SYNTHESES AND FRET BINDING DETERMINATION OF VARIOUS
SUBSTITUTED 2-AMINOBENZIMIDAZOLE INHIBITORS TARGETING
THE HEPATITIS C VIRUS IRES SUBDOMAIN IIA

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in
Chemistry

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The Undersigned Faculty Committee Approves the

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Syntheses and FRET Binding Determination of Various Substituted 2-
Aminobenzimidazole Inhibitors Targeting the Hepatitis C Virus IRES

Subdomain IIa

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DEDICATION

To the three people who made this master's degree a reality. To my parents, whose hard work has resonated through me every day and kept me pushing for my own accomplishments. I would not be anywhere near the person I am today without their never-ending love, support, strength and persistence to better myself in every aspect of life. To my fiancé for his endless encouragement on continued research, weekend lab work, and the fight for desired results. This Master’s degree could not have been achieved without these three loving and caring individuals.
Do not go where the path may lead;  
go instead where there is no path and leave a trail.  

- Ralph Waldo Emerson
ABSTRACT OF THE THESIS

Syntheses and FRET Binding Determination of Various Substituted 2-Aminobenzimidazole Inhibitors Targeting the Hepatitis C Virus IRES Subdomain IIa

by

Urszula Aneta Milewicz
Master of Science in Chemistry
San Diego State University, 2015

The life-threatening virus, Hepatitis C (HCV), creates problems in the health of millions of people worldwide and is a leading cause of liver cancer. Previous treatments to clear the virus have included a dual or regimen of interferon ribavirin as well as the availability of new antiviral drugs. Unfortunately, current treatments are associated with serious side effects and only effective in select genotypes. A new therapeutic treatment was recently approved by the FDA and shows promise in being a curable solution. However, with the reality that viruses continually mutate, creation of a library of compounds shown to inhibit the virus is vital. The Bergdahl group has recently published a novel total synthesis of a class of benzimidazole compounds, which has shown to prevent replication of the virus in a unique way by binding to the conserved internal ribosome entry site (IRES) of the HCV RNA preventing initiation of translation. The route was structured for a late introduction of diversity to allow for ease in synthesis of diverse derivatives. In addition, an X-ray crystal structure of the bound inhibitor-RNA complex has been achieved. The crystal structure allows for guided structural modifications of analog compounds to improve binding affinity to the IRES and consequently drug effectiveness. Once tested, the recently gained bioassay and Förster resonance energy transfer (FRET) has continuously directed the diversity introduced in the compounds. Currently, around 20 analogs have been synthesized in the Bergdahl group and have shown excellent activity (EC_{50} = 3-90 \mu M) against the virus. Many structure-binding relationships of alkylamino-substituted benzimidazole ligands were established. The inhibitors have provided valuable data towards the synthesis of additional compounds in anticipation of more powerful molecules for the treatment of HCV. Future derivatives are expected to act more efficiently due to more drastic changes to the backbone of the structure without compromising the toxicity to the human body. The data-driven approach allows for continuation of optimization of binding affinity. This will also allow selectivity in the syntheses of drugs with the hope of having readily available and cost effective treatments for the HCV virus.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>INTRODUCTION TO COMPOUNDS ACTING AGAINST HEPATITIS C</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.1 Background of Hepatitis C</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1.2 Discovery of Hepatitis C</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1.3 Investigation of Current Hepatitis C Treatments</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>1.3.1 Early HCV Treatments</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1.3.2 Treatment with Boceprevir and Telaprevir</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>1.3.3 Treatment with Faldaprevir</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>1.3.4 Treatment with Simeprevir</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>1.3.5 Treatment with Sofosbuvir</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>1.3.6 Treatment with Daclastavir</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>1.4 Previous Synthesis and Investigations of Dyhydropyranobenzimidazole (DPI)</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>1.4.1 Introduction</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>1.4.2 Seth et al. Route</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>1.4.3 Bergdahl Group Route</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>1.4.4 Investigation of Recently Reported 1-Aryl-2- Aminobenzimidazole Compounds</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>1.5 Significance of Research</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>1.6 Arrangement of the Thesis</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>2 SYNTHESES OF DIHYDROPYRANOBENZIMIDAOLE</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>2.1 Divergent Synthetic Strategy</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>2.2 Recent Improvements in Synthesis</td>
<td>28</td>
</tr>
</tbody>
</table>
3 ENGINEERING A DIRECTED SYNTHESIS TOWARDS NOVEL HCV INHIBITORS ................................................................. 30
  3.1 Crystal Structure-Directed Approach ......................................................... 30
    3.1.1 Optimization of Amine-Phosphate Salt Bridges and Ligand Interactions .................................................. 35
    3.1.2 Synthesis of Conformationally Restricted Analogs .................................. 36
  3.2 Observed Crystal Structure Interactions ................................................... 36
  3.3 Late-Stage Introduction of Diversity ........................................................ 37
  3.4 Creation of Diverse Library ........................................................................ 39
4 APPROACHES TO DIVERSIFICATION OF ANALOGS ........................................ 42
  4.1 Use of Secondary Amines ........................................................................... 42
  4.2 Use of Primary Amines ................................................................................ 49
5 LIBRARY CREATION OF SYNTHESIZED ANALOGS ........................................ 59
  5.1 Benzyl-Methylamine Derivative ................................................................. 59
    5.1.1 Initial Synthetic Approaches ................................................................ 59
    5.1.2 Cleavage of Benzyl Group .................................................................... 60
  5.2 Phenethyl-Butylamine and Dibutyl Compounds ........................................... 61
  5.3 Piperidine Derivative .................................................................................. 62
  5.4 Morpholine Derivative ............................................................................... 65
  5.5 Variation in Side Chain Arm Length ........................................................... 66
  5.6 Piperazine-Like Derivatives ....................................................................... 69
  5.7 Control Compound Analogs ...................................................................... 71
    5.7.1 Elimination of Amino Terminus ............................................................ 73
    5.7.2 Replacement of Amino Terminus with Alcohol Group ......................... 74
  5.8 Secondary Amines; the RMeNH Analog ...................................................... 76
    5.8.1 Current Approach ................................................................................. 77
    5.8.2 Initial Synthetic Approach .................................................................... 77
    5.8.3 The Undesired Cyclization Reaction ................................................... 78
    5.8.4 Alternate Synthetic Proposal ............................................................... 80
  5.9 The Phenethylamine Compound ................................................................ 81
  5.10 Additional Compounds ............................................................................. 82
  5.11 Investigation of Methyl Group Appearance .............................................. 82
6 BIOLOGICAL ACTIVITY ................................................................................. 89
LIST OF FIGURES

Figure 1.1. Estimated sources of HCV infection. .................................................. 2
Figure 1.2. Worldwide prevalence of the Hepatitis C virus. ..................................... 4
Figure 1.3. Chemical structure of Ribavirin. .......................................................... 6
Figure 1.4. TMC647055 NS5B inhibitor ................................................................. 8
Figure 1.5. Chemical structure of Boceprevir ......................................................... 9
Figure 1.6. Chemical structure of Telaprevir ......................................................... 9
Figure 1.7. Chemical structure of Faldaprevir ....................................................... 10
Figure 1.8. Chemical structure of Simeprevir ....................................................... 11
Figure 1.9. Chemical structure of Sofosbuvir ....................................................... 12
Figure 1.10. Chemical structure of Daclastavir ................................................... 12
Figure 1.11. Original 2-aminobenzimidazole compound screened to have the highest selectivity and affinity for the IRES subdomain IIa. ....................... 15
Figure 1.12. 2-Amino-benzimidazole inhibitor with a 6-(dimethylamino)propyloxy side chain increasing the binding affinity of the molecule. .................... 16
Figure 1.13. Selected benzimidazole derivatives displaying binding affinity (MS KD), replicon activity (EC50), and cellular toxicity (MTT). ....................... 17
Figure 1.14. Route of Seth et al. dihydropyranobenzimidazole compound of interest. .... 17
Figure 1.15. Structures and IC50 values of nine 1-aryl-substituted-2-aminobenzimidazole compounds published by the Hermann group .................. 19
Figure 1.16. HCV RNA organization displaying untranslated regions and the IRES element found in 5’ UTR; S= structural gene region, NS= nonstructural gene region. ......................................................... 21
Figure 2.1. Retrosynthetic analysis of the chroman ring. ..................................... 25
Figure 2.2. Total synthetic approach of the target benzimidazole compound by the Bergdahl laboratory. ................................................................. 26
Figure 2.3. 1H NMR study showing correct regioselectivity of nitrated compound. .... 27
Figure 2.4. Three-step synthesis of Boc-methylpropane-1,3-diamine (2.12). ............ 28
Figure 3.1. A. Secondary structure of the 5’ UTR of HCV genotype 1B; boxed region indicates subdomain IIa (sequence displayed to the right). B. Crystal structure of subdomain IIa. ......................................................... 31
Figure 3.2. Representation for the mode of action of a conformational change from bent to linear in HCV RNA upon introduction of benzimidazole inhibitor (5)........32
Figure 3.3. Observed binding interactions of inhibitor 1.10 with IRES of HCV RNA........33
Figure 3.4. Graphical representation of novel binding mode of HCV original inhibitor (1.10)..................................................................................................................................35
Figure 3.5. Introduction of diversity into the benzimidazole backbone via S_NAr...............38
Figure 4.1. Proposed diversity in the final HCV inhibitor; X = 0, 1, 2, 3, 4. .........................42
Figure 4.2. First alkylation step in the diversification of analogs; n = 3 and 4; X = Cl or Br. .................................................................43
Figure 4.3. Successful route in synthesis of diverse free amines shown with N-methylbenzylamine. ........................................................................................................................................44
Figure 4.4. Successful secondary amines in the S_N2 reaction. ..................................................44
Figure 4.5. Attempted secondary amines in the S_N2 reaction ....................................................45
Figure 4.6. Successful S_N2 reaction using morpholine. ..........................................................45
Figure 4.7. Successful synthesis with morpholine substrate and extended chain length......46
Figure 4.8. High-yielding S_N2 approach using piperidine and in the presence of NaI. ....46
Figure 4.9. Good-yielding S_N2 route with piperidine and extended chain length. ........46
Figure 4.10. Attempted mono-Boc protection of piperazine. ..................................................47
Figure 4.11. S_N2 reaction between compound 4.1 and N-Boc-piperazine in good yield.................................................................48
Figure 4.12. S_N2 reaction between compound 4.9 and N-Boc-piperazine in good yield........................................................................................................................................48
Figure 4.13. Chen et al. attempted base-promoted N-alkylation; X = Cl, Br. .........................49
Figure 4.14. Successful synthesis of desired secondary amine using Cho methodology. ..........................................................................................................................................50
Figure 4.15. S_N2 reaction with previously synthesized secondary amine 4.17. .................51
Figure 4.16. Successful reductive amination via route of Cho.................................................51
Figure 4.17. Attempted reductive amination with acetaldehyde via method of Cho. ..........51
Figure 4.18. Successful synthesis of secondary amine using Salvatore methodology. ....52
Figure 4.19. Successful S_N2 route of compound 4.22. ..........................................................52
Figure 4.20. Gabriel synthesis followed by corresponding aldehyde formation. .................52
Figure 4.21. Reductive amination procedure using route of Abdel-Magid group..............53
Figure 4.22. Attempted Boc-protection via method of Brass et al. of amine 4.26. ..............54
Figure 4.23. Imine formation followed by reduction to secondary amine 4.28.................55
Figure 4.24. Attempted Boc-protection of secondary amine via a traditional method........55
Figure 4.25. Attempted reductive amination procedure using the method of McMills.........55
Figure 4.26. Attempted reductive amination using 3-bromoprop-1-ene via method of
McMills..................................................................................................................56
Figure 4.27. Attempted reductive amination in water via method of Etchells and
Simion........................................................................................................................57
Figure 4.28. Attempted TEMPO and BAIB assisted reductive amination........................57
Figure 5.1. Initial proposal of benzyl-methyl derivative synthesis.................................60
Figure 5.2. Predicted structure of product after palladium-catalyzed reduction...............61
Figure 5.3. Synthetic route of a phenethylbutyl-amine derivative.................................62
Figure 5.4. Route for the creation of a dibutyl-containing analog.................................63
Figure 5.5. Overall synthetic route for creation of piperidine-containing analog..............63
Figure 5.6. Piperidine derivative synthesis with additional carbon in side chain arm.......65
Figure 5.7. Synthetic scheme for the final morpholine inhibitor in 23.1% total yield........66
Figure 5.8. Proposed method to synthesize the four-carbon side chain with
morpholine terminus..............................................................................................67
Figure 5.9. Incorporation of freshly synthesized amine into chroman core (2.7) for the
generation of a shorter side chain arm inhibitor...................................................68
Figure 5.10. Attempted route for the synthesis of a four-carbon chain length side arm......68
Figure 5.11. Proposed synthesis of a 4-carbon chain length side arm............................69
Figure 5.12. Proposed variations of series of piperazine-like derivatives; X = 1, 2, 3;
Y = 1, 2. ..................................................................................................................69
Figure 5.13. Synthesis of functionalized free amine (5.38) followed by its coupling to
chroman (2.7) to yield two-carbon, 6-membered ring final inhibitor......................70
Figure 5.14. Attempted approach to selectively Boc-protect the secondary amine of 2-
(piperazin-1-yl)ethan-1-amine...............................................................................71
Figure 5.15. Synthesis of functionalized free amine followed by incorporation to
chroman (2.7) to yield three-carbon, 6-membered ring final inhibitor.....................72
Figure 5.16. Synthesis of functionalized free amine 5.46 followed by incorporation to
chroman (2.7) to yield four-carbon, 6-membered ring final inhibitor......................73
Figure 5.17. Creation of a final analog with the elimination of the amino terminus..........74
Figure 5.18. Proposed synthetic approach to analog 5.57 having an alcohol side chain.....75
Figure 5.19. Reduction and cyclization followed by amide reduction providing 5.59......75
Figure 5.20. Proposed synthesis to yield the desired alcohol 5.61.................................76
Figure 5.21. Alternative route to RMeNH analog by Mr. David Schmit.........................77
Figure 5.22. Attempted route for construction of RMeNH-containing derivative. ..........78
Figure 5.23. Structures for the final two compounds in synthesis of RMeNH
derivative............................................................................................................79
Figure 5.24. Attempt to cleave Boc-group with TFA........................................80
Figure 5.25. Attempted approach to the phenethylamine derivative. ..............81
Figure 5.26. Attempted cyclization of the phenethylamine analog....................82
Figure 5.27. Conformationally restricted inhibitors currently synthesized by Mr.
David Schmit........................................................................................................83
Figure 5.28. Undesired methylation of the piperidine derivative........................83
Figure 5.29. Undesired methylation of the dimethylamino 2-carbon side chain
derivative............................................................................................................84
Figure 5.30. Published synthesis of generating a methylated derivative in place of an
amino group by the Dibrov group.......................................................................84
Figure 5.31. Test substrate demonstrating the quality of the Pd-catalyst..............86
Figure 5.32. Successful reduction of 2-nitroaniline and attempted cyclization of
benzene-1,2-diamine..........................................................................................87
Figure 5.33. Successful reduction of 2-nitroaniline and attempted cyclization of 2-
nitroaniline...........................................................................................................87
Figure 6.1. Inhibitor 5.69 showing no potency against the HCV infected cells.......93
Figure 6.2. Original inhibitor 1.10 showing excellent potency against the HCV
infected cells........................................................................................................94
Figure 6.3. Inhibitor 5.80 showing relatively good potency against the HCV infected
cells.....................................................................................................................95
Figure 7.1. Activity of all HCV inhibitors synthesized to date..............................99
Figure 7.2. Two, three, and four-carbon chain length comparison of final analog
structures............................................................................................................101
Figure 7.3. Piperazine derivatives ranging from two to four-carbon tethers............102
Figure 7.4. Comparison of final inhibitors containing piperidine, morpholine,
piperazine, and methylimidazoline as side chain terminus..................................103
Figure 7.5. Side-by-side comparison of three final analogs with corresponding EC_{50}
values...................................................................................................................106
Figure 8.1. Initial approach proposed to incorporate an amino acid, such as histidine..109
Figure 8.2. Alternative approach proposed to incorporate the amino acid, histidine..109
Figure 8.3. S_{N}Ar proposal with pure undesired isomer of chroman 2.7.............110
Figure 8.4. Proposal for additional three piperazine-containing final compounds with
varying side chain length....................................................................................111
Figure 8.5. Proposed synthesis for a one-carbon containing side chain inhibitor. ................................. 112
Figure 8.6. Proposed synthesis for a five-carbon-containing side chain inhibitor................................. 113
Figure 8.7. Electrophilic halogenation approach to introduction of halogen atoms. X + N-fluorobenzenesulfonimide, sulfuryl chloride, or pyridinium bromide perbromide. ................................................................. 114
Figure 8.8. General synthetic scheme for the synthesis of 2-aminobenzoxazole compounds. ................................................................. 115
Figure A.1. Compound 5.1. ........................................................................................................... 125
Figure A.2. Compound 5.5. ........................................................................................................... 125
Figure A.3. Compound 5.6. ........................................................................................................... 126
Figure A.4. Compound 5.7. ........................................................................................................... 127
Figure A.5. Compound 5.10. ....................................................................................................... 127
Figure A.6. Compound 5.14. ....................................................................................................... 128
Figure A.7. Compound 5.15. ....................................................................................................... 129
Figure A.8. Compound 5.16. ....................................................................................................... 130
Figure A.9. Compound 5.18. ....................................................................................................... 131
Figure A.10. Compound 5.22. .................................................................................................... 131
Figure A.11. Compound 5.23. .................................................................................................... 132
Figure A.12. Compound 5.24. .................................................................................................... 133
Figure A.13. Compound 5.28. .................................................................................................... 134
Figure A.14. Compound 5.29. .................................................................................................... 134
Figure A.15. Compound 5.30. .................................................................................................... 135
Figure A.16. Compound 5.39. .................................................................................................... 136
Figure A.17. Compound 5.43. .................................................................................................... 137
Figure A.18. Compound 5.47. .................................................................................................... 137
Figure A.19. Compound 5.50. .................................................................................................... 138
Figure A.20. Compound 5.51. .................................................................................................... 139
Figure A.21. Compound 5.62. .................................................................................................... 139
Figure A.22. Compound 5.55. .................................................................................................... 140
Figure A.23. Compound 5.58. .................................................................................................... 141
Figure A.25. Compound 4.17. .................................................................................................... 142
Figure A.26. Compound 5.70. .................................................................................................... 142
Figure A.27. Compound 5.71. .................................................................................................... 143
Figure B.33. $^1$H NMR of compound 5.55.................................................................177
Figure B.34. $^1$H NMR of compound 5.58.................................................................178
Figure B.35. $^1$H NMR of compound 4.17.................................................................179
Figure B.36. $^1$H NMR of compound 5.70.................................................................180
Figure B.37. $^1$H NMR of compound 5.71.................................................................181
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ</td>
<td>chemical shift</td>
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<tr>
<td>µM</td>
<td>micromolar</td>
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<td>$^1$H NMR</td>
<td>proton nuclear magnetic resonance</td>
</tr>
<tr>
<td>$^{13}$C NMR</td>
<td>carbon nuclear magnetic resonance</td>
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<tr>
<td>Ac</td>
<td>acetyl</td>
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<tr>
<td>ACN</td>
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<td>Boc</td>
<td>tert-butoxycarbonyl</td>
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<tr>
<td>Boc$_2$O</td>
<td>di-tert-butyl dicarbonate</td>
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<tr>
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<td>Centers for Disease Control and Prevention</td>
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<tr>
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<td>d</td>
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<td>DABCO</td>
<td>1,4-diazabicyclo[2.2.2]octane</td>
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<td>ee</td>
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CHAPTER 1

INTRODUCTION TO COMPOUNDS ACTING AGAINST HEPATITIS C

1.1 BACKGROUND OF HEPATITIS C

The Hepatitis C (HCV) virus infects the liver and can cause chronic infection with symptoms lasting from a few days to life-long illness. An estimated 150 million people worldwide are known to have a chronic infection. In many of these individuals, the chronic infection will lead to the possibility of liver cancer and/or liver cirrhosis. It is estimated that the number of people living with HCV-related liver failure and liver cancer will triple in the next 15 years because of low treatment rates.

In 2007, shocking data became available that the rapid growth of HCV deaths had surpassed HIV-related mortality. HCV has now infected almost four times as many people as HIV. HCV is roughly ten-fold more concentrated in blood in comparison to HIV. In addition, cirrhosis and chronic liver disease are among the top fifteen leading causes of death in the U.S. The life-threatening virus is caused by blood to blood contact with infected blood, although the natural history of HCV is not completely understood. The most likely circumstance of infection is through contaminated needles such as in drug use, rarely in unclean hospital settings, but also common in unsafe medical procedures in poor countries (Figure 1.1). A published report from 1991 reports a documented 3.7% transmission rate in medical personnel of 110 needle-stick exposures. Additionally, in the 1990’s HCV was seen to develop in approximately 10% of all blood transfusions, which translated to blood transfusion causing 15,000 new cases of HCV per year. Sexual transfer of the disease between partners or from mother to fetus were actually less common. The HCV virus is able to survive outside of the body at room temperature for no less than 16 hours and up to four days.

Once infected, the virus incubates inside a patient’s body for roughly 60 days. A percentage (40-75%) of infected individuals will never experience symptoms and will clear the virus without treatment. Of those who do not clear the virus, a minor percentage will
never experience symptoms making early detection nearly impossible. However, 86% of people are likely to develop chronic HCV and may experience symptoms of fever, fatigue, decreased appetite, nausea, anemia, depression, vomiting, jaundice (10% of patients) and abdominal pain. It is well known that treatment of HCV infection during the acute phase instead of chronic phase leads to a better response rate among patients. Until a treatment option is started, the virus will continue produce approximately $10^{10}$ to $10^{12}$ virons each day. The virus is known to have a rapid rate of replication with a half-life of roughly only two to five hours.

The diagnosis of HCV consists of screening a patient for anti-HCV antibodies followed by a nucleic acid test for conformation. If infected, it is imperative to find out the genotype of the virus, with the possibility of more than one genotype simultaneously infecting a patient. The virus may also be responsible for immunological disorders seen in chronically infected HCV patients as the virus may replicate in peripheral blood mononuclear cells.
During the rapid phylogenetic evolution, six common genotypes of HCV (1-6) and over 100 sub-genotypes (1A,1B,2A,2B,3A, etc.) have been observed.\textsuperscript{12} The difference is sequence between genotypes ranges between 31-33\% and 20-25\% between subgenotypes.\textsuperscript{12} Given the high heterogeneities in viral sequence, treatment therapies will not have similar effectiveness for all HCV genotypes and subtypes. Worldwide, the most prevalent form of HCV is genotype 1 and as a result, most drugs under development are designed for genotype.\textsuperscript{13} In the United States and Europe, subtypes 1a and 1b are most common, while subtype 1b exists in roughly 73\% of HCV cases.\textsuperscript{14} Subtypes 2A and 2B are common in Europe, Japan, and North America, while 2c is predominantly seen in northern Italy.\textsuperscript{14} Genotype 3A is mostly observed within drug users in Europe and the U.S., genotype 4 is responsible for the majority of cases within Africa and the Middle East, while genotypes 5 and 6 are predominantly confined to South Africa and Hong Kong, respectively.\textsuperscript{14}

A laboratory test is crucial as there are several treatment options and each is heavily dependent on the genotype of HCV. Next, an assessment of liver damage is performed and various treatment options are explored. The risk of liver cirrhosis within the first 20 years of the infection is 15-30\%. HCV is currently the fastest-growing cause of cancer related deaths. Though HCV is most prevalent in developing countries of Central and East Asia as well as North Africa and Europe, it still affects thousands of people worldwide (Figure 1.2).\textsuperscript{15} HCV related liver disease deaths number approximately 500,000 per year.\textsuperscript{16} Eradicating HCV infection worldwide will only be possible through universal access to HCV testing and new effective therapy approaches.\textsuperscript{16}

2014 marks the 25\textsuperscript{th} anniversary of HCV cloning by Michael Houghton and his colleagues at Chiron and Daniel Bradley at Centers for Disease Control and Prevention (CDC).\textsuperscript{17} Antiviral medicines have been recently explored and seem to show effectiveness in a percentage of cases as well as showing signs of reducing the development of liver cancer and cirrhosis. However, due to the seriousness of this disease and lack of vaccine, it is crucial that research be continued in hopes of finding long-lasting and effective permanent treatment options.
1.2 DISCOVERY OF HEPATITIS C

1975 marks the year that Michael Houghton, Qui-Lim Choo, George Kuo, and Dr. D.W. Bradley (CDC) demonstrated that most post-blood transfusion hepatitis cases were not caused by Hepatitis A nor B viruses. The new strain of virus was initially classified non-A, non-B (NANB) Hepatitis which was identified with various serological tests against serological markers of Hepatitis A and B. Findings demonstrated that NANB Hepatitis did not possess any Hepatitis A or B serological markers. After hundreds of millions of bacterial cDNA clones from infected chimpanzee were screened, a single c-DNA clone was successfully isolated that was shown to be derived from new flavi-like virus (HCV) using novel immunoscreening method by using HCV antibodies. Key viral encoded enzymes essential to the life of the virus were then targets of vigorous drug development activities. In 1988, Chiron Corporation announced the discovery of viral antigens for post-transfusion
and the data was published in 1989.\textsuperscript{18} At that time, NANB Hepatitis was the first virus that had been discovered via molecular cloning without direct use of biological methods.\textsuperscript{14} In 1991, the complete HCV genome was established by Choo \textit{et al.}\textsuperscript{14}

\section*{1.3 Investigation of Current Hepatitis C Treatments}

In rare cases, the infected person’s immune system will successfully clear the virus without any treatment. Otherwise, treatment options must be explored. Throughout the years, extensive research has been devoted to discovering alternative treatments for HCV. However, a vaccine remains unavailable and there is no known cure for HCV. The treatments which are explored depend most importantly on the genotype of the virus, as each unique genotype of the virus requires different treatments. The most standard treatment of the virus has been an immunostimulatory regimen of antiviral therapy drugs with interferon-\(\alpha\) and the nucleoside analog ribavirin, which has been shown effective against all genotypes of the virus. Both approaches work by inhibiting viral replication and enhancing the body’s immune response to eradicate the virus. Unfortunately, this type of treatment is accompanied by serious side effects, forcing many patients to abandon the treatment. Other shortcomings of the treatment include non-responsiveness as well as developing resistance to the treatment.\textsuperscript{12} Triple combination with direct-acting antiviral drugs now on the market have extremely low availability due to cost. In addition, because of the high genomic heterogeneity within the subtypes of HCV, direct-acting antiviral agents will not show similar levels of efficiency.

Ongoing research has made other therapy options possible. There have been tremendous advances in the approval of anti-HCV small molecules which show promise of eliminating some of the severe side effects experienced with interferon/ribavirin treatments.\textsuperscript{17} Many of these novel drugs act in different ways, and in more direct routes, but are only effective for certain genotypes. There remains an ongoing need for HCV research as most of the current drugs on the market have complicated injection and pill regimens (up to 18 tablets per day) and require treatment for up to one year to be effective. Although the low cost of manufacturing these drugs is appealing, high prices set by pharmaceutical companies make them unaffordable for most patients, especially patients outside of the U.S.\textsuperscript{1} Thus, there
remains an unmet medical necessity for safe and effective treatment choices which deliver a high cure rate with a short duration time.

1.3.1. Early HCV Treatments

Historically, the most popular form of HCV treatment was a combination of antiviral therapy with interferon and ribavirin drugs. Interferons are proteins in the glycoprotein class that are both made and released by a cell in response to a pathogen. These proteins are used for their unique ability to protect cells from viral replication by activating protective defenses of the immune system which help eliminate pathogens from the cell. Secondly, the guanosine analog, ribavirin acts as a nucleoside inhibitor to put an end to viral RNA synthesis (Figure 1.3). Similarly, ribavirin interferes with the metabolism of RNA via several unknown mechanisms, preventing viral replication. What is known is that ribavirin causes hyper mutations in the RNA-dependent replication of viruses causing them to not replicate further. However, ribavirin is now considered generic and reserved for use only when newer, direct-acting anti-viral medications are unavailable.

![Chemical structure of Ribavirin](image)

Figure 1.3. Chemical structure of Ribavirin.

Though there are instances where this approach can be life-saving, the side effects caused by this combination of treatments is so severe that often patients withdraw from treatment after only a few doses. The most severe side effect includes flu-like symptoms which persist for weeks to months of the treatment plan. Because the pain associated with interferon and ribavirin is unmanageable to a large number of patients, it cannot be considered an effective route to treating HCV. More recently, new treatments have been used paired, tripled, or quadrupled with interferon, but still yield similar side effects.\(^\text{19}\) Therefore,
these nonspecific therapies have largely been abandoned and direct-acting antiviral agents are gaining popularity. Three major classes of therapies are now available, including polymerase (RNA-dependent RNA polymerase NS5B), protease (nonstructural NS3) inhibitors, NS5A protein, and direct-acting molecules that interact with host cell protein to inhibit HCV replication.

Because of success with HIV treatment, NS3/4A protease and NS5B polymerase were the next targets to be explored. NS4B is a more recent target, it is a 27-kDa integral membrane protein which is thought to act as an endoplasmic reticulum-localized scaffold for the complexes needed for HCV virus replication. These first-generation NS3/4A protease inhibitors were first approved in 2011. In addition, there is a high demand for robust protease inhibitors which can withstand resistance due to the emergence of drug-resistant mutants and not being able to treat all genotypes of the virus.

Polymerase inhibitors bind to less conserved allosteric sites of NS5B and therefore have dissimilar resistance summaries in each genotype of HCV. Molecular assays have been the most useful tools for genotyping HCV which can be done by analyzing the sequence of the NS5B region, which is crucial in clinical decision making. Researchers also say that analysis of the NS5B region may be useful for tracing the source of HCV infection. Published studies show that the probability of a sustained virological response (SVR) to antiviral therapy is heavily based on genotype, suggesting that treatment should be personalized to the specific genotype. Allosteric inhibition of NS5B can be achieved through binding at several sites on the enzyme. The molecules gained interest as NS3/4A and NS5B enzymes are readily recapitulated in biochemical assays so screening and evaluation of lead compounds is heavily facilitated. One such selective indole NS5B inhibitor is TMC647055 (Figure 1.4). NS5B is a cell-permeating, RNA-dependent RNA polymerase (RdRp) that synthesizes both positive and negative-strand genomic RNA and has shown initial cross-genotypic coverage. Genotype 1B infection results showed a mean IC\textsubscript{50} of 34 nM, however, genotypes 1A, 1B, 3A, 4A, and 6A ranged from 27 to 113 nM. In addition, genotype 2A and 2B IC\textsubscript{50} values were 200-fold higher.

As treatment with the newest anti-viral medications remains prohibitively expensive for most people, there is a need for more affordable medicines. Almost 75% of all HCV patients reside in economically deprived areas where the possibility of obtaining antiviral
medication is quite low. Therefore, patients are left only with the option of continued interferon and ribavirin treatments. Furthermore, the emergence of viral strains resistant to protease and polymerase inhibitors has been increasing, leaving the necessity for other targeted treatments to be explored.

1.3.2. Treatment with Boceprevir and Telaprevir

In addition to the numerous possibilities mentioned above, the protein NS3 has recently been targeted by Boceprevir and Telaprevir. Both compounds are in the class of antiviral drugs known as protease inhibitors and were developed specifically against HCV genotype 1. These molecules act by binding to the HCV nonstructural NS3 active site and are a good targeted approach for treating HCV, though only effective for genotype 1 of the virus and slightly against genotypes 3 and 4. Significantly weaker antiviral activity is observed for patients with genotype 2 and 3 HCV. Victrelis, commonly known as Boceprevir, was synthesized by Merck and Co. and gained FDA approval in May 2011 (Figure 1.5).

The drug VX-950, more commonly known as Telaprevir, Incivek, or Inivo, was co-discovered by Johnson and Johnson and Vertex Pharmaceuticals and gained FDA approval in May 2011 (Figure 1.6).

However, Boceprevir is known to have a medium antiviral resistance and medium genetic barrier to resistance. To be effective, both of these drugs need to be administered with pegylated interferon (INF)-alfa ribavirin, which as discussed carries serious side effects.
In addition, the Boceprevir dosage is thirty times higher than that of the recently discovered drug Daclastavir. The cost of Telaprevir treatment is estimated to be $189,000 per treatment period, making it unaffordable for many infected individuals.

In Telaprevir trials, participants received treatment either alone, in a dual regimen with ribavirin, or in a triple/quadruple regimen with both ribavirin and pegylated interferon alfa-2a (Pegasys). The dual treatment suffered from low effectiveness with a significant number of viral breakthroughs and was discontinued early. In the triple regimen, patients received 1.125 g of Telaprevir twice a day and 400 mg of VX-222 twice a day plus ribavirin. When HCV RNA was not cleared by 8 weeks, patients received pegylated interferon/ribavirin alone for 24 additional weeks. The quadruple treatment showed the best results, yet still with significant side effects. The most common adverse events were diarrhea (50%), rash (37%), nausea and itching (30%), fatigue (24%), and mild anemia (15%).
Patients with genotype 1A took considerably longer to clear the virus in comparison to those with genotype 1B.¹⁹

1.3.3 Treatment with Faldaprevir

Faldaprevir is a small, non-macrocyclic molecule inhibitor of HCV NS3 protease which was synthesized by Boehringer Ingelheim (Figure 1.7). Faldaprevir possesses favorable bioavailability, safety, as well as *in-vivo* potency as a treatment for HCV.²⁶ The newest synthetic route was broken up into three key intermediates: a dipeptide acid, a thiazole-quinoline, and an aminoester cyclopropane; however, because of the complexity of the molecule, the total synthesis is still quite lengthy.²⁶

![Figure 1.7. Chemical structure of Faldaprevir.](image)

Multiple reasons have made Faldaprevir a less attractive drug candidate. Most importantly, it must be administered with pegylated interferon, is less effective than other antiviral drugs on the market and has a longer length of therapy. Interestingly, Boehringer Ingelheim retracted FDA submission for this drug as of June 2013 and is discontinuing its use.³

1.3.4 Treatment with Simeprevir

Simeprevir (also known as TMC435 and Olysio) was developed by Johnson & Johnson as a NS3/4A protease inhibitor and was approved in late 2013 (Figure 1.8). Simeprevir is administered as one capsule once daily with pegylated interferon and ribavirin for the treatment of HCV genotype 1 and 4. This drug is also combined with other direct-
acting antiviral agents, such as Daclastavir and Sofosbuvir. The effect on activity (IC$_{50}$) has been demonstrated to be 0.5 mM for genotype 1A and 1.4 nM for genotype 1B.\textsuperscript{20} In one study, four deaths occurred in the treatment groups, although they were judged to be unrelated to treatment. Other common adverse effects were rash (28%), influenza-like illness (26%), itching (22%), and nausea (22%).\textsuperscript{20}

1.3.5 Treatment with Sofosbuvir

Sofosbuvir, marketed under the brand name Sovaldi, was discovered at Pharmasset and developed by Gilead Sciences (Figure 1.9). This complex drug acts as a nucleotide analog polymerase inhibitor and was FDA approved in December 2013. Sofosbuvir has been administered without interferon for patients with genotype 2 and 3 HCV. In clinical trials, treatment usually lasted between 12 and 24 weeks and most commonly incorporated ribavirin. Genotype 1 and 4 patients underwent concurrent doses of interferon.

A dual combination of Sofosbuvir and Simeprevir has been shown in patients with genotype 1 to have a sustained virological response of 92-94%.\textsuperscript{2} Although these drugs are not marketed as a dual approach, it shows promise for patients who do not tolerate interferon and ribavirin.\textsuperscript{2} However, the price of Sofosbuvir limits its use in developing countries, with a typical treatment cost of $84,000 to $168,000.\textsuperscript{27}
1.3.6 Treatment with Daclastavir

The newest FDA approved drug on the market is BMS-790052, commonly known as Daclastavir (Figure 1.10). This treatment was developed by Bristol-Myers Squibb in 2014.

Daclastavir acts by inhibiting the nonstructural protein NS5A. Studies suggest that Daclastavir causes the quick degradation of HCV RNA by targeting not only one, but two crucial steps in the viral replication process of HCV. BMS-790052 is currently one of the most potent inhibitors of the multi-functioning NS5A protein which has a large role in the replication of HCV.25

The synthesis of Daclastavir consisted of over 13 steps.28 Compared to similar analogs, Daclastavir showed higher oral bioavailability in rat, dog, and monkey pharmacokinetic studies. EC$_{50}$ values for Daclastavir were found to be 50 pM for genotype 1A and 9 pM for genotype 1B.28 Based upon this and other animal studies Daclastavir is considered the preferred compound of interest.28
A 2-drug regimen of Daclatasvir and Asunaprevir (an NS3/4A protease inhibitor) is currently awaiting FDA approval in the U.S. This therapeutic approach led to 82-90% SVR in people who were previously treated with genotype 1B and patients who were unable to tolerate interferon. The major drawback is that the treatment is still quite lengthy (24 weeks). In addition, though nanomolar potency was observed, the emergence of drug-resistant mutants is a severe problem.

Daclastavir is currently considered a reasonable treatment alternative, yet is still not effective for all genotypes. The probability of the drug withstanding future mutations of the virus is also unlikely. The fact is that viruses are known to have a relatively rapid mutation rate. Although some have labeled Daclastavir as a “cure”, it would be unwise to discontinue research on alternative ways to treat the virus. Daclastavir has only been FDA approved in Japan by Ministry of Health, Labour and Welfare (FDA equivalent) and is awaiting approval in Europe and the U.S. With a cost for this treatment of approximately $1,000 per pill, Daclasavir is an unrealistic treatment option for most people around the world, especially in countries that lack health care.

1.4 Previous Synthesis and Investigations of Dihydropyranobenzimidazole (DPI)

Various routes in the synthesis of the benzimidazole compound of interest will be discussed herein.

1.4.1 Introduction

In order to demonstrate that the synthesis of HCV inhibitors presented by the Bergdahl group in 2011 was novel and offers advantages over previous efforts, it was necessary to explore preceding work in this class of compounds. Among several publications, none clearly revealed a route designed with a library production in mind. Without this crucial development, it was difficult to study trends and the structure-activity relationship of the benzimidazole class of molecules. A new synthetic route allows for the investigation of structural modifications on the activity of the molecules. In addition, prior publications suffered from low yielding final products, making large scale synthesis of these drug molecules unlikely. Preceding syntheses also incorporated difficult steps and used harsh conditions, which are not ideal in terms of making these treatments viable for large scale
pharmaceutical development. The recent total synthetic approach presented by the Bergdahl group is not only the highest yielding of all published methods, but also shows many improvements in the synthetic scheme.\textsuperscript{29} All steps are relatively simple to carry out. The individual reactions are high yielding and are mostly crystalline solids, allowing for easy purification on silica or basic alumina. Moreover, the approach allows for the diversification of the molecule in a straightforward fashion.

1.4.2 Seth \textit{et al.} Route

In 2005, Seth \textit{et al.} reported a new class of molecules that bind to the IRES element in an innovative mode of action with sub-micromolar affinity, making this class attractive for new HCV therapies.\textsuperscript{30} The newly discovered class of molecules consists of a substituted benzimidazole structure fused to a pyran or furan ring. Out of over 180,000 compounds being screened for their affinity to subdomain IIa, the most suitable compound for HCV treatment in the study was found to be the 2-amino-1-(3-(dimethylamino)propyl)-substituted benzimidazole compound.\textsuperscript{30} A high throughput screening method based on mass spectroscopy was used as a tool to guide SAR.

Until the publication of Seth \textit{et al.}, treatment of HCV with small drug molecules acting against the IRES site had been an unexplored approach to therapy.\textsuperscript{30} This was in part due to the difficulty of studying molecules which bind specifically and selectively to structured RNA targets as well as the lack of medicinal chemistry studies explaining how small molecules recognize and interact with specific RNA. To develop the screening method, Seth \textit{et al.} constructed a 29-mer RNA fragment, which acted as the RNA model target for newly synthesized benzimidazole analogs. In addition, to demonstrate the importance of the IRES, the site was mutated in several ways. Consistent with the hypotheses, the forms of mutated IRES subdomain IIa showed no ability to replicate the virus. Of the initial compounds screened, the benzimidazole molecule, shown in Figure 1.11, was found to possess the highest selectivity and binding affinity with a $K_D$ of less than 100 $\mu$M.

For the optimization of this compound, it was important to first examine which functional groups had a large impact on SAR. Results showed that when the dimethylamino head group was replaced with a proton, selectivity and affinity both decrease drastically. When the group was replaced with a pyrrolidino and diethylamine groups, results showed a
Figure 1.11. Original 2-aminobenzimidazole compound screened to have the highest selectivity and affinity for the IRES subdomain IIa.

lower affinity for the subdomain IIa. Likewise, replacement with morpholino and dibutyl groups caused a total loss of binding, most likely due to the bulkiness of these groups. Another interesting finding was the reduction of chain length between the dimethylamino head group and the benzimidazole ring which caused a two-fold reduction of affinity. The importance of the dimethylamino group for optimal RNA binding was verified.

Testing an additional 150 analogs with both electron withdrawing and electron donating groups on the ring showed that only a methoxy substituent demonstrated a slight increase in affinity. There was a belief that there may be insufficient room in the RNA binding pocket to accommodate such substitutions on the rings. The optimization was interesting with respect to the position on the molecule that was three carbons away from the nitrogen that the dimethylamino carbon chain was attached to. Several substituents were evaluated at this position and only one 6-(dimethylamino)propyloxy side chain (Figure 1.12) provided a remarkable 10-fold improvement in binding affinity over the previous compound as well as a 5-fold selectivity increase. The compound resulted in a $K_D$ around 10 µM.

The study evaluated whether all three cationic side chains played a crucial role in the interactions between compound and RNA. Elimination of the NH$_2$ group, removal of the three carbon chain with dimethylamino headgroup, and moving the 6-(dimethylamino)propyloxy side chain to the adjacent carbon 7 within the molecule were all performed independently. In all cases, the compound’s affinity for the IRES subdomain IIa binding was lost, indicating the importance of all three groups in the drug’s action. One alteration that did increase binding affinity was replacing the dimethylamino headgroup with a polycationic group. Unfortunately, similar compounds are unsuitable as therapeutic agents due to their
high polarity. Seth et al. also demonstrated the synthesis and examined the change in activity when the pyranose in the molecule was altered into a tetrahydrofuran structure.\(^\text{30}\) Results demonstrated that the pyranose derivative had much higher binding affinity.

A proposed alternative strategy was to constrain the two relatively long side chains of benzimidazole compound shown in Figure 1.12. Presumably the change in entropy would be lower, allowing the interactions with the IIa subdomain to be larger. As expected, all analogs synthesized in this matter showed significant improvements in binding with minimal toxicity. The best compound tested by Seth et al. was the benzofuran structure, which is likely to be attributed to the analog’s enhanced cellular penetration. This compound not only possessed the highest \(K_D\) 40-mer binding affinity (0.86 \(\mu\)M), but also had the best replicon activity (\(EC_{50} = 3.9 \mu\)M) and lowest cellular toxicity in comparison to the two other compounds as seen in Figure 1.13.

SAR relationship studies of these various structures revealed the key interactions responsible for target recognition by the translation inhibitors as well as confirmed the importance of a rigid core scaffold for ligand affinity to the IRES subdomain IIa.\(^\text{30}\) However, disadvantages of Seth’s synthetic approach include a complicated, lengthy and low yielding synthesis (under 4%), troublesome purifications, and a lack of adaptability for the creation of a library of analogs.\(^\text{30}\) Seth’s route to the benzopyran structure is shown in Figure 1.14, beginning with the treatment of substituted difluorobenzyl bromide 1.1 with diethyl malonate and sodium hydride followed by a reduction with LAH to yield diol 1.2. Next, the diol underwent acylation and nitration via fuming nitric acid of the phenyl ring to attain intermediate 1.3. \(S_NAr\) displacement of the \textit{ortho}-fluoro-substituent was performed with
Figure 1.13. Selected benzimidazole derivatives displaying binding affinity (MS K\textsubscript{D}), replicon activity (EC\textsubscript{50}), and cellular toxicity (MTT).

Figure 1.14. Route of Seth et al. dihydropyranobenzimidazole compound of interest.

dimethylpropane diamine to access substituted aniline 1.4. After deprotection and ring closure were completed with the addition of methanol and potassium carbonate, the chroman nucleus 1.5 was reduced to aniline 1.6 via a palladium catalyzed hydrogenation. The aniline was then exposed to benzoyl isothiocyanate which induced a cyclization, followed by the treatment with EDC to form the dehydrated molecule 1.7. Under acidic conditions, the
benzoyl group was then eliminated to yield intermediate 1.8. The final product 1.9 was obtained after mesylation and subsequent incorporation of dimethylamino group. Both benzimidazole and benzofuran compounds were synthesized via similar methods, each consisting of twelve steps.

### 1.4.3 Bergdahl Group Route

Our group recently published a total synthesis of the target anti-Hepatitis C benzimidazole compound. The synthetic scheme had an impressive total yield of 10% and consisted of only nine steps. Of the three main compounds investigated by Seth et al., the (±)-benzopyran structure was chosen as the synthetic goal to minimize difficulty in the separation of diasteromers, which would not have been avoided during the synthesis of the other two compounds, making the creation of analogs much more troublesome. The most potent benzofuran compound possesses two stereocenters, while the chosen benzopyran compound possesses only one stereocenter. In comparison to the previous method of Seth et al., the major adjustment of our approach was the early incorporation of the chroman backbone into the synthesis. Putting in place the skeleton of the molecule made it possible to incorporate various amino side chains later in the synthesis, which facilitated the production of HCV derivatives.

The various amino side chains were chosen based upon hypothesized activity with the IRES subdomain IIa of the HCV RNA, as suggested by the observed binding interactions in the co-crystal structure published by the Hermann and Bergdahl groups. Variation of several structural trends was proposed by the two groups in order to observe direct correlation between structural changes and activity of the inhibitor. One proposed trend was to create analogs with varying carbon length within the side chain arm of the compound. Another route is currently to vary the heterocyclic ring size at the terminus of the side chain. With multiple analogs already synthesized, and more going through the synthetic pipeline, the goal is continued creation of improved examples in this class of molecules via the presented library optimized route. With this approach, high yielding reactions make it possible to obtain enough material for testing in bioassay and FRET studies, as well as for attaining x-ray crystallography with the bound RNA at a future date. Data gathered from these assays will continue to direct ongoing HCV research in the Bergdahl group.
1.4.4 Investigation of Recently Reported 1-Aryl-2-Aminobenzimidazole Compounds

Collaborators at UCSD have recently published aryl substituted aminobenzimidazole inhibitors synthesized in their laboratory.\textsuperscript{32} The basis for these structures, which have similarities to the inhibitors investigated in this thesis, was the publication by Seth \textit{et al.} in 2005.\textsuperscript{30} Out of the nine aryl substituted aminobenzimidazole structures that were created, two showed no activity towards subdomain IIa, while the remaining seven resulted in EC\textsubscript{50} values ranging between 74 and 250 µM.\textsuperscript{32} The FRET assays were performed in triplicate; chemical structures and corresponding affinity results with standard deviations are presented in Figure 1.15.

![Chemical structures and IC\textsubscript{50} values](image)

**Figure 1.15.** Structures and IC\textsubscript{50} values of nine 1-aryl-substituted-2-aminobenzimidazole compounds published by the Hermann group.
1.5 Significance of Research

Only one total synthesis of the target aryl-substituted aminobenzimidazole inhibitor of interest has been published since 2011. This study demonstrated that this class of molecules acts on the HCV RNA in a novel way that shows much promise for future HCV treatments. HCV is a positive sense single-stranded RNA (ssRNA) virus belonging to the Flaviviridae family. This small and enclosed virus contains a highly conserved internal ribosome entry site (IRES) in the 5’ untranslated region of the viral RNA which is comprised of 340 nucleotides which extends from position 40 through 372 of the viral RNA genome, which was carefully mapped out using dicistronic reporter assays.

The IRES is a highly structured RNA, first discovered in 1988, which acts by mediating translation initiation of the virus. The secondary structure of the IRES displays two major domains (II and III) whose function is encoded in conserved structural motifs in these two major domains and is vital for transcription initiation. The organization of these domains is for the most part conserved among related viruses of the Flaviviridae family. IRES elements are observed in over twenty well-known viral genomes, but are also seen in cellular messenger RNA’s (mRNA’s). Poliovirus, Rhinovirus, Encephalomyocarditis virus, Foot-and-mouth disease virus, classical swine fever virus, Bovine viral diarrhea virus, Human immunodeficiency virus (HIV), and the Hepatitis A and C are among viruses seen to have an IRES, while IRES-containing mRNA’s include the fibroblast growth factor, transcription and translation factors, oncogenes, transporters and receptors, as well as activators and inhibitors of apoptosis.

The HCV IRES domain II is comprised of subdomain IIa as well as subdomain IIb. Currently, the IRES subdomain IIa is the most advanced target for small molecule inhibition of HCV translation mainly due to its unique function and high conservation and is the shortest among IRESs currently discovered. Compounds that target this highly conserved region of HCV have significantly improved efficacy treating different genotypes and subtypes with an aim to discover a universal cure of the HCV. Such a binding pocket is currently unknown in any natural RNA target other than bacterial riboswitches. Other key features of the virus contain a single open reading frame of about 9,000 nucleotides as well as a very short 3’-untranslated region (UTR) (Figure 1.16).

The IRES subdomain Ila sequence, consisting of 65 nucleotides, is unquestionably crucial for the translation and replication of HCV as the IRES is responsible for mediating the initiation of viral-RNA in a can-independent manner. Once introduced, aminobenzimidazole small molecules act by inhibiting function of the IRES by capturing an extended conformation of subdomain Ila RNA, which consequently blocks translation initiation. Consequently, it has been established that IRES function is governed by structure. The ability to target this highly conserved sequence of the RNA, the IRES site, is appealing as it allows not only for the direct targeting of that specific site by the inhibitors, but cross-genotypic activity. This approach is different than anything that has been previously attempted. The blocking of the IRES site itself is anticipated to open the door to a large array of treatment options and has already been shown to be a promising target in the future of anti-HCV antiviral therapies. An advantage of this targeted treatment method is that the newly synthesized translational inhibitors are anticipated to demonstrate a high barrier of resistance due to targeting a highly conserved area in the genome.

After reviewing the existing total synthesis published by the Seth group, it was decided that a synthetic approach allowing for a late stage introduction of diversity would be
advantageous. The rationale behind this was to be able to alter the activity levels by changing the basicity of the side chain nitrogen, and thereby gain a better understanding of the interactions between the RNA genome. It was also necessary to improve the existing synthesis in terms of yield, number of steps, and to simplify the synthetic approach to make the route feasible in higher scales. Further modifications of existing analogs are vital to address possible rapid mutation of the HCV virus as it is known that the replication and mutation rates of HCV are much higher in comparison to HIV. According to researcher Bartenschlager, HCV mutates rapidly caused by a high error rate in RNA-dependent RNA polymerase activity. These mutations produce a large amount of variants of the virus, also known as quasispecies. A study conducted on a patient over 13 years (1977 – 1990) shows different regions of the HCV genome evolving at different rates. Published data by Ogata et al. estimated the mutation rate of the HCV genome to be roughly $1.92 \times 10^{-3}$ nucleotide substitutions per site per year using direct-sequencing of reverse-transcribed, PCR-amplified HCV genomic RNA as direct sequencing of the entire genome is not practical. The value is roughly a million-fold higher compared to the rate of replication of chromosomal DNA in prokaryotes and eukaryotes.

The potential HCV translational inhibitors presented in this thesis are unique in that they target the highly conserved region of the RNA and act in a novel way. As the virus mutates over time, current treatment approaches treatments will no longer be effective and exploration of other modes of inhibition will be necessary. It has been demonstrated that preexisting drug mutations are prevalent in similar viruses, validating an urgent need for unique antiviral agents directed at distinct HCV targets. With the continued generation of a large library of analogs, the goal is to provide sufficient information to understand some of these key interactions and expedite the discovery of new drugs that will save the lives of many people around the world. There is high likelihood that this class of benzimidizole compounds will be effective in treating other diseases which act based upon a similar mechanism of action, therefore expanding the scope of these therapeutic targets. Such benzimidazole scaffolds have already been recognized as useful platforms for the synthesis of biologically active ligands for similar RNA targets, the HIV TAR element, and microRNA’s.
Shortly after the novel synthesis presented by the Bergdahl laboratory, the analog bound to the HCV RNA was co-crystallized; showing the size of the binding pocket along with how the molecule orients itself inside of the binding pocket. The crystal structure then allowed the diversification of analogs based upon observed interactions of the original compound to the IRES of the HCV. Increased understanding of structure and mode of binding provide a unique opportunity to explore parameters for induction in the IRES subdomain. With this knowledge, it was possible to interactively vary structural changes to the molecules in a direct fashion. With each newly synthesized molecule, structure activity relationship studies and FRET studies were conducted in anticipation of increasing interactions with the RNA and consequently lowering IC\textsubscript{50} values.

The goal of synthesizing more potent and effective drug molecules is the driving force for this ongoing research. By publishing a library of structures of the synthesized analogs and their respective EC\textsubscript{50} values, there is an expectation that this research will shed light on the effectiveness of drug treatments and future drug design against HCV. The fourteen analogs synthesized have already shown remarkable activity against HCV cells and the EC\textsubscript{50} values gained have demonstrated several trends which may affect the molecules affinity for the IRES, which are discussed in Chapter 7.

It is important to note the difference between IC\textsubscript{50} and EC\textsubscript{50}, which are both used throughout this thesis. An IC\textsubscript{50} (half maximal inhibitory concentration) value refers to the effectiveness in inhibition of a specific biological process. The actual value indicates how much drug compound (inhibitor) is needed to inhibit that particular process by half. IC\textsubscript{50} values are most commonly used when analyzing biological function assays. On the other hand, an EC\textsubscript{50} (half maximal effective concentration) value refers to the concentration of a drug which induces a response halfway and is a common method to measure of drug’s potency. For example, discussions of recent antiviral drugs express IC\textsubscript{50} values for the drugs, while in the case of FRET testing, the change of FRET signal is measured as a result of binding to virus RNA. The value at which FRET quenching half way is referred to as the EC\textsubscript{50} (half of the total effect observed). Overall, IC\textsubscript{50} and EC\textsubscript{50} values complement each other very well indeed.

The central aim of this research was to understand the IRES binding site to a larger extent and interactively continue to modify ligands in a way as to optimize the binding...
interactions between the analog and the RNA. As more analogs continue to be synthesized, there is no doubt this diverse library will expand the way HCV treatments are thought about and be a tool in related pharmacological studies.

1.6 ARRANGEMENT OF THE THESIS

The research of this thesis is comprised of data for the creation of a large library of benzimidazole inhibitors to act against HCV. The data presented is the collection of synthetic strategies used towards the creation of seven diverse analogs. The total number of analogs synthesized by the Bergdahl group to date is fourteen, seven of which were synthesized according to the chemistry in this thesis. The remaining analogs were created by a coworker in the laboratory. Chapter 1 presents the background and prior investigations of compounds known to act against HCV, as well as an overview of goals and methods for the present research. Chapter 2 describes the recent synthesis of an aryl-substituted aminobenzimidazole inhibitor for HCV published by the Bergdahl group in 2011. Using the established synthetic approach as a foundation, the introduction of diversity is detailed in Chapter 3. Chapter 4 discusses the differences between the incorporation of primary and secondary amines into the synthetic approach. The majority of the novel research is presented in Chapter 5, with detailed synthetic strategies for the synthesis of six diverse derivatives. Chapter 6 summarizes the relevant biological activity of the 7 synthesized compounds, followed by a summary of all 14 structures synthesized to date and structure activity relationships in Chapter 7. The thesis concludes in Chapter 8 with future research directions. Experimental procedures and $^1$H and $^{13}$C NMR spectra are presented in Appendix A (Figures A.1-A.27) and Appendix B (Figures B.1-B.37), respectively.
CHAPTER 2

SYNTHESIS OF
DIHYDROPYRANOBIENZIMIDAZOLE

2.1 DIVERGENT SYNTHETIC STRATEGY

With the anticipation of developing a better synthetic strategy for the synthesis of the target dihydropyranobenzimidazole, a retrosynthetic scheme (Figure 2.1) was first developed based upon a similar chroman ring previously synthesized utilizing methods by Loiodice et al.40

The ten step total synthesis of the relevant anti-HCV compound recently presented by the Bergdahl group in 2011 is outlined in Figure 2.2.29 The synthetic approach began with a commercially available 2-chloro-6-fluorobenzaldehyde 2.1 in which the fluoro-substituent was displaced by the hydroxide in DMSO. The selectivity for displacing the fluoro-substituent compared to the chloro-substituent can be explained by solvent dependability as well as differences in basicity of the leaving group halogen. In a polar, aprotic solvent, such as DMSO, the fluoro-substituent has a stronger bond with the potassium cation in comparison to the less-electronegative chloro-substituent. The size of the fluorine atom, in comparison to the chlorine atom, might also favor a S_NAr type reaction at C-F bond connection. In the second step, the synthesized 2-chloro-6-hydroxybenzaldehyde 2.2 was converted into the desired chroman backbone via a Baylis-Hillman reaction with acrolein and
DABCO which proceeded in good yield to give 5-chloro-2H-chromene-3-carbaldehyde 2.3. The chroman aldehyde underwent a Tollens oxidation in the presence of silver oxide and was oxidized to the corresponding 5-chloro-2H-chromene-3-carboxylic acid 2.4. For the previous step, the Ag₂O was freshly prepared in-situ in a mixture of water, ethanol, and excess NaOH. Next, the chroman ring was reduced to 5-chlorochromane-3-carboxylic acid 2.5 under classical conditions using freshly prepared sodium amalgam and NaOH in quantitative yield. The coupling reaction of the prepared chroman ring 2.5 with EDC and dimethylamine in the presence of HOBt and N-methylmorpholine was carried out via the method of Yoshikawa et al. to yield 5-chloro-N,N-dimethylchromane-3-carboxamide (2.6) in excellent yield. 41 The nitration of 2.6 was carried out with finely powdered sodium nitrate in TFA to yield a 50% mixture of a 2:1 diastereomeric ratio in which the desired isomer 2.7 dominated in which the nitro group was introduced para to the activating alkoxy-substituent. The minor isomer generated was the ortho-substituted nitro group to the activating alkoxy group. Efforts to improve the yields by switching to nitronium tetrafluoroborate unfortunately lead to a
reversal of regioselectivity. A study was done by the Bergdahl laboratory to confirm the regiochemistry of desired isomer 2.7 via $^1$H NMR studies.\textsuperscript{29} The study consisted of easily separating the diastereomers using flash chromatography and performing a palladium-catalyzed hydrodechlorination/reduction of 2.7 to the corresponding analine (2.13) (Figure 2.3). Because extensive $^1$H NMR studies revealed only one pair of ortho-coupled aryl protons after reduction, the correct regioisomer 2.13 was identified. The coupling data revealed $J_{ab} = 8.5$ Hz, while $J_{bc}$ and $J_{ac}$ were not observed.\textsuperscript{29}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2.3.png}
\caption{$^1$H NMR study showing correct regioselectivity of nitrated compound.}
\end{figure}

Conformational analysis of inhibitor (±)-1.10 suggested that structures are very similar and both enantiomers would exhibit identical binding with the IRES. Incorporation of diversity to carboxamide 2.7 was accomplished by a nucleophilic aromatic substitution reaction (S\textsubscript{N}Ar) in which the commercially available mono-Boc-protected $N$-methyl-1,3-propanediamine displaced the activated chloro-substituent in good yield to give carboxamide 2.8. The Boc-protected $N$-methyl-propanediamine (2.12) was synthesized according to Figure 2.4. The primary amine of the readily available $N$-methylpropane-diamine was selectively protected as its trifluoroacetate (2.10) with trifluoroethylacetate in quantitative yield followed by the Boc-protection of the secondary amine in 98%. In the last step, compound 2.11 was refluxed in the presence of potassium carbonate in methanol to selectively remove only the trifluoroacetate protecting group leading to the $N$-methyl-$N$-Boc-propane-diamine (2.12) in 98%.
A hydrogenation of 2.8 with palladium catalyst followed by the immediate cyclization of the compound with a freshly prepared solution of BrCN in ethanol yielded the corresponding 2-amino-substituted benzimidazole compound 2.9 via the method of Lo et al. in good yield.\(^4^2\) Lastly, the desired final compound 1.10 was obtained via LAH reduction which not only reduced the dimethylamide carbonyl, but also the Boc-group of the alkylamino side chain to the corresponding methyl group in a 89% yield. Compound 1.10 was synthesized as a mixture of enantiomers with no need for separating the enantiomers as both had identical activity.

### 2.2 Recent Improvements in Synthesis

Because of the necessity of large amounts of the benzimidazole backbone to carry out the total synthesis of derivatized novel compounds, the initial seven steps of the total synthesis were repeated many times on different occasions. Advantages were discovered for specific reactions by optimizing reaction conditions. This work was conducted after the submission of the Bergdahl group publication.\(^2^9\) Most importantly, the reaction conditions of the nitration reaction in the sixth step of the synthesis were enhanced to maximize the yield as well as simplify the work-up procedure. Initially, the step consisted of stepwise addition of reagents at specific temperatures with adequate time in between and allowing the nitration reaction to stir for twelve hours below ambient temperature at 18 °C, followed by quenching and tedious exactions with 5% KH\(_2\)PO\(_4\). Instead, the reaction was capped tightly and allowed to stir for exactly eighteen hours at ambient temperature under an NO\(_2\) gas atmosphere. It is hypothesized that the NO\(_2\) gas formed during the reaction being trapped inside the reaction vessel aided in the progress of the reaction as opposed to previously having the possibility of escaping. The yield for the nitration step was increased from 55% to an impressive 88%.
Second, the number of equivalents of lithium aluminum hydride in the last step of the total synthesis was explored. Because the target molecule published by the Bergdahl group contained a Boc-group which needed to be reduced simultaneously with the dimethylamide carbonyl, using 37 equiv of LAH was deemed appropriate. Theoretically, in the absence of a Boc-group within the new HCV derivatives a much lower quantity of LAH should be adequate and would be favorable. When much less LAH was used (10 equiv), good yields were produced demonstrating that there is no need to go above 10 equiv of LAH in the absence of a Boc-group in the molecule of interest.

Lastly, during the cyclization of carboxamide (2.8) with BrCN, the solvent used after the Pd-catalyzed reduction was substituted from ACN to ethanol. It was speculated that the common acetamide contaminants in commercially purchased anhydrous ACN could be responsible for substitution of the NH$_2$ group with a methyl group.
CHAPTER 3

ENGINEERING A DIRECTED SYNTHESIS
TOWARDS NOVEL HCV INHIBITORS

3.1 CRYSTAL STRUCTURE-DIRECTED APPROACH

The three dimensional structure of the HCV RNA, which is illustrated in Figure 3.1, reveals an overall L-shaped architecture around the RNA internal ribosome entry site (IRES), which is supported by NMR and cryoelectron microscopy, but did not reveal any insight to binding mechanisms. The bent architecture is an important factor for the correct positioning of the initiation codon in the viral mRNA, along with directing loop 11b towards the ribosomal E site in proximity of the active site.

To acquire a crystal structure of subdomain IIa RNA-inhibitor complex, the original analog 1.10 was crystallized together with HCV RNA in optimally derived salt conditions. Upon inhibitor binding to the IRES element, RNA architecture was seen to adopt a linear shape, causing a halt in transcription of the virus. While the stereochemistry of inhibitor 1.10 was not seen in the bound crystal structure, when compound 1.10 was crystallized independently, observed enantiomers were determined sufficiently similar to permit for binding with target RNA of both isomers via observed hydrogen bonding and stacking interactions.

This can be described as a “ligand-captured” conformational change. Figure 3.2 shows a conformational switch diagram of the addition of a benzimidazole inhibitor (5) to the RNA. Benzimidazole (5) is in the same class as the synthesized analogs in this thesis and is therefore an excellent model to exemplify the mode of action on subdomain IIa. When an inhibitor is present in the system it established equilibrium between the two conformational states of the RNA, which are depicted on the right of Figure 3.2.

The usual bent shape of the HCV RNA (shown in the middle), which is stabilized by Mg ions, is absolutely crucial for proper positioning of subdomain IIa on the ribosome. The equilibrium pointing towards the bottom exemplifies the structural switch of RNA with
arginine and/or guanosine capturing the straightened conformation, thus leading to inhibition of viral protein synthesis. Additionally, to the right of the diagram, a diaminopiperidine derivative (6) is also shown to disrupt IRES function. The derivative does so by competing with the structural Mg\(^{2+}\) ions to bind to the IRES and lock the viral RNA in a bent state. Inhibition of the subdomain IIa function is likely caused by the constriction of RNA to a bent state which prevents ribosomal release.

The global conformational changes upon ligand binding were confirmed by NMR analysis, but the details of this novel mode of inhibition of the HCV genome need to be
Figure 3.2. Representation for the mode of action of a conformational change from bent to linear in HCV RNA upon introduction of benzimidazole inhibitor (5). Source: Dibrov, S. M.; Parsons, J.; Carnevali, M.; Zhou, S.; Rynearson, K. D.; Ding, K.; Garcia Sega, E.; Brunn, N. D.; Boerneke, M. A; Castaldi, M. P.; Hermann, T. J. Med. Chem. 2014, 57, 1694–1707. Studied more extensively. The extended conformation created a deep cavity in which the inhibitor was tightly engulfed (Figure 3.3).\textsuperscript{31} The strong binding interactions between the inhibitor and the RNA allowed for the compound to be rooted deep inside of the binding pocket, while the two dimethylamino groups of the molecule pointed towards the opening of the pocket (Figure 3.3).\textsuperscript{31} There appeared to be enough space to replace the methyl groups with larger substituents in order to improve binding. The successful completion of the crystal structure of inhibitor 1.\textsuperscript{10} bound to the HCV RNA coupled with the synthetic advantage of attaching variable side chains at a late stage in the synthesis developed by the Bergdahl laboratory has enhanced the ability to synthesize highly targeted compounds with refined SAR (Figure 3.3).\textsuperscript{29,31} A basic understanding of the mode of binding provides a first-hand opportunity to explore structural optimizations of analogs that are required for conformational induction of the IRES subdomain. Future substituents with improved properties will be chosen based upon the crystal structure published by Hermann et al.\textsuperscript{31}
3.1.1 Optimization of Amine-Phosphate Salt Bridges and Ligand Interactions

As seen in the high resolution crystal structure, it is known that the amino terminus of the side chain arm in compound 1.10 lies outside of the hydrophobic cavity. This carbon tether can be readily altered to improve the salt bridge strength between the analog and the phosphate backbone of the HCV RNA. Salt bridges are small and non-covalent stabilizing interactions which combine both hydrogen bonding as well as electrostatic interactions. Using the x-ray crystal structure as a model, research was aimed at increasing the nucleophilicity of the side chain arm nitrogen in anticipation of increasing hydrogen bond strength. At the same time, it was important not to increase hydrophobicity of the ligand for optimal activity. Ongoing efforts to optimize analogs have influenced the diversification of upcoming compounds. Numerous compounds have demonstrated excellent EC\textsubscript{50} values showing significance in the future of HCV treatments by means of this novel mode of inhibition (Figure 3.4).

3.1.2 Synthesis of Conformationally Restricted Analogs

Conformational restriction is commonly utilized to increase the affinity of a ligand for the target. Before analog 1.10 binds to the RNA backbone of HCV, the side chain arm is known to be floppy and in free rotation. Once the inhibitor forms interactions with the subdomain IIa, entropy of the ligand-RNA complex is significantly lowered as a result of the stabilization gained from the interactions. Gibbs free energy ($\Delta G = \Delta H - T\Delta S$) provides a framework for the possibility of increasing the strength of the binding interactions by varying the disorder of the un-complexed ligands. According to the change in Gibbs free energy, when the change in disorder (entropy; $\Delta S$) is smaller, the change in Gibbs free energy ($\Delta G$) becomes less negative, resulting in a more thermodynamically favorable reaction. The desire is to increase ligand affinity for the RNA by pre-constraining the side chain arm which would generate a smaller change in entropy. Binding affinity is anticipated to increase significantly by conformationally restricting the side chain of the analog. Altering such entropic considerations is known to heavily influence salt bridges on the surface of proteins. Conformationally restricted derivatives have already demonstrated outstanding binding-affinity results and ongoing work is directed towards the synthesis of additional derivatives of this nature. Synthetic routes of all analogs can be seen in Chapter 5 and inhibition levels are discussed in Chapter 7.

3.2 Observed Crystal Structure Interactions

The x-ray crystallography image obtained between the original inhibitor 1.10 and the HCV RNA was acquired at 2.2 Å resolution and has been serving as a fundamental guide for the synthesis of novel analogs. The deep ligand binding pocket, has proved to be truly intricate and sophisticated. The visual representation allows for the ability to see most of the drug molecule fitting tightly into this deep RNA cavity, while the free rotating side chain can be observed slightly outside of the pocket. High salt concentrations used during the sample preparation were discovered to be responsible for the co-crystallization of inhibitor 1.10 with the RNA complex. Similarly, magnesium ions played a large role in the stabilization of RNA folding as the phosphate atoms of the RNA backbone are stabilized by two Mg$^{2+}$ ions. Studies showed that in the absence of magnesium ions, RNA was not be able to fold and as a result, the ligand binding was not successful. Even at a much higher concentration of
inhibitor, the EC<sub>50</sub> value without Mg<sup>2+</sup> ions was measured to be 117 ± 8 µM in comparison to the 3.4 ± 0.3 µM for the original compound (1.10). Binding of benzimidazole compounds was not affected by presence of excess competitor RNA and salt, including magnesium ions, which indicates a very specific IRES-compound interaction not governed by electrostatic components.

Interactions extracted from the 3D image were both fascinating and groundbreaking; considerable information was gained from the detailed understanding of the bound cocrystallized structure of the complexed crystal structure and binding mechanisms. Many key features were revealed and among them, several prominent interactions were visibly observed. There were two salt bridges formed between the inhibitor and the RNA phosphate backbone, and more specifically, to the guanine heterocycle nucleobase of the guanine-cytosine in the C58-G110 base pair. One of these hydrogen bonds originated at the NH<sub>2</sub> group and hydrogen bonds with one of the nitrogen atoms of guanine. The second originates at a protonated nitrogen atom that is part of the chroman core (2.7) and hydrogen bonds with the carbonyl oxygen atom of guanine. Third, it is apparent that there are π stacking interactions of the benzene ring in the molecule with A53 and the G52-C111 pair. These stacking interactions can be visualized by observing the benzene ring in between the “ceiling” and “floor” of the RNA ligand cavity and therefore has π interactions with both the top and bottom of the pocket, which significantly stabilize the molecule in place. Additional binding sites are seen within both dimethylamino groups with the protonated form of the dimethylamino terminus of inhibitor 1.10 forming a salt bridge with the phosphate backbone of the RNA. To exemplify the specificity of these interactions and the significance of subdomain IIa, an interesting FRET study was published by Dibrov et al. The experiment demonstrated that when the location of guanine and cytosine base pairs of the RNA construct were interchanged, no interactions were observed with the HCV inhibitor. Likewise, replacement of the NH<sub>2</sub> group with a methyl group, discussed extensively in Chapter 5.8, led to the expected loss of activity of the molecule.

### 3.3 Late-Stage Introduction of Diversity

In the seventh step of the total synthesis, diversity was introduced into the molecule via nucleophilic aromatic substitution (S<sub>N</sub>Ar) as shown in Figure 3.5. The previously
synthesized chroman backbone (2.7) was introduced to a freshly prepared free amine with desired structural characteristics. The reaction was allowed to stir in the presence of N-Methyl-2-pyrrolidone (NMP) at 75 °C between one and three nights tightly capped. Although the S_N_Ar step has a relatively slow reaction rate with completion taking two to three nights at ambient temperature, there were several advantages in this particular step in the synthesis. In instances where the reaction was stirred for only 24 hours, yields were significantly lower. Alternatively, when the reaction was allowed to react for four nights, yields also decreased. It was hypothesized that at short reaction times, the substrates did not have enough time to react, when the reaction went too long, degradation of the product is certainly possible.

An important advantage to this step was the success of the reaction with all functionalized free amines to yield the predicted products. In addition, the reaction was high yielding with the majority of yields ranging from 85% to 99%. The manipulations were simple and only consisted of combining the chroman nucleus 2.7 with various free amines in the presence of NMP, while the work-up entailed a simple extraction with diethyl ether. The ability to recover unreacted starting material 2.7, in the event that the S_N_Ar reaction did not go to completion, was a benefit to this approach since the chroman nucleus was particularly precious at this stage in the overall synthesis. Often up to five equivalents of free amine were commonly used for this reason, which is further discussed in Chapter 5. Nonetheless, it was extremely time and cost efficient to be able to collect both leftover, unreacted pure isomers of 2.7 from the reaction in the same step as the purification of the product. Lastly, the bright yellow oil appearance of the generated product was advantageous, which was constant with all functionalized amines used. The yellow product was easily spotted on thin-layer
chromatography (TLC) and likewise was purified without difficulty on flash-chromatography and high-performance liquid chromatography (HPLC). Similarly, the disappearance of bright yellow color amidst the palladium reaction conditions was a quick and useful indicator that the reduction was complete.

3.4 CREATION OF DIVERSE LIBRARY

When deciding the functional diversification of analogs, a highly systematic and rational approach was used. The original 2-aminobenzimidazole core was left intact, while only the side chain was manipulated. Initially, it was proposed that modifying one of the methyl groups of the dimethylamino tether of molecule 1.10 into an electron-donating group would aid in making the outer nitrogen more nucleophilic. There was anticipation that a phenyl-methylamine analog would cause the EC$_{50}$ value to decrease accordingly. Due to unforeseen difficulties with synthesizing this analog, a similar inhibitor was considered due to its availability. A phenethyl-butylamine derivative was proposed to observe the effects of having an aromatic ring in near proximity of the binding nitrogen. Full syntheses of all proposed analogs are discussed to great extent in Chapters 5 and 7.

Next, a dibutyl-amino derivative was suggested in order to examine the differences between activities compared to the original inhibitor 1.10. A hypothesis was formed that predicted these analogs would be extremely similar in binding affinity as the structures are comparable. It was questioned whether observed activity would actually be similar to compound 1.10 to validate that the same interactions were occurring as were previously seen.

After work was done towards the synthesis of aliphatic derivatives, it was confirmed that rings and heterocyclic ligands would provide more attractive binding affinities based upon results acquired from several aliphatic and ring-containing derivatives. A piperidine ring derivative was synthesized next as it was predicted that its nitrogen atom would be relatively nucleophilic and similar as with the original dimethyl analog. Meanwhile, an identical synthetic scheme was employed to synthesize a morpholine derivative to understand what an additional oxygen atom in the heterocycle would do to binding affinity.

The next three analogs to be synthesized were chosen based on results gathered from the piperidine and morpholine derivatives. Once it was known that a morpholine-containing compound had much higher binding affinity than that of the piperidine ring, it was assumed
that the oxygen atom in the heterocycle was directly or indirectly participating in the interactions with the RNA. Either way, the addition of one oxygen atom in the side chain was the only difference in the two inhibitors and caused activity to increase nearly three-fold. The extra atom in the ring was assumed to have a significant role in binding activity and synthesizing a six membered ring with a nitrogen atom replacing the oxygen would potentially lower the EC$_{50}$ value even further.

There was also interest in studying the effect that side chain length had on the binding affinity inside of the binding pocket. Since the original molecule 1.10 had a chain length of three carbon atoms between the core nitrogen atom and “outer” nitrogen in the molecule, the variations that were interesting to study were the actual number of carbon atoms within the chain. The shorter, two-carbon, chain was successfully synthesized, while the longer, four-carbon, chain is currently in progress. Six additional piperazine compounds were proposed for further study of the heterocyclic derivatives, along with attention to chain length variations in hopes of acquiring valuable information about the behavior of the heterocycles within the analogs.

Two unique control compounds were designed to verify that the amino terminus on the side chain had an essential interaction with the IRES subdomain IIa; it was deemed necessary to investigate potency of such a derivative. The complete elimination of the amino terminus left the terminus of the side chain arm as a carbon atom. In addition, the substitution of the amino head group for an alcohol functional group was proposed to examine whether the alcohol proton would have higher hydrogen bond strength with RNA phosphate than the amino hydrogen bond. As discussed earlier, even with increasing amounts of acquired data, predicting activity strengths continued to be quite difficult. However, each novel analog contributed a more detailed understanding of the binding pocket and mechanism of the inhibition.

The methyl-NH secondary amine head group remains one of the most interesting compounds left to be synthesized and is currently underway. The methyl-NH analog is anticipated to shed light to the understanding of the binding affinity. This compound is significant as it will be the first analog to possess a secondary amine side chain in comparison to all tertiary amines. The original belief was that this analog was synthesized, but due to unforeseen circumstances, the analog cyclized in an undesirable location and did
not yield the methyl-NH-substituted derivative. Previous efforts to synthesize this particular analog are discussed in Chapter 5.7. A unique procedure to generate the desired inhibitor has been strategized and synthesis of this important inhibitor is currently under way.
CHAPTER 4

APPROACHES TO DIVERSIFICATION OF ANALOGS

4.1 USE OF SECONDARY AMINES

Amines have been very important functional groups in the realm of organic chemistry for decades. They have been commonly employed in commercial drugs and medicines, while occurring naturally in many amino acids, nucleic acids, and alkaloids.\(^45\) Because amines are of such significance, especially their role in reductive amination methods, much work has been directed toward their synthesis. Reductive amination has gained much popularity and is now known as one of the most valuable reactions for the generation of primary, secondary, and tertiary amines.\(^46\)

A wide range of aliphatic amine derivatives were initially proposed for synthesis, as they could lead to promising data. Proposed R\(_1\) groups to be introduced for the creation of the initial ten analog library were as follows: Me, Et, Pr, i-Pr, n-Bu, i-Bu, sec-Bu, t-Bu, neo-Pent, Bn; secondary library consisting of ten analogs was proposed to have the following R\(_1\) groups: Ph, Tol, 3,5-di-Me-Ph, 4-Cl-Ph, 4-MeO-Ph, 4-F-Ph, 3,5-di-F-Me, 3,5-di-MeO-Ph, F-5Ph, and Nap (Figure 4.1).

![Figure 4.1](image-url)  
Figure 4.1. Proposed diversity in the final HCV inhibitor; X = 0, 1, 2, 3, 4.
A useful synthetic approach has been found to employ secondary amines for the creation of desired HCV analogs. The phthalimide group has proved to be a highly useful tool in the synthesis of diverse free amines. The route was found suitable not only for the reactions with highly functionalized amines, but it was also found to be easily de-protected, leaving the desired primary amine in good yields. Phthalimide was initially reacted with a dihalide alkyl chain of varying lengths via Gabriel synthetic methods (Figure 4.2).

Interestingly, a chloride leaving group proved to work the best in these reactions as seen by higher yields in comparison to a bromide leaving group.

![Figure 4.2. First alkylation step in the diversification of analogs; n = 3 and 4; X = Cl or Br.](image)

Predominantly, 1-bromo-3-chloro-propane starting material (n = 3) was used for the majority of the analogs as it was hypothesized that a three carbon chain length would provide maximum activity against HCV. Later, the scope was expanded to a four-carbon chain length, using 1-bromo-4-chloro-butane, to be able to directly observe the effects that a longer side chain arm may have on drug activity. The 2-(3-chloropropyl)isoindoline-1,3-dione (n = 3) and 2-(4-chlorobutyl)isoindoline-1,3-dione (n = 4) products were generated in 80% and 77%, respectively. Alternatively, 2-(3-bromopropyl)isoindoline-1,3-dione provided significantly higher reactivity during the creation of the piperidine analog; it was synthesized in 83%.

The initial advantage to this route was being able to use a common initial method to construct analogs of different chain lengths by simply varying one substrate, allowing for a streamlined synthesis. Phthalimide alkyl chain compounds were then subjected to an $S_N$2 type nucleophilic substitution reaction using the desired secondary amines, such as piperidine and morpholine. Substitution reactions were carried out with many substrates which allowed for easy optimizations. It was expected that most amines could be introduced at this point for further diversification of analogs. Initially, $N$-methyl-benzylamine was used due to its
availability to examine the effectiveness of the reaction. The reagents were refluxed in acetone and provided good yields ranging from 80% to 92% (Figure 4.3). Intermediate 4.2 was then refluxed with hydrazine in methanol to give desired free amine 4.3 in excellent yield (92%).

![Figure 4.3. Successful route in synthesis of diverse free amines shown with N-methylbenzylamine.](image)

The difference between suitable and non-suitable secondary amines for this method were important to be noted. Non-suitable secondary amines were those which would participate in elimination instead of substitution reactions. Since both reactions share similar conditions, such as having a good leaving group, it is often the case that the reactions compete with each other. The factors influencing substitution require a good nucleophile and the use of polar, aprotic solvents which have been known to increase nucleophilicity. On the other hand, factors which favor elimination were an antiperiplanar β–hydrogen, a strong base and are heightened by α and β–branching and higher temperature.

The chemistry was performed on various secondary amines shown in Figure 4.4 and Figure 4.5. As in the case of methylbenzylamine, piperidine, morpholine, and N-Boc-piperazine, all were good nucleophiles and substitution occurred as expected (Figure 4.4). On the other hand, pyrrole, lithium diisopropylamide (LDA), imidazole, and N-tert butylisopropylamine, were stronger bases and elimination was likely the favored reaction (Figure 4.5).

![Figure 4.4. Successful secondary amines in the S_N2 reaction.](image)
The initial attempts to construct the morpholine derivative consisted of using acetone as a solvent, as it was known to work previously with N-methylbenzylamine. Despite having attempted the reaction in acetone, DMF, and THF, it was ultimately discovered that the order in which reagents were added were crucial for the success of the reaction. It was revealed that dissolving starting material (4.1) in acetone and adding it dropwise to a solution of stirring morpholine and NaI in acetone, proceeded to give product 4.4 in 72-86% (Figure 4.6). Over time, it was observed that the yields in acetone would increase as reaction times were lengthened suggesting that the reaction was slower than anticipated. This experimental data supported the range of yields accumulated for this reaction; 72% was obtained when the reaction was allowed to reflux for one night, while an 86% yield was achieved after allowing the reaction to reflux for three nights.

The morpholine substrate was also introduced into a longer chain phthalimide molecule (4.5), according to the protocol of Hay et al., and refluxed in DMF for one night yielding 4.6 in a 78% yield (Figure 4.7).

In Figure 4.8, the piperidine derivative was synthesized according to a method described by Bothmann et al. which suggested that the reaction be ran in butanone which generated the desired product (4.8) in 81%. Similarly to the reaction with morpholine, it was observed that yields ranged from 44-56% when the reaction was conducted for one night.
Figure 4.7. Successful synthesis with morpholine substrate and extended chain length.

Figure 4.8. High-yielding SN2 approach using piperidine and in the presence of NaI.

and jumped to 81% when allowed to react for two nights. In pursuit of the product, the reaction was attempted in acetone but only starting material 4.7 was observed.

When the route was expanded to react with imide (4.5) in butanone, via method of Valacci et al., yields ranged from 44-75% for compound 4.9 for one night and two nights, respectively (Figure 4.9).\(^{53}\)

Figure 4.9. Good-yielding SN2 route with piperidine and extended chain length.

Despite multiple attempts, the secondary amines, did not generate the desired products. The decreased non-reactivity of these substrates is likely due to the substrates undergoing elimination reactions. In the case of LDA, because the substrate is known to have strong base characteristics, it was unable to undergo substitution. The reaction with imidazole was attempted according to the method of Ratel et al., as well as by Press et al.
Due to the stable resonance structures of imidazole, it is possible that the aromaticity would alter it to be a non-promising nucleophile for the reaction. Likewise, pyrrole was unsuccessful in the $S_N^2$ reaction because of similar aromatic arguments. When the reaction was attempted with $N$-tert-butylisopropylamine, the large amount of bulkiness is a likely argument for the explanation of the unsuccessful reaction. Discovery of a publication by Abdel-Magid et al. confirmed this theory by reporting that among secondary amines, cyclic amines, such as morpholine, reacted faster than acyclic amines such as diethylamine, while sterically hindered amines, such as diisopropylamine, did not react even after several days. 

The next series of compounds were all synthesized according to various published routes. The proposed amines to be synthesized were $N$-Boc-piperazine ring derivatives with chain lengths varying from two to four carbons in length. Initially, it was planned to selectively Boc-protect the inexpensive and readily available piperazine to yield the $N$-Boc-piperazine (4.10) (Figure 4.10). The method of Dighe et al. called for solventless reaction of piperazine and BOC$_2$O for 2 minutes at 100 °C. The publication reported an 88% yield with the exact substrates, but after several attempts, only starting materials were present. Mono $N$-Boc-piperazine was then purchased in order to shorten the overall synthetic route for these analogs.

![Figure 4.10. Attempted mono-Boc protection of piperazine.](image)

Reaction conditions for the mono-Boc protection were optimized in several ways including: heating both materials until they dissolved prior to the microwave reaction described by the Dighe method, allowing the reaction to react for five times longer, increasing the power level, as well as a reaction scale up. In all attempts, only starting material (piperazine) was recovered.
An attempt to synthesize product 4.11 was performed by combining compound 4.1 and N-Boc-piperazine in the presence of dimethylacetamide (DMA) for one night using method of Jones et al., resulting in a 37% yield. In order to optimize yield, a change in the solvent was believed to increase success because it was previously seen in the synthesis of the piperidine functionalized amine. Butanone was attempted and showed a large increase in yield to 75% (Figure 4.11).

![Figure 4.11. S_N2 reaction between compound 4.1 and N-Boc-piperazine in good yield.](image)

Next, to expand the scope of carbon tether length, N-Boc-piperazine was reacted with molecule 4.9 in butanone to give 4.12 in a 66% yield (Figure 4.12).

![Figure 4.12. S_N2 reaction between compound 4.9 and N-Boc-piperazine in good yield.](image)

An interesting route for the base-promoted N-alkylation using various formamides as the N-sources in mild conditions without the use of a catalyst was described by Chen et al. Although N,N-dimethylformamide was not attempted by our group, published data reports the product being formed after just 3 hours, while other formamides are reported to yield products in 12 hours. An attempt was made with both benzylchloride and benzylbromide in
the presence of KOH and the reaction was reacted in neat water for 3 hours (Figure 4.13). Both times, the resulting inseparable mixture of products was troublesome to interpret, and product 4.13 was not observed. Replacement of the benzyl substrate for the alkyl electrophile, bromomethyl-cyclohexane, had not change on the outcome of the reaction. It appeared that either N-methylformamide was not an effective formamide for this approach or that these reactions did not go to completion and additional time would yield to desired C-N bond formation.

![Figure 4.13. Chen et al. attempted base-promoted N-alkylation; X = Cl, Br.](image)

Overall, multiple functionalized side chains were acquired using the methods discussed in this section. A conclusion was made that solvents were substrate specific and could not be optimized for general use in the SN2 nucleophilic substitution reactions. Although secondary amines had proved to be useful reactants towards the creation of HCV compounds, there was a desire to develop a method in which primary amines could be used for many reasons discussed in Chapter 4.2. The main reason was the greater availability of primary amines which would direct the synthetic approach towards lowering the cost and making the synthetic scheme more practical on a large scale. Secondary amines are slightly more basic than primary amines and they are therefore somewhat better nucleophiles. Thus, primary amines appear unsuitable for the established route because of their reduced nucleophilicity compared to secondary amines in SN2-type reactions.

### 4.2 Use of Primary Amines

With the desire of synthesizing secondary amines from primary amines easily and rapidly, numerous methods were explored. While many routes were troublesome and/or low-yielding, two methods stood out for their promising future in analog diversification. One of those routes consisted of synthesizing the desired secondary amines from simple primary amines and then introducing those amines into the previous established SN2 route. The
method of Cho et al. was explored for its simplicity and solventless technique in the creation of secondary amines. Successful synthesis of amine 4.16 entailed the solventless grinding of phenethylamine with acetaldehyde in 30% (Figure 4.14). During the synthesis, the imine intermediate (4.14) was isolated and verified by $^1$H NMR analysis.

![Figure 4.14. Successful synthesis of desired secondary amine using Cho methodology.](image)

These particular substrates were not tested in the publication of Cho et al., yet proved to give the desired products. During the investigation of the final product it was discovered that there was an extra broad $^1$H NMR signal in the product. After further examination, it was predicted that a second intermediate was being generated in the reaction. The intermediate was projected to be a boron complex (4.15) with the amine as a result of using sodium borohydride and boric acid. This boron complex possessed an NH, which would cause the broad $^1$H NMR signal due to the rapid intermolecular exchange of such a proton. The disappearance of the boron complex and appearance of product 4.15 could easily be verified via $^1$H NMR after refluxing the intermediate complex 4.16 in methanol for 16 hours. The yield of the product was rather low which was expected because of the nature of this reaction; grinding with mortar and pestle yielded a significant loss of product.

Using the freshly made amine (4.16), the next step of the synthesis was then successfully performed (Figure 4.15). In an attempt to increase the yield, it was seen that reflux in THF yielded the product 4.17 in 50%, while reflux in acetone gave the product in a 77% yield.

The method was also attempted with phenethylamine and phenylacetaldehyde to demonstrate its effectiveness of this method with other substrates (Figure 4.16). Identical conditions generated the desired product (4.18) in 27%, yet without the appearance of a boron-complex in this case.
Next, the same method was attempted with an aliphatic aldehyde (acetaldehyde) and benzylamine, but the reaction did not prove to be effective and product 4.19 was not formed (Figure 4.17). It was later realized that the boiling point of acetaldehyde was 20.2 °C, making it extremely likely that all of the acetaldehyde reagent evaporated during the overnight imine formation, which was open to air. It is highly likely that this route would prove to be successful if an alternative aliphatic amine was used. Nonetheless, this approach shows promising outcomes for the construction of various secondary amines needed in the future.
suppress overalkylations as well as promote primary amine alkylation in good yields.\textsuperscript{60} The group tested many bases, solvents and drying agents to optimize the selectivity of efficient mono-\(N\)-alkylation of various amines.\textsuperscript{60} Simple substrates were initially chosen to attempt the Cesium Effect under the method of Salvatore \textit{et al.}\textsuperscript{60} Because of observed difficulties in the accidental cleavage of the benzyl group, phenethylamine was used. Phenethylamine was reacted with butylbromide in the presence of cesium hydroxide monohydrate and allowed to react at ambient temperature for 20 hours (Figure 4.18). Purification and \(^1\text{H} \text{NMR} \) analysis showed the presence of two products; one was the desired product (4.20) and one was of the overalkylated compound (4.21). The publication stated that the ratio of desired product to overalkylated product was 9:1, however, experimentally, the ratio was observed to be 3:1 in favor of the desired product.\textsuperscript{60} The yield of only 40\% of the desired product 4.20 was observed and the remaining mass balance is made up by the overalkylated product. Regardless, this route opens up the door to the synthesis of extensive number of desired, aliphatic secondary amine ligands in a relatively simple way.

![Figure 4.18. Successful synthesis of secondary amine using Salvatore methodology.](image)

Secondary amine 4.20 was subsequently introduced into the established alkylation step to yield the desired phthalimide protected amine (4.22) in a 70\% yield (Figure 4.19).

![Figure 4.19. Successful S\textsubscript{N}2 route of compound 4.22.](image)
Throughout the course of this experimentation, an alternative reductive amination procedure was explored using sodium triacetoxy-borohydride (NaBH(OAc)$_3$). Sodium triacetoxy-borohydride was identified as a reducing agent in 1989 and has been considered to be the most suitable for selective reductive amination of aldehydes.$^{45}$ Sodium triacetoxyborohydride has now become one of the most effective, superior, and convenient reagents for reductive amination protocols.$^{46}$ The reagent is a relatively mild reducing agent in comparison to sodium borohydride and sodium cyanoborohydride and avoids the generation of toxic by-products.$^{46}$ Its mild properties can be explained by considering the steric and electron-donating effects of the three acetoxy groups which act to stabilize the reducing agent. Choosing a suitable reducing agent is critical to reaction success, especially in direct reductive amination reactions in which the imine intermediate is not isolated in the process.$^{46}$

Initially, alcohol $4.23$ was generated using phthalic anhydride and 3-aminopropan-1-ol in good yield via method of Pascale et al. and successfully converted to the corresponding aldehyde $4.24$ using Dess–Martin periodinane (DMP) in 70% based on publication via Klein et al. (Figure 4.20).$^{61,62}$

![Figure 4.20. Gabriel synthesis followed by corresponding aldehyde formation.](image)

The chemical literature contains several papers that demonstrate DCE being the solvent of choice due to its increased yields and decreased reaction times in comparison to similar solvents, such as THF, DMF, and ACN.$^{46}$ After the route was optimized, product $4.25$ was attained in 29% using the former route of Abdel-Magid et al. (Figure 4.21).$^{45}$ The difficult manipulations coupled with low yielding results led us to continue exploring alternative routes for the efficient synthesis of functionalized primary amines. The same reaction conditions were applied using sodium borohydride as the reducing reagent in this reaction, yet the desired product was not formed.
Two attempts were made to protect compound 4.25 with di-tert-butyl dicarbonate (Boc₂O). First, compound 4.25 was reacted with triethanolamine and DMAP in anhydrous DCM, followed by the addition of Boc₂O according to the methodology of Brass et al. (Figure 4.22).63 Subsequent work-up and analysis showed only unreacted starting material 4.26, which was likely caused by time-worn reagents.63

In addition, compound 4.25 was allowed to react with 30% triethylamine (Et₃N) in anhydrous MeOH, followed by the addition of Boc₂O. Similar results to the previous attempt were attained and no product (4.26) was observed. There is a possibility that the secondary nitrogen of 4.25 is attacking the carbonyl group of the phthalimide group and that an equilibrium of those two products exists, making it difficult to Boc-protect the amine.

The methodology described by Graham et al. consisted of reacting aldehyde 4.25 with a primary amine, such as the simple butylamine, and charging the reaction with anhydrous magnesium sulfate in triethylamine.64 The protocol successfully yielded imine 4.27 in 32%; the imine intermediate was verified via ¹H NMR analysis. Subsequently, imine 4.27 was next treated with sodium acetoxyborohydride in the presence of glacial acetic acid to yield the desired product (4.28) in 42% (Figure 4.23).45
Figure 4.23. Imine formation followed by reduction to secondary amine 4.28.

The next step was to introduce a Boc-group to compound 4.28, making it a suitable secondary amine for the future S_N_Ar step. The Boc-group was introduced in a very traditional manner by adding Boc₂O to the reaction and letting it stir at ambient temperature (Figure 4.24). Unfortunately, this approach did not yield the desired product (4.29) and only starting material 4.28 was recovered upon work-up. Addition of potassium carbonate and the optimization of solvent (DCM:ACN) conditions was believed to yield a successful Boc-protection via method of Kang et al., but only starting materials were seen. It is possible that the secondary nitrogen is attacking the carbonyl of the phthalimide group and that an equilibrium of those two products exists, preventing interaction with the Boc₂O reagent.

Figure 4.24. Attempted Boc-protection of secondary amine via a traditional method.

Alternatively, compound 4.30 was achieved successfully in 56% yield using phthalic anhydride and previously synthesized N-Boc-1,3-diamine-propane. Compound 4.30 was then treated with benzylbromide in the presence of sodium hydride and anhydrous DMF according to the method described by McMills et al. but product 4.31 was not observed (Figure 4.25).

Figure 4.25. Attempted reductive amination procedure using the method of McMills.
After some optimizations, $^1$H NMR analysis suggested the disappearance of the phthalimide group. The actual product formed using $^1$H NMR spectra was predicted to be Boc-protected $N$-benzyl-$N$-Boc-butan-1-amine hydrate. The harsh conditions generated by NaH seem to have caused the opening up of the phthalimide group, which was removed during the reaction. To further investigate this occurrence, another substrate (allyl bromide) was chosen to perform the reaction an additional time (Figure 4.26). Once again, the cleavage of phthalimide was observed via $^1$H NMR analysis without the generation of desired product 4.32. Such a reaction was deemed unsuitable for the desired approach. It is predicted that water may be contaminating the anhydrous DMF causing the formation of NaOH and $H_2$ gas in situ, in which case NaOH would certainly be likely to attack the carbonyl of phthalimide causing the group to open up. To confirm this occurrence, NaH in DMF will be combined with compound 4.30 to verify that these conditions destroy the substrate.

![Figure 4.26. Attempted reductive amination using 3-bromoprop-1-ene via method of McMills.](image)

Another approach which was explored consisted of producing an imine intermediate by reaction of a primary amine and aldehyde, which was proposed to be subsequently reduced to the corresponding secondary amine. First, phenethylamine was mixed with propionaldehyde in water at ambient temperature for 3 hours under the route of Etchells *et al.* and Simion *et al.*, but did not yield the desired product (4.33) (Figure 4.27). It is possible that this approach only works with aromatic aldehydes, as supported by the two publications. An alternative reductive amination method utilized the reagents TEMPO (a catalyst for the oxidation of primary alcohols to aldehydes) and a stoichiometric ratio of BAIB (compound necessary to regenerate TEMPO)
Figure 4.27. Attempted reductive amination in water via method of Etchells and Simion.

(Figure 4.28). The publication by Guerin et al. reported this method with octanol and benzylamine as substrates, but octanol was the only substrate the publication presented.70 Alcohol 4.23 was allowed to stir at ambient temperature in the presence of Tempo and BAIB via method of Guerin et al. until the complete conversion of alcohol.70 Benzylamine and NaBH₄ were then introduced into the reaction and the reaction was allowed to stir for 20 hours, yet product 4.35 was not formed.70

Figure 4.28. Attempted TEMPO and BAIB assisted reductive amination.

The route presented by Guerin et al. was advantageous as it would make the total synthesis shorter because the intermediate aldehyde would be generated in situ and would not need to be separately isolated and purified.70 The challenge of optimizing reaction and work-up conditions proved to be a major disadvantage using this approach. After several purification attempts, product still showed too many impurities and only slight signs of the desired product via ¹H NMR analysis. Several other methods were attempted, but are not recommended for their complexity, difficult manipulations, and difficulty in product formation.
Overall, the ability to generate particularly designed secondary amines from primary amines was not a simple task. However, with all methods explored, two remain as being compatible with a large variety of substrates. The methods of Salvatore et al. and Cho et al. have been optimized and it has been established that these routes show promising possibilities for future synthesis of functionalized amines. The publication of Cho et al. provides the possibility for generating a large diversity of functionalized aromatic secondary amines (and likely aliphatic amines), while the route of Salvatore et al. makes available the synthesis of a variety of functionalized aliphatic secondary amines. These diversified amines will be introduced to the chroman core in order to generate final analogs with desired functionality.
CHAPTER 5

LIBRARY CREATION OF SYNTHESIZED ANALOGS

5.1 BENZYL-METHYLAMINE DERIVATIVE

After the successful synthesis and testing of original inhibitor (1.10), it was decided that similar compounds should be designed in a way to increase binding with the IRES element by interactively refining SAR. Among the first modifications initiated was the replacement of one methyl group on the dimethylamino side chain arm with a benzyl group. The thought process consisted of replacing an electron donating group in hopes of making the adjacent nitrogen a better base (or nucleophile) and increase hydrogen bonding interactions with the virus RNA.

5.1.1 Initial Synthetic Approaches

The synthetic route of the benzyl-methyl analog was pursued with the generation of imide (4.1) which was then used as an electrophile in an S_N2 reaction with methybenzylamine to yield compound 4.2 in 92%, as previously mentioned in Chapter 4. Phthamidide deprotection via route of Wu et al. of 4.2 led to the S_NAr step with the freshly synthesized free amine 4.3 and the chroman core 2.7 (Figure 5.1). During the S_NAr step for each of the analogs synthesized, the free amine was used in a 5 equivalent excess over the chroman compound 2.7. Unless an external base is added, this is expected since the first equivalent would be rendered inactive once protonated, forming the hydrochloride salt due to the hydrogen chloride generated in situ. The remaining four equivalents of the base were used to ensure completion of the reaction with the desired isomer since the reaction was relatively slow. The subsequent two manipulations were proposed to include reduction of the nitro group, followed by cyclization of the molecule using cyanogen bromide and lastly reduction of the amide group with LAH (Figure 5.1). Surprisingly, once compound 5.1 was subjected to palladium reduction and BrCN cyclization, 1H NMR analysis did not support formation of the desired product (5.2) due to the elimination of the benzyl group.
Figure 5.1. Initial proposal of benzyl-methyl derivative synthesis.

5.1.2 Cleavage of Benzyl Group

After careful examination of the $^1$H NMR data, it was observed that the aromatic signals corresponding to the benzyl group were absent in the proton spectra. The disappearance of these proton signals in the aromatic region hinted towards the disconnection of the benzyl group attached to the amine. After brief investigation, it was realized that palladium catalyst in presence of hydrogen gas (H$_2$) has the ability to readily remove benzyl groups indeed. Benzyl group eliminations have been known to be a smooth reaction. It was also learned that the presence of substituents on the aromatic ring affect the rate of benzyl ether hydrogenolysis in various ways. Soon after, it was confirmed the palladium catalyst was, in fact, responsible for the benzyl cleavage during the reduction step producing compound 5.4 (Figure 5.2). Since there was no need for isolation and purification of
intermediate compound after reduction and before cyclization, reduction and cyclization steps were performed back to back to minimize unnecessary exposure of the compounds to moisture in the air. As a result, $^1$H and $^{13}$C NMR data was not gathered for the reduced intermediate, which, when done in the future, will provide evidence to support the cleavage during that particular step. Therefore, with the complete disappearance of aromatic protons coupled with the widely established benzyl-group removal during Pd-catalyzed hydrogenation conditions, a new strategy was required to be employed for the construction of such an analog.

Because the route for reduction of the nitro group with palladium was already optimized to a large extent, it was decided that the reduction step itself would not be altered, and instead, a unique route would have to be developed at a later time.

5.2 Phenethyl-Butylamine and Dibutyl Compounds

The generation on the phenethyl-butylamine derivative began with the synthesis of phenethylbutyl-amine 4.20 (Figure 5.3). The following two steps consisted of an $S_{N}2$ reaction, which went in a 70% yield, followed by the deprotection of phthalimide to yield compound 5.6 in 96% via methods described in Chapter 4.2. Next, $S_{N}Ar$ with chroman 2.7 gave molecule 5.7 in quantitative yield. Compound 5.7 is currently awaiting cyclization and reduction (Figure 5.3). The final step of reducing the amide in the molecule will give the desired product 5.9. Up to this point, the seven step convergent synthesis has yielded the desired compound 5.7 in a 37.1% total yield, with two steps remaining to finish the overall total synthesis.
Next, the synthesis of final compound (5.12) was initiated in a relatively straightforward manner. N,N-dibutylamino-1,3-diamino-propane was exposed to the SNAr reaction with the chroman nucleus (2.7) which proceeded to generate compound 5.10 in quantitative yield. The generation of the correct product was confirmed via ¹H NMR in a quantitative yield based upon the desired starting material isomer of 2.7. A method was designed on HPLC to effectively separate the compound from the remaining undesired isomer of 2.7. The synthetic route currently has just two steps remaining for the completion of analog 5.12 (Figure 5.4).

### 5.3 Piperidine Derivative

The overall synthetic route towards the piperidine-containing derivative is displayed in Figure 5.5. First, a three step synthesis was designed to generate the functionalized free amine 5.13, discussed in Chapter 4.1. Gabriel synthesis yielded compound 4.7 in 83%, which was followed up by introducing piperidine to the molecule to give compound 4.8 in 81% (Figure 5.5). In an attempt to simplify manipulations, the common hydrazine deprotection conditions that had previously been successful in good yields were substituted with a method described by Vooturi et al. The publication described deprotecting the same imide (4.8) using
Figure 5.4. Route for the creation of a dibutyl-containing analog.

Figure 5.5. Overall synthetic route for creation of piperidine-containing analog.

a much simpler and less time-consuming procedure without the need for HCl and NaOH. However, the yield obtained (36%) was significantly lower compared to the previous approach, thus the phthamilide group was deprotected under the usual hydrazine conditions to give the functionalized free amine 5.13 in 87%.
Next, amine 5.13 was introduced to compound 2.7 and underwent a smooth SNAr type reaction and generated a bright yellow oil (5.14) in 97%. In order to ensure complete consumption of the substrate, the reaction is usually allowed to react for up to three days with the functionalized free amine being introduced which will displace the chloro-substituent. The final two steps consisted of cyclization of the molecule as well as final reduction of the amide, which were both successfully carried out to form inhibitor 5.16, in 33% and 88%, respectively (Figure 5.5). The nine step synthesis had an excellent overall yield of 10.6%. In terms of yield, it is important to note that reactions were only performed once or twice at the most and therefore yields will be increased once reaction conditions are optimized, when higher yields are necessary for a large-scale production. Interestingly, when free amine (5.13) was combined with the mixture of chroman isomers (2.7), two products were formed, hypothesized to be a result of the amine reacting with both isomers. TLC supported this theory by showing two noticeably different bright yellow product spots with an Rf difference of roughly 0.3. In addition, 1H NMR signals also confirmed the presence of more than one product. No success was seen in the separation of these two compounds with the use of flash chromatography and HPLC. Although evidence supported the formation of two products of the corresponding isomers, it was previously established that the reactivity of the undesired 2.7 isomer is considerably lower, and it had never been observed to undergo an SNAr type reaction having the nitro group in the para position relative to the chloro-substituent. While sterics of the nitro group are a slight consideration, the electronic effects are more relevant. When the nitro group is ortho to the chlorine in 2.7, as in the case of the desired isomer, the nitro group accepts electron density much more readily from the benzene π system, making the π system more electrophilic which is favorable for the subsequent SNAr reaction. The functionalized free amine is then able to displace the chloro-substituent in the ortho-nitro isomer significantly easier in comparison to the para-nitro isomer in which the nitro group is much further away from the chloro-substituent.

Separation of isomers of the chroman backbone (2.7) was then performed prior to the reaction in order to react the amine with solely the desired isomer. As hypothesized, the pure isomer reacted with the free amine (5.13) and led to one bright yellow product which was easily purified via flash chromatography and further synthesis was continued without any difficulty. In order to ensure the unreactive nature of the undesired isomer 2.7, a proposal for
attempting an S_N_Ar reaction with free amine \textbf{5.13} and the pure undesired isomer of \textbf{2.7} is outlined in Chapter 8.2. Extensive characterization of such a product will give us better knowledge and understanding of the chemistry taking place during the S_N_Ar reaction and give us an increased understanding of directing groups at work.

Variations in chain lengths within the piperidine derivative were explored for the interest in understanding the free rotating nature of the carbon side chain. First, the synthesis of a longer alkyl side chain was initiated with Gabriel synthesis which provided compound \textbf{4.5} in 77\%. Next, S_N_2 reaction with piperidine gave intermediate \textbf{4.9} in 44\% followed by the deprotection of phthalimide in 90\% to give amine \textbf{5.17} (Figure 5.6). The amine was then reacted with chroman \textbf{2.7} to yield the S_N_Ar product (\textbf{5.18}) in a 48\% yield. If this derivative is of interest in the future, only two steps remain to acquire the final analog. In order to concentrate our efforts towards derivatives of higher potency levels, further optimizations of the side chain of the piperidine derivative have not yet been performed. Instead, efforts to explore heterocyclic groups begun.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5_6.png}
\caption{Figure 5.6. Piperidine derivative synthesis with additional carbon in side chain arm.}
\end{figure}

\section*{5.4 MORDERLINE DERIVATIVE}

The morpholine-containing ligand was created to explore heterocyclic rings and the activity that these rings may possess against HCV. The synthesis of final inhibitor \textbf{5.24} proceeded without any complications and consisted of all high-yielding reactions. Previously seen effectiveness of secondary amines in the already established Gabriel synthetic method was carried out in 83\% (Figure 5.7). Next, morpholine was introduced via S_N_2 reaction in to
Form molecule 4.4 in 86%, which was subject to phthalimide group removal in a 47% yield forming the desired morpholine-containing free amine 5.21 (Figure 5.7). The remaining three steps of the synthesis all went extremely well, especially the cyclization step that afforded an excellent 81% yield. An advantageous was that all intermediates had simple purification methods. A remarkably high overall yield (21.3%) was accomplished for the nine step synthesis of the final analog (5.24).

The synthesis of three analogs in this series was originally proposed, which included the two-carbon and four-carbon variations in chain length. The synthetic route in Figure 5.8 will be continued with four additional steps, which will yield the final inhibitor (5.25). Likewise, the two-carbon side chain morpholine analog can be prepared using a similar approach.

**5.5 VARIATION IN SIDE CHAIN ARM LENGTH**

From information gathered from the original inhibitor (1.10) having the highest potency against the IRES subdomain IIa, it was determined that variations of chain length would be an interesting study.43 Inhibitors possessing shorter and longer carbon-chain length were synthesized and will create an increased understanding of the necessary parameters for designing substrates with optimal binding.
Figure 5.8. Proposed method to synthesize the four-carbon side chain with morpholine terminus.

The synthesis of a two-carbon side chain analog with a dimethylamino-substituted terminus was initiated. The methodology reported by Liu et al. was used to simultaneously protect both terminus of the diamine starting material. Thus, N-methylpropane-1,3-diamine was first exposed to ethyl trifluoroacetate and subsequently treated with Boc₂O yielded the desired product (5.26) in 70% (Figure 5.9). Compound 5.26 was then deprotected via the method described by Urakami et al. by using potassium carbonate in methanol to give amine 5.27 in 68%. This route proved to be advantageous as it eliminated one protecting step during the synthesis of the amine, making the synthetic route more economical. Next, the remaining three reactions were performed to yield final inhibitor 5.30 (Figure 5.9). The majority of yields were high-yielding resulting in 11.2% for the nine step synthesis of analog 5.30.

In order to provide a more comprehensive understanding of the length of the side chain, and directly compare the compounds with identical terminus functionality, a dimethylamino 4-carbon tether compound was proposed. Thus, a route was devised which included making the functionalized amine (5.34) according to the method of Gavande et al. This consisted of an initial Boc-protection of the readily available 4-aminobutanoic acid in 82% to give compound 5.31 (Figure 5.10). The proposed next step was to simultaneously reduce the carboxylic acid to the corresponding alcohol as well as reduce the
Figure 5.9. Incorporation of freshly synthesized amine into chroman core (2.7) for the generation of a shorter side chain arm inhibitor.

Figure 5.10. Attempted route for the synthesis of a four-carbon chain length side arm.

Boc-group to provide compound 5.32, which would then be subjected to a second Boc-protection and replacement of the alcohol group with an amine group to give compound 5.34 (Figure 5.10).

However, due to low-yielding reactions, we developed a superior approach to ligand 5.37, illustrated in Figure 5.11. 4-Aminobutanoic acid is a suitable starting material which can be reduced to the corresponding alcohol (5.35) using sodium borohydride and I$_2$.
Figure 5.11. Proposed synthesis of a 4-carbon chain length side arm.

according to the method of McKennon \textit{et al.}\textsuperscript{[76]} Next, $N$-Boc-protection of the amine is proposed to give intermediate \textbf{5.36}. The final three steps are proposed to replace the alcohol with a better leaving group (Br), followed by the incorporation of phthalimide and lastly the cleavage of the phthalimide with hydrazine to generate the primary amine product \textbf{5.37}. A second Boc-group attached to the opposite amine terminus was established to be highly advantageous in order to change the basicity of the opposite terminus nitrogen atom, simple removal of the Boc-group in the last step provides a desired increase in basicity of the terminus nitrogen atom for the increased binding with IRES subdomain IIa.

\textbf{5.6 PIPERAZINE-LIKE DERIVATIVES}

Synthesis of the piperazine-like-containing analogs was initiated after results from the binding studies of the corresponding piperidine and morpholine compounds. The presence of the additional heteroatom showed a significant increase in binding of the morpholine analog to the HCV IRES. Figure 5.12 shows the proposal of six various piperazine-like free diamine side chains to be used in the $S_N$Ar coupling to obtain six corresponding inhibitors having additional heteroatoms available for binding.

Figure 5.12. Proposed variations of series of piperazine-like derivatives; $X = 1, 2, 3$; $Y = 1, 2$. 
Currently, only the two last steps in making these compounds remain for each of the three finals analogs (X = 1, 2, and 3) with Y = 2 to be fully synthesized and will be under examination for IRES binding affinities (Figure 5.12). Below is the synthetic route for the two-carbon-containing (X = 1) and 6-membered ring (Y = 2) final analog (Figure 5.13). A method described by Han et al. was explored by refluxing 1-(2-aminoethyl)piperazine in the presence of benzaldehyde, which temporarily protected the primary amine, followed by the addition of Boc₂O and workup led to the successful synthesis of compound 5.38 in 64%. Next, compound 5.38 underwent a S_N_Ar reaction with compound 2.7 to give the desired carboxamide 5.39 in 85%. The remaining two steps in the synthetic route, consist of a cyclization reaction and amide reduction, which then will yield the functionalized analog 5.41 (Figure 5.13).

![Synthetic route](image)

**Figure 5.13. Synthesis of functionalized free amine (5.38) followed by its coupling to chroman (2.7) to yield two-carbon, 6-membered ring final inhibitor.**

In an attempt to improve the yield of the formation of the generation of free amine 5.38, method of Laduron et al. was also explored. This involved the selective Boc-protection of the secondary amine nitrogen atom in the presence of a primary amine using azeotropic distillation. Thus, 1-(2-aminoethyl)piperazine was refluxed in methyl isobutyl ketone (MIBK) to form the corresponding imine, which then allowed the secondary amine to react with an alkylating or acylating agent, in this case, Boc₂O. The reagents were refluxed until the generation of water from the reaction had come to a stop, which signified the completion
of imine formation, at which point, Boc$_2$O was added in one portion (Figure 5.14). While the paper reported the product : diacyl ratio of 97:3, work-up led only mainly the recovery of starting material.

![Figure 5.14. Attempted approach to selectively Boc-protect the secondary amine of 2-(piperazin-1-yl)ethan-1-amine.](image)

The three carbon chain length ($X = 2$) and 6-membered ring ($Y = 2$) amine (Figure 5.12) was synthesized using a different approach beginning with an $N$-Boc-piperazine coupling in an S$_N$2 reaction with compound 4.1 to generate molecule 4.11 in 75% (Figure 5.15). Next, phthalimide deprotection led to free amine 5.42 in an excellent 91% yield. The S$_N$Ar step went in 74% followed by the cyclization and amide reduction to generate the final inhibitor 5.45.

The four-carbon chain length ($X = 3$) and 6-membered ring ($Y = 2$) amine in Figure 5.12 was created in a very similar approach to the previously described amine 5.42. Thus, the $N$-Boc-piperazine and compound 4.9 were coupled via an S$_N$2 reaction which led to compound 4.12 in a 66% yield (Figure 5.16). Next, phthalimide deprotection led to free amine 5.46 in 87%. The subsequent S$_N$Ar coupling gave intermediate 5.47 in a 63% yield. This analog will be followed by the cyclization and reduction of the amide (Figure 5.16). The syntheses of the remaining three compounds in the piperazine series for $Y=1$ and $X=1, 2,$ and 3 compounds is proposed in Chapter 8.

### 5.7 Control Compound Analogs

A proposal was initiated to construct analogs which would prove the importance of the presence of amino terminus of the side chain arm extended from the backbone of the molecule. By designing such analogs and testing corresponding EC$_{50}$ values, it would be possible to access the significance of the amino terminus in terms of potency towards HCV. In the absence of the amino group, it is predicted that activity would decrease considerably, with a high probability of a total loss in potency. An initial proposal consisted of the
Figure 5.15. Synthesis of functionalized free amine followed by incorporation to chroman (2.7) to yield three-carbon, 6-membered ring final inhibitor.
Figure 5.16. Synthesis of functionalized free amine 5.46 followed by incorporation to chroman (2.7) to yield four-carbon, 6-membered ring final inhibitor.

synthesis of two analogs; one analog with the complete elimination of the amino terminus leaving a methyl terminus, and the other side chain undergoing a replacement of the amino group for an alcohol terminus.

5.7.1 Elimination of Amino Terminus

For the creation of the alkyl chain analog with a carbon terminus, the readily available and inexpensive butylamine was directly introduced into the chroman nucleus to generate compound 5.50 in an 83% yield (Figure 5.17). The remaining three steps led to the desired final inhibitor 5.62 in a 4.7% total overall yield over the nine steps without any optimization of the three last reactions in the overall sequence.

During the purification of the S_NAr product (5.50), it was interesting to observe that the chroman molecule (2.7) and compound 5.50 had identical R_f values. An effective purification method was successfully developed using HPLC and the final two steps were smoothly performed. FRET binding and ex-vivo cell assay results of analog 5.62 are in-progress.
5.7.2 Replacement of Amino Terminus with Alcohol Group

Likewise, substitution of the nitrogen atom at the terminus of the side chain arm for an oxygen atom was predicted to have a potential decrease in binding affinity for the IRES. Synthesis of such an analog was anticipated to provide essential data towards deeming the nitrogen atom crucial. There is a slight possibility that hydrogen bonding with an oxygen atom would lead to a stronger binding affinity, which would be truly fascinating if this would be the case, but it is difficult to predict. The knowledge gained from the binding affinity results of compound 5.57 are expected to be very valuable.

The creation of the desired free amine 5.54 was possible in just three steps. Thus, phthalic anhydride reacted first with 3-aminopropan-1-ol to yield isoindoline-dione 4.23 in 45% followed by introduction of 4.23 in the presence of benzylbromide and sodium hydride which successfully formed intermediate 5.53 in 41% (Figure 5.18). Next, the usual phthalimide deprotection was isolated in quantitative yield and the free amine 5.54 was ready for an S_N_Ar reaction upon which the product (5.55) was formed in 98%. The generation of structure 5.56 was expected upon palladium reduction and cyclization, which would be later reduced to final analog 5.57 (Figure 5.18). However, it was noted that the benzylic aromatic proton signals remained in the ^1_H NMR spectrum after the Pd-catalyzed hydrogenation. It was expected that the benzyl group would have been eliminated for this reason, as previously observed during the synthesis of the N-methyl-N-benzylamine analog (5.3).

Instead, after the Pd-catalyzed hydrogenation of compound 5.55; the benzyl group remained attached on the ether oxygen to provide compound 5.58 in 24% (Figure 5.19). The yield of 5.58 was rather low, and no other products were isolated after the work-up procedure. After purification, intermediate 5.58 was reduced with LAH to provide analog 5.59 in 70% of the crude product (Figure 5.19).
Figure 5.18. Proposed synthetic approach to analog 5.57 having an alcohol side chain.

Figure 5.19. Reduction and cyclization followed by amide reduction providing 5.59.

Although benzyl cleavages are known to be relatively fast, it is anticipated that if the Pd-catalyzed hydrogenation is conducted with a different catalyst (e.g. Pd(OAc)$_2$), the benzyl cleavage should be successful. Moreover, it was learned that nonsubstituted benzyl ether cleavages, such as this one, have a comparatively lower relative rates in comparison to more substituted benzyl ethers or benzyl amines.$^{72}$

It was decided that another attempt would be made to remove the benzyl group, for instance by taking advantage of a different catalyst. It is also known that treatment with LAH occasionally can cleave benzyl ether groups, and is seldom the practice because of the reagents’ reactivity to many other functional groups.$^{79}$ Since it was already established that the molecule of interest would survive treatment with LAH, method of Kutney $et$ $al.$ was attempted.$^{79}$ Compound 5.58 was refluxed overnight in the presence of 40 equiv of LAH in THF. After purification, it was determined that the amide group was successfully reduced in 70%, but the benzyl ether still remained. Nevertheless, the undesired compound 5.59 was
synthesized in 6.2% overall yield in nine steps. The benzyl ether-containing compound was not submitted for IRES binding testing due to the presence of the large, hydrophobic benzyl group which would prevent strong binding interactions with the active site.

As indicated previously, an alternative approach must be used in order to detach the benzyl group from the ether oxygen atom. Benzyl ether groups were found to be deprotected in high yields using a single electron oxidant such as 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ). For example, \(p\)-methoxy benzyl ether can be cleaved with DDQ because the attached methoxy group is observed to stabilize intermediates because of its resonance. More recently, simple benzyl ethers have been successfully cleaved with the use of DDQ using photo-irradiation. Alternatively, the cleavage of benzyl ethers is also possible using strong acids, but this method is limited to acid-insensitive substrates. Additional methods will be explored in the near future to successfully cleave the benzyl group to generate final analog 5.61 (Figure 5.20).

Figure 5.20. Proposed synthesis to yield the desired alcohol 5.61.

5.8 SECONDARY AMINES; THE RMENH ANALOG

The secondary amine ligand arm remains of utmost importance in the analysis of the interactions that are taking place inside and outside of the RNA binding pocket. By altering the side chain of the original molecule to possess a secondary amine group, three carbon chain length away from the cyclized nitrogen atom, it was hypothesized that the increase in the nitrogen atom’s basicity would cause a significantly stronger bond for the HCV RNA subdomain IIa. The basis of initiating synthesis of this particular inhibitor was that the basicity of a secondary amine is slightly higher than that of a tertiary amine. This argument can be supported by \(pK_a\) values of a tertiary amine being roughly 9.81 and a secondary amine
roughly 10.73, with the higher pKₐ signifying the more basic compound. As a result of the bond between the more basic nitrogen atom and the HCV RNA being stronger, the active site would be conformationally more captured therefore lowering the EC₅₀ value and increasing the effectiveness of the compound.

5.8.1 Current Approach

Because the significance of such an analog remains high, a unique route was initiated for the synthesis of this compound. The newest direction, designed by Mr. David Schmit in the laboratory, involves using N-methylpropane-1,3-diamine as the starting material. Protection of the primary amine using ethyl trifluoroacetate and subsequent protection of the secondary amine using N-[2-(Trimethylsilyl)ethoxycarbonyloxy]succinimide (Teoc-OSu), yielded compound 5.62 in 10%. The trifluoroacetate group was then deprotected to yield the free primary amine (5.63) in 89% (Figure 5.21). After obtaining the S_N_Ar product (5.64), the continued cyclization and reduction remain.

![Reactions Diagram]

Figure 5.21. Alternative route to RMeNH analog by Mr. David Schmit.

5.8.2 Initial Synthetic Approach

The original synthetic approach, the synthetic route began with the readily available phthalimide which led to the desired free amine 4.3 in only three steps. With the successful synthesis of this ligand at hand, the final three steps of the synthesis consisted of an S_N_Ar reaction, followed by a stepwise reduction, then cyclization and lastly the reduction of the amide to yield the hypothesized product 5.67 (Figure 5.22). Because it was previously
established that the Pd-catalyzed hydrogenation was able to remove a benzyl group off a benzylamine, it was decided that the formerly unexpected disconnection approach would be used as an advantage in efforts to synthesize the RMeNH derivative. Interestingly, the proposed compound was initially believed to have been synthesized, but once it was tested, showed no activity in FRET binding studies. To further validate these results, biological cell assays also showed no potency in the HCV cell line. As it turns out, compound 5.65 was not formed, but instead an unexpected cyclized guanidine product was formed (5.68). The formation of this product is discussed in Section 5.8.3.

5.8.3 The Undesired Cyclization Reaction

After the palladium catalyzed benzylamine cleavage, compound 5.1 was immediately subjected to cyclization and amide reduction (Figure 5.22). This three step type sequence has been conducted many times and the thought was that the synthesis of compound 5.67 turned out according to plan. Unfortunately, compound 5.67 was not the product formed but rather
compound 5.69 (Figure 5.23). Needless to say, this problem was realized when the FRET study results showed a total loss of IRES binding activity. Initially, these results were extremely surprising as hydrogen bonding was hypothesized to be heightened as a result of the secondary amine at the side chain terminus. Consequently, it was deemed highly unlikely for such a molecule to possess absolutely no binding activity with the IRES element and determined that the structure of the product (5.67) must have cyclized incorrectly.

![Figure 5.23. Structures for the final two compounds in synthesis of RMeNH derivative.](image)

Although it was difficult to foresee the cyclization taking place in another location in the molecule, it was concluded that it was the case. It was determined that the BrCN cyclization was performed on an unintended part of the molecule and likely caused the structures of the final two analogs to change drastically. Because of the similarities between the observed and predicted structure (5.67), both compounds yielded $^1$H and $^{13}$C NMR spectra that were, for the most part, indistinguishable. The exact mass of hypothesized product (5.67) as well as the predicted actual structure (5.69) was identical (317.22 g/mol) and the two final compounds (5.68 and 5.69) were produced (Figure 5.23). Predicted structures 5.68 and 5.69 match the $^1$H and $^{13}$C NMR and MS results. However, structures cannot be a complete guarantee until more extensive characterization studies can be done. In the section of future work, outlined in Chapter 8, the cyclization products are discussed further. The overall total yield for the nine step synthesis of structure 5.69 was 17.7%.

With such knowledge, it became clear that a side chain arm possessing a secondary amine will not, yield the desirably-cyclized product. Due to the differences in nucleophilicity (between a secondary vs a tertiary amine), put preference on an alternative cyclization mechanism. An alternative synthesis will be devised accordingly and is anticipated to yield the desired RMeNH analog (6.57) with ease.
5.8.4 Alternate Synthetic Proposal

A completely unique route for the synthesis of compound 5.67 was devised. Using the established synthetic route of the original inhibitor, compound 2.9 was generated. However, instead of reducing the Boc-group in the final reduction step, it was anticipated that the Boc-group can be cleaved in an additional step following the cyclization to yield compound 5.66. Final reduction of the amide would then give the desired product (5.67) (Figure 5.24). Thus, compound 2.9 was exposed to trifluoroacetic acid (TFA) for the anticipated cleavage of the Boc-group to provide the corresponding secondary amine. The acidic conditions unfortunately destroyed the product in some way because $^1$H NMR analysis did not resemble the desired product. Several complimentary methods provide the possibility of cleaving the Boc-group amidst other functionality in the molecule. For example, Wipf et al. reported cleavage of Boc-group amongst a highly functionalized complex molecule using 0.005 N HCl in Et$_2$O/DCM at ambient temperature for 1.5 hours. Currently, only TFA has been attempted (Figure 5.24), but alternative milder reducing agents will certainly be attempted in the future.

![Figure 5.24. Attempt to cleave Boc-group with TFA.](image)

Once the synthesis of this fascinating compound (5.67) is complete, the analog will shed much light in terms of binding interactions with the virus RNA. The functionality of a secondary amine at the side chain terminus will determine whether potency is increased. Once the IRES RNA binding studies have been completed, additional secondary amine derivatives will be explored.
5.9 THE PHENETHYLAMINE COMPOUND

Similarly, as previously illustrated, synthesis of a phenethylamine compound (5.73) was also under development. All synthetic intermediates were formed prior to the realization of the unexpected cyclization complication. During the synthesis, it was anticipated that the correct cyclized products were being formed (Figure 5.25).

However, $^1$H NMR proton signals being very similar and MS being identical for the expected and actual compounds made the synthesis more complex than realized. In reality, the cyclization occurred at an undesired location in the molecule to yield a predicted structure (5.75) (Figure 5.26). This is similar as previously discussed in the synthesis of the RMeNH analog 5.69. Likewise, the structures have not been characterized and there is need for more
Figure 5.26. Attempted cyclization of the phenethylamine analog.

NMR studies to completely verify the structure of the predicted compounds. The undesired product (5.75) had a total yield of 12.4% over nine steps.

5.10 ADDITIONAL COMPOUNDS

As mentioned previously, an additional approach for increasing ligand-RNA affinity is synthesizing conformationally restricted derivatives. Figure 5.27 outlines the structures of inhibitors that have been synthesized by Mr. David Schmit. The EC₅₀ values and activity levels are discussed in detail in Chapter 7.

5.11 INVESTIGATION OF Methylene GROUP APPEARANCE

During the synthesis of several compounds, as for instance seen in the piperidine and the two-carbon side chain analogs, an undesired methylation product was observed (Figure 5.28 and Figure 5.29). Nonetheless, the problem was not too severe pertaining to this research since desired products were isolated in quantities which made it possible to submit the analogs for RNA binding testing. Although reaction conditions for the reduction and cyclization steps were kept unchanged for all analogs, the levels of methylation occurrence, it would occur very randomly. For example, even with cyclization reactions performed on the same day, one was the morpholine compound and the other was the piperidine compound; results showed a large extent of methylated piperidine compound while no methylated morpholine product was observed. These results were quite perplexing and the cause of the methylation has not yet been pin-pointed. During the investigation of the perplexing methyl signal, numerous hypotheses were predicted and later tested. Both compounds 5.84 and 5.86 were confirmed by ¹H NMR.

When the additional signal in the ¹H NMR spectrum was observed at 2.5 ppm was observed for the first time, the belief was that the signal could be an unknown impurity and synthesis of the synthesis was continued in hopes of being able to separate the impurity in the
Figure 5.27. Conformationally restricted inhibitors currently synthesized by Mr. David Schmit.

Figure 5.28. Undesired methylation of the piperidine derivative.
Figure 5.29. Undesired methylation of the dimethylamino 2-carbon side chain derivative.

Subsequent purification method. However, the perplexing signal remained in the $^1\text{H}$ NMR even after an additional purification and could not be separated from the product. After observing the same signal in the analysis of the $^1\text{H}$ NMR spectrum of an additional functionalized derivative, it was presumed that the methyl group was directly attached to the molecule. In addition, in all cases, the additional signal was roughly integrated to three protons supported the argument of the perplexing peak being a methyl group.

With multiple NMR experiments, such as HSQC, HSMBC, NOSEY, and COSY, full characterization of compound 5.83 was possible. The connection of the methyl group to the molecule, where the amino group was previously observed was confirmed. Figure 5.30 illustrates the intentional methylation of the benzimidazole scaffold using ethyl acetimidate hydrochloride as a methylating reagent. Comparison of proton shifts of undesired methylated molecule 5.83 with published $^1\text{H}$ NMR signals from Dibrov et al. is identical, which also supports the correct prediction of the methylated analog 5.88.$^{31}$

Figure 5.30. Published synthesis of generating a methylated derivative in place of an amino group by the Dibrov group.
Secondly, it was believed that using ethyl acetate as a solvent might be causing the methylation. The main reason for this assumption was the comparison of proton signals of the accidentally methylated structure to the purposeful replacement of the amino group with a methyl group as seen in Figure 5.30. The publication by Dibrov et al. described the removal of several crucial functional groups on the original inhibitor 1.10 in order to prove their significance in activity. As outlined in Chapter 3.2, replacement of the NH$_2$ group with a methyl group led to complete loss of potency of the inhibitor. Dibrov et al. synthesized this compound according to the normal Pd-catalyzed reduction. When the reaction was complete, the palladium was filtered off and ethyl acetimidate hydrochloride was added to the mixture. Formation of intermediate 5.87 led to reduction with LAH to yield the final methylated derivative (5.88) (Figure 5.30). It is crucial to note the similarities in structure between ethyl acetimidate hydrochloride and ethyl acetate, which only differ in one atom (nitrogen atom compared to an oxygen atom, respectively). It is expected that because of the similarities in structure, ethyl acetate could have similar functionality and behavior in order to methylate the molecule via a similar mechanism as the previously used ethyl acetimidate reagent.

However, it was also confirmed there was physically no ethyl acetate in contact with any of the reactions throughout the previous steps and yet a large ratio of the methylated product was still observed after the cyclization reaction. This led us to believe it would be highly unlikely that ethyl acetate is not the culprit in the methylaing reaction. A marginal possibility was that ethyl acetate was contaminating the sample at some step of the reaction due to not properly dried test tubes or glassware, argon lines, or deuterated chloroform, which were all determined to be unlikely. As a precaution, measures were taken to keep the intermediates away from ethyl acetate at all cost in all steps of the synthesis for the production of future derivatives.

A third hypothesis was similar in that it was still believed that the Pd-catalyzed hydrogenation might be incomplete, which could have been caused by the palladium reagent losing its effectiveness over time. If the nitro compound was not getting reduced efficiently, it would be difficult to analyze what was causing the methylation. In an effort to test this hypothesis, and inexpensive test substrate was used to demonstrate the quality the palladium
catalyst (Figure 5.31). The test substrate chosen was 2-nitroaniline for its simplicity, availability, and similarity to compound of interest.

![Figure 5.31. Test substrate demonstrating the quality of the Pd-catalyst.](image)

Upon reduction of 2-nitroaniline for three hours, results showed complete disappearance of starting material and product was seen in 80%. The reaction was conducted in ethanol so it was concluded that both the palladium catalyst was effectively working and that ethanol was a suitable solvent for the reduction. Now that the reduction was verified to be effective and numerous factors were eliminated in terms of possible causes of the methylation, the cyclization reaction was then further investigated. There was a belief that the number of equivalents of cyanogen bromide used might have an effect on the outcome. The number of equivalents was increased from 0.9 equiv to 1 equiv and later to 1.2 equiv, yet no significant change in products or yields was observed. An alternative culprit is the cyanogen bromide reagent itself, it might have lost its effectiveness as it is known to be moisture-sensitive and must be stored in dry conditions. However, after purchasing a second batch of cyanogen bromide reagent, the methylation of derivatives continued to be seen in large yields.

Lastly, it may be questioned why the solvent used during cyclization was switched from the original ACN solvent which gave a good yield in the Bergdahl group publication.29 The reason for the adjustment was a precaution taken to protect the reaction from acetamides. The usual contaminants in commercial ACN are known to include water, acetamidate, NH₄OAc, and NH₃.82 As discussed earlier, ethyl acetimidate hydrochloride was used as a reagent to cause the intended replacement of amino group with a methyl group. Therefore, Ethyl acetimidate hydrochloride could be an unwanted contaminant in the cyclization reaction in which there is need to prevent the appearance of methyl group appearance in the molecule. However, since the methylated product was so prevalent when using ethanol as a solvent, a solvent comparison needed to be performed. Even though it was verified that the
palladium reduction was working effectively, the reduction was still performed before the cyclization as to mimic the actual approach that would be necessary in the desired route with our priceless intermediates. Anhydrous ACN, 80:10 ACN:H₂O, and anhydrous methanol were all attempted as a solvent during the cyclization of benzene-1,2-diamine using methods described by Huigens et al. and Garg et al., but did not yield the desired product (Figure 5.32). ⁸³,⁸⁴

![Figure 5.32. Successful reduction of 2-nitroaniline and attempted cyclization of benzene-1,2-diamine.](image)

To further investigate, previously synthesized 1,2-diaminobenzene was reacted with freshly purchased cyanogen bromide in ethanol for 15 hours; the desired product was not observed (Figure 5.33). Although there did not appear to be a methyl signal in the ¹H NMR results, the reaction was extremely indecipherable and showed no signs of product. The test substrate was determined ineffective and the need of discovering an effective solvent remains. In order to rule out the effect of traces of Pd-catalyst while conducting the cyclization reaction, the cyclization will be attempted in THF rather than in ethanol.⁷²

![Figure 5.33. Successful reduction of 2-nitroaniline and attempted cyclization of 2-nitroaniline.](image)

Further investigations need to be performed to track down the cause for the methylation. From all of these manipulation, it can be assumed that the solvent is a crucial factor to the success of the cyclization reaction. Currently, the methylation dilemma has not been sorted out, but we are exceptionally close to pin-point the cause since most factors have been eliminated as causes of the methylation.
Nonetheless, while conditions are not refined for the cyclization, obtaining non-methylated product is clearly possible. Furthermore, desired products were being formed which was vital for the structure-activity relationship studies. These compounds were provided for our collaborators conducting the *in-vitro* and *ex-vivo* studies (FRET assay and biological assay testing).
CHAPTER 6

BIOLOGICAL ACTIVITY

6.1 INTRODUCTION

Although it is very difficult to predict how effective a drug molecule will be based upon its structure, once similar compounds were tested, it was more feasible to look for trends and begin predicting potency. The inhibitors synthesized in the Bergdahl laboratory undergo two separate approaches in testing. The FRET studies test the binding affinity that the inhibitors have for the target HCV IRES domain IIa RNA, while the cell assay studies consist of exposing live Hepatitis C cells with increasing concentrations of the compounds tested. It is vital to acknowledge that the FRET study and SAR experiments are complimentary to each other. A total of 14 compounds have been synthesized to date by the Bergdahl group. There are reported FRET assay results for 14 of these compounds and cell assay results for 12 of the compounds. Three derivatives are currently undergoing testing and the results will be made available in the near future.

6.2 FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET) ASSAY

Once novel inhibitors were synthesized, there was a need for relatively quick and efficient analysis to be performed to identify which ligands were more suitable for the advancement of antiviral therapies. The IRES is responsible for the assembly of functional ribosomes at the start codon which in turn bypasses the initiation of virus translation. Upon infection, the HCV IRES element acts by binding to the host cell’s 40s ribosomal subunit with high affinity upon infection. Once bound, the virus initiates translation in a 5’ cap-independent manner. The binding of certain benzimidazole inhibitors was discovered to cause a conformational change in the IIa domain effectively preventing IRES from functioning. Fortunately, an effective assay was developed by the Hermann group, which is based upon a Förster (fluorescence) resonance energy transfer (FRET) method made to investigate the molecular mechanism of ligand-captured conformational change in the IRES. The FRET testing of compounds was designed in a way so it could determine how
effective the derivatives were in blocking transcription of the virus. The assay technique relied on monitoring changes in fluorescence that indicated rearrangement of RNA conformation upon ligand binding. By using fluorescence labeling and structure information, it was possible to observe viral RNA levels being reduced after benzimidazole inhibitors targeted subdomain IIa. Previous studies reported a ligand “induced” conformational change, but recent evidence shows the RNA executing the “capturing” of the ligand.

To construct such a screening method, first, a key adenine (A54) was chosen based upon its suitability in the subdomain IIa to be replaced with a fluorescent nucleobase analog (2-aminopurine) so that it would be possible to simultaneously monitor RNA folding and metal-ion binding. The 5’ terminus of the IRES was labeled with a pair of fluorescent cyanine dyes. A distance of approximately 7 Å was able to maximize FRET sensitivity to widening of the RNA. Compounds were identified based upon ability to induce the widened interhelical angle in the cyanine dye labeled subdomain target which reduces FRET signal. As a result, minor changes in the overall conformation of the native IIa subdomain structure were converted into changes in FRET signal.

During the optimization of RNA folding, ionic conditions were also tested and resulted in the strongest RNA folding occurring at a concentration of 2 mM MgCl₂. A publication by Parsons et al. explained that magnesium ions controlled the increase in fluorescence triggered by the presence of the benzimidazole inhibitors. These findings suggest there must be at least a partial competition between inhibitor binding and metal binding. Inhibitor binding was seen to displace at least one of the magnesium ions which confirms the likelihood of the binding site being in close proximity of structural magnesium ion sites. In the absence of magnesium ions, the IRES internal loop does not fold and as a result FRET was not detected. With the controlled addition of magnesium ions, FRET could be observed in a dose-dependent manner.

Using the FRET assay, the activity of compound 1.10 was tested against the HCV IRES domain IIa RNA target. The EC₅₀ value was observed to be 3.4 ± 0.3 µM, which was very similar to the mass-spectrometry based assay which reported a Kᵰ 40-mer binding affinity of 0.86 µM and replicon EC₅₀ value of 3.9 µM. Once the FRET methodology was established, investigation of novel benzimidazole inhibitors was initiated. It was confirmed
that the benzimidazole compound of interest 1.10 behaved as an HCV-specific translation inhibitor via conformational induction at the IIa subdomain. The mechanism of action could be clearly understood by observing the quenching of fluorescence signal upon binding of the inhibitor to the IRES. More simply, when higher concentrations of inhibitor were required for FRET quenching, the binding was weaker, which was supported by acquired data.85

Based upon outcomes of the FRET assay, the effective concentration value (EC$_{50}$) was established. Results have demonstrated significant advances in the area of targeted HCV therapy as this is the first example of a conformational mechanism of a small drug molecule which has targeted RNA outside the bacterial ribosome.85 Exploration of the RNA IRES site is a new and unique drug target that shows much promise as being an effective treatment against the Hepatitis C virus. Future derivatives will explore changes in binding as structure, hydrophobicity, and basicity of the inhibitors are optimized.

6.3 BIOLOGICAL CELL ASSAY

To test the effectiveness of the inhibitors, liver tissue cells, known as hepatocytes, were infected with JFH1 (genotype 2A) strain of Hepatitis C Virus (HCV). Following infection, cells were treated with increasing concentrations of the tested drug molecule. These cells were then examined for the presence of HCV capsid protein by both fluorescence microscopy and fluorescence activated cell sorting (FACS). The results obtained have been used to influence the structures of future drug molecules and demonstrate the importance of synthesizing additional inhibitors. Additional inhibitors will continue refining SAR and may be effective against all genotypes encountered throughout the world.

Below is a general procedure for how the compounds were tested by collaborators in the SDSU Biology Department. On day one, Naïve Huh 7.5.1 cells were split into a twelve well tissue culture plate with 100,000 cells per well. The cells were kept in Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) + Penicillin-Streptomycin- L-Glutamine (PS-LG) and incubated at 37 °C with 5% CO$_2$ injection. On day two, the cells were infected with JFH1 (genotype 2A) or JFH1 green fluorescent protein (GFP) virus; a low multiplicity of injection (MOI). Immediately after adding the virus-containing media to the cells, the appropriate concentration of benzimidazole compound was added to the appropriate well. Each twelve well plate was set up to contain one naïve Huh
7.5.1 sample as negative control, and one untreated JFH1 infected sample as background infectivity control. The remaining ten wells were treated with a different concentration of the compound being tested. The concentrations tested were 100 nM, 500 nM, 1 μM, 2 μM, 3 μM, 4 μM, 5 μM, 6 μM, 8 μM, and 10 μM.

On day three, the viral media was removed from each well and replaced with DMEM w/ 10% FBS + PS-LG. The appropriate concentrations of compound were added to the fresh media in each well. On the fifth day, the media was changed again and the compound was replenished in the corresponding wells. During day seven, the cells were detached from the plate (trypsinized) and fixed in 2 - 3% paraformaldehyde (PFA) for 15 minutes. The samples were then spun down at 1500 RPM for 5 minutes and resuspended in phosphate buffered saline (PBS). JFH1-GFP virus-infected samples were readily determined by flow cytometry analysis due to the presence of green fluorescent protein embedded in the viral proteome. Samples using wild type JFH1, which cannot be readily recognized by fluorescence, required further technical procedures such as permeabilizing the cellular membrane with 100% methanol for 10 minutes followed by treatment with 5% bovine serum albumin (BSA) for 35 minutes to prevent non-specific binding of Core antibody used for staining. The samples were then stained with the primary antibody (mouse-Core) in PBS for 2 hours at ambient temperature or overnight at 4 °C. The primary antibody was washed off using PBS. The secondary antibody (anti-mouse Alexa Fluor 488) was added in PBS and allowed to incubate in darkness at ambient temperature for 35 minutes. The secondary antibody was washed off and the cells were resuspended in PBS and analyzed by flow cytometry. The advantage to using the new JFH1-GFP virus is the ability to analyze toxicity more rapidly since the virus now produces GFP itself and the additional staining steps are no longer necessary. On the other hand, JFH-1 represents a more natural model for HCV infection.

Not only are these experiments able to access how effective viral inhibition is, but they are also able to provide toxicity studies of the drugs. Toxicity was measured directly based upon the cell death count. Flow cytometry was used as a method for counting dead cells which float to the top of the media. Flow cytometry, which is an innovative laser-based technology, is able to detect each individual cell based on its fluorescent parameters. Death can be addressed by many different cellular parameters. Our collaborators have used propidium iodide (PI) to analyze the percentage of dead cells in each experimental set-up.
Supt1 cells were treated with the drug molecule for two days followed by staining them with PI. This stain acts by binding to DNA and only stains dead cells as it is unable to enter cellular membrane of living cells. These cells are not fixed with paraformaldehyde since this aldehyde causes cell death. PI fluoresces in the PerCP channel of the flow cytometer, analysis consists of counting fluorescent cells to assess the population of dead cells. The advantage of using Supt1 cells in comparison to the previously used Huh 7.5.1 cell line to measure toxicity levels is accuracy. Accuracy of the experiment increases tremendously as Supt1 cells are non-adherent. The problem with adherent Huh 7.5.1 cells is the dead cells are detaching from the plate and becoming part of the media resulting in an inaccurate death cell count.

There are several advantages to these experiments. Foremost, there is a major correlation between the cell assays and FRET studies. For example, data gathered from both experiments testing compound 1.10 showed many similarities and was agreeable. Likewise, data analysis for compound 5.69 showed no activity in the FRET study and was seen to not have any potency in the cell assay study, supporting the first study (Figure 6.1).88

![Figure 6.1. Inhibitor 5.69 showing no potency against the HCV infected cells. Source: Evans, A.; Wolkowicz, R. San Diego State University, San Diego, CA. Unpublished work, 2014.](image-url)
Figure 6.1 shows no decrease in HCV infected cells during treatment with all concentrations of the drug molecule. This data supports the inactivity of compound 5.69 against HCV, which was also supported with FRET studies discussed in Chapter 6.2. Biological cell assays have given us excellent preliminary data from the compounds already tested. Efforts are being made towards repeating each test in triplicate for each of the compounds to increase the accuracy for these cell assays. Additionally, several of the compounds led to false positives during the assays, repeating those would allow for data to be more statistically relevant. Due to time constraints, the experiments were only performed once for each inhibitor so precision was limited, with the exception of testing of analog 1.10 which was done in triplicate. Significant data was gathered from experiments performed on compound 1.10 and the results serve as exceptional data for the HCV project (Figure 6.2).

![Inhibitor 1.10 (Bergdahl_1) Treated JFH1-GFP Huh 7.5.1 Cells](image)

**Figure 6.2. Original inhibitor 1.10 showing excellent potency against the HCV infected cells.** Source: Evans, A.; Wolkowicz, R. San Diego State University, San Diego, CA. Unpublished work, 2014.

From Figure 6.2 it can be observed that the inhibitor was most effective at a concentration of 8 µM. There was also a good deal of potency at very low inhibitor concentrations of 100 nM and 500 nM, however, these could be false positives. A significant increase in potency occurred in concentrations greater than 1 µM. Toxicity studies showed that toxicity increased with inhibitor concentration. This preliminary data indicated that
compound \textbf{1.10} was effective in preventing JFH1 infection and possessed cytotoxicity only at high concentrations. Additional testing will be done with higher doses of the drug (above 10 \( \mu \text{M} \)) as well as with higher percent GFP expression.

Both inhibitors \textbf{1.10} and \textbf{5.80} showed very promising results. Compound \textbf{5.80} yielded the second most potent results out of all compounds currently tested, but showed even lower levels of toxicity in comparison to inhibitor \textbf{1.10} (Figure 6.3).\textsuperscript{88} For compound \textbf{1.10} and \textbf{5.80} there was slight cell death, but overall both inhibitors seem to have rather low toxicity levels.

![Inhibitor 5.80 (DJS_2_80) Treated JFH1-GFP Huh 7.5.1 Cells](image)

\textbf{Figure 6.3.} Inhibitor 5.80 showing relatively good potency against the HCV infected cells. Source: Evans, A.; Wolkowicz, R. San Diego State University, San Diego, CA. Unpublished work, 2014.

For several of the inhibitors analyzed, a reverse trend with low GFP at low concentrations and high GFP at high concentrations was observed. These negative results were difficult to analyze because of the lack of repetition of the experiments and it was difficult to propose how these results were possible. Ongoing work is conducted in the biology department to further elucidate proper conditions, such as time and concentration to maximize the validity of these results. In addition, current efforts are directed towards performing additional trials for each of the drug compounds. Overall, the preliminary data gained from biological assays and toxicity studies was very insightful and provided
complementary data to the FRET studies. With results from both assays, it was possible to analyze the direction of future novel inhibitors of HCV.
CHAPTER 7

OBSERVED RELATIONSHIPS AND TRENDS BETWEEN ANALOGS

7.1 SUMMARY OF ALL DERIVATIVES

Extensive efforts have been made to create a library of compounds related to inhibitor 1.10 for the future treatments of HCV with three main directions for increasing activity. This thesis focuses on optimizing the amine-phosphate salt bridge between the analog and the subdomain IIa of HCV, while the two remaining directions include designing conformationally restricted analogs as well as introducing a halogen atom to replace one or both of the aryl protons seen in the final compound. Several parts of the benzimidazole backbone have the potential of being altered in order to increase affinity binding. Currently, modifications solely to the side chain arm of the benzimidazole structure have been under exploration and scrutiny. More specifically, this thesis focuses on the optimization of the amine-phosphate salt bridges via ligand interactions observed in the crystal structure. The main idea being to increase the basicity of the terminus nitrogen atom of the side chain arm to allow for stronger and more stabilizing hydrogen bonding interactions with the phosphate backbone of the virus. The other approach of making structural modifications includes synthesizing conformationally restricted analogs currently under investigation by a co-worker in the laboratory. Both directions are predicted to greatly increase affinity of the new compounds.

At this point, it is necessary to discuss the term of hydrogen bonding. These words can be somewhat misleading since the hydrogen bonding interaction is not actually a bond in a normal sense but rather a quite strong electrostatic force “bond” between a proton and an electronegative element, such as oxygen, nitrogen or a halogen. More appropriately, the interaction is a dipole-dipole attraction force. In the case of observed interactions in the bound crystal structure of inhibitor 1.10 and the phosphate backbone of the virus RNA, intermolecular hydrogen bonds can be observed between the synthesized analog and the virus.
Upon completion of synthesis of the analogs, these compounds were submitted for binding testing against the IRES-IIa subdomain via FRET assay specially designed by our collaborators at the University of California, San Diego. The FRET assay itself is discussed in detail in Chapter 6.2. Several compounds produced outstanding data, while few analogs have yet to undergo testing against HCV-infected liver cells for anti-viral activity and cytotoxicity by collaborators in the Biology Department at San Diego State University, as discussed in Chapter 6.3. Analogs with various side chain arm ligands have been synthesized and have all demonstrated promising activity against HCV in both binding affinity and cell line studies. All analogs synthesized, to date, are summarized in Figure 7.1 with corresponding molecular weights and EC$_{50}$ values. The derivatives are listed in order of decreasing EC$_{50}$ value, resulting in increasing activity for the HCV IRES element.

With the ongoing collection of data, it was possible to explore how different structural modifications affect the mode of viral inhibition by which these compounds act and allowed us to continue to interactively refine the structure-activity relationships. The collection of FRET results suggests trends between the different analogs, however, reliable trends cannot be extracted from such a small set of data pool and some correlations do not hold true for all inhibitors. The synthesis of additional compounds is ongoing in order for additional SAR data. Nonetheless, the current data set provides excellent preliminary results to begin establishing SAR. Future modification of compounds is underway and is expected to demonstrate better EC$_{50}$ values. There is hope that more trends can be established in the future and interactions can be refined with the increasing number of analogs synthesized. Investigations of each series of analogs and comparison of affinities are discussed in this chapter. Valuable information about binding mechanisms has been gained from this library of derivatives. Recently, additional plans have been made to modify the backbone of the inhibitors so the compounds retain the most significant functional groups, but the structures will be modified to see whether the EC$_{50}$ values decrease further. The next proposed directions for the research project are suggested in Chapter 8. In the future, it would be useful to co-crystallize a few compounds with high potency, such as the morpholine analog (5.24), with the HCV RNA to verify that the expected binding interactions are taking place, along with observation of any new interactions with diverse molecules.
7.2 Side Chain Length Comprised of Two, Three, and Four Carbons

The desire to examine difference in side chain arm length was one of the major parameters under investigation of refining SAR of the benzimidazole drug molecules. It was thought to be beneficial to study the fluctuations in binding-affinity depending on the distance of functionalized amino groups from the HCV IRES binding site. It is, however,
quite challenging to analyze the actual location of the functional group at the end of the side chain arm. It would make sense that the shorter the chain length, the closer the terminus group would be to the virus RNA and because of this closer proximity, it would allow for increased bonding interactions. However, because the entire side chain has so much free rotation associated with it, it is very difficult to predict how the carbon tether is rotating and bending in the vicinity of the virus binding pocket. Thus, it is not suitable to conclude that side chain length showing decreased potency is because of the distances of the ligand to the RNA. This challenge held constant for all synthesized analogs. In addition, the amino terminus nitrogen is protonated in the bound crystal structure due to the pH of the environment which gives this area of the molecule a tetrahedral geometry around the terminus nitrogen. It remains unclear whether the nitrogen atoms are rotating in the molecule.

Synthesis of side chain lengths ranging from one methylene to five methylene groups between the core of the molecule and terminus functional amino group was proposed. It was planned to introduce a Boc-protecting group to all chain length variations, including original compound 1.10, which would later be easily reduced to give a methyl amine. As seen in the synthesis of compound 1.10, a Boc-protecting group made the overall synthesis simpler. Once a Boc-group is introduced to the molecule, the molecule no longer behaves as an amine, allowing the necessary cyclization of the molecule to occur with ease, which would otherwise be much more troublesome. This Boc-group is also relatively unreactive to most nucleophiles and bases which it may be exposed. The Boc-group not only allowed for easier manipulations of the substrates, but it also reduced the basicity of the nitrogen throughout the last three steps of the total synthesis, which is highly advantageous. Furthermore, once it is reduced in the final step of the synthesis, it is truly beneficial that it increases that nitrogen’s basicity to intensify the strength of interactions with the virus and in turn halt translation of the virus.

With the successful synthesis of compounds 5.30, 1.10, and 7.1 shown in Figure 7.2, it was possible to directly compare the effect that chain length had on potency of the inhibitors. Shortening the side ligand chain length had negative impact on the affinity and binding of the derivative to the viral RNA. Results obtained from the FRET studies show that altering the side chain tether by only one carbon in length had a large impact on the effectiveness of the compound. The original inhibitor 1.10 demonstrated an EC50 value of 3.4
µM, while shortening the carbon tether to compound (5.30) gave an EC$_{50}$ value of 75 µM (Figure 7.2).

This approximately 22-fold decrease in activity was difficult to support with a possible justification. Based upon other derivatives created by a co-worker on the project, it was likely that when the distance between the two nitrogen atoms was increased to three carbons in length apart, the compounds retain activity. This SAR is consistent with a series of N-pyrrolidine compounds synthesized by Mr. David Schmit in the Bergdahl group.$^{80}$ The three carbon side chain analog 5.77 yielded an IC$_{50}$ value of 25 µM, while the shorter carbon chain analog 5.46 gave an IC$_{50}$ value of 58 µM. By reducing the carbon chain length leads to a decrease in activity by a factor of 2.3, although not as drastic as with the dimethyl compound (22-fold). When the dimethylamino (5.30) group was substituted for the N-pyrrolidine group (5.46), the EC$_{50}$ value decreased from 75 µM to 58 µM. On the contrary, when comparing the longer carbon chain of the same groups, when the dimethylamino (1.10) group was substituted for the N-pyrrolidine group (5.77), the EC$_{50}$ value increased from 3.4 µM to 25 µM. Consequently, for the 2-carbon ligand, the N-pyrrolidine group (5.46) showed a slight factor of 1.3 in inhibitory increase over the dimethylamino group (5.30), while in the 3-carbon ligand, the reverse was observed; there was a 7.4-fold decrease in inhibition of the N-pyrrolidine group over the dimethylamino group. Increasing the 3-carbon chain with one methylene group to a 4-carbon chain length within the N-pyrrolidine group does not show a drastic change in activity; compound 5.78 showed roughly the same activity (EC$_{50} = 22$ µM) in comparison to compound 5.77 (EC$_{50} = 25$ µM). Comparison of these two analogs with the two and four-carbon dimethylamino side chain will be discussed once synthesized in the near

Figure 7.2. Two, three, and four-carbon chain length comparison of final analog structures.
future. When briefly looking at the EC$_{50}$ values of $N$-methylpiperidine of a one-carbon ligand (5.80) (21 µM) and $N$-methylpiperidine without a carbon chain (5.81) (50 µM), binding affinity is seen to decrease with the addition of a carbon atom.

The piperazine analog series (Figure 7.3) were also constructed with a side chain length study in mind. By comparing additional molecules with various functionalities, it was possible to more accurately determine the optimal length of the carbon side chain to be used with the generation of future compounds. When the remaining one, four, and five-carbon dimethylamino derivatives are synthesized, a series of five compounds will be made available for direct comparison of chain length and it will be possible to study whether the change is consistent and holds true as an overall trend. Once successful, direct comparison between these five structures would yield a general trend which could be established for the optimal side chain to use in the synthesis of future analog derivatizations.

![Figure 7.3. Piperazine derivatives ranging from two to four-carbon tethers.](image)

Although general trends are challenging to observe at this early stage of the SAR study, it does appear that shortening the chain length of the HCV inhibitors has a drastically negative effect on the binding affinity. This may be due to the inability of the side chain to make contact with the phosphate backbone of the viral RNA once the inhibitor is held inside the binding pocket formed. In contrast, lengthening the chain seems to have little impact on the binding, with the exception of the methylpiperizine derivatives.

### 7.3 HETEROCYCLIC DERIVATIVES

Previous research has shown that the nature of heterocycles can change the antibacterial activity of the molecule. Based upon previously determined EC$_{50}$ values, it was seen that molecules with rings containing a nitrogen atom in the terminus of the carbon
tether showed promising activity and therefore, piperidine and morpholine compounds were chosen to examine the role that heterocycles would play in translation inhibition of HCV. Synthesizing morpholine was anticipated to give a good starting point in terms of heterocyclic rings, while a piperidine analog would serve as an excellent control based upon the similarity in structure with morpholine (Figure 7.4).

![Figure 7.4. Comparison of final inhibitors containing piperidine, morpholine, piperazine, and methylimidazoline as side chain terminus.](image)

The piperidine derivative yielded some of the most surprising results in terms of EC$_{50}$ activity against HCV. The EC$_{50}$ value was expected to be much better (lower) than most of derivatives containing aliphatic groups in the side chain terminus, however, results showed a remarkably high EC$_{50}$ value of 89 µM. This was a significant decrease in comparison to the original inhibitor 1.10 by a factor of about 26. Although the potency of compound 5.16 is the lowest (highest numerical value) within the entire library, it still provides an excellent control model for the addition of additional electron donating groups into the ring which are anticipated to increase the nitrogen’s basicity facilitating binding with the IRES. In addition, the piperazine derivative will allow us the ability to directly access the effect on EC$_{50}$ values of additional heterocycle derivatives. The explanation of such a low affinity for the IRES might have to do with limited space of binding possibility.

Even though it appears that these tertiary amines are pointing out and away from the active site, it was interesting to observe that the inclusion of an additional oxygen atom in the 6-membered ring (N-morpholine) (5.24) (EC$_{50}$ = 35 µM) was able to increase binding affinity nearly 3-fold. The presence of an oxygen atom certainly increases hydrophilicity of the ring and potentially adds a second site for additional hydrogen bonding. However, it is difficult to tell which phenomena is leading to the increased binding capacity. If acting directly, there might be an additional binding possibility for hydrogen binding outside the
HCV IRES subdomain, which we are not able to identify in the original crystal structure. To support this hypothesis, it is seen that there is a second phosphate atom in the crystal structure in the near vicinity of the end of the side chain arm. There is high interest to co-crystallize this analog with the IRES to observe whether an additional binding interaction could be seen. Until an additional co-crystalized structure of derivative 5.24 and the IRES is made available, it is not possible to see whether this hypothesis holds true, which is suggested in future work (Chapter 8). If acting indirectly, the oxygen could be acting as an electron donating group in the ring, allowing the nearby nitrogen to have a greater hydrogen bond with the IRES element. Either way, the addition of one oxygen atom in the side chain was the sole variation in the two inhibitors and caused activity to increase nearly 3-fold, demonstrating its impact on binding affinity.

Since it was assumed that the additional oxygen atom caused the increase in activity, it was hypothesized that synthesizing a six-membered ring with a nitrogen atom replacing the oxygen could theoretically give an even lower EC$_{50}$. To further investigate the predictions, piperazine and imidazoline inhibitors were synthesized and are currently under investigation for FRET results (Figure 7.4). The thought behind including two nitrogen atoms in the ring was two-fold. First, the “outermost” nitrogen could potentially be better electron donor to the “inner” nitrogen atom which could bind more strongly with the HCV IRES and second, the additional nitrogen atom could be forming an extra interaction with the virus RNA backbone. Once the synthesis of the six piperazine compounds is finished and EC$_{50}$ values are obtained, it will be possible to draw more conclusions in terms of binding possibility.

When the 3-carbon tether $N$-piperidine group (5.16) was substituted for the $N$-pyrrolidine group 3-carbon tether (5.77), the EC$_{50}$ value increased from 25 to 89 µM, showing a 3.6-fold in inhibitory increase of the $N$-pyrrolidine group. Because of this observed decrease in binding affinity of compound 5.16, there is a possibility that space may be an issue in the vicinity of the binding pocket. More space is necessary to accommodate the larger heterocyclic 6-membered ring size (5.16) in the active site of the HCV IRES subdomain IIa, in comparison to the 5-membered ring size (5.77). Furthermore, the differences in inhibition activity could potentially be due to more sterically demanding chair conformations of the $N$-piperidine structure compared to the flatter $N$-pyrrolidine ring.
7.4 Nitrogen, Carbon, and Oxygen Containing Derivatives

As previously mentioned in Chapter 5, derivatives were synthesized with the elimination of the amino terminus on the side chain to act as controls in the SAR study. Two such analogs were strategically designed to investigate the significance of the amino group being present at that position in the molecule. First, complete elimination of the dimethylamino terminus led to a butyl side chain on the chroman structure. Secondly, a substitution of the amino terminus for an alcohol terminus led to an alcohol analog.

Amine atoms are known to be much more basic than the corresponding oxygen atoms and should therefore theoretically participate in stronger hydrogen bonding interactions. However, the alcohol group was also anticipated to exhibit hydrogen bonding with the RNA to an unknown extent. The alcohol does not need to be protonated to form an interaction, but if it is protonated, it becomes a stronger acid and therefore a weaker base. Likewise, interactions involving amines are known to be the stronger in comparison to binding with other atoms such as oxygen and sulfur. Along with amines being intrinsically strong, ammonium ions are largely stabilized by hydrogen bonding in water. On the other hand, sulfur and oxonium ions both have relatively lower intrinsic stability to that of amines. Though the intrinsic stability of sulfur is higher than an oxonium ion, the oxonium ion allows for a much longer stabilization in water which usually leads differences in basicity to be medium-dependent, which can sometimes even lead to reversals in base strength. These circumstances are challenging to predict as they are more complex than expected and it is difficult whether this will be the case.

It is noteworthy that this scientific justification may not be directly correlated to the interactions within HCV inhibitors and the RNA and may not be a useful tool to predict current and future structure activity relationships. It does, however, give general magnitudes in strength of various hydrogen bonding interactions. The strength of an N-H proton hydrogen binding to an oxygen atom has an enthalpy of approximately 8 kJ/mol. When comparing this finding to an O-H proton hydrogen bonding to an oxygen atom, it is found to have an approximate enthalpy of 21 kJ/mol.

Initial hypothesis will be drawn from these binding constants by comparing original inhibitor 1.10, possessing a dimethylamino terminus, to that of the in-progress alcohol...
compound (5.57) (Figure 7.5). These general values are of bonds with an oxygen atom, while the HCV drug molecules bind to phosphate in the RNA backbone. Knowing this, it was hard to predict whether the potency of the alcohol-containing inhibitor (5.57) would decrease significantly due to a loss of the significant N(CH₃)₂ group, or whether these strength predictions would cause stronger binding affinity between the analog and subdomain IIa. Synthesis of the alcohol derivative is awaiting two final steps and once studies are performed, results are expected to determine which hypothesis is most valid. Thus while the general binding constants may not be absolutely pertinent to the HCV system, it is scientifically justifiable to state that an O-H hydrogen bond is almost three times stronger than an N-H hydrogen bond.

![Figure 7.5](image.png)

**Figure 7.5. Side-by-side comparison of three final analogs with corresponding EC₅₀ values.**

This section will be discussed once activities of the compounds are made available to the Bergdahl group (Figure 7.5). The EC₅₀ value obtained will demonstrate whether the nitrogen atom at the terminus plays a crucial role in the hydrogen bonding to the phosphate backbone of the HCV RNA.

### 7.5 Non-Suitable Compounds

Throughout the refinement of SAR studies, several aniline-containing and aniline-like substrates were proposed for their attractive structural characteristics and therefore a possible heightened affinity for the IRES. Although some interesting binding interactions were hypothesized, the use of aniline substrates continued to be avoided as this class of molecules was known to exhibit high toxicity in the human body. Aniline-containing compounds have
been observed to cause damage to the oxygen-transfer protein, hemoglobin; acting in a pathway which cannot be interrupted in the body. Investigation of these aniline-related compounds have shown cancer-causing characteristics in rats.\(^4\) Consequently, no aniline-like compounds were explored for potential activity.

**7.6 CONCLUSION**

Overall, numerous effective inhibitors have been synthesized ranging from 4.7% to 23.1% total overall yields. Individual routes to the relatively rapid synthesis of these small drug molecule drugs have not yet been optimized. Once time-permitting, optimization of each step, especially the second-to-last cyclization step in the synthesis, was anticipated to increase total yields at least two-fold. Initial SAR trends have been investigated and will continue to be expanded upon. Even though none of the novel analogs recently synthesized have surpassed the potency of the original molecule 1.10 \((\text{EC}_{50} = 3.4 \, \mu\text{M})\), data collected has led in the right direction to manipulate structural parameters which are anticipated to give higher binding affinity. Current compounds show \(\text{EC}_{50}\) values ranging from 3.4 \(\mu\text{M}\) to 89 \(\mu\text{M}\). The second most potent inhibitor after the original compound was determined to be the methylpiperidine 1-carbon tether analog (5.80) followed by the pyrrolidinone 4-carbon analog (5.83) with \(\text{EC}_{50}\) values of 21 \(\mu\text{M}\) and 22 \(\mu\text{M}\), respectively. On the contrary, it was not surprisingly that no activity was seen with the two compounds which cyclized at the wrong location and have a methyl group substituted for the necessary 2-aminobenzimidazole group (7.69 and 7.79). Although general trends are difficult to observe at this early stage of the SAR study, preliminary results were able to give a good basis for future studies and shine light on which factors have a drastic effect on binding. It was found that varying the side chain arm length, ring size, and heteroatom substitutions in rings had a significant effect on \(\text{EC}_{50}\) values.
CHAPTER 8

FUTURE WORK

8.1 NOVEL LIGANDS

Knowledge of the detailed binding mechanisms of the HCV inhibitors synthesized in the laboratory has already been expanded with the research completed. The suggested upcoming work on the HCV project will be continued by other members in the Bergdahl group with the ongoing goals of expanding knowledge of the detailed binding mechanisms of the inhibitors and the IRES as well as discovering additional novel therapeutic candidates. This section outlines the proposed free amines to be synthesized as the functionalized side chain arm of additional derivatives in the near future. The amines chosen continue to be guided by the co-crystalized inhibitor and IRES structure, but are also heavily influenced by new results from binding affinity studies of novel analogs which have been made available. In general, it cannot be guaranteed that certain alterations in the molecule will affect the binding affinity in a positive or negative manner, but sophisticated preliminary hypotheses continue to be inferred about the interactions taking place between the inhibitors and the HCV RNA subdomain IIa.

A truly fascinating category of substrates to investigate involve the incorporation of amino acids into the side chain of the HCV inhibitors. The S_NAr step can be carried out with chosen amino acids which would be advantageous as they already possess a free amine terminus. The use of this class of compounds is appealing as they are readily available and inexpensive. It is also valuable that amino acids do not possess any threat of toxicity in the human body. The initial amino acid to test would be histidine for its attractive structural characteristics of possessing an imidazole and for its importance in many biochemistry applications. Reducing the carboxylic acid of histidine to the corresponding alcohol is anticipated to yield the desired primary amine 8.1 which can be coupled with the chroman core (2.7) to give the anticipated S_NAr product 8.2, as seen in Figure 8.1. The final two steps would be completed as previously outlined with reduction of the nitro group and subsequent cyclization with BrCN, followed by amide reduction to give compound 8.3.
An alternative approach would be to reduce the carboxylic acid after the $\text{SN}_\text{Ar}$ step as seen in Figure 8.2. There is difficulty in predicting which method would yield more successful results. Once successful, other amino acids, such as tryptophan and arginine can be examined in hopes of synthesizing more potent derivatives.

Other innovative ideas include pre-constraining the ligands in order to increase binding affinity by minimizing the entropic barrier. If an olefin was successfully included within the side chain, the derivative would be much more constrained than any analog that has been previously synthesized. It would also be interesting to study whether both the $\text{Z}$- and $\text{E}$-isomers would have similar or different activity in the FRET binding assays. Nevertheless, it would be synthetically challenging to create such a compound as the final three steps in the synthesis. Further alterations may focus on extended analysis of aliphatic ligands which are likely to be constructed using the method of Salvatore et al.\textsuperscript{60} This technique has proved to be extremely beneficial as it has eliminated problematic over-alkylation and opens the door for an endless quantity of secondary amines to be rapidly synthesized.

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**Figure 8.1.** Initial approach proposed to incorporate an amino acid, such as histidine.

**Figure 8.2.** Alternative approach proposed to incorporate the amino acid, histidine.
It would also be beneficial to co-crystalize additional compounds with the IRES subdomain IIa and analyze that observed interactions are consistent with the original bound crystal image. A great analog candidate for crystallization would be the morpholine-containing derivative. If co-crystallized, it would be possible to validate an extra binding interaction between the oxygen atom in the morpholine ring and the HCV RNA target. Overall, co-crystallizing additional derivatives would give us a better understanding of binding mechanisms with the IRES.

**8.2 Further Investigation of Piperidine and NH Analogs**

As mentioned in Chapter 5.7, once the isomer mixture of chroman 2.7 was reacted with the piperidine-functionalized propylamine, two hypothesized isomer (NO$_2$ group being *ortho* and *para* to the displaced chloro-group) products with an $R_f$ difference of roughly 0.3 were observed, yet were difficult to separate despite several attempts. The presence of more than one product was also confirmed via $^1$H NMR. Due to lack of time, further efforts were not made to separate the two products. However, once a method is developed that can separate the two, additional information may be gained about the significance in activating and deactivating groups of the $S_N$Ar reaction. Alternatively, the same functionalized piperidine propylamine free amine (5.13) can be reacted with the pure undesired isomer of compound 2.7 to observe whether the $S_N$Ar reaction proceeds to any extent (Figure 8.3). The reactivity of the undesired 2.7 isomer is believed to be extremely low and NMR studies would need to be done to fully characterize the structure from this proposed test reaction.

**Figure 8.3.** $S_N$Ar proposal with pure undesired isomer of chroman 2.7.
In addition, it would be advantageous to investigate the undesired cyclization of molecules 5.69 and 5.75 in more detail. Additional NMR studies must be performed to verify that the structure is as mechanistically predicted and that the cyclization occurred at the location which was predicted. The predicted structure of 5.69 correlates to the \(^1\)H NMR spectra as well as the lack of activity of analog 5.69 observed in binding-affinity testing. FRET and biological assays are not expected to show zero activity for a properly cyclized NH analog. It is very unlikely that functionality in the correctly cyclized compound would result in a total lack of activity in these assays which lead us to believe that the cyclization occurred in the incorrect location. The predicted structures in Figure 5.23 cannot be guaranteed until they are fully characterized by NMR techniques. Confirming the structure of the compound would also give more insight on the SAR of the analog.

### 8.3 Continuation of Additional Piperazine Compounds

As discussed in Chapter 7.3, piperazine containing derivatives are very interesting to study because the heterocyclic functionality is suspected to increase binding affinity with the IRES subdomain IIa. In chapter 5.5, six various piperazine compounds were proposed. Currently, the three 6-membered ring analogs only need to be cyclized and reduced to yield the final analogs and the three 5-membered in Figure 8.4 need to be synthesized. After successful synthesis of these additional three compounds, direct comparison among the various side chain arm lengths as well as ring sizes will be studied. Thus, it will be possible to determine at which position in the ring the nitrogen atom has optimal binding as well as which is the optimal ring size for binding.

![Figure 8.4. Proposal for additional three piperazine-containing final compounds with varying side chain length.](image-url)
8.4 CONTINUATION OF VARYING SIDE CHAIN LENGTH

There is great potential in further investigating the various aspects of the inhibitors, for example, the distance between the side chain head group and the benzimidazole core. Once additional inhibitors of varying chain lengths become available for comparison, it will be informative to examine to what extent this particular variable affects binding affinity. Once direct assessments can be made, it will be possible to definitively establish which carbon chain length is optimal for future derivatives.

8.4.1 One Carbon Side Chain Proposal

A proposed synthesis of a one-carbon side chain length with a dimethyl amino head can be synthesized according to the approach outlined in Figure 8.5. The dimethyl amino group is proposed in order for chain length to be the only variable being tested. Also, since the two and three-carbon side chain analogs have already been synthesized, it will be possible to directly compare structures and quantify the differences in EC₅₀ values. It will be interesting to observe whether there is a trend in carbon side chain length on the translational inhibition by these compounds. If data is found to be consistent with what we have already observed, and the binding affinity is decreased to a larger extent as compared to the two-carbon side chain derivative, it will be possible to state that there is a trend between side chain length and SAR. Currently, both the two and three-carbon side chain derivatives have been successfully synthesized. Work towards the four-carbon-containing inhibitor is underway and is expected to be completed in the near future.

Figure 8.5. Proposed synthesis for a one-carbon containing side chain inhibitor.
8.4.2 Five Carbon Side Chain Proposal

By synthesizing the five-carbon side chain analog, there would be a comprehensive set of five inhibitors which would give us a complete set of data to establish preliminary structure-activity relationships (Figure 8.6). These results would likely conclude the optimization of side chain length within the dimethylamine analog.

![Figure 8.6. Proposed synthesis for a five-carbon-containing side chain inhibitor.](image)

8.5 Creation of Aryl-Ring Substituted Compounds

There is potential in synthesizing aryl-substituted 2-aminobenzimidazole inhibitors. This can be accomplished by adding halogen atoms to the two un-substituted positions on the backbone ring of the chroman nucleous in hopes of increasing binding affinity. The x-ray crystal structure of the inhibitor and IRES complex exposes a small amount of space available located adjacent to the two aryl protons in the compound. Progress towards introduction of a small aryl atom, such as a fluoro- or chloro-substituent, is anticipated to begin in the near future. By synthesizing the initial derivative with a halo-substituent in either of the two positions, it will be possible to see whether there is, in fact, enough space to accommodate such an atom (Figure 8.7). Testing of this inhibitor will show whether the data
Figure 8.7. Electrophilic halogenation approach to introduction of halogen atoms. X + = N-fluorobenzenesulfonylimide, sulfuryl chloride, or pyridinium bromide perbromide.

attained had a significant influence on the strength of binding affinity and whether other similar substituents should be explored. If the studies yield promising results, additional small atoms will substitute the aryl hydrogen atoms.

**8.6 EXPLORATION OF DRUG LIKE 2-AMINO-BENZOXAZOLE AND 2-AMINO-INDOLE STRUCTURES**

It is necessary to keep in mind the findings of Seth et al. and to retain all significant functional groups present in the molecule. In the past years, variations within the side chain have been heavily explored. Future work will consist of alterations being made to the core of the compounds in hopes of an increase in potency. The proposed general synthesis of a benzoxazole derivative is presented in Figure 8.8. There is uncertainty as to whether the molecule will be stable to LAH in the last step. It is known that oxazoles are oftentimes sensitive to LAH, but the belief is that the 2-amino-oxazole group may be able to protect itself by acting as a stable enolate after the NH proton is removed. On the other hand, if synthesis proves to be troublesome, a milder reducing agent may be used.

**8.7 COMPUTATIONAL CHEMISTRY**

With the benefit of a computational chemistry, from a computational chemist who recently joined the HCV project, there will be additional pieces of information guiding the synthesis of future analogs. It will be very interesting to observe which innovative functional groups may be added or altered in the final inhibitors to increase hydrogen bonding.
Figure 8.8. General synthetic scheme for the synthesis of 2-aminobenzoxazole compounds.

interactions within the bound crystal structure. It is anticipated that several improvements to make the drug bind more strongly to the highly conserved region of the RNA will come as a result of incorporating computational chemistry within this project.

8.8 CONCLUSIONS

As discussed in previous chapters, HCV is a serious liver disease killing millions of people each year and consequently remains a major health burden. The research presented in this thesis, as well as ongoing efforts, may one day influence the treatment options for people who suffer from the Hepatitis C virus. Much time can be saved in the future by understanding the favorable interactions with subdomain IIa of the IRES. In addition,
structural parameters of the analogs have been optimized to be most effective on the virus by binding to the highly conserved IRES element.

Much work has been made towards the creation of a library of novel inhibitors acting against the IRES subdomain IIa from the time the optimized total synthesis was made available by the Bergdahl group. Throughout the synthesis of numerous HCV drug molecules discussed in this thesis, significant accomplishments have been achieved. Some approaches led to interesting results and some reactions yielded unexpected, yet fascinating results. Considerable information was extracted from the testing performed on recently synthesized analogs, but not surprisingly, additional information remains to be discovered. With the new knowledge gained, future work has been proposed to help benefit the investigation of the detailed mode of inhibition of the HCV virus. Novel anti-HCV analogs will continue to be screened for activity as well as toxicity in cell assays. As the hunt for novel drugs continues, the co-crystal structure will continue to drive the structurally-guided discovery of translational inhibitors acting against HCV.
REFERENCES


APPENDIX A

EXPERIMENTAL
**General.** Flash chromatography was performed using Biotage SP1 using prepackaged silica columns as well as hand columns. $^1$H and $^{13}$C and other spectroscopy experiments were recorded at 25 °C. TLC was performed using EMD Silica Gel 60 F$^{254}$ plates. All air sensitive reactions were conducted under argon atmosphere and in septum-capped oven-dried glassware unless otherwise specified. Chemical yields are based on purified material (>95% by $^1$H NMR spectroscopy). NMR spectra were recorded at 25 °C on either Varian 400, 500, or 600 MHz instruments using CDCl$_3$ and DMSO as solvents and TMS as an internal standard ($\delta = 0$ ppm). 400 MHz, 500 MHz, 600 MHz $^1$H NMR and 100 MHz, 126 MHz, 151 MHz NMR $^{13}$C standards: CDCl$_3$, $\delta = 7.26$ ppm and $\delta = 77.36$ ppm. Coupling patterns are abbreviated as s (singlet), d (doublet), t (triplet), q (quartet), quin (quintet), sex (sextet), sep (septet), m (multiplet), J (coupling). Proton assignments were obtained from COSY, DEPT, HMQC and HMBC spectra. Mass spectra were recorded with the help of Dr. Hermann’s laboratory on an LCMS at the University of California, San Diego. Flash Chromatography was conducted using silica gel (Sorbent Standard Grade, 60 Å, 230-400 mesh).

**FRET.** FRET experiments were performed on a Spectra Max Gemini monochromator plate reader at 25 °C. The RNA construct used at 100nM in 10mM Hepes buffer of pH = 7 with 2mM MgCl$_2$. Emission filters were set at 550 nm and 670 nm and the data was analyzed and FRET calculated.

**Chemicals.** Diethyl ether (Et$_2$O) and tetrahydrofuran (THF) were distilled from sodium benzophenone ketyl and were collected when the indicator became deep blue or purple. Methylene chloride (CH$_2$Cl$_2$) was distilled from calcium hydride. All reagents and solvents were purchased from Aldrich, Fisher Scientific, or BACHe unless otherwise specified.
5-((3-(Benzyl(methyl)amino)propyl)amino)-N,N-dimethyl-6-nitrochromane-3-carboxamide (5.1). A mixture of previously prepared carboxamide (2.7) (1 equiv, 1.06 g, 3.73 mmol), 4.3 (5 equiv, 3.32 g, 18.6 mmol), and N-methylpyrrolidinone (3.5 mL) were heated in a sealed tube at 75 °C for 48 hours. The reaction mixture was poured into a 1:1 mixture (250 mL) of Et₂O : H₂O and the mixture was stirred for 30 minutes. The mixture was washed with H₂O (4 x 50 mL), dried with Na₂SO₄, and evaporated in vacuo. The crude product was purified by flash chromatography (silica gel, 60-100% EtOAc/hexane). The product was isolated as a bright yellow oil in 94% yield. ¹H NMR (400 MHz, CDCl₃) δ 7.97 (d, J = 9.5 Hz, Ar-H, 1H), 7.72 (bt, J = 5.9 Hz, NH, 1H), 7.39 – 7.08 (m, Ph, 5H), 6.35 (d, J = 9.5 Hz, Ar-H, 1H), 4.47 – 4.32 (dt, J = 3.1 Hz, OCH₂, 1H), 4.18 – 4.04 (m, OCH₂, 1H), 3.48 – 3.45 (m, NHCH₂, 2H), 3.20 (dd, J = 12.6 Hz, CH, 1H), 3.12 (s, N(CH₃)₂, 3H), 3.05 – 2.92 (s, partially hidden m, N(CH₃)₂, OCH₂CHCH₂, 4H), 2.78 (m, OCH₂CHCH₂, 1H), 2.42 (dt, J = 6.8 Hz, NHCH₂CH₂CH₂, 2H), 2.16 (s, NCH₃, 3H), 2.06 – 2.03 (s, NCH₃Ph, 2H), 1.83 – 1.70 (m, CH₂CH₂CH₂, 2H).

2-(3-(Butyl(phenethyl)amino)propyl)isoindoline-1,3-dione (5.5). A mixture of 4.1 (1 equiv, 0.0590 g, 0.264 mmol) in acetone (1.8 mL) was added dropwise to a solution of 4.20 (2.15 equiv, 0.100 g, 0.567 mmol) and NaI (3 equiv, 0.119 g, 0.792 mmol) in acetone (1.8 mL).
The reaction mixture was refluxed for 24 hours. The reaction was quenched with H₂O (10 mL) and extracted with Et₂O (3 x 10 mL). The organic layers were combined and dried over MgSO₄ and concentrated in vacuo. The residue was purified on silica (0-35% EtOAc/hexanes) to yield a light yellow oil product in 69%. ^1H NMR (400 MHz, CDCl₃) δ 7.88 – 7.79 (dd, J = 5.4, 3.0 Hz, Ar-H, 2H), 7.74 – 7.64 (dd, J = 5.4, 3.0 Hz, Ar-H, 2H), 7.32 – 7.11 (m, Ph, 5H), 3.79 – 3.67 (t, J = 7.3 Hz, PhthNC₂H₂, 2H), 2.76 – 2.62 (2m, CH₃CH₂CH₂CH₂N, CH₂CH₂N(CH₂)₂, 4H), 2.56 (t, J = 7.1 Hz, PhCH₂, 2H), 2.52 – 2.37 (t, J = 7.1 Hz, NCH₂CH₂Ph, 2H), 1.90 – 1.78 (quin, J = 14.6 Hz, NCH₂CH₂CH₂, 2H), 1.40 (m, CH₂CH₂CH₃, 2H), 1.36 – 1.21 (m, CH₂CH₂, 2H), 0.97 – 0.83 (t, J = 7.2 Hz, CH₃, 3H). ^13C NMR (126 MHz, CDCl₃) δ 168.35, 140.80, 133.80, 132.26, 128.73, 128.28, 125.80, 123.11, 77.28, 55.90, 53.65, 51.68, 36.58, 33.59, 29.34, 26.33, 20.67, 14.09, 14.01.

**Figure A.3. Compound 5.6.**

*N-Butyl-N-phenethyl-1,3-diaminopropane (5.6)* Compound 5.5 (1 equiv, 0.0561 g, 0.154 mmol) was dissolved in 1 mL MeOH. Hydrazine (2 equiv, 0.00985 g, 0.307 mmol) was added dropwise to the stirring reaction and refluxed at 95 °C for 3 hours. The reaction was cooled and the solvent was evaporated in vacuo. A 1:1 mixture of H₂O : EtOH (2 mL) was added followed by the slow addition of 1M aqueous HCl until pH=1. Precipitate was removed via filtration and rinsed with H₂O. The solution was made basic with 1N NaOH and the aqueous was extracted with Et₂O (4 x 10 mL), dried over Na₂SO₄ and evaporated in vacuo to yield desired colorless oil in 97% yield. ^1H NMR (400 MHz, CDCl₃) δ 7.23 (m, Ph, 5H), 2.71 (m, CH₂CH₂Ph, NH₂, 6H), 2.50 (m, H₂NCH₂CH₂CH₂N, NCH₂CH₂CH₂CH₃, 4H), 1.59 (quin, J = 7.0 Hz, H₂NCH₂CH₂CH₂N, 2H), 1.43 (m, H₂NCH₂CH₂CH₂N, 2H), 1.31 (m, CH₂CH₂CH₃, 4H), 0.91 (t, J = 7.2 Hz, NCH₂CH₂CH₂CH₃, 3H). ^13C NMR (100 MHz, CDCl₃) δ 140.85, 128.69, 128.25, 125.80, 77.22, 56.06, 53.84, 51.95, 40.82, 33.54, 29.35, 20.69, 14.06.
5-((3-(Butyl(phenethyl)amino)propyl)amino)-N,N-dimethyl-6-nitrochromane-3-carboxamide (5.7). A mixture of previously prepared carboxamide (2.7) (1 equiv, 0.00850 g, 0.0299 mmol), 5.6 (5 equiv, 0.0350 g, 1.41 mmol), and N-methylpyrrolidinone (0.052 mL) was heated in a sealed tube at 75 °C for 48 hours. The reaction mixture was poured into a 1:1 mixture (50 mL) of Et₂O : H₂O and stirred for 30 minutes. The mixture was extracted with Et₂O (4 x 10 mL), dried over Na₂SO₄, and evaporated in vacuo. The crude product was purified by flash chromatography (silica gel, 0-5% MeOH/DCM). The product was isolated as a bright yellow oil in 96% yield. ¹H NMR (500 MHz, CDCl₃) δ 7.99 (d, J = 9.4 Hz, Ar-H, 1H), 7.70 (bs, NH, 1H), 7.33 – 7.12 (m, Ph, 5H), 6.35 (d, J = 9.4 Hz, Ar-H, 1H), 4.41 (dd, J = 10.9 Hz, OCH₂, 1H), 4.12 (dd, J = 10.5 Hz, OCH₂, 1H), 3.48 (q, J = 7.0 Hz, NHCH₂, 2H), 3.38 (m, CH, 1H), 3.20 – 3.06 (s, N(CH₃)₂, 3H), 3.06 – 2.93 (s, partially hidden m, N(CH₃)₂, CCH₂, 4H), 2.85 – 2.75 (m, CCH₂, 1H), 2.69 (m, NCH₂CH₂Ph, 2H), 1.72 (m, NHCH₂CH₂CH₂, 2H), 1.34 – 1.24 (m, CH₃CH₂CH₂, 4H), 1.21 (t, J = 7.0 Hz, N(CH₂)₃, 6H), 0.90 (t, J = 7.3 Hz, CH₃, 3H).

5-((3-(Dibutylamino)propyl)amino)-N,N-dimethyl-6-nitrochromane-3-carboxamide (5.10). A mixture of previously prepared carboxamide (2.7) (1 equiv, 0.200 g, 0.702 mmol), N,N-
dibutyl-1,3-diaminopropane (5 equiv, 0.654 g, 3.51 mmol), and N-methylpyrrolidinone (1.22 mL) was heated in a sealed tube at 75 °C for 48 hours. The reaction mixture was poured into a 1:1 mixture (200 mL) of Et₂O : H₂O and the reaction was stirred for 30 minutes. The mixture was extracted with Et₂O (4 x 20 mL), dried with Na₂SO₄, and evaporated *in vacuo*. The crude product was purified by flash chromatography (silica gel, 40-50% Et₂O /DCM). The product was isolated as a bright yellow oil in 70% yield. ¹H NMR (400 MHz, CDCl₃) δ 7.95 (d, J = 9.8 Hz, Ar-H, 1H), 7.54 (bt, J = 5.5 Hz, NH, 1H), 6.39 (d, J = 9.5, Ar-H, 1H), 4.41 (td, J = 10.7, 5.6 Hz, OCH₂, 1H), 4.20 – 4.07 (m, OCH₂, 1H), 3.61 – 3.38 (m, CH, 1H), 3.33 – 3.20 (m, NHCH₂, 2H), 3.15 (s, N(CH₃)₂, 3H), 3.10 – 2.95 (s, *partially hidden* m, N(CH₃)₂, CCH₂, 5H), 2.94 – 2.70 (m, N(CH₂)₃, 6H), 2.07 (m, CH₂CH₂CH₂, 2H), 1.77 – 1.53 (m, (CH₂)₂(CH₂)₂(CH₃)₂, 4H), 1.48 – 1.22 (sex, J = 7.3 Hz, (CH₂)₂(CH₃)₂, 4H), 1.06 – 0.79 (t, J = 7.3 Hz, (CH₂)₂(CH₃)₂, 6H). ¹³C NMR (126 MHz, CDCl₃) δ 171.45, 160.25, 149.34, 131.40, 126.57, 110.70, 109.19, 74.68, 67.46, 54.04, 51.24, 49.45, 46.28, 37.52, 35.65, 35.34, 30.64, 28.85, 28.80, 27.89, 20.68, 17.91, 14.03.

*Figure A.6. Compound 5.14.*

**N,N-Dimethyl-6-nitro-5-((3-(piperidin-1-yl)propyl)amino)chromane-3-carboxamide (5.14)**

A mixture of previously prepared carboxamide (2.7) (1 equiv, 0.0821 g, 0.288 mmol), 5.13 (5 equiv, 0.205 g, 1.44 mmol), and N-methylpyrrolidinone (0.500 mL) was heated in a sealed tube at 75 °C for 48 hours. The reaction mixture was poured into a 1:1 mixture (100 mL) of Et₂O : H₂O and the mixture was stirred for 30 minutes. The mixture was washed with H₂O (4 x 20 mL), dried with Na₂SO₄, and evaporated *in vacuo*. The crude product was purified by flash chromatography (silica gel, 10-20% MeOH/DCM). The product was isolated as a bright yellow oil in 97%. ¹H-NMR (400 MHz, CDCl₃) δ 7.97 (d, J = 9.4 Hz, Ar-H, 1H), 7.66 (bt, J = 5.8 Hz, NH, 1H), 6.35 (d, J = 9.4 Hz, Ar-H, 1H), 4.43 – 4.39 (ddd, J = 11.2, 10.8, 2.9 Hz, OCH₂, 1H), 4.16 – 4.09 (m, OCH₂, 1H), 3.46 – 3.37 (m, CH, 1H), 3.23 – 3.11 (s, *partially*...
hidden m, N(CH₃)₂, NHCH₂, 4H), 3.05 – 2.95 (s, partially hidden m, N(CH₃)₂, NHCH₂, CHCH₂, 5H), 2.85 – 2.77 (m, CHCH₂, 1H), 2.35 (t, J = 7.2 Hz, CH₂N(CH₂)₂, 6H), 1.81 – 1.72 (q, J = 7.0 Hz, CH₂CH₂CH₂, 2H), 1.61 – 1.53 (m, N(CH₂)₂(CH₂)₂CH₂, 4H), 1.46 – 1.39 (m, N(CH₂)₂(CH₂)₂CH₂, 2H).

Figure A.7. Compound 5.15.

2-Amino-N,N-dimethyl-1-(3-(piperidin-1-yl)propyl)-1,7,8,9-tetrahydrochromeno[5,6-d]imidazole-8-carboxamide (5.15). To a flask under argon containing 10% palladium on carbon (0.4 equiv, 0.119 g, 0.113 mmol) was added a solution of 5.14 (1 equiv, 0.110 g, 0.281 mmol) in absolute EtOH (15 mL). The mixture was stirred for 4 hours under 1 atmosphere of hydrogen at rt. The mixture was filtered through Celite. The Celite was washed absolute EtOH (3 x 5 mL) and all filtrates were combined. To this solution was added cyanogen bromide (1 equiv, 0.0298 g, 0.281 mmol) in a solution of absolute EtOH, and the mixture was stirred at rt for 15 hours under argon. The solvent was concentrated in vacuo, and the residue was dissolved in 1:1 mixture of DCM : saturated NaHCO₃ solution (70 mL) and the mixture was stirred for 20 minutes. The mixture was extracted with DCM (2 x 20 mL), dried over Na₂SO₄, and evaporated. The residue was purified by flash chromatography (basic alumina, 0-6% MeOH/Et₂O). The pure product was isolated as a light red crystalline solid in 32% yield. ¹H-NMR (400 MHz, CDCl₃) δ 7.15 (d, J = 8.5 Hz, Ar-H, 1H), 6.65 (d, J = 8.5 Hz, Ar-H, 1H), 6.12 (bs, NH₂, 2H), 4.38 – 4.30 (ddd, J = 10.7, 2.7, 2.0 Hz, OCH₂, 1H), 4.24 – 4.14 (m, ArNCH₂, 1H), 4.14 – 4.04 (m, ArNCH₂, 1H), 3.99 – 3.89 (dd, J = 10.7, 10.7 Hz, OCH₂, 1H), 3.55 – 3.45 (dd, J = 15.4 Hz, 11.2 Hz, ArCH₂, 1H), 3.34 – 3.23 (m, CH, 1H), 3.21 – 3.12 (s, partially hidden m, N(CH₃)₂, ArCH₂, 4H), 3.02 (s, (NCH₃)₂, 3H), 2.50 – 2.20 (m, partially hidden m, CH₂N(CH₂)₂, 6H), 2.12 – 1.90 (m, partially hidden m, CH₂CH₂CH₂, 2H), 1.61 (m, N(CH₂)₂(CH₂)₂, 4H), 1.50 (m,
N(CH$_2$)$_2$(CH$_2$)$_2$CH$_2$, 2H). $^{13}$C-NMR (151 MHz, CDCl$_3$) δ 171.83, 155.47, 148.37, 136.84, 132.06, 115.14, 110.62, 103.70, 66.59, 53.62, 52.91, 39.97, 37.22, 35.95, 35.71, 30.31, 28.13, 25.72, 24.43, 24.28.

**Figure A.8. Compound 5.16.**

$^8$-((Dimethylamino)methyl)-1-(3-(piperidin-1-yl)propyl)-1,7,8,9-tetrahydrochromeno[5,6-d]imidazol-2-amine (5.16). Compound 5.15 (1 equiv, 0.0280 g, 0.0726 mmol) was dissolved in distilled THF (1.5 mL) under argon. Lithium aluminum hydride (36 equiv, 0.0992 g, 2.61 mmol) was carefully added and the mixture was stirred at 60 °C for 5 hours. The reaction was quenched with careful dropwise addition of 0.106 mL of water, 0.106 mL of 15% aq. NaOH solution, followed by 0.320 mL of water. The mixture was filtered through Celite and washed with anhydrous THF. The filtrate and washings were combined, dried over Na$_2$SO$_4$, and evaporated in vacuo. The residue was purified by flash chromatography (basic alumina, 0-10% MeOH/DCM). The pure product was isolated as a light tan crystalline solid in 88% yield. $^1$H-NMR (600 MHz, CDCl$_3$) δ 7.12 (d, $J = 8.6$ Hz, Ar-$H$, 1H), 6.62 (d, $J = 8.6$ Hz, Ar-$H$, 1H), 6.04 (bs, NH$_2$, 2H), 4.28 – 4.23 (ddd, $J = 10.6$, 2.4, 1.4 Hz, OCH$_2$, 1H), 4.21 – 4.07 (m, CNCH$_2$, 2H), 3.83 (dd, $J = 10.6$, 6.8 Hz, OCH$_2$, 1H), 3.24 – 3.16 (dd, $J = 15.5$, 5.0 Hz, ArCH$_2$, 1H), 2.84 – 2.77 (dd, $J = 15.5$, 7.0 Hz, 1H), 2.43 – 2.34 (m, N(CH$_2$)$_2$, 4H), 2.34 – 2.23 (m, partially hidden m, CHCH$_2$N(CH$_3$)$_2$, CH$_2$CH$_2$N, 5H), 2.24 (s, N(CH$_3$)$_2$, 6H), 2.06 – 1.98 (m, ArNCH$_2$CH$_2$, 2H), 1.65 – 1.57 (m, N(CH$_2$)$_2$(CH$_2$)$_2$, 4H), 1.53 – 1.47 (m, N(CH$_2$)$_2$(CH$_2$)$_2$CH$_2$, 2H). $^{13}$C-NMR (151 MHz, CDCl$_3$) δ 155.23, 149.10, 136.68, 132.16, 115.03, 110.82, 103.96, 68.48, 61.77, 53.68, 53.01, 46.02, 39.98, 30.57, 28.03, 25.78, 25.64, 24.34. [M+H]$^+$: calc. for C$_{21}$N$_5$OH$_3$, 371.52; found, 372.40.
**Figure A.9. Compound 5.18.**

*N,N-Dimethyl-6-nitro-5-((4-(piperidin-1-yl)butyl)amino)chromane-3-carboxamide (5.18).* A mixture of previously prepared carboxamide (2.7) (1 equiv, 0.172 g, 0.604 mmol), 5.17 (5 equiv, 0.472 g, 3.02 mmol), and *N*-methylpyrrolidinone (0.851 mL) was heated in a sealed tube at 75 °C for 48 hours. The reaction mixture was poured into a 1:1 mixture (200 mL) of Et₂O : H₂O and the mixture was stirred for 30 minutes. The mixture was extracted with Et₂O (4 x 20 mL), dried with Na₂SO₄, and evaporated in vacuo. The crude product was isolated as a bright yellow oil in 48% yield. ¹H NMR (400 MHz, CDCl₃) δ 7.98 (d, *J* = 9.4 Hz, Ar-*H*, 1H), 7.67 (bt, *J* = 5.5 Hz, NH, 1H), 6.36 (d, *J* = 9.5 Hz, Ar-*H*, 1H), 4.49 – 4.34 (dt, *J* = 10.8 Hz, OC₃H₂, 1H), 4.12 (dd, *J* = 10.7, 7.1 Hz, OC₃H₂, 1H), 3.42 (m, CH, 1H), 3.26 – 3.09 (s, partially hidden m, N(C₃H₃)₂, 3H), 3.09 – 2.92 (s, partially hidden m, N(CH₃)$_2$, CCH₂, 4H), 2.79 (dd, *J* = 11.2, 3.0 Hz, CCH₂, 1H), 2.29 (bt, partially hidden t, *J* = 7.1 Hz, N(CH₂)$_2$, 4H), 1.57 (m, NHCH₂CH₂CH₂CH₂N(CH$_2$)$_2$(CH₂)$_2$, 10H), 1.42 (m, (CH₂)$_2$CH₂, 2H).

**Figure A.10. Compound 5.22.**

*N,N-Dimethyl-5-((3-morpholinopropyl)amino)-6-nitrochromane-3-carboxamide (5.22).* A mixture of previously prepared carboxamide (2.7) (1 equiv, 80.94 mg, 0.284 mmol), 5.21 (5 equiv, 0.205 g, 1.42 mmol), and *N*-methylpyrrolidinone (0.4 mL) were heated in a sealed tube at 75 °C for 48 hours. The reaction mixture was poured into a 1:1 mixture (100 mL) of Et₂O : H₂O and the mixture was stirred for 30 minutes. The mixture was washed with H₂O (4 x 10 mL), dried with Na₂SO₄, and evaporated in vacuo. The crude product was purified by
flash chromatography (silica gel, 60-100% EtOAc/hexane). The product was isolated as a bright yellow oil in 94% yield. $^1$H-NMR (400 MHz, CDCl$_3$) δ 7.98 (d, $J = 9.4$ Hz, Ar-H, 1H), 7.81–7.41 (bs, NH, 1H), 6.37 (d, $J = 9.4$ Hz, Ar-H, 1H), 4.44 – 4.39 (ddd, $J = 11.0, 3.3, 2.7$ Hz, OCH$_2$, 1H), 4.15 – 4.08 (dd, $J = 11.0, 11.0$ Hz, OCH$_2$, 1H), 3.69 (t, $J = 4.7$ Hz, O(CH$_2$)$_2$, 4H), 3.48 – 3.40 (ddd, $J = 12.5, 7.0, 6.7$ Hz, CHCH$_2$C, 1H), 3.25 – 3.18 (ddd, $J = 12.5, 7.0, 6.7$ Hz, CHCH$_2$C, 1H), 3.13 (s, NCH$_3$, 3H), 3.09 – 2.95 (s, partially hidden m, NCH$_3$, NHCH$_2$ 5H), 2.82 – 2.77 (m, CH, 1H), 2.44 – 2.35 (m, partially hidden m, N(CH$_2$)$_2$, CH$_2$N(CH$_2$)$_2$, 6H), 1.79 – 1.71 (q, $J = 6.85$ Hz, CH$_2$CH$_2$CH$_2$, 2H).

Figure A.11. Compound 5.23.

2-Amino-N,N-dimethyl-1-(3-morpholinopropyl)-1,7,8,9-tetrahydrochromeno[5,6-d]imidazole-8-carboxamide (5.23). To a flask under argon containing 10% palladium on carbon (0.4 equiv, 0.119 g, 0.112 mmol) was added a solution of 5.22 (1 equiv, 0.110 g, 0.280 mmol) in absolute EtOH (15 mL). The mixture was stirred for 4 hours under 1 atmosphere of hydrogen at rt. The mixture was filtered through Celite. The Celite was washed with absolute EtOH (3 x 5 mL) and all filtrates were combined. To this solution was added cyanogen bromide (0.9 equiv, 0.267 g, 0.252 mmol) in a solution of absolute EtOH, and the mixture was stirred at rt for 15 hours under argon. The solvent was concentrated in vacuo, and the residue was dissolved in 1:1 mixture of DCM : saturated NaHCO$_3$ solution (70 mL) and the mixture was stirred for 20 minutes. The mixture was extracted with DCM (2 x 20 mL), dried over Na$_2$SO$_4$, and evaporated. The residue was purified by flash chromatography (basic alumina, 0-5% MeOH/EtOAc). The pure product was isolated as a tan crystalline solid in 81% yield. $^1$H NMR (400 MHz, CDCl$_3$) δ $^1$H-NMR (400 MHz, CDCl$_3$) δ 7.15 (d, $J = 8.5$ Hz, Ar-H, 1H), 6.66 (d, $J = 9.13$ Hz, Ar-H, 1H), 5.87 (bs, NH$_2$, 2H), 4.38 – 4.32 (ddd, $J = 10.8, 3.1, 2.0$ Hz, OCH$_2$, 1H), 4.25 – 4.16 (m, ArNCH$_2$, 1H), 4.16
– 4.07 (m, ArNCH₂, 1H), 3.94 (dd, J = 10.8, 10.8 Hz, OCH₂, 1H), 3.74 (t, J = 4.6 Hz, O(CH₂)₂, 4H), 3.57 – 3.49 (dd, J = 15.4, 11.2 Hz, ArCH₂, 1H), 3.33 – 3.24 (m, CH, 1H), 3.20 – 3.12 (s, partially hidden ddd, NCH₃, ArCH₂, 4H), 3.03 (s, NCH₂, 3H), 2.51 – 2.44 (m, N(CH₂)₂, 4H), 2.42 – 2.26 (m, partially hidden m, NCH₂, 2H), 2.14 – 1.94 (m, CH₂CH₂CH₂, 2H).

Figure A.12. Compound 5.24.

8-((Dimethylamino)methyl)-1-(3-morpholinopropyl)-1,7,8,9-tetrahydrochromeno[5,6-d]imidazol-2-amine (5.24). Compound 5.23 (1 equiv, 0.510 g, 0.130 mmol) was dissolved in distilled THF (2.8 mL) under argon. Lithium aluminum hydride (36 equiv, 0.178 g, 4.68 mmol) was carefully added and the mixture was stirred at 60 °C for 4 hours. The reaction was quenched with careful dropwise addition of 0.190 mL of water, 0.190 mL of 15% aqueous sodium hydroxide solution, followed by 0.573 mL of water. The mixture was filtered through Celite and washed with anhydrous THF. The filtrate and washings were combined, dried over Na₂SO₄, and evaporated in vacuo. The residue was purified by flash chromatography (basic alumina, 100% EtOAc). The pure product was isolated as light tan crystalline solid in 81% yield. ¹H-NMR (600 MHz, CDCl₃) δ 7.14 (d, J = 8.4 Hz, Ar-H, 1H), 6.64 (d, J = 8.4 Hz, Ar-H, 1H), 5.79 (s, NH₂, 2H), 4.28 – 4.23 (ddd, J = 10.6, 2.5, 1.6, OCH₂, 1H), 4.19 – 4.13 (m, ArNCH₂, 2H), 3.83 (dd, J = 10.6, 6.3 Hz, OCH₂, 1H), 3.75 (t, J = 4.7 Hz, O(CH₂)₂, 4H), 3.24 – 3.14 (dd, J = 15.8, 4.7 Hz, ArCH₂, 1H), 2.86 – 2.77 (dd, J = 15.8, 6.9 Hz, ArCH₂, 1H), 2.52 – 2.44 (bt, J = 4.7 Hz, N(CH₂)₂, 4H), 2.39 – 2.22 (m, partially hidden m, CHCH₂N(CH₃)₂, CH₂N(CH₂)₂, 5H), 2.25 (s, N(CH₃)₂, 6H), 2.04 (m, CH₂CH₂CH₂, 2H). ¹³C-NMR (151 MHz, CDCl₃) δ 154.65, 149.26, 136.55, 132.08, 115.21, 111.07, 103.98, 68.48, 66.70, 61.74, 53.11, 53.00, 46.03, 39.99, 30.55, 27.47, 25.60. [M+H]⁺: calc. for C₂₀N₅O₂H₃, 373.49; found, 374.42.
Figure A.13. Compound 5.28.

*t-Butyl (2-((3-(dimethylcarbamoyl)-6-nitrochroman-5-yl)amino)ethyl)(methyl)carbamate (5.28).* A mixture of previously prepared carboxamide (2.7) (1 equiv, 0.152 g, 0.534 mmol), 5.27 (5 equiv, 465 mg, 2.67 mmol), and N-methylpyrrolidinone (0.925 mL) was heated in a sealed tube at 75 °C for 48 hours. The reaction mixture was poured into a 1:1 mixture (200 mL) of Et₂O : H₂O and the mixture was stirred for 30 minutes. The mixture was extracted with Et₂O (4 x 20 mL), dried over Na₂SO₄, and evaporated in vacuo. The crude product was purified by flash chromatography (silica gel, 10-20% Et₂O/DCM). The product was isolated as a bright yellow oil in 94% yield. ¹H-NMR (400 MHz, CDCl₃) δ 7.97 (d, J = 8.7 Hz, Ar-H, 1H), 7.49 (bs, NH, 1H), 6.39 (d, J = 8.7 Hz, Ar-H, 1H), 4.42 (m, NCH₂CH₂Boc, 2H), 4.24 – 4.00 (m, OCH₂, 1H), 4.00 – 3.90 (m, OCH₂, 1H), 3.60 – 3.35 (m, partially hidden m, CH₂C, CH, 3H), 3.5 – 3.21 (m, NHCH₂CH₂, 1H), 3.20 – 3.08 (s, partially hidden m, N(CH₃)₂, NHCH₂CH₂, 4H), 3.08 – 2.93 (m, N(CH₃)₂, 3H), 2.93 – 2.76 (s, CH₃NBoc, 3H), 1.50 – 1.33 (m, Boc, 9H). ¹³C-NMR (100 MHz, CDCl₃) δ 223.23, 159.78, 148.08, 126.36, 77.18, 67.33, 37.25, 35.65, 35.20, 30.62, 29.55, 28.35, 27.85, 17.65.

Figure A.14. Compound 5.29.

*t-Butyl (2-(2-amino-8-(dimethylcarbamoyl)-8,9-dihydrochromeno[5,6-d]imidazol-1(7H)-yl)ethyl)(methyl)carbamate (5.29).* To a flask under argon containing 10% palladium on carbon (0.4 equiv, 0.171 g, 0.161 mmol) was added a solution of 5.28 (1 equiv, 0.170 g,
0.402 mmol) in absolute EtOH (20 mL). The mixture was stirred for 4 hours under 1 atmosphere of hydrogen at rt. The mixture was filtered through Celite. The Celite was washed with absolute EtOH (3 x 5 mL) and all filtrates were combined. To this solution was added cyanogen bromide (1.0 equiv, 0.445 g, 0.402 mmol) in a solution of absolute EtOH, and the mixture was stirred at rt for 15 hours under argon. The solvent was concentrated in vacuo, and the residue was dissolved in 1:1 mixture of DCM : saturated NaHCO₃ solution (70 mL) and the mixture was stirred for for 20 minutes. The mixture was extracted with DCM (2 x 20 mL), dried over Na₂SO₄, and evaporated. The residue was purified by flash chromatography (basic alumina, 0-5% MeOH/DCM). The pure product was isolated as a dark red crystalline solid in 33% yield. ¹H-NMR (400 MHz, CDCl₃) δ 7.14 (d, J = 8.5 Hz, Ar-H, 1H), 6.65 (d, J = 8.5 Hz, Ar-H, 1H), 5.27 (bs, NH₂, 2H), 4.38 – 4.23 (m, partially hidden m, OCH₂, ArNCH₂, 2H), 4.18 – 4.10 (ddd, J = 15.5, 6.4, 6.4 Hz, ArNCH₂, 1H), 3.94 (dd, J = 10.6, 10.6 Hz, OCH₂, 1H), 3.56 – 3.34 (m, partially hidden m, ArCH₂, NCH₂, 3H), 3.32 – 3.23 (m, CH, 1H), 3.18 (s, CH₃, 3H), 3.14 – 3.04 (bm, NCH₂, 1H), 3.02 (s, CH₃, 3H), 2.78 (s, BocNCH₃, 3H), 1.46 (s, Boc, 9H). ¹³C-NMR (126 MHz, CDCl₃) δ 171.70, 154.18, 148.69, 136.13, 131.65, 115.45, 110.98, 103.44, 80.49, 77.26, 66.57, 50.19, 41.82, 37.26, 36.40, 35.91, 35.78, 28.30, 24.46.

Figure A.15. Compound 5.30.

1-(2-(Dimethylamino)ethyl)-8-((dimethylamino)methyl)-1,7,8,9-tetrahydrochromeno[5,6-d]imidazol-2-amine (5.30). Compound 5.29 (1 equiv, 0.0440 g, 0.110 mmol) was dissolved in distilled THF (2.17 mL) under argon. Lithium aluminum hydride (37 equiv, 0.148 g, 3.90 mmol) was carefully added and the mixture was stirred at 60 °C for 5 hours. The reaction was quenched with careful dropwise addition of 0.154 mL of H₂O, 0.154 mL of 15% aqueous NaOH solution, followed by 0.463 mL of H₂O. The mixture was filtered through Celite and washed with anhydrous THF. The filtrate and washings were combined, dried over NaSO₄, and evaporated in vacuo. The residue was purified by flash chromatography.
(basic alumina, 0-4% MeOH/DCM). The pure product was isolated as a light red crystalline solid in 96% yield. $^1$H-NMR (400 MHz, CDCl$_3$) $\delta$ 7.14 (d, $J$ = 8.6 Hz, Ar-$H$, 1H), 6.62 (d, $J$ = 8.6 Hz, Ar-$H$, 1H), 5.60 (bs, NH$_2$, 2H), 4.27 – 4.22 (ddd, $J$ = 10.8 Hz, 2.7, 1.4 Hz, OCH$_2$, 1H), 4.24 – 4.07 (m, partially hidden m, ArNCH$_2$, 2H), 3.84 – 3.78 (dd, $J$ = 10.8, 7.6 Hz, OC$_2$H$_2$, 1H), 3.20 – 2.95 (m, partially hidden m, ArCH$_2$, CHCH$_2$, CH$_2$NMe$_2$, 4H), 2.78 – 2.66 (m, partially hidden m, ArCH$_2$, CH$_2$NMe$_2$, 3H), 2.32 (s, N(CH$_3$)$_2$, 6H), 2.24 (s, N(CH$_3$)$_2$, 6H). $^{13}$C-NMR (151 MHz, CDCl$_3$) $\delta$ 155.15, 149.25, 136.30, 132.50, 115.27, 110.83, 103.82, 68.42, 61.67, 61.62, 46.00, 45.98, 43.18, 30.56, 26.43. [M+H]$^+$: calc. for C$_{17}$N$_5$OH$_{27}$, 317.43; found, 318.29.

Figure A.16. Compound 5.39.

t-Butyl 4-((3-(dimethylcarbamoyl)-6-nitrochroman-5-yl)amino)ethyl)piperazine-1-carboxylate (5.39). A mixture of previously prepared carboxamide (2.7) (1 equiv, 0.263 g, 0.926 mmol), 5.38 (5 equiv, 1.060 g, 4.63 mmol), and N-methylpyrrolidinone (1.2 mL) was heated in a sealed tube at 75 °C for 48 hours. The reaction mixture was poured into a 1:1 mixture (200 mL) of Et$_2$O : H$_2$O and the mixture was stirred for 30 minutes. The mixture was washed with H$_2$O (4 x 50 mL), dried with Na$_2$SO$_4$ and evaporated in vacuo. The crude product was purified by HPLC (20-70% ACN : H$_2$O). The product was isolated as a bright yellow oil in 85% yield. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.96 (d, $J$ = 9.5 Hz, Ar-$H$, 1H), 7.88 (bt, NH, 1H), 6.37 (d, $J$ = 9.4 Hz, Ar-$H$, 1H), 4.41 (dd, $J$ = 10.8 Hz, OCH$_2$, 1H), 4.19 – 4.04 (m, OCH$_2$, 1H), 3.42 (bm, partially hidden m, CH, BocN(CH$_2$)$_2$, 5H), 3.14 (s, partially hidden m, N(CH$_3$)$_2$, CCH$_2$, 3H), 3.10 – 2.93 (s, partially hidden m, N(CH$_3$)$_2$, NHCH$_2$, 5H), 2.82 (dd, $J$ = 11.3 Hz, 8.7 Hz, CCH$_2$, 1H), 2.64 – 2.20 (m, (CH$_2$)$_2$(CH$_2$)$_2$NCH$_2$, 6H), 1.45 (s, Boc, 9H).
**Figure A.17. Compound 5.43.**

*t-Butyl 4-((3-(dimethylcarbamoyl)-6-nitrochroman-5-yl)amino)propyl)piperazine-1-carboxylate (5.43).* A mixture of previously prepared carboxamide (2.7) (1 equiv, 0.0420 g, 0.142 mmol), 5.42 (5 equiv, 0.0173 g, 0.711 mmol), and N-methylpyrrolidinone (0.146 mL) was heated in a sealed tube at 75 °C for 48 hours. The reaction mixture was poured into a 1:1 mixture (100 mL) of Et₂O : H₂O and the mixture was stirred for 30 minutes. The mixture was extracted with Et₂O (4 x 20 mL), dried with Na₂SO₄, and evaporated *in vacuo.* The crude product was purified by flash chromatography (silica gel, 0-15% MeOH/Et₂O). The product was isolated as a bright yellow oil in 74% yield. **¹H NMR (400 MHz, CDCl₃) δ**

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1H NMR (400 MHz, CDCl₃) δ 7.98 (d, J = 9.4 Hz, Ar-H, 1H), 7.68 (bs, N-H, 1H), 6.37 (d, J = 9.4 Hz, Ar-H, 1H), 4.49 – 4.33 (dt, J = 10.8 Hz, OCH₂, 1H), 4.11 (dd, J = 10.5 Hz, OCH₂, 1H), 3.55 – 3.33 (m, partially hidden m, NHCH₂, BocN(CH₂)₂, 6H), 3.31 – 3.16 (m, CH, 1H), 3.12 (s, N(CH₃)₂, 3H), 3.07 – 2.95 (s, partially hidden m, N(CH₃)₂, CCH₂, 4H), 2.88 – 2.72 (m, CCH₂, 1H), 2.34 (bt, partially hidden bt, J = 6.8 Hz, N(CH₂)₃, 6H), 1.75 (quin, J = 6.7 Hz, CH₂CH₂CH₂, 2H), 1.44 (s, Boc, 9H).
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**Figure A.18. Compound 5.47.**

*t-Butyl 4-((3-(dimethylcarbamoyl)-6-nitrochroman-5-yl)amino)butyl)piperazine-1-carboxylate (5.47).* A mixture of previously prepared carboxamide (2.7) (1 equiv, 0.118 g, 0.414 mmol), 5.46 (5 equiv, 0.529 g, 2.07 mmol), and N-methylpyrrolidinone (0.540 mL) was heated in a sealed tube at 75 °C for 48 hours. The reaction mixture was poured into 200
mL of a 1:1 mixture of Et₂O : H₂O and the mixture was stirred for 30 minutes. The mixture was extracted with Et₂O (4 x 20 mL), dried with Na₂SO₄, and evaporated in vacuo. The crude product was purified by flash chromatography (silica gel, 0-10% MeOH/DCM). The product was isolated as a bright yellow oil in 63% yield. ¹H NMR (400 MHz, CDCl₃) δ 7.98 (d, J = 9.4 Hz, Ar-H, 1H), 7.70 (bs, NH, 1H), 6.36 (d, J = 9.5 Hz, Ar-H, 1H), 4.42 (dd, J = 10.8 Hz, OCH₂, 1H), 4.16 – 4.04 (m, OCH₂, 1H), 3.51 – 3.35 (m, partially hidden m, BocN(CH₂)₂, NHCH₂, 6H), 3.21 – 3.09 (s, partially hidden m, N(CH₃)₂, CH, 4H), 3.09 – 2.94 (m, 2 partially hidden m, N(CH₃)₂, CCH₂, 4H), 2.84 – 2.71 (m, CCH₂, 1H), 2.47 – 2.26 (m, partially hidden m, N(CH₂)₃, 6H), 1.71 – 1.51 (m, NHCH₂CH₂CH₂CH₂, 4H), 1.46 (s, Boc, 9H).

Figure A.19. Compound 5.50.

5-(Butylamino)-N,N-dimethyl-6-nitrochromane-3-carboxamide (5.50). A mixture of previously prepared carboxamide (2.7) (1 equiv, 0.131 g, 0.460 mmol), butylamine (5 equiv, 0.227 mL, 2.30 mmol), and N-methylpyrrolidinone (0.647 mL) was heated in a sealed tube at 75 °C for 48 hours. The reaction mixture was poured into a 1:1 mixture (100 mL) of Et₂O : H₂O and the mixture was stirred for 30 minutes. The mixture was extracted with E₂O (4 x 20 mL), dried with Na₂SO₄, and evaporated in vacuo. The crude product was purified by flash chromatography (HPLC purification, 35-65% ACN/H₂O). The product was isolated as a bright yellow oil in 83% yield. ¹H NMR (400 MHz, CDCl₃) δ 7.99 (d, J = 9.4 Hz, Ar-H, 1H), 7.68 (bt, J = 4.4 Hz, NH, 1H), 6.35 (d, J = 9.4 Hz, Ar-H, 1H), 4.49 – 4.32 (dt, J = 10.7 Hz, OCH₂, 1H), 4.19 – 4.04 (m, OCH₂, 1H), 3.41 (ddt, J = 12.2, 7.0, 5.1 Hz, CH, 1H), 3.22 – 3.06 (s, partially hidden m, N(CH₃)₂, CCH₂, 4H), 3.06 – 2.90 (s, partially hidden m, N(CH₃)₂, NHCH₂, 5H), 2.88 – 2.71 (m, CCH₂, 1H), 1.67 – 1.49 (m, CH₂CH₂CH₃, 2H), 1.46 – 1.27 (m, CH₂CH₂CH₃, 2H), 0.92 (t, J = 7.3 Hz, CH₂CH₂CH₃, 3H). ¹³C NMR (100 MHz, CDCl₃) δ 171.44, 159.94, 149.16, 126.36, 110.79, 108.97, 67.30, 48.08, 37.25, 35.66, 35.33, 33.17, 27.90, 19.94, 13.74.
2-Amino-1-butyl-N,N-dimethyl-1,7,8,9-tetrahydrochromeno[5,6-d]imidazole-8-carboxamide (5.51). To a flask under argon containing 10% palladium on carbon (0.4 equiv, 0.132 g, 0.124 mmol) was added a solution of 5.50 (1 equiv, 0.109 g, 0.310 mmol) in absolute EtOH (15 mL). The mixture was stirred for 4 hours under 1 atmosphere of hydrogen at rt. The mixture was filtered through Celite. The Celite was washed with absolute EtOH (3 x 5 mL) and all filtrates were combined. To this solution was added cyanogen bromide (0.9 equiv, 0.0296 g, 0.279 mmol) in a solution of absolute EtOH, and the mixture was stirred at rt for 15 hours under argon. The solvent was concentrated in vacuo, and the residue was dissolved in 1:1 mixture of DCM: saturated NaHCO₃ solution (70 mL) and the mixture was stirred for 20 minutes. The mixture was extracted with DCM (2 x 20 mL), dried over Na₂SO₄, and evaporated. The residue was purified by flash chromatography (basic alumina, 0-50% MeOH/Et₂O). The pure product was isolated as a light red crystalline solid in 25% yield. ¹H NMR (400 MHz, CDCl₃) δ 7.18 (d, J = 8.5 Hz, Ar-H, 1H), 6.68 (d, J = 8.5 Hz, Ar-H, 1H), 4.61 – 4.28 (dt, partially hidden bs, J = 10.8 Hz, OCH₂, NH₂, 3H), 4.15 – 3.89 (m, partially hidden m, NHCH₂, CCH₂, 3H), 3.55 – 3.42 (dt, J = 15.3 Hz, OCH₂, 1H), 3.26 (tdd, J = 10.9, 5.2, 3.3 Hz, CH, 1H), 3.18 (s, N(CH₃)₂, 3H), 3.11 (ddd, J = 15.4, 5.2, 2.0 Hz, CCH₂, 1H), 3.02 (s, N(CH₃)₂, 3H), 1.86 – 1.66 (m, CH₂CH₂CH₃, 2H), 1.50 – 1.34 (m, CH₂CH₂CH₃, 2H), 0.96 (t, J = 7.3 Hz, CH₂CH₃, 3H).

Figure A.20. Compound 5.51.

Figure A.21. Compound 5.62.
1-Butyl-8-((dimethylamino)methyl)-1,7,8,9-tetrahydrochromeno[5,6-d]imidazol-2-amine (5.62). Compound 5.51 (1 equiv, 0.024 g, 0.0759 mmol) was dissolved in distilled THF (1.6 mL) under argon. Lithium aluminum hydride (37 equiv, 0.106 g, 2.81 mmol) was carefully added and the mixture was stirred at 60 °C for 5 hours. The reaction was quenched with careful dropwise addition of 0.111 mL of H₂O, 0.111 mL of 15% aqueous NaOH solution, followed by 0.335 mL of H₂O. The mixture was filtered through Celite and washed with anhydrous THF. The filtrate and washings were combined, dried over Na₂SO₄, and evaporated in vacuo. Purification via flash chromatography gave the desired beige, crystalline solid in 60% yield. ¹H NMR (400 MHz, CDCl₃) δ 7.15 (d, J = 8.6 Hz, Ar-H, 1H), 6.65 (d, J = 8.5 Hz, Ar-H, 1H), 4.40 (d, NH₂, 2H), 4.26 (dp, J = 10.5 Hz, OCH₂, 1H), 4.08 – 3.98 (bt, J = 8.2 Hz, NHCH₂, 2H), 3.85 (ddd, J = 10.8 Hz, OCH₂, 1H), 3.69 (m, CH, 1H), 3.15 (dd, J = 15.8 Hz, CCH₂, 1H), 2.79 (dd, J = 15.8 Hz, CCH₂, 1H), 2.37 – 2.14 (s, partially hidden m, N(CH₃)₂, CHCH₂N, CHCH₂N, 8H), 1.81 – 1.65 (m, CH₂CH₂CH₃, 2H), 1.49 – 1.36 (m, CH₂CH₂CH₃, 2H), 0.98 (t, J = 7.3 Hz, CH₂CH₃, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 152.69, 149.45, 135.74, 132.03, 115.38, 111.17, 104.18, 68.44, 61.57, 45.95, 44.16, 33.12, 30.48, 25.63, 19.96, 13.74.

Figure A.22. Compound 5.55.

5-((3-(Benzylxy)propyl)amino)-N,N-dimethyl-6-nitrochromane-3-carboxamide (5.55). A mixture of previously prepared 2.7 (1 equiv, 0.252 g, 0.885 mmol), 5.54 (5 equiv, 0.731 g, 4.42 mmol), and N-methylpyrrolidinone (0.900 mL) was heated in a sealed tube at 75 °C for 48 hours. The reaction mixture was poured into a 1:1 mixture (200 mL) of Et₂O : H₂O and the mixture was stirred for 30 minutes. The mixture was extracted with Et₂O (4 x 20 mL), dried with Na₂SO₄, and evaporated in vacuo. The crude product was purified by HPLC (10-60% ACN/H₂O). The product was isolated as a bright yellow oil in 98% yield. ¹H NMR (400 MHz, CDCl₃) δ 7.98 (d, J = 9.4 Hz, Ar-H, 1H), 7.68 (bt, J = 5.6 Hz, 1H), 7.42 – 7.16 (m, Ph,
5H), 6.40 – 6.31 (d, J = 9.4 Hz, Ar-H, 1H), 4.43 (m, partially hidden m, OCH$_2$Ph, OCH$_2$, 3H), 4.19 – 4.01 (m, OCH$_2$, 1H), 3.62 – 3.44 (m, NHCH$_2$CH$_2$CH$_2$, 4H), 3.39 – 3.21 (m, CH, 1H), 3.10 (s, N(CH$_3$)$_2$, 3H), 3.09 – 2.90 (s, partially hidden m, N(CH$_3$)$_2$, CCH$_2$, 4H), 2.88 – 2.72 (m, CCH$_2$, 1H), 1.98 – 1.78 (quin, J = 6.1 Hz, CH$_2$CH$_2$CH$_2$, 2H).

**Figure A.23. Compound 5.58.**

2-Amino-1-(3-(benzylkoxy)propyl)-N,N-dimethyl-1,7,8,9-tetrahydrochromeno[5,6-d]imidazole-8-carboxamide (5.58). To a flask under argon containing 10% palladium on carbon (0.4 equiv, 0.111 g, 0.105 mmol) was added a solution of 5.55 (1 equiv, 0.108 g, 0.261 mmol) in absolute EtOH (15 mL). The mixture was stirred for 4 hours under 1 atmosphere of hydrogen at rt. The mixture was filtered through Celite. The Celite was washed with absolute EtOH (3 x 5 mL) and all filtrates were combined. To this solution was added cyanogen bromide (0.9 equiv, 0.024 9 g, 0.235 mmol) in a solution of absolute EtOH, and the mixture was stirred at rt for 15 hours under argon. The solvent was concentrated in vacuo, and the residue was dissolved in 1:1 mixture of DCM : saturated NaHCO$_3$ solution (70 mL) and the mixture was stirred for 20 minutes. The mixture was extracted with DCM (2 x 20 mL), dried over Na$_2$SO$_4$, and evaporated. The residue was purified by flash chromatography (basic alumina, 0-10% MeOH/Et$_2$O). The pure product was isolated as a light gray crystalline solid in 34% yield. $^1$H NMR (500 MHz, CDCl$_3$) δ 7.43 – 7.28 (m, Ph, 5H), 7.17 (d, J = 8.5 Hz, Ar-H, 1H), 6.67 (d, J = 8.5 Hz, Ar-H, 1H), 5.09 (bs, NH$_2$, 2H), 4.61 – 4.47 (bs, OCH$_2$Ph, 2H), 4.36 (t, J = 14.4 Hz, OCH$_2$CH, 1H), 4.20 (m, NCH$_2$CH$_2$CH$_2$, 2H), 4.01 – 3.86 (t, J = 10.7 Hz, OCH$_2$CH, 1H), 3.62 – 3.42 (m, partially hidden m, CH$_2$CH$_2$O, CH, 3H), 3.34 – 3.21 (m CCH$_2$, 1H), 3.21 – 3.09 (s, partially hidden m, N(CH$_3$)$_2$, CCH$_2$, 4H), 3.01 (s, N(CH$_3$)$_2$, 3H), 2.16 – 1.96 (m, CH$_2$CH$_2$CH$_2$, 2H).
2-(3-(Benzyl(phenethyl)amino)propyl)isoindoline-1,3-dione (4.17). Amine (4.16) (2.15 equiv, 0.123 g, 0.588 mmol) and NaI (3 equiv, 0.123 g, 0.820 mmol) were dissolved in THF (2 mL). Isoindoline (4.1) (1 equiv, 0.611 g, 0.273 mmol) was dissolved in THF (2 mL) and added dropwise to the stirring mixture. The reaction was refluxed for 24 hours. The reaction was quenched with H$_2$O (10mL) and extracted with Et$_2$O (3 x 5mL). Biotage chromatography purification of the residue (5-25% EtOAc/Hexane with 1% Et$_3$N) gave the pure product in 30 % yield. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.83 (dd, $J = 5.5$, 3.0 Hz, Ar-H, 2H), 7.71 (dd, $J = 5.4$, 2.8 Hz, Ar-H, 2H), 7.22 (m, Ph, 10H), 3.70 (bt, $J = 7.4$ Hz, PhthNCH$_2$, 2H), 3.64 (bs, NCH$_2$Ph, 2H), 2.73 (m, PhCH$_2$N(CH$_2$)$_2$, 4H), 2.58 (bt, $J = 7.1$ Hz, NCH$_2$CH$_2$Ph, 2H), 1.87 (quin, $J = 7.3$ Hz, CH$_2$CH$_2$CH$_2$, 2H).

Figure A.26. Compound 5.70.

N-Benzyl-N-phenethyl-1,3-diaminopropane (5.70). The previously synthesized 4.17 (1 equiv, 0.0801 g, 0.203 mmol) was dissolved in 1 mL MeOH. Hydrazine (2 equiv, 0.0130 mL, 0.407 mmol) was added dropwise to the stirring reaction and refluxed at 95 °C for 3 hours. The reaction was cooled and the solvent was evaporated in vacuo. A 1:1 mixture of H$_2$O : EtOH (1 mL) was added followed by the slow addition of 2N aqueous HCl until pH=1. The reaction was heated to 110 °C and refluxed for 30 minutes. Precipitate was removed via filtration and rinsed with H$_2$O. The solution was made basic with 1N NaOH and the aqueous was extracted with Et$_2$O (4 x 10 mL), dried with Na$_2$SO$_4$ and evaporated in vacuo to give the
pure product as a colorless oil in 81% yield. $^1$H NMR (400 MHz, CDCl$_3$) δ 7.22 (m, Ph, 10H), 2.73 (m, PhCH$_2$CH$_2$NCH$_2$Ph, 4H), 2.66 (bt, $J = 6.7$ Hz, H$_2$NCH$_2$, 2H), 2.53 (t, $J = 6.8$ Hz, NCH$_2$CH$_2$Ph, 2H), 1.59 (quin, $J = 6.8$ Hz, H$_2$NCH$_2$CH$_2$CH$_2$N, 2H), 1.44 (bs, partially hidden m, NH$_2$, CH$_2$CH$_2$CH$_2$N(CH$_2$)$_2$, 4H).

![Figure A.27. Compound 5.71.](image)

t-Butyl (2-((3-(dimethylcarbamoyl)-6-nitrochroman-5-yl)amino)ethyl)(methyl)carbamate (5.71). A mixture of previously prepared carboxamide (2.7) (1 equiv, 0.152 g, 0.534 mmol), 5.70 (5 equiv, 0.465 g, 2.67 mmol), and N-methylpyrrolidinone (0.925 mL) was heated in a sealed tube at 75 °C for 48 hours. The reaction mixture was poured into a 1:1 mixture (200 mL) of Et$_2$O : H$_2$O and the mixture was stirred for 30 minutes. The mixture was extracted with Et$_2$O (4 x 20 mL), dried with Na$_2$SO$_4$, and evaporated in vacuo. The crude product was purified by flash chromatography (silica gel, 10-20% Et$_2$O/DCM). The product was isolated as a bright yellow oil in 94% yield. $^1$H NMR (400 MHz, CDCl$_3$) δ 7.96 (d, $J = 9.6$ Hz, Ar-H, 1H), 7.64 (bt, $J = 5.3$ Hz, NH, 1H), 7.44 – 7.00 (m, Ph, 10H), 6.34 (d, $J = 9.5$ Hz, Ar-H, 1H), 4.46 – 4.37 (m, OCH$_2$, 1H), 4.13 (dd, $J = 12.4$, 8.8 Hz, OCH$_2$, 1H), 3.63 (s, NCH$_2$Ph, 2H), 3.41 – 3.35 (t, $J = 7.1$ Hz, CCH$_2$, 2H), 3.30 (m, CH, 1H), 3.12 – 3.07 (s, N(CH$_3$)$_2$, 3H), 3.06 – 3.00 (s, N(CH$_3$)$_2$, 3H), 2.99 – 2.89 (m, 2H), 2.86 – 2.81 (m, partially hidden m, NHCH$_2$CH$_2$CH$_2$N, CCH$_2$, 3H), 2.79 – 2.65 (m, NCH$_2$CH$_2$Ph, 4H), 2.07 – 1.95 (quin, $J = 6.8$ Hz, NHCH$_2$CH$_2$CH$_2$, 2H).
APPENDIX B

$^1\text{H}$ NMR AND $^{13}\text{C}$ NMR SPECTRA
Figure B.1. $^1$H NMR of compound 5.1.
Figure B.2. 13C NMR of compound 5.5.
Figure B.3. $^1$H NMR of compound 5.5.
Figure B.4. $^{13}$C NMR of compound 5.6.
Figure B.5. $^1$H NMR of compound 5.6.
Figure B.6. $^1$H NMR of compound 5.7.
Figure B.7. $^{13}$C NMR of compound 5.10.
Figure B.8. $^1$H NMR of compound 5.10.
Figure B.9. $^1$H NMR of compound 5.14.
Figure B.10. $^{13}$C NMR of compound 5.15.
Figure B.11. $^1$H NMR of compound 5.15.
Compound 5.16

Figure B.12. $^{13}$C NMR of compound 5.16.
Figure B.13. $^1$H NMR of compound 5.16.
Figure B.14. $^1$H NMR of compound 5.18.
Figure B.15. $^1$H NMR of compound 5.22.
Figure B.16. $^1$H NMR of compound 5.23.
Figure B.17. $^{13}$C NMR of compound 5.24.
Figure B.18. $^1$H NMR of compound 5.24.
Figure B.19. $^{13}$C NMR of compound 5.28.
Figure B.20. $^1$H NMR of compound 5.28.
Figure B.21. $^{13}$C NMR of compound 5.29.
Figure B.22. $^1$H NMR of compound 5.29.
Figure B.23. $^{13}$C NMR of compound 5.30.
Figure B.24. $^1$H NMR of compound 5.30.
Figure B.25. $^1$H NMR of compound 5.39.
Figure B.26. $^1$H NMR of compound 5.43.
Figure B.27. $^1$H NMR of compound 5.47.
Figure B.28. $^{13}$C NMR of compound 5.50.
Figure B.29. $^1$H NMR of compound 5.50.

Compound 5.50
Figure B.30. $^1$H NMR of compound 5.51.

Compound 5.51
Figure B.31. $^{13}$C NMR of compound 5.62.
Figure B.32. $^1$H NMR of compound 5.62.
Figure B.33. $^1$H NMR of compound 5.55.
Figure B.34. $^1$H NMR of compound 5.58.
Figure B.35. $^1$H NMR of compound 4.17.
Figure B.36. $^1$H NMR of compound 5.70.
Figure B.37. $^1$H NMR of compound 5.71.