7, 128.5, 127.8, 125.2, 68.0, 51.6, 29.4.

Benzyl 5-bromo-7-sulfamoylindoline-1-carboxylate (64b): To a round bottom flask of 64 dissolved in glacial AcOH was added Br$_2$ dropwise with stirring. The reaction was allowed to stir until complete as determined by TLC and quenched by pouring in water. To the aqueous portion was added sodium thiosulfate and the precipitate was filtered by vacuum filtration. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 7.80 (d, $J = 2.0$ Hz, 1H), 7.69 (dd, $J = 2.1$, 1.1 Hz, 1H), 7.45–7.38 (m, 2H), 7.41–7.30 (m, 3H), 7.34–7.24 (m, 1H), 7.21 (ddt, $J = 6.0$, 4.6, 3.2 Hz, 0H), 7.16 (s, 2H), 5.20 (s, 2H), 4.48 (s, 0H), 4.10 (t, $J = 7.9$ Hz, 2H), 3.12–3.05 (m, 2H).
1-((Benzyl oxy)carbonyl) indoline-7-sulfonic acid (67): N-Cbz-7-sulfonamide indoline 64 (0.3 mmol) was added to a sealed test tube flushed with Argon. The sulfonamide was dissolved by the addition of anhydrous 1,4 dioxane (0.375 M). The reaction was heated to 65°C until most solid dissolved with stirring. Isoamyl nitrite was added to the test tube via syringe in two portions. After the first addition evolution of N₂ was observed. After addition of the second portion the reaction was heated to 85°C to which the reaction turned a yellow-orange color. The reaction was heated at reflux for 2 hours and solvent was removed by rotary evaporation. The remaining sticky residue was chased with DCM, a brown liquid remained and reaction was triturated by addition of Et₂O. The product filtered was removed and an off white solid remained giving 47% yield with 98+% purity by HPLC. ¹H NMR (300 MHz, CDCl₃) δ 7.96 (d, 1 H), 7.25 (t, 1 H), 7.45-7.43 (m, 5 H), 7.50 (d, 1 H), 5.32 (s, 2 H), 4.29 (t, 2 H), 3.12 (t, 2 H).

Benzyl 7-(N-acetylsulfamoyl) indoline-1-carboxylate (76a): A solution of acid chloride or anhydride (1.25 eq) and DMAP (10 mol %) in DCM was cooled to 0°C, a separate solution of N-Cbz-7-sulfonamide indoline 58 and Et₃N in DCM was placed on ice. The solution of acid chloride was slowly added to the solution of sulfonamide dropwise over ten minutes while maintaining a temperature close to 0°C. After complete addition, the reaction was warmed to rt with continued stirring. After completion of the reaction as determined by TLC the reaction was quenched by addition of water and the aqueous portion was extracted with DCM. The organic layers were combined and washed with 1 M HCl and brine and dried of Na₂SO₄ and solvent was removed via rotovap. The solid was purified via trituration by dissolving in EA, cooling on ice and crashing out with Et₂O. ¹H NMR (300 MHz, DMSO D₆) δ 11.65 (s, 1 H), 7.70 (d, 1 H), 7.53 (d, 1 H), 7.24 (t, 2 H), 7.32 (d, 2 H), 7.37 (m, 5 H), 5.40 (s, 2 H), 4.13 (t, 2 H), 3.08 (t, 2 H), 1.93 (s, 3 H). ¹³C NMR (126 MHz, Chloroform-d) δ 168.5, 155.5, 140.1, 136.7, 135.8, 130.4, 129.9, 129.1, 128.8, 128.6, 128.2, 124.8, 68.8, 68.8, 51.4, 31.0, 29.4, 24.5.
**Benzyl 7-(N-benzoysulfamoyl)indoline-1-carboxylate (76b):** Indoline 76b was synthesized using the general procedure for 76a using benzoic anhydride to produce an off white solid. $^1$H NMR (300 MHz, DMSO-D$_6$)

![Chemical structure of 76b](image)

**Benzyl 7-(N-(4-methoxybenzoyl)sulfamoyl)indoline-1-carboxylate (76c):** Indoline 76c was synthesized using the general procedure for 76a using anisoyl chloride to produce an off white solid. $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 8.19 (d, 1 H), 7.96 (d, 2 H), 7.42-7.30 (m, 6 H), 7.2 (t, 1 H), 6.93 (d, 2 H), 5.32 (s, 2 H), 4.20 (t, 2 H), 3.87 (s, 3 H), 3.05 (t, 2 H).

![Chemical structure of 76c](image)

**Benzyl 7-(N-(thiazole-5-carbonyl)sulfamoyl)indoline-1-carboxylate (76d):** Indoline 76d was synthesized using the general procedure for 76a using thiazole-5-carbamoyl chloride to produce an off white solid. (49%). $^1$H NMR (500 MHz, DMSO-D$_6$)

![Chemical structure of 76d](image)

**Benzyl 7-(N-(isoxazole-5-carbonyl)sulfamoyl)indoline-1-carboxylate (76e):** Indoline 76e was synthesized using the general procedure for 76a using isoxazole-5-carbamoyl chloride to produce an off white solid. (70%)

![Chemical structure of 76e](image)

**Benzyl 7-(N-(3-methylbutanoyl)sulfamoyl)indoline-1-carboxylate (76f):** Indoline 76f was synthesized using the general procedure for 76a using isovaleric acid to produce an off white solid.
Benzyl 7-(N-(morpholine-4-carbonyl)sulfamoyl)indoline-1-carboxylate (78a): To a solution of N-Cbz-7-sulfonamide Indoline 64 (0.3 mmol) in anhydrous THF (0.2 M) was added carbonyl chloride (0.33 mmol) and reaction was allowed to stir at rt. NaH 60% dispersion in mineral oil (0.375 mmol) was added and effervescence was observed. The vails were then purged with N2 and allowed to stir at rt for 4 days. HPLC showed starting material still present to which 0.5 more equivalence of NaH was added. The reaction was quenched by addition of water, the aqueous layer was extracted with EA three times and the organic layers were washed with brine, dried over MgSO4 and concentrated. (94%) 

Benzyl 7-(N-(pyrrolidine-1-carbonyl)sulfamoyl)indoline-1-carboxylate (78b): Synthesized with the general procedure of 78a using pyrrolidine carbonyl chloride. (91%).

Benzyl 7-(N-(4-methylpiperazine-1-carbonyl)sulfamoyl)indoline-1-carboxylate (78c): Synthesized with the general procedure of 78a using 4-methyl-1-piperidine carbonyl chloride ca. 45% HPLC.

N-(1-(Morpholine-4-carbonyl)indolin-7-yl)sulfonylacetamide (79d): Synthesized with the general procedure of 78a using N-morpholine-carbomoyl chloride.
N-((1-(Isoxazole-5-carbonyl)indolin-7-yl)sulfonyl)acetamide (79e): Synthesized with the general procedure of 78a using isoxazole-5-carbonyl chloride.

\[
\text{\includegraphics[width=0.5\textwidth]{image.png}}
\]

7-(N-Acetylsulfamoyl)-N,N-dimethylindoline-1-carboxamide (79f): Synthesized with the general procedure of 78a using N,N-dimethyl-carbonyl chloride.

\[
\text{\includegraphics[width=0.5\textwidth]{image.png}}
\]

1-(Tert-butoxycarbonyl)indoline-7-sulfonic acid (83): To a three-neck round bottom flask fitted with a stir bar was added N-Boc indoline followed by degassing with N₂. The indoline was dissolved in anhydrous THF and cooled to -78 °C. Next, the addition of TMEDA followed by addition n-BuLi slowly addition at -78 °C with continued stirring for one hour and slow warming to rt. Reaction was allowed to stir over night. To a second solution of sulfur trioxide trimethyl amine complex (STTAC) in THF under nitrogen at -78°C was added the first mixture slowly over time to which a yellow color appeared. This was allowed to stir for 2 hours at -78°C and warmed to room temperature with continued stirring overnight. The thick white solution was concentrated to remove solvent and 4 equivalents of HCl was added and the organic portion was extracted with EA an concentrated in vacuo. 45% yield by HPLC.

\[
\text{\includegraphics[width=0.5\textwidth]{image.png}}
\]

Indoline-1-sulfonic acid (87): To a three-neck round bottom flask with a stir bar was added indoline dissolved in hexane. The flask was flushed with nitrogen and cooled to -78°C. Next, the addition of n-BuLi followed by warming to room temperature with continued stirring overnight. The reaction went from clear to gelatinous. The reaction was cooled back down to -78 °C, diluted with Et₂O and CO₂ was introduced through a drying tube by needle places directly under the surface of the solvent. Excess CO₂ was allowed to escape and reaction was stirred at room temperature overnight. The reaction was then diluted with THF and cooled to -78 °C and TMEDA followed by n-BuLi was added and the reaction was allowed to stir. A second round-bottom flask with THF and STTAC at -78°C was purged with Nitrogen and the addition of the first mixture was added via syringe and allowed to stir for 18 hrs. The reaction was then
concentrated, washed with water and brine, dried over MgSO4 and concentrated to produce a reddish-pink colored solid.

\[
\text{N-Isopentylindoline-7-sulfonamide (93a): } ^1\text{H NMR (500 MHz, Chloroform-d) } \delta 7.60 \text{ (dt, } J = 8.0, 1.0 \text{ Hz, 1H)}, 7.44 \text{ (dq, } J = 7.4, 1.2 \text{ Hz, 1H)}, 7.17 \text{ (t, } J = 7.7 \text{ Hz, 1H)}, 4.29 \text{ (dd, } J = 9.1, 8.1 \text{ Hz, 2H)}, 3.98 - 3.88 \text{ (m, 2H)}, 3.34 \text{ (ddt, } J = 9.4, 8.1, 1.1 \text{ Hz, 2H)}, 1.75 - 1.64 \text{ (m, 3H)}, 1.26 \text{ (d, } J = 1.7 \text{ Hz, 1H)}, 1.01 - 0.93 \text{ (m, 6H),}
\]

\[
\text{N-Propylindoline-7-sulfonamide (93b): } ^1\text{H NMR (300 MHz, Acetone-d6) } \delta 7.60 \text{ (d, } J = 7.6 \text{ Hz, 3H)}, 7.27 \text{ (s, 1H)}, 4.29 \text{ (s, 2H)}, 3.82 \text{ (s, 2H)}, 3.41 \text{ (t, } J = 8.2 \text{ Hz, 3H)}, 2.05 \text{ (s, 2H)}, 1.75 \text{ (s, 3H)}, 1.74 \text{ (d, } J = 7.5 \text{ Hz, 0H)}, 1.30 \text{ (s, 2H)}, 0.94 \text{ (t, } J = 7.5 \text{ Hz, 1H).}
\]
N-Benzylindoline-7-sulfonamide $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 7.70 (d, $J = 8.0$ Hz, 1H), 7.64 (d, $J = 7.4$ Hz, 1H), 7.40 – 7.23 (m, 6H), 4.99 (s, 2H), 4.24 – 4.17 (m, 2H), 3.41 – 3.28 (m, 1H).
APPENDIX G

NMR SPECTRA FOR CHAPTER FIVE
1-Acetyl-5-bromo-N-isopentylindoline-7-sulfonamide (49a)
1-Acetyl-5-bromo-N-propylindoline-7-sulfonamide (49b)
1-Acetyl-5-bromo-N-benzylindoline-7-sulfonamide (49c)
8-Bromo-2-propyl-5,6-dihydro-[1,2,4]thiadiazino[6,5,4-h]indol-3(2H)-one 1,1-dioxide (53b)
5-Bromo-N-propyldolone-7-sulfonamide (54b)
5-Bromo-N-propyldoline-7-sulfonamide (54b)
5-Bromo-N-benzylindoline-7-sulfonamide (54c)
5,6-Dihydro-[1,2,4]thiadiazino[6,5,4-h]indol-3(2H)-one 1,1-dioxide (56)
5,6-Dihydro-[1,2,4]thiadiazino[6,5,4-h]indol-3(2H)-one 1,1-dioxide (56)
2-Isopentyl-5,6-dihydro-[1,2,4]thiadiazino[6,5,4-h]indol-3(2H)-one 1,1-dioxide (57a)
2-Propyl-5,6-dihydro-[1,2,4]thiadiazino[6,5,4-h]indol-3(2H)-one 1,1-dioxide (57b)
2-Benzyl-5,6-dihydro-[1,2,4]thiadiazino[6,5,4-h]indol-3(2H)-one 1,1-dioxide (57c)
Indoline-7-sulfonamide (58)
Indoline-7-sulfonamide (58)
1-Acetylpindoline-7-sulfonamide (59)
1-Acetylindoline-7-sulfonamide (59)
$N$-([Indolin-7-ylsulfonyl]acetamide (63):
1-((Benzyloxy)carbonyl)indoline-7-sulfonamide (64)
Benzyl 5-bromo-7-sulfamoylindoline-1-carboxylate (64b):
1-((Benzzyloxy)carbonyl)indoline-7-sulfonic acid (67)
Benzyl 7-\((N\text{-acetyl)sulfamoyl})\)indoline-1-carboxylate (76a)
Benzyl 7-(N-acetyl)sulfamoyl)indoline-1-carboxylate (76a)
**Benzyl 7-(N-benzoysulfamoyl)indoline-1-carboxylate (76b)**

![Chemical Structure of Benzyl 7-(N-benzoysulfamoyl)indoline-1-carboxylate (76b)](image)

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<th>Chemical Shift (ppm)</th>
<th>Integration</th>
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<tbody>
<tr>
<td>B (m)</td>
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<td>4</td>
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<tr>
<td>A (m)</td>
<td>7.39</td>
<td>3</td>
</tr>
<tr>
<td>E (s)</td>
<td>5.29</td>
<td>2</td>
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<tr>
<td>F (s)</td>
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<td>2</td>
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<tr>
<td>G (m)</td>
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<td>1</td>
</tr>
<tr>
<td>H (s)</td>
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<td>1</td>
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<tr>
<td>J (s)</td>
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<td>1</td>
</tr>
<tr>
<td>K (s)</td>
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<td>1</td>
</tr>
<tr>
<td>L (s)</td>
<td>0.08</td>
<td>1</td>
</tr>
</tbody>
</table>

**NMR Spectrum**

![NMR Spectrum](image)
Benzyl 7-(N-(4-methoxybenzoyl)sulfamoyl)indoline-1-carboxylate (76c)
Benzyl 7-(N-(thiazole-5-carbonyl)sulfamoyl)indoline-1-carboxylate (76d)
Benzyl 7-(N-(isoxazole-5-carbonyl)sulfamoyl)indoline-1-carboxylate (76e):
Benzyl 7-(N-(3-methylbutanoyl)sulfamoyl)indoline-1-carboxylate (76f)
Benzyl 7-(N-(3-methylbutanoyl)sulfamoyl)indoline-1-carboxylate (76f)
Benzyl 7-(N-(morpholine-4-carbonyl)sulfamoyl)indoline-1-carboxylate (78a)
Benzyl 7-(N-(pyrrolidine-1-carbonyl)sulfamoyl)indoline-1-carboxylate (78b)
N-((1-(Morpholine-4-carbonyl)indolin-7-yl)sulfonyl)acetamide (79d)
$N$-({1-(Morpholine-4-carbonyl)indolin-7-yl}sulfonyl)acetamide (79d)
$N-((1\text{-}(\text{Isoxazole-5-carbonyl})\text{indolin-7-yl})\text{sulfonyl})\text{acetamide (79e)}$
$N-$((1-(Isoxazole-5-carbonyl)indolin-7-yl)sulfonyl)acetamide (79e)
7-\((\text{N-acetylsulfamoyl})\)-\(\text{N,N-dimethylindoline-1-carboxamide}\) (79f)
7-(N-acetylsulfamoyl)-N,N-dimethylindoline-1-carboxamide (79f)
N-Isopentylindoline-7-sulfonamide
N-Propylindoline-7-sulfonamide
$N$-Benzylindoline-7-sulfonamide
BIBLIOGRAPHY


VITA

Dr. Tahirah K. I. Heath matriculated at Clark Atlanta University in Atlanta, Georgia in 2006. Dr. Heath began research under advisement of Dr. Conrad Ingram working with zeolite nanoparticles. In 2008, while at Clark Atlanta University, she received a NOAA funded research scholarship and later continued research in NSFM (nano-scale functional materials) in the cancer research center at CAU. Dr. Heath graduated with her Bachelor of Science degree in Chemistry in December 2010. In August 2012, Dr. Heath began her Ph.D. studies at Loyola University Chicago in Chicago, Illinois and joined Dr. Daniel P. Becker’s laboratory in January of 2013. Dr. Heath began interdisciplinary research with synthetic organic chemistry and enzymology in the area of medicinal chemistry where she presented at many local and national conferences, and became a co-inventor on two patents. In October 2017, Dr. Heath was an invited speaker at the regional Midwest Enzyme Chemistry Conference (MECC) at Loyola University Chicago. Dr. Heath became a co-author of her first publication in November 2017 in the ACS journal *Biochemistry*, and the first-author of her second published academic paper, which was accepted to PLOS ONE, as well as a leading, or co-author on several additional manuscripts in preparation.