Benzoisoxazolone-Based Scaffolds for Development of Diversity Driven Small Molecule Libraries: An Effective Tool to Identify Novel Molecules Targeting Key Therapeutic Applications

by

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Abstract

The screening of compound libraries has repeatedly demonstrated its effectiveness as a research tool in drug discovery. Parallel synthesis is a powerful approach for the assembly of compound libraries possessing a wide range of structural and functional group diversity for evaluation against both new and established biological targets.

This thesis project was focused on the development of four amino and/or carboxy-substituted benzoisoxazolone (B to E) as privileged scaffolds for the parallel synthesis of diversity driven libraries to identify isoform selective inhibitors of Rho-kinase (ROCK) for the treatment of diabetic cardiovascular disease and different cancers, as well as potential anti-HIV agents that block HIV pre-mRNA alternative splicing, and inhibitors of protein arginine methyltransferase 4 (PRMT4) for the treatment of cancer.

In the context of developing a synthesis to the C5-amino substituted benzoisoxazolone scaffold B, the general methodology to construct the benzoisoxazolone ring system found in all four scaffolds is described. This involved reaction of a requisite salicylic acid derivative with hydroxylamine followed by dehydrative cyclization to assemble the isoxazolone ring. Also described in Chapter 2 was the synthesis of the C4-amino substituted scaffold C. The crucial issue encountered was control of chemoselectivity during dehydrative ring closure. The intent was that scaffold B and C-based libraries would contain type 2 ROCK inhibitors. The concept described in Chapter 3 was the “formal” use of the C5-carboxy substituted benzoisoxazolone scaffold D to generate novel molecules that mimic the tetracyclic indole anti-HIV agent IDC16, through conservation of the central (B and C-rings) in its structure. Based on the results of virtual screening/docking experiments on PRMT4, chapter 4 describes the synthesis of the 5-carboxy-7-amino substituted benzoisoxazolone scaffold E and its functionalization such that the benzyl substituents introduced at N-2 and the 5-benzamide motif interact with hydrophobic residues along the PRMT4 substrate binding site, and that the appending side chain at position 7, incorporating a urea sub-element, binds
strongly in the arginine binding region in PRMT4. Overall, the synthesis of scaffolds B to E on a multigram scale has been achieved, opening the way for diversity driven parallel synthesis of novel compound libraries.
Lay Summary

The application and usefulness of the privileged structures, which are molecular frameworks, has emerged as an effective approach in an attempt to find novel biologically active molecules. Central to the success of this approach is having access to novel starting compounds with drug-like properties, which incorporate multiple activated sites in their structure where parallel synthesis can be carried out. Benzoisoxazolone and its derivatives show a wide range of therapeutic effects including anti-inflammatory, antioxidant, antitumor, antiviral and antimicrobial activity. This research project was focused on the development of benzoisoxazolone-based compounds with potential as isoform selective inhibitors of Rho-kinase (ROCK) for the treatment of diabetic cardiovascular disease and different cancers, as anti-HIV agents that block HIV pre-mRNA alternative splicing, and as inhibitors of protein arginine methyl transferase4 for the treatment of cancer.
Preface

The research in this thesis was approved by the Biosafety Committee of the UBC Office Research Ethics, certificate number B15-0030.

The research described in this dissertation was carried out by the author under the guidance and supervision of Dr. David Grierson in the Department of Pharmaceutical Sciences, University of British Columbia, between September 2011 and June 2017. Dr. David Grierson and the author are responsible for designing of the synthetic strategy. The thesis was thoroughly edited by Dr. David Grierson and he also contributed a tremendous amount of effort to a portion of Chapter 1 and the last paragraph of Chapter 2.

The evaluation of the isoform selective Rho kinase inhibitors was in collaboration with the Dr. Kath MacLeod laboratory. The compounds synthesized as anti-HIV agents were tested by Dr. Peter Cheung at the BC Center for Excellence in HIV/AIDS. The design and biological testing of PRMT4 inhibitors were in collaboration with the Dr. Adam Frankel and Dr. Tara Leah Klassen laboratories.

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<th>Definition</th>
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<tbody>
<tr>
<td>3'ss</td>
<td>3' Splice site</td>
</tr>
<tr>
<td>5'ss</td>
<td>5' Splice site</td>
</tr>
<tr>
<td>Å</td>
<td>Angstrom</td>
</tr>
<tr>
<td>Act</td>
<td>Activating group</td>
</tr>
<tr>
<td>ADAM</td>
<td>Alkenyl diarylmethane</td>
</tr>
<tr>
<td>aDMA</td>
<td>Asymmetric dimethylarginine</td>
</tr>
<tr>
<td>ADMET</td>
<td>Absorption, distribution, metabolism, excretion and toxicity</td>
</tr>
<tr>
<td>AdoHcy</td>
<td>S-adenosyl-L-homocysteine</td>
</tr>
<tr>
<td>AdoMet</td>
<td>S-adenosyl methionine</td>
</tr>
<tr>
<td>AGC</td>
<td>Protein kinase A, G and C</td>
</tr>
<tr>
<td>AIBN</td>
<td>Azobisisobutyronitrile</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>Ala</td>
<td>Alanine</td>
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<tr>
<td>ALLINIs</td>
<td>Allosteric integrase inhibitors</td>
</tr>
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<td>Androgen receptor</td>
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<td>Anti-retroviral therapy</td>
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<td>ASF1</td>
<td>Alternative splicing factor 1</td>
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<td>Asparagine</td>
</tr>
<tr>
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<td>Aspartic acid</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>B/C/P</td>
<td>build/couple/pair</td>
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<tr>
<td>BINA</td>
<td>Biphenylindanone A</td>
</tr>
<tr>
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<tr>
<td>c-AMP</td>
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<tr>
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<td>Dimethylformamide</td>
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<tr>
<td>DMG</td>
<td>Direct metalation group</td>
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<tr>
<td>DOS</td>
<td>Diversity-oriented synthesis</td>
</tr>
<tr>
<td>E1cB</td>
<td>Elimination unimolecular conjugate base</td>
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</tr>
<tr>
<td>E2F1</td>
<td>Transcription factor E2F1</td>
</tr>
<tr>
<td>EC\textsubscript{50}</td>
<td>Half maximal effective concentration</td>
</tr>
<tr>
<td>EDCI</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>Env</td>
<td>Envelope protein</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ESE</td>
<td>Exonic splicing enhancer</td>
</tr>
<tr>
<td>ESS</td>
<td>Exonic splicing silencer</td>
</tr>
<tr>
<td>Et\textsubscript{3}N</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>EtOAc</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-Aminobutyric acid</td>
</tr>
<tr>
<td>Gag</td>
<td>Group-specific antigen</td>
</tr>
<tr>
<td>GAR</td>
<td>Glycine- and arginine-rich</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GI\textsubscript{50}</td>
<td>Growth inhibition of 50%</td>
</tr>
<tr>
<td>Gln</td>
<td>Glutamine</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Gly</td>
<td>Glycine</td>
</tr>
<tr>
<td>HBTU</td>
<td>2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate</td>
</tr>
<tr>
<td>Het</td>
<td>Heterocycle</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>hnRNP</td>
<td>hnRNP</td>
</tr>
<tr>
<td>HOAc</td>
<td>Acetic acid</td>
</tr>
<tr>
<td>HOAt</td>
<td>1-Hydroxy-7-azabenzotriazole</td>
</tr>
<tr>
<td>HTS</td>
<td>High-throughput screening</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>Hyc</td>
<td>Hydrophobic channel</td>
</tr>
<tr>
<td>Hyp</td>
<td>Hydrophobic pocket</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IDC16</td>
<td>Indole compound 16</td>
</tr>
<tr>
<td>InSTIs</td>
<td>Integrase strand transfer inhibitors</td>
</tr>
<tr>
<td>IOPY</td>
<td>Iodopyridinone</td>
</tr>
<tr>
<td>IRS-1</td>
<td>Insulin receptor substrate-1</td>
</tr>
<tr>
<td>ISE</td>
<td>Intronic splicing enhancer</td>
</tr>
<tr>
<td>ISS</td>
<td>Intronic splicing silencer</td>
</tr>
<tr>
<td>kb</td>
<td>Kilo-base pair</td>
</tr>
<tr>
<td>Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>MAOB</td>
<td>Monoamine Oxidase B</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Breast cancer cell line</td>
</tr>
<tr>
<td>mCPBA</td>
<td><em>meta</em>-Chloroperoxybenzoic acid</td>
</tr>
<tr>
<td>MEM</td>
<td>Methoxyethoxymethyl ether</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>Met</td>
<td>Methionine</td>
</tr>
<tr>
<td>mGluR2</td>
<td>Metatropic glutamate receptor 2</td>
</tr>
<tr>
<td>MLL</td>
<td>Mixed lineage leukaemia</td>
</tr>
<tr>
<td>mM</td>
<td>Millimetre</td>
</tr>
<tr>
<td>MMA</td>
<td>Monomethylarginine</td>
</tr>
<tr>
<td>MOM</td>
<td>Methoxymethyl acetal</td>
</tr>
<tr>
<td>MOR</td>
<td>μ-opioid receptors</td>
</tr>
<tr>
<td>MS</td>
<td>Multiply spliced</td>
</tr>
<tr>
<td>NaBH&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Sodium borohydride</td>
</tr>
<tr>
<td>NBS</td>
<td>N-Bromosuccinimide</td>
</tr>
<tr>
<td>Nef</td>
<td>Negative Regulatory Facto</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>NMM</td>
<td>N-methylmorpholine</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NNRTI</td>
<td>Non-Nucleoside Reverse Transcriptase Inhibitors</td>
</tr>
<tr>
<td>NRTIs</td>
<td>Reverse transcriptase inhibitors</td>
</tr>
<tr>
<td>Oxone</td>
<td>Potassium peroxymonosulfate</td>
</tr>
<tr>
<td>PAM</td>
<td>Positive allosteric modulator</td>
</tr>
<tr>
<td>Phe</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>PK</td>
<td>Pharmacokinetics</td>
</tr>
<tr>
<td>POCl₃</td>
<td>Phosphoryl chloride</td>
</tr>
<tr>
<td>Pol</td>
<td>DNA polymerase</td>
</tr>
<tr>
<td>PPh₃</td>
<td>Triphenylphosphine</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>PRMT</td>
<td>Protein arginine methyl transferase</td>
</tr>
<tr>
<td>PTC</td>
<td>Premature termination codon</td>
</tr>
<tr>
<td>PTCR</td>
<td>Premature termination codon readthrough</td>
</tr>
<tr>
<td>Rev</td>
<td>Response element lentivirus</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated, coiled-coil-containing protein kinase</td>
</tr>
<tr>
<td>RRE</td>
<td>Rev response element</td>
</tr>
<tr>
<td>RRM</td>
<td>RNA recognition motif</td>
</tr>
<tr>
<td>SAH</td>
<td>S-Adenosyl homocysteine</td>
</tr>
<tr>
<td>SAM</td>
<td>S-Adenosyl methionine</td>
</tr>
<tr>
<td>Sam68</td>
<td>Src-Associated substrate in Mitosis of 68 kDa</td>
</tr>
<tr>
<td>SAR</td>
<td>Structural activity relationship</td>
</tr>
<tr>
<td>sDMA</td>
<td>Symmetric dimethylarginine</td>
</tr>
<tr>
<td>SF2</td>
<td>Pre-mRNA-splicing factor 2</td>
</tr>
<tr>
<td>SH3</td>
<td>SRC Homology 3</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>SI</td>
<td>Selectivity Index</td>
</tr>
<tr>
<td>S_N2</td>
<td>Bimolecular nucleophilic substitution</td>
</tr>
<tr>
<td>S_NAr</td>
<td>Nucleophilic aromatic substitution</td>
</tr>
<tr>
<td>snRNA</td>
<td>Small nuclear RNA</td>
</tr>
<tr>
<td>snRNP</td>
<td>Small nuclear ribonucleoprotein</td>
</tr>
<tr>
<td>SOCl_2</td>
<td>Thionyl chloride</td>
</tr>
<tr>
<td>SR</td>
<td>Serine-Arginine</td>
</tr>
<tr>
<td>SRE</td>
<td>Splicing regulatory element</td>
</tr>
<tr>
<td>SRPK</td>
<td>Serine/threonine-protein kinase</td>
</tr>
<tr>
<td>SRSF1</td>
<td>Serine/arginine-rich splicing factor 1</td>
</tr>
<tr>
<td>SRSF10</td>
<td>Serine and arginine rich splicing factor 10</td>
</tr>
<tr>
<td>SS</td>
<td>Singly spliced</td>
</tr>
<tr>
<td>ST7</td>
<td>Suppressor of tumorigenicity protein 7</td>
</tr>
<tr>
<td>Tat</td>
<td>Trans-Activator of Transcription</td>
</tr>
<tr>
<td>TBD</td>
<td>1,5,7-Triazabicyclo[4.4.0]dec-5-ene</td>
</tr>
<tr>
<td>TFFH</td>
<td>Tetramethylfluoroformamidinium Hexafluorophosphate</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin-layer chromatography</td>
</tr>
<tr>
<td>TMS</td>
<td>Tetramethylsilane</td>
</tr>
<tr>
<td>TMSCN</td>
<td>Trimethylsilyl cyanide</td>
</tr>
<tr>
<td>TMSOEt</td>
<td>Ethoxytrimethylsilane</td>
</tr>
<tr>
<td>Trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>UHP</td>
<td>Urea hydrogen peroxide</td>
</tr>
<tr>
<td>US</td>
<td>Unspliced</td>
</tr>
<tr>
<td>Vif</td>
<td>Viral infectivity factor</td>
</tr>
<tr>
<td>Vpr</td>
<td>Viral Protein R</td>
</tr>
<tr>
<td>Vpu</td>
<td>Viral Protein U</td>
</tr>
<tr>
<td>------</td>
<td>-----------------</td>
</tr>
<tr>
<td>μM</td>
<td>Micromolar</td>
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First and foremost, I would like to extend my sincere gratitude to my supervisor, Professor David Grierson, for sharing his extensive knowledge with me in the past six years. I am indebted of his instructive advice and useful suggestions throughout my PhD studies. I am deeply grateful of his patient instruction, insightful criticism and expert guidance in the completion of my degree as well as this thesis. Without his invaluable and generous help, the present thesis would not have been accomplished. Additionally, I would like to thank all my committee members including my chair: Dr. Adam Frankel, Dr. Urs Hafeli, Dr. Laurel Schafer and Dr. Stelvio Bandiera (chair) for their constructive comments and valuable advice throughout the years of my research.

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To my parents
Chapter 1

Introduction

1.1 Drug discovery: Natural product sources and the HTS-compound library approach

Drug discovery, which encompasses the fields of (medicinal) chemistry, molecular biology, biochemistry and pharmacology/pharmacognosy, is a lengthy, complicated and expensive process that begins with the identification of hit/lead compounds through in vitro and in vivo screens. Continued optimization of lead compounds through the stage of phase I-III clinical trials focuses on corrections to their drug-like properties, including modulation of their chemical stability, cell permeability, absorption, distribution, metabolism, excretion and toxicity/bioactivation (ADMET)\(^1\). Historically, natural products have been a major source of leads for the drug development\(^2\). They cover a wide range of therapeutic indications with great diversity and complexity of chemical structure. These include the macrolide antibiotic erythromycin, isolated from the bacteria \textit{Saccharopolyspora erythraea}\(^3\); galantamine, an Alzheimer drug isolated from daffodils in 1951\(^4\); the anticancer drugs paclitaxel (Taxol)\(^5\) and vinblastine\(^6\), extracted from the yew tree and the periwinkle plant, respectively; the immunosuppressant drug ciclosporin\(^7\), first isolated from fungus \textit{Tolypocladium inflatum} in 1971, and the cardiac drug digoxin isolated from foxglove plant in 1930\(^8\) (Figure 1).

Although the exploration of natural sources continues, today’s efforts in drug discovery are increasingly oriented in new directions that couple technologies for high throughput screening and protein structure determination to strategies for rapid synthesis of new chemical entities and the \textit{in silico} study of their interaction with different drug targets.

In this inventive climate, a modern paradigm in the pharmaceutical industry is to systematically reevaluate both existing “in-house” libraries, issuing from different SAR-driven drug discovery programs, and diversity (non SAR) driven libraries, prepared by
combinatorial/parallel synthesis, against a wide array of both new and already established therapeutic targets. An underlying motivation behind this strategy to discover new hits/leads for drug development is to “let molecules talk”, i.e., reveal useful/unexpected information about the binding options possible in an enzyme/receptor binding pocket or surface (protein-protein association/interaction inhibitors). This approach is of particular interest when searching for biologically active molecules against targets for which little or no useful mechanistic/structural data is available. In fact, in these situations compound library screening is one of very few options possible to identify new groundbreaking avenues for drug development.

**Figure 1:** Natural products as source of drugs.
As medicinal chemists, our laboratories contribution to the discovery of new biologically active compounds is to use diversity driven parallel synthesis as a means to construct an in-house library of drug-like compounds. This library has found use as a translational research tool in collaborative programs with partner biology laboratories to explore new therapeutic areas. As described in chapters 2-4 of this thesis, a crucial aspect of parallel synthesis is to have access to novel (privileged) scaffolds, which, depending on the nature of the appendages attached to them, give rise to new unexplored molecules displaying affinity toward an intended/unintended therapeutic target. As further discussed in chapter 4, the combination of parallel synthesis with virtual screening has proven to be a valuable method to guide the design of molecules targeting protein arginine methyltransferase 4 (PRMT4).

The discovery of new drug-like hits and leads against novel therapeutic targets in this manner has two important outcomes: It generates a new research orientation with the opportunity to generate new knowledge, and it creates the opportunity to be a leader in a new field rather than a follower.

1.2 “In-House” Compound Libraries

Development of HTS technologies, based on fluorescence (cf. ELISA) and other robust detection methods, tremendously advanced our ability to inexpensively carry out large-scale assays to evaluate small molecules (10,000-100,000 compounds and greater per test) for their biological activity against a wide range of therapeutic targets\textsuperscript{11, 12}. One response of “big pharma” to this capability was the creation of proprietary, i.e. “in-house” libraries. Indeed, decades of drug discovery research by the pharmaceutical industry on different drug discovery programs resulted in the accumulation of very large compound collections of molecules belonging to multiple compound classes. Some estimates indicate that the development of a new clinically approved drug requires the synthesis and evaluation of up to 10,000 analogues of an initial “hit” structure (Figure 2). The 9999 molecules that don’t make it to market for each application become members of the “in house” library. Continued
screening of these compounds against different drug targets has proven to be an effective strategy to reveal new biological applications, which are often unrelated to the reason for which they were initially synthesized (this identifies the true potential/value of a molecule). An advantage to re-screening in-house molecules is that, generally, the physico-chemical and drug-like properties of these refurbished molecules are already known. Considerable time can, thereby, be economized in bringing them down the discovery pipeline, and the chances of them failing in clinical trials are considerably diminished. Approximately 90% of molecules, identified as hits/leads in screening programs, fail as drug candidates in the later stages of drug development.

Figure 2: Steps involved in new drug discovery.

A potential inherent limitation to the exploitation of industrial sector in-house libraries is that the components issue from specific SAR-driven research programs, and consequently, there can be a relatively high degree of similarity between molecules within the same structural class (Figure 3).

The development of opioid derived drugs illustrates this latter point. Over the past century a lot of effort has been directed toward modulating the structure of morphine, isolated from the opium poppy (*Papaver somniferum*), such that its desired analgesic properties are separated from undesirable side effects (nausea, constipation, respiratory depression, and drug
tolerance/dependence$^{13}$. Following the traditional SAR process a conservative iterative structural change was made to the periphery of the morphine skeleton and the consequence this had on activity was used to decide/determine further modifications that could be made. Changes that were studied included modifying the nature of the $N$-methyl group, functionalizing the C-3/6 hydroxyl groups, reducing the C-ring double bond, exchanging the C-14 hydrogen atom by an OH group, and cutting out the C-4,5-bridging oxygen atom$^{14,15}$. On the positive side, this resulted in the discovery of the important opioid agonist drugs Hydromorphone$^{14}$, Oxycodone$^{16}$, and opioid antagonist Naloxone to treat opioid overdose$^{17}$. More infamous was the discovery of the street drug heroin (3,6-diaceylmorphine)$^{18}$. In terms of in house library content, the downside to SAR driven research is that the hundreds to thousands of failed morphine analogues all bear a close resemblance to the parent compound morphine. This creates a certain redundancy within an in-house library.

![Figure 3: SAR of Morphine.](image)

1.3 Combinatorial and Parallel Synthesis – Diversity-Driven Libraries

Combinatorial and parallel synthesis methodologies have been created to permit the rapid construction of diversity driven compound libraries. Using the formation of an amide bond as
an example, the combinatorial chemistry strategy involves the reaction of a varied series of amines with a series of different acid components in a single reaction vessel\textsuperscript{19}. Under these conditions, all possible combinations of amine-acid coupled products are, in principle, formed\textsuperscript{20}. The advantage of the combinatorial chemistry strategy is that a large library can be generated in a single experiment. Testing the reaction mixture allows rapid determination of whether any of the products formed show activity. The difficulty in this approach is that, when an active compound is revealed in the biological assay, it is necessary to deconvolute the library, i.e. separate the desired product from the large number of other product molecules in the mixture. Strongly inspired by the work by Merrifield on solid support synthesis of peptides, separation of the product mixture from the reactants, reagents, and side products produced in the reaction can be achieved by carrying out the reaction on polystyrene and other solid phase resins\textsuperscript{21}. However, the problem of separating the individual product molecules from each other remains.

Today, the combinatorial chemistry approach to hit discovery has largely been surpassed by parallel synthesis. Looking again at the synthesis of a library of amides, parallel synthesis corresponds to carrying out simultaneously, \textit{but in separate reaction vessels}, the reaction of one acid in a set of acids with one amine in a set of amines\textsuperscript{22}. In a $40 \times 40$ (acid) protocol this would correspond to running 1600 parallel reactions, corresponding to all combinations of reactants. In this way a distinct product is present in every reaction vessel, and the problem of deconvolution is eliminated. The robotic technology created around HTS can be adapted to running such large numbers of reactions.

An illustration of the power of parallel synthesis is the development of compound libraries that place emphasis on compound diversity. As initially conceived, diversity-driven compound libraries were purposely directed toward exploring “chemical space”, rather than the SAR style optimization of molecules relative to a specific therapeutic target/hit\textsuperscript{23}. Indeed, once again inspired by what Nature has achieved, a guiding principle has been to generate
library components with widely different structural-functional group organizations and widely different complexity. In addition, the idea was to create molecules that had not previously been evaluated for their biological potential.

One example, among many others, that illustrates the principles and requirements of parallel synthesis in diversity-oriented synthesis (DOS) is the work carried out by Schreiber et al to elaborate on solid phase a library of 29,400 complex polycyclic compounds (Figure 4)\textsuperscript{24}. Based on research developed by Fallis and co-workers\textsuperscript{25}, a triene intermediate such as 2, obtained in two steps from the resin loaded aromatic aldehyde 1, was used as a substrate for two sequential Diels-Alder (D-A) reactions\textsuperscript{24}. Indeed, the triene system in 2 is unique in that its 1,3-diene component can undergo a Diels-Alder reaction to produce a product possessing, itself, a newly formed 1,3-diene system that reacts in a D-A mode. For instance, reaction of 2 with the tetra-substituted dienophile 2,5-diphenyl-1,4-benzoquinone produced the monocycloaddition product 3. When maleimide (as the second dienophile) was introduced into the reaction medium, the second D-A reaction occurred, giving the tetracycle product 4. In the reaction of 2 with less encumbered disubstituted dienophiles, such as maleamide, the \textit{bis}-cycloaddition product 5 were formed directly. In-total, 63 di-, tri-, and tetra-substituted dienophiles were engaged in sequential Diels-Alder reactions with the 41 resin loaded trienes.

Three characteristics of this DOS approach were: i) the preparation of the complete library prior to submitting it to biological testing, ii) the use of solid phase synthesis as a means to manage the production/purification of a large number of molecules on a micro scale (“one bead – one stock solution tactic”), and iii) the use of a robust reaction which is amenable to parallel synthesis. Indeed, other than the fact that heating is generally required to promote the Diels-Alder reaction, it is a high yielding process, which accomplishes a lot of chemistry in a single step (creation of a ring, introduction of stereochemistry and positioning of a double bond).
Figure 4: A cycloaddition approach with triene 2 to obtain a library of polycyclic compounds by reacting with different di-, tri-, and tetra-substituted dienopiles.

Once liberated from their solid support with HF-pyridine (and then TMSOEt) and diluted to 10mM stock solutions in DMF, evaluation of these 29,400 structurally complex polycyclic products in an array of protein-binding and phenotypic assays revealed that some of them display specific and potent activities.24

Another example that emphasizes the impact of the DOS approach to drug-discovery is illustrated by the recent and spectacular discovery by Schreiber et al of a novel macrocyclic antimalarial agent.26-27. Outlined in Figure 5, they used the olefin metathesis reaction as a central component in a “build/couple/pair” (B/C/P) version of DOS to construct a library of medium-sized (14-membered) macrocyclic compound 11 from a common linear intermediate (6-9). Compound 11 contains three appendage-diversification sites which allow a rich collection of novel compounds with varied functionalities to be prepared. Compound ML238 was identified as a nanomolar inhibitor of *P. falciparum* (GI50=0.54 nM) through screening
this novel compound library\textsuperscript{27}.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5}
\caption{Overview of the B/C/P strategy of DOS yielded compound 11 with three appendage sites and the potent antimalarial agent ML238.}
\end{figure}

As illustrated in Figure 6, the DOS approach can also be used in more SAR-like manner in advanced drug discovery efforts to optimize lead compounds. The question addressed was whether introduction of a polar heterocyclic component at the C5 position in the iodopyridinone (IOPY) anti-HIV agent 12 would enhance its activity against drug resistant HIV mutant strains\textsuperscript{28}. Without knowing which heterocyclic motif would produce the optimal effect, the strategy chosen was to react the chloromethyl compound 13 with a diverse series of nitrogen, oxygen and sulfur nucleophiles in the presence of weak base. Under these conditions a 236-membered library was rapidly assembled, and tested. Compound 14a (indicated in blue circle in Figure 6) proved to be one of the most potent NNRTI type anti-HIV agents described to date. Unfortunately, this compound failed during ADMET studies, due to problems with clearance.
Figure 6: Parallel synthesis to make compound library with anti-HIV activity.

In a further illustration of work from our laboratory (Figure 7), parallel synthesis was used to prepare a diversity library containing molecules designed to be structural mimics of the tetracyclic indole compound IDC16 (a known cytotoxic agent). The objective was to discover non-cytotoxic compounds that would, like IDC16, block HIV replication through perturbation of the function of host cell SR (Serine-Arginine rich) protein SRSF1\textsuperscript{29}. This SR protein plays a crucial role in the alternative splicing of HIV pre-mRNA in HIV infected cells\textsuperscript{30}. 
Figure 7: Development of IDC16 mimics by parallel synthesis, and the active compounds 15-18 that contain an amide linker and a common benziosothiazole motif are confirmed from the preliminary anti-HIV testing.

In the absence of any structural/mechanistic information on SRSF1, the idea pursued was to construct a library of ring-opened molecules where the central (B/C) rings in IDC16 were replaced by seven selected spacer motifs (Figure 7)\textsuperscript{31}. The only constraint imposed was that all new molecules should maintain the overall outline of IDC16 when projected onto a 2D surface. Of the 256 mimics tested against wild type HIV, and strains resistant to all the current anti-HIV drugs used in ART therapy, activity was found for the four molecules 15-18, which possess an amide spacer unit and a common benziosothiazole motif as the D-ring.
replacement \(^{31}\). To place things in perspective, this discovery, which forms the foundation for the exploration of an entirely new strategy to treat HIV infection, would not have occurred if we had chosen to explore our hypothesis using the traditional (iterative) SAR approach, as compound 19, which most closely mimics the structure of IDC16 did not display any activity.

A second point of importance issuing from this work was that although amide bond formation (arguably one of the most important reactions in organic synthesis) is highly efficient for reactions involving nucleophilic amines (cf. amino acids), these reactions are often low yielding for coupling of non-nucleophilic amino substituted heterocycles (a consequence of the decreased electron density on the reacting nitrogen in these systems due to resonance delocalization). Indeed, the preparation of diheteroaryl amides in the library of IDC16 mimics proved to be difficult due to the presence of residual starting materials, the formation of by-products generated from the coupling reagents used, and competing side reactions, etc. To counter this problem our laboratory has developed a highly efficient amide bond synthesis protocol for parallel synthesis (Figure 8), which is based on the greater reactivity of the amine anion generated \textit{in situ} from N-TMS heterocyclic amines and the differential solubility between the amide products and the less polar activated acid (acid fluoride) and TMS amines used as reactants \(^ {32}\). Indeed, using acetonitile as the solvent for these reaction, results in selective precipitation of the desired amide product 20 in pure form.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure8.png}
\caption{Parallel synthesis of diheteroaryl amides 20 through acid fluoride and TMS-amine intermediate.}
\end{figure}
1.4 (Privileged) Scaffolds in drug discovery/Parallel synthesis

The use of structural elements (scaffolds) that display drug-like properties, that are activated toward reaction at several positions in their structure, and that become an integral part of the biologically active molecule being synthesized, is an important tactic in drug discovery research. The term “Privileged Scaffold” further refers to the fact that certain scaffolds can interact separately with more than one type of receptor or enzyme target depending upon the pattern and nature of the functional groups attached to it. A wide range of polyfunctionalized heterocycles and other systems have been studied and exploited for their potential use as (privileged) scaffolds in drug discovery. Recently, a European consortium of industrial and academic laboratories has been created (and heavily funded) to design additional/new scaffolds for drug research/compound library synthesis.

Nature has once again led the way by showing us the economy that can be achieved in using privileged scaffolds to carry out diverse cellular functions. For instance, the 19 derivatives of the glycine scaffold are the constituents of all proteins. Further, polyhydroxylated hydrocarbons (sugars) are exploited in both a myriad of biological process and as components of cellular architecture, and multiple compounds built upon the steroid nucleus display crucial biological properties.

The development of molecules based on the benzodiazepine (Figure 9) and purine ring systems (Figure 10) are just two examples of the use of privileged scaffolds in the development of synthesis-derived drugs.

The discovery of benzodiazepines as psychoactive drugs for treatment of anxiety was a case of serendipity (or irrational drug design). It did, however, motivate intense SAR studies on this class of compounds. As a consequence of making a wide range of peripheral modifications to this system, molecules were identified that are effective in other therapeutic areas, including drugs for the treatment of peptic ulcers (Pirenzepine) and HIV/AIDS.
(Nevirapine)\textsuperscript{37, 38}.

**Figure 9:** Benzodiazepine: a privileged scaffold in drug design.

Purine-based molecules are of considerable significance in biology, corresponding to two of the DNA/RNA building blocks (adenine and guanine), the energy producing molecule ATP, the secondary messenger c-AMP/GMP, and molecules such as caffeine. Taking advantage of the fact that conditions exist for the orthogonal (chemo-selective) functionalization at the C-2/8 (transition metal coupling), C-6 (S\textsubscript{N}Ar displacement), and C-9 (alkylation/acylation) centers, it is possible to vary greatly the nature of the functionalities at these positions\textsuperscript{39}. This is illustrated in the solid phase synthesis of the 2-acetylenylpurine based CDK1/CDK2 inhibitor 21 (Figure 10a)\textsuperscript{40}. As indicated in the diagram, by introducing different combinations of functionality onto the 4 indicated positions of the purine ring, molecules displaying very different biological activities are obtained (Figure 10b/c)\textsuperscript{41}. 
Figure 10: a) Synthesis of 2-acetylenylpurine based CDK1/CDK2 inhibitor 21. b) Potential applications of purine scaffold derivatives. c) Representative compounds of purine scaffold identified for different protein targets through high-throughput screening assay.
1.5 In Silico (Virtual) screening in drug discovery

Thanks to tremendous advances in computer technology and techniques in X-ray crystallography, there are an ever-increasing number of protein structures for which structural data is available. Further, in cases where a protein structure has not as yet been determined, the available data on protein fragments permits construction of more and more reliable homology models. As a consequence of virtual screening – the docking of compound libraries into an enzyme active/receptor binding sites - has become a powerful method for evaluating physical/virtual compound libraries for potential hit compounds. Indeed, using different algorithms/scoring functions, the binding free energy for different binding modes (conformations) can be predicted and the most likely binding modes can be ranked/prioritized. In this way, the “top virtual hits” can be selected for synthesis, or in the case of existing library “cherry picked” as candidates for biological testing. The latter scenario is a cost effective alternative to screening an entire library.

A successful example is the discovery of analgesic compound PZM21 by virtual screening (Figure 12). Like morphine, this molecule binds to the μ opioid receptor. However, unlike morphine, and all morphine like compounds, it displays reduced side effects (nausea, respiratory depression and constipation).

Recent studies showed that opioid analgesia results from $G_{\alpha i}$ coupling at the μ opioid receptor (MOR), while several potential serious side effects (respiratory and gastrointestinal dysfunction) are related to β-arrestin recruitment (Figure 11). Unbiased ligands such as morphine are engaged in both pathways whereas biased ligands can dissociate one pathway from the other. A recent experiment showed that the μ opioid receptors agonist morphine displays increased efficacy and duration of analgesic response with reduced side effects in β-arrestin-2 knockout mice, suggesting that G protein biased μ opioid receptor agonists would be more efficacious with reduced adverse events.
Figure 11: G-protein biased ligands can promote G-protein coupling and evade β-arrestin recruitment. (Figure is adapted from reference 43.)

Over 3 million commercially available lead-compounds were computationally docked at the binding pocket of MOR to seek new MOR agonists via a structure-based approach. Compound 22 was identified (Figure 12) as a novel small-molecule chemotype (structure distinct from other known opioid ligands) that was highly selective toward MOR, but induces minimal β-arrestin recruitment\(^{42}\). Structure-based optimization of compound 22 by adding a phenolic hydroxyl group yielded PZM21. PZM21 showed improved affinity (\(K_i\)) to 1 nM with undetectable β-arrestin recruitment in the assays. The binding interactions between PZM21 and MOR include hydrogen bonds between the urea and Gln124 and Tyr326, hydrophobic interactions between the \(N\)-methyl group and Met151 and Trp293, and an ionic bond with Asp147.
Figure 12: Discovery of G protein biased μ opioid receptor ligand PZM21 through virtual screening and its binding interactions with MOR. (Figure is adapted from reference 42.)

1.6 Thesis project: Benzoisoxazolone-based scaffolds for development of diversity driven small molecule libraries

In our laboratory, compound library synthesis and screening has been used to identify new avenues for the treatment of several major pathologies. These include novel anti-HIV agents that act on HIV pre-mRNA alternative splicing (collaborations: Dr. Peter Cheung and Dr. Richard Harrigan, BC center for Excellence in HIV/AIDS; Dr. Benoit Chabot, University de Sherbrooke; and Dr. Alan Cochrane, University of Toronto); novel isoform selective inhibitors of Rho kinase (ROCK1/2) for treatment of diabetic cardiovascular disease (collaboration: Dr. Kath MacLeod, Pharmaceutical Sciences, UBC); inhibitors of protein arginine methyltransferases (PRMT’s) in an epigenetic approach to cancer treatment (collaboration: Dr. Adam Frankel, Pharmaceutical Sciences, UBC); novel compounds that affect translational readthrough (genetic diseases and cancer) (collaboration: Dr. Michel Roberge, Life Science Centers, UBC); and novel anti-cancer agents targeting Bcl-x splicing (collaboration: Dr. Benoit Chabot, U de Sherbrooke). Crucial to these efforts are the development of robust-minimum waste strategies for parallel synthesis and access to novel
privileged scaffolds on which to build new chemical entities.

In the context of these programs, this thesis research project has been focused almost exclusively on developing efficient multi gram scale syntheses of the four amino/carboxy substituted 1,2-benzoisoxazolone systems B to E (Figure 13), as privileged scaffolds for the synthesis of diversity driven libraries. Further, during the course of this work several small exploratory libraries (6-20 compounds each) were prepared for evaluation as isoform selective inhibitors of Rho-kinase (ROCK), potential anti-HIV agents, and PRMT inhibitors.

A search of SciFinder, the electronic database created by the American Chemical Society, identified 829 “structures” for the 1,2-benzoisoxazolone system A. Alternatively, doing the search for the 1,2-benzoisoxazolone scaffold A by “reaction” identified 1721 hits, indicating that the same structure occurs in multiple publications. In contrast, the specific benzoisoxazolone derivatives B to E, of interest to us, where the appended substituents corresponds to NH, have received little attention, as indicated by the number of SciFinder hits: (B) 16; (C) 1; (D) 4; and (E) 0. These numbers indicate that there is considerable room to investigate the utility of these systems as scaffolds/privileged scaffolds to construct biologically active compounds.

Figure 13: The benzoisoxazolone ring system A and the amino/carboxy substituted benzoisoxazolones scaffolds B-E. Positions for introduction of diversity are indicated with blue arrows.
As background, a number of benzoisoxazolone-based compounds have been developed for therapeutic applications, including use as anti-inflammatory, antioxidant, antitumor, antiviral, and antimicrobial agents\textsuperscript{45-48}. For example, the selected compounds 23 to 25 (Figure 14) have been shown to inhibit the activity of phospholipases, and may find use to treat hepatic lipase and endothelial lipase mediated diseases\textsuperscript{45}.

![Figure 14](image1.png)

**Figure 14:** Selected structures for the treatment or prevention of disease mediated by phospholipases.

A novel series of 2-allylbenzo[d]isoxazol-3(2H)-ones were synthesized and their anticancer activity *in vitro* was evaluated using the HT-29 human colon cancer cells line. Three of them 26 to 28 (Figure 15) showed potent anticancer activity against HT-29 colon cancer cells (IC\textsubscript{50}=8.86 μM, 16.04 μM, 28.94 μM, respectively) compared to the standard drug 5-flurouracil (IC\textsubscript{50}=64.42 μM)\textsuperscript{46}.

![Figure 15](image2.png)

**Figure 15:** Novel benzoisoxazolone derivatives showed *in vitro* anticancer activities.

Further, to circumvent the metabolic liability, the aromatic ester function in the alkenyldiaryl methane (ADAM) class of non-nucleoside reverse transcriptase inhibitors (NNRTIs), one strategy was to replace the aromatic ester function by an isoxazolone system\textsuperscript{49}. This provided the hydrolytically stable ADAMs compounds 29 and 30 (Figure 16),
which maintain anti-HIV activity with decreased cytotoxicity\(^47\).

**Figure 16**: Replacement of the metabolically labile methyl ester moieties in the ADAMs with isoxazolone results in novel ADAMs.

Finally, 30-((2-cyclopentyl-6,7-dimethyl-1-oxo-2,3-dihydro-1H-inden-5-yloxy)methyl)-biphenyl-4-carboxylic acid (BINA) was identified through high-throughput screening as a metatropic glutamate receptor 2 (mGluR2) positive allosteric modulator (PAM), suggesting its use for the treatment of cocaine and other psychomotor stimulant addictions\(^50\). However, BINA lacks potency for mGluR2 both in vitro and in vivo and has poor pharmacokinetics (PK) properties. Modification of the indanone ring of BINA by incorporating N/O-heteroatoms led to new positive allosteric modulator 31 (Figure 17), which is more potent (EC\(_{50}\) value decreased from 0.51nM to 0.31nM)\(^48\). It also significantly improved the PK properties with excellent oral bioavailability and brain penetration.

**Figure 17**: The modification of BINA led to compound 31, which is more potent and has improved PK properties.
1.7 Research objectives

The objectives defined for this thesis research have their origin in preliminary results obtained for several ongoing projects in the laboratory.

**Objective 1: Benzoisoxazolone scaffolds B and C: Identification of isoform selective Type 1/Type 2 Rho-kinase inhibitors**

In a collaboration with Dr. Kath MacLeod to design isoform selective inhibitors of Rho kinase (ROCK 1/2) as probes to study their involvement in diabetic cardiovascular disease, it was found that the two pyridopyrazolone compounds SKK293 and 297, prepared by Dr. Stoyan Karagiosov (Research Associate in our laboratory), displayed selective inhibition of ROCK1 at concentrations comparable to the known “type 1” ROCK1/2 inhibitor Y27632. Our initial goal was to establish whether the related benzisoxazolone 32, obtained by functionalization of the exocyclic amino group in scaffold B would also represent a new family of ROCK inhibitors (Figure 18).

![Figure 18: Structures of pyridopyrazolone compounds and benzisoxazolone compound 32.](image)

To further explore the structural requirements for the development of type 1/type 2 inhibitors of the two ROCK isoforms, the preparation of a library of related compounds 33, from the isomeric C4-amino substituted benzoisoxazolone scaffold C was envisaged (Figure 19). In these structures the benzoisoxazalone motif still binds to the hinge residue, but the geometry of the overall molecule has been altered. This may better favor type 2 H-bonding of the
amide carbonyl in 33 to the backbone NH of the DFG sequence, in the DFG out conformation, and hydrophobic interactions between the terminal (hetero) aromatic motif in 33 and the allosteric pocket.

Figure 19: Design of C4-amino substituted benzisoxazolones 33 and their proposed type 2 binding mode in Rho kinase.

Objective 2: Benzoisoxazalone scaffolds D: Novel inhibitors of alternative splicing events crucial to HIV

In collaboration with Dr. Peter Cheung, Dr. Benoit Chabot and Dr. Alan Cochrane, compound 1C8, a ring opened mimic of IDC16, is under development as an anti-HIV/AIDS agent, which blocks HIV alternative splicing of HIV pre-mRNA\textsuperscript{31}. Interestingly, whereas IDC16 blocks the function of SRSF1, 1C8 targets the SR protein SRSF10\textsuperscript{51}. As HIV pre-mRNA alternative splicing is critically dependent on SR proteins, we are continuing the search for SRSF1 inhibitors through further manipulation of the structure of IDC16. The
concept being developed is to explore different modifications of the A and D rings of IDC16, through attachment of different motifs to the benzoisoxazolone scaffold D, which substitute for the central B/C-rings found in IDC16 (Figure 20).

Figure 20: Superposition of IDC16 and the benzoisoxazolone-based compound 34.

As seen from the superposition of the benzoisoxazolone derivative 34 onto IDC16, the 2D structure of the benzoisoxazolone-based IDC16 mimics very closely the outline of IDC16. Note in particular that the pendant side chains in structure 34 can adopt a conformation such that the terminal amino groups overlap with the A- and D-ring nitrogen atoms in IDC16, which are believed to play an important role in interactions with the human splicing factor SRSF1.

Objective 3: Benzoisoxazalone scaffold E: Small molecule library synthesis to identify bioactive compounds targeting PRMTs

Arginine methylation is a post-translational modification found on both nuclear and cytoplasmic proteins, which is catalyzed by protein arginine N-methyltransferases (PRMT’s)\textsuperscript{52}. Overexpression of PRMT1 is implicated in bladder and lung cancer cells\textsuperscript{53}, and
overexpression of PRMT4 has been observed in both breast and prostate cancers. With an established understanding of PRMT structural characteristics and function, both parallel synthesis and virtual screening were used to design benzoisoxazolone-based PRMT4 inhibitors possessing different urea substituted side chains (Figure 21) (collaboration with Dr. Adam Frankel and co-workers and Dr. Tara Leah Klassen, Pharmaceutical Sciences, UBC). The in silico activity found for the MAOB inhibitor GPS383 (synthesized in our laboratory) serves as the foundation for this research.

**Figure 21:** Structures of arginine and GPS383, proposed compound 35 including a guanidine group which can reinforce H-binding with PRMT4.
Chapter 2

C5 and C4-amino substituted benzoisoxazolone-based scaffolds B and C: Identification of isoform selective Rho-kinase inhibitors

2.1 Protein kinases

There are 518 protein kinases encoded in the human genome and they account for approximately 2% of all human genes\textsuperscript{55}. They comprise one of the largest families of phosphotransferases and play an important role in regulating enzyme activity by catalyzing the transfer of the phosphate group of adenosine triphosphate (ATP) to the hydroxyl group of serine/threonine, or tyrosine residues (Figure 22a)\textsuperscript{56, 57}.

![Figure 22: a) Phosphorylation of protein substrate by protein kinases. b) Chemical structures of Fasudil and Imatinib.](image)

Abnormal expression of protein kinases is associated with major pathologies, including cancer, diabetes and inflammation\textsuperscript{57, 58}. Development of kinase inhibitors began in earnest in the early 1990’s and this led to the approval in 1995 (Japan) of Fasudil, a Rho kinase inhibitor used to treat cerebral vasospasm and Imatinib (Gleevec) in 2001, the first targeted anti-cancer agent for the treatment of chronic myelogenous leukemia (CML) (Figure
Today there are 28 kinase inhibitors approved by US FDA on the market, and many others are in advanced stages of drug development.  

2.2 Rho-kinase  

Rho kinase or ROCK is associated with multiple physiological/pathophysiological processes. Indeed, over 20 downstream substrates are phosphorylated by ROCK, many of which are involved in cellular contractility, cell migration, actin-filament dynamics and cell adhesion. In collaboration with Dr. Kath MacLeod, whose research focuses on the study of the implication of Rho kinase (ROCK) in diabetic cardiomyopathy, our laboratory embarked on a project to design isoform selective ROCK inhibitors (PhD thesis Dr. Safwat Mohamed). Rho-associated protein kinase (ROCK), a member of the AGC family of kinases, is a serine/threonine protein kinase that was discovered as a downstream effector of Rho in 1995. The two isoforms of ROCK (ROCK1 and ROCK2) share an overall 65% sequence similarity in their amino-acid sequence and 92% sequence similarity in their kinase domain. Given this high degree of similarity in their kinase domains, it was initially assumed that the two ROCK isoforms would share the same substrates. However, experiments have shown that the two ROCK isoforms have important and distinct physiological/pathophysiological roles. Relevant to our project, Dr. MacLeod’s lab found that increased activation of the RhoA/ROCK pathway plays a critical role in the contractile dysfunction associated with diabetic cardiomyopathy based on the finding that acute administration of ROCK inhibitors improved ventricular function in vivo in diabetic rats, and that of the two ROCK isoforms, ROCK2 may be particularly important in diabetic cardiomyopathy. Further, insulin resistance plays a critical role in type 2 diabetes, as the activity of the RhoA/ROCK pathway is increased in the skeletal muscles of the insulin-resistant Zucker obese rat. It was suggested that ROCK is responsible for the abnormalities in insulin signalling through phosphorylation of serine of the insulin receptor substrate-1 (IRS-1). Long-term treatment with Rho-kinase inhibitors can prevent the
phosphorylation of IRS-1 and correct glucose and lipid metabolism\textsuperscript{73}. These findings and others using knock-out mice provide a solid argument for the development of isoform selective inhibitors of ROCK. However, this has proven to be a formidable challenge.

2.3 Kinase (ROCK kinase) architecture

In common with essentially all known protein kinases, the kinase core of ROCK1 and ROCK2 is composed of two lobes/domains (Figure 23): a small N-terminal lobe containing five anti-parallel β sheet strands and the C-helix, and a largely α-helical C-terminal lobe\textsuperscript{74}. The two lobes are connected by a short loop containing three crucial “hinge” residues\textsuperscript{75}. The ATP binding pocket is situated at the interface between the two lobes and the adenine ring of ATP forms donor/acceptor H-bonds with the amide backbone NH and C=O of the hinge residues. The peptide substrate interacts primarily with the “activation loop”, a 25-30 amino acid segment that corresponds to a key regulator of kinase activity\textsuperscript{74, 76}. In its open conformation the activation loop permits binding of both ATP and the peptide substrate in the enzyme binding-catalytic site. At the beginning of the activation loop is a conserved three-residue Asp-Phe-Gly (DFG) sequence that contributes one of the catalytic aspartate residues (Asp216/232). The catalytic site (phosphate binding) residues of both ROCK enzymes are Asn203/219, Asp198/214, Asp216/232 and Lys105/121. The Lys105/121-Glu124/140 (C-loop) pair, a conserved feature of the catalytically active kinase conformation, also plays a crucial role in positioning the triphosphate motif\textsuperscript{77-79}. 
2.4 Activation Loop conformations - Type 1/Type 2 kinase inhibitor models

The kinase activation loop can adopt two extreme conformations that are in equilibrium (Figure 24a): the open (active) conformation and the closed (inactive) conformation\(^8\). In the activation loop open state the DFG sequence adopts the “in” (pointing into the catalytic site) conformation\(^8\). In this conformation the catalytic residues are correctly oriented/positioned for the enzyme-catalyzed reaction to occur (Figure 24b, left)\(^8\). In contrast, in the activation loop closed conformational state, the DFG motif is shifted away by approximately 10 Angstroms from its position in the catalytic site (DFG “out” conformation)\(^8\). In the closed conformational state the catalytic site is disorganized and binding of both ATP and the peptide substrate is blocked (Figure 24b, right).

**Figure 23:** General kinase architecture and residues numbering for the components at the ATP binding site in two ROCK isoforms.
Figure 24: a) Structural difference between activation loop open (left) and closed (right) conformations, b) organization of ATP binding and kinase catalytic site (left) and type 2 inner binding pocket (right). (Figure is adapted from reference 81 and 82.)

The majority of small-molecule kinase inhibitors developed to date target the ATP binding site in the activation loop open conformation. Referred to as type 1 kinase inhibitors, they mimic ATP binding to the hinge residues and are ATP competitive\(^{76}\). However, essentially all inhibitors of this class bear no structural resemblance to ATP. This is a consequence of the fact that the geometry/composition of the catalytic site between kinases is highly conserved\(^{83}\). Developing inhibitors that project uniquely into the adenine, ribose sugar and phosphate binding regions would, thus, not display kinase selectivity, i.e. would not distinguish the intended target kinase from all other kinases. It is fortunate, therefore, that two zones/pockets
exist in the ATP binding site that are not used in ATP binding. As the size, geometry, and composition of these non-conserved pockets varies between kinases, projecting motifs in the inhibitor structure into these regions is an important strategy to design kinase selective type 1 kinase inhibitors/drugs\textsuperscript{81}. The proximity of the small inner hydrophobic selective pocket (Hyp1) and the outer hydrophobic channel (Hyc1), which connects to the polar solvent exposed surface of the enzyme, is depicted in the type 1 binding model (Figure 25a)\textsuperscript{65}. Using dasatinib to illustrate type 1 inhibitor binding (Figure 25b), one sees that it forms two H-bonds to the hinge and projects the polar components in its structure toward the Hyc1 channel/solvent exposed surface and the smaller aryl motif toward the inner Hyp1 pocket\textsuperscript{84}.

**Figure 25:** a) Composition of the type 1 ATP/kinase inhibitor binding site: adenine-binding region, sugar-binding region and phosphate-binding region, hydrophobic region I and II, and gatekeeper residue; b) Type 1 model showing bound Dasatinib. (Figure is adapted from reference 65.)
Figure 26: Representation of the composition of the type 2 kinase inhibitor binding site with the inhibitor imatinib. (Figure is adapted from reference 74.)

As the activation loop closes, leading to the inactive kinase conformation, there is a concomitant and significant enlarging of the inner pocket to include the DFG sequence and the Lys-Glu pair (Figure 24b, right)\textsuperscript{61, 76, 77}. Further, there is creation of a hydrophobic region (allosteric site) toward the one the extremity of this pocket\textsuperscript{83}. Imatinib, and other type 2 kinase inhibitors, bear functionality that interacts with this kinase region being formed during the open→closed transition, and consequently the inhibitor ultimately captures and stabilizes the closed state. Type 2 kinase inhibitors are typically elongated/extended molecules possessing a signature amide/urea function flanked by two aromatic (hydrophobic), which interact with the residual Hyp pocket and the allosteric site, respectively. As seen for imatinib in the type 2 binding model (Figure 26), H-bonding to the hinge region is conserved and H-bond contacts are made between the amide function and the Glu carbonyl oxygen and the backbone Gly NH of the DFG sequence.

2.5 Type1/Type 2 Rho-kinase inhibitors

Fasudil, the first approved kinase inhibitor, and its active metabolite hydroxyfasudil bind in the ATP site in Rho kinase (ROCK) such that the homopiperazine motif interacts either with
the ribose (R) or the catalytic phosphate (P) regions, respectively (Figure 27a)\textsuperscript{76}. Fasudil displays no isoform selectivity (ROCK1: IC\textsubscript{50}=1.2μM; ROCK2: IC\textsubscript{50}=0.82μM). Another pyridine-based compound Y-27632 (ROCK1: IC\textsubscript{50}=140nM; ROCK2: IC\textsubscript{50}=220nM) (Figure 27b), competes for the ATP binding site and is slightly more potent than fasudil, but it also inhibits both ROCK1 and ROCK2\textsuperscript{85}. Note, as there is no contact with the Hyp1 and Hyc1 pockets for fasudil/hydroxyfasudil, they are also non-selective inter-kinase inhibitors, possessing affinity for a relatively broad spectrum of kinases.

\textbf{Figure 27:} a) Fasudil and hydroxylfasudil (pyridinone tautomer) in complex with ROCK1 and their binding in the 3D representation of the type 1 model; b) Molecular structures of three classic ROCK inhibitors. (Figure is adapted from reference 76.)
Over the past decade, intensive effort has been made to design newer and more potent Rho kinase inhibitors that are both inter-kinase and isoform selective. The progress of this research has been described in several excellent reviews\textsuperscript{55, 86-90}. Considering the almost 100\% sequence identity in the kinase active site of ROCK1/2, particular attention has been directed toward developing type 2 ROCK inhibitors. Indeed, as the length and composition of the activation loop varies between kinases, the idea was that differences in the folded/closed conformation could represent an opportunity to achieve discrimination between the isoforms. However, the conclusion drawn from detailed structural analysis of type 2 kinase inhibitors targeting other kinases suggests that this paradigm is too simplistic and possible incorrect. Further, it has been revealed through multiple crystallographic studies of ROCK-inhibitor complexes that basically all known ROCK inhibitors, irrespective of their size and structural complexity, bind to ROCK via the type 1 (activation loop open) mode.

The current generation of ROCK inhibitors with enhanced potency/ROCK selectivity falls into defined classes, according to the nature of the hinge-binding elements. Selected examples are shown in Figure 28 for compounds built on the pyridine, indazole, pyrazole, and aminofurazane scaffolds\textsuperscript{86, 91-94}. Interestingly, although most/all of these compounds have an extended “type 2-like” structure, they are all type 1 kinase inhibitors. Indeed, it is amazingly that although the aminofurazane compound 49 has a long extended structure, including a central diarylamide motif (see the red box), which is typical of type 2 kinase inhibitors, this molecule is a type 1 inhibitor with a subnanomolar IC\textsubscript{50} value. It projects all the functionality attached to the hinge binding motif into the hydrophobic channel (Hyc1) and out onto the solvent exposed surface\textsuperscript{91}.

A noted exception to general tendency for ROCK inhibitors to have an elongated/linear structure, the presence of the dihydropyridinone motif in compound 41 serves to direct the terminal aryl group at an oblique angle\textsuperscript{95}.
Figure 28: Selected Rho-kinase inhibitors organized according to their hinge-binding group: pyridine, indazole, pyrazole and aminofurazane (the hinge-binding atoms are indicated in blue).
Despite a large amount of research, up until recently only one molecule, Slx-2119, was reported to display isoform selectivity toward ROCK2 (Figure 29) (ROCK1: \( IC_{50}=24\mu M \); ROCK2: \( IC_{50}=0.11\mu M \))^96. As no structural data has become available concerning the origin of the selectivity observed, it was presumed (by us) that the presence of the central quinazoline ring was a determining feature. However, replacement of this bicyclic motif by a pyrimidine ring, as in 50 and 51, resulted in more potent ROCK2 inhibitors with a nearly equivalent selectivity index (SI: \( IC_{50}\)ROCK1/\( IC_{50}\)ROCK2=90 to 170)^97. Note that compounds 52 and 53, where the indazole motif has been replaced by a phenyl substituted pyrazole and pyridine motif, and the “right side” isoindole subunit is retained are even more ROCK2 selective (SI > 5000)^97. Structural data for these new inhibitors in complex with ROCK2 is still missing. Hence, it is not known whether this molecule is a type 1 or type 2 ROCK inhibitor. Be that as it may, the activity determined for these interesting compounds does define a direction to be taken to achieve isoform selectivity.

![Molecular structures of isoform selective ROCK2 inhibitors.](image)

**Figure 29:** Molecular structures of isoform selective ROCK2 inhibitors.

### 2.6 Project objective and design strategy

Based on preliminary indications (one point assay at 10\( \mu M \) concentration) that the
pyridopyrazolone compounds SKK293/297 are equipotent to the reference non selective ROCK inhibitor Y27632 and they appeared to be ROCK1 selective, our objective was to determine whether the corresponding 5-aminobenzoisoxazolone scaffold B, in which the “pyridine-like” nitrogen in the SKK compounds becomes an “aniline-type” substituent on the ring, would provide new opportunities to design isoform selective ROCK inhibitors, and in particular those selectively inhibiting ROCK2 (Figure 30).

In such molecules, the internal amide motif can interact with the DFG subunit, and introduction of diverse components on the right side of the molecule will enable us to optimize interaction with the allosteric site. An additional option to explore interactions with the DFG motif and the allosteric site using scaffold B would be to alter the nature of the linker unit, as illustrated for the urea type compounds 32 where n=0.

**Figure 30:** Structures of C5-substituted benzoisoxazolone-based compounds 32 and the representation of the kinase/inhibitor binding sites.
In a further exploration of the benzoisoxazolone system as a platform for designing Rho kinase inhibitors, the isomeric C4-amino substituted benzoisoxazolone scaffold C can be used to prepare compounds of general formula 33 (Figure 31). In these structures the benzoisoxazolone motif can still bind to the hinge residue, but the geometry of the overall molecule has been altered.

**Figure 31:** Design of C4-amino substituted benzisoxazolones 33 and their proposed type 2 binding mode with Rho kinase.

### 2.7 Chemistry

#### 2.7.1 Synthesis of the benzisoxazolone ring system

There are a number of different approaches possible to construct the benzisoxazolone ring system A. However, almost universally followed are the two approaches illustrated in Figure 32. In these strategies the pivotal hydroxamic acid intermediate 55 or 57 are prepared from readily available carboxylic acid precursors and are activated toward ring closure by either conversion of the hydroxamic acid OH in 55 to a good leaving group (a) or by reaction of
compound 57 with base to induce an intramolecular $S_N$Ar reaction (b)\textsuperscript{99}. In the former cyclization process, the hydroxamic acid OH activation has been achieved using a variety of reagents and conditions (reaction with CDI\textsuperscript{100}, SOCl\textsubscript{2}\textsuperscript{46}, PPh\textsubscript{3}/DIAD (Mitsunobu conditions)\textsuperscript{101}, PPh\textsubscript{3}/EDCI\textsuperscript{102}, and NaOCl\textsuperscript{103}, etc).

![Diagram](https://example.com/diagram.png)

**Figure 32:** The two different approaches to construct the benzisoxazolone ring system A.

2.7.2 Synthesis of the C5-amino substituted benzoisoxazolone scaffold B and its application to the preparation of a limited library of isoform compound selective Rho-kinase inhibitors 32

Expanding the SciFinder structure search concerning scaffold B (Figure 13) to include all cases where the C5-substituent corresponds to a nitrogen atom identified 33 hits, approximately half of which corresponded to molecules where the nitrogen atom is part of a ring, and the other half to cases where the nitrogen atom corresponded to a nitro group. Indeed, there was only 1 citation where the substituent corresponded to NH\textsubscript{2}\textsuperscript{104}. This indicated to us that the primary goal in the synthesis of scaffold B was to obtain the C5-nitro substituted compound 59, and to subsequently find means to selectively reduce the NO\textsubscript{2} function without concomitant cleavage of the isoxazolone N-O bond. Our synthesis of scaffold B thus uses the readily available and inexpensive 5-nitro salicylic acid derivative 58 as the starting material (Figure 33).
Figure 33: Strategy to construct scaffold B from 5-nitro salicylic acid derivative 58.

As it is well established that hydroxylamine reacts efficiently with esters to give the corresponding hydroxamic acids\(^\text{105}\), compound 58 was first converted to its methyl ester 60 by reaction with methanol containing concentrated sulfuric acid (Figure 34)\(^\text{106}\). According to expectation, reaction of ester 60 with hydroxylamine hydrochloride provided the hydroxamic acid intermediate 61 in high yield. Dehydrative cyclization of 61 through reaction with CDI at 60°C in THF gave the benzoisoaxazolone intermediate 59 (71% overall yield for the three steps). In hindsight, and as described further on for the preparation of scaffold C (see Figures 51, from 98 to 99), it should be possible to synthesize compound 59 directly from the salicylic acid precursor 58 using CDI as both the acid activating group to obtain 61 and the reagent for its cyclization to 59.

Figure 34: Synthesis of compound 59 from 5-nitrosalicylic acid 58.
With intermediate 59 in hand, the crucial step involving reduction of the nitro group was studied under different conditions. Initially, the idea was to use catalytic hydrogenation condition using palladium on carbon as the catalyst\(^{107}\). However, it was found that both nitro group reduction and cleavage of the N-O bond in the isoxazolone ring occurred giving 62 (Figure 35). The structure of compound 62 was confirmed by \(^1\)H NMR and mass spectrometry (the singlet appears at 11.79 ppm corresponding to phenolic OH proton; the two peaks appear at 8.13 ppm and 7.63 ppm corresponding to the amide NH\(_2\)). Similarly, concomitant opening of the isoxazolone ring was observed when 59 was reacted with iron powder, in either a mixture of glacial acetic acid, ethanol and water under ultrasonic irradiation for 1h at 30°C\(^{108}\), or in concentrated hydrochloric acid at room temperature\(^{109}\). Other conditions using sodium borohydride and copper(II) sulfate pentahydrate in methanol at 70°C\(^{110}\) or sodium borohydride and nickel(II) acetate tetrahydrate in aqueous solution at room temperature\(^{111}\) were also explored, but, again, they resulted in the cleavage of the isoxazolone ring.

**Figure 35:** Reduction of the nitro group in compound 59 under different conditions.

Ultimately, chemoselective nitro group reduction was achieved in 54% yield by reacting 59 with stannous chloride in hydrochloric acid at room temperature for 7 days\(^{104}\) (Figure 36a). The mechanism for the nitro group to amine reduction with SnCl\(_2\) is shown in Figure 36b\(^{112}\).
In this process, the acid acts as a proton donor and the stannous ions serve as electron donors for the reduction step. This reaction was optimized by reacting 59 with a mixture of SnCl₄ and SnCl₂ in the presence of concentrated hydrochloric acid at room temperature for 4 hours. Scaffold B was successfully obtained under this mild condition in 61% yield as free amine.

Figure 36: a) Reduction of the nitro group in compound 59 by using stannous chloride or the mixture of stannous chloride and stannic chloride. b) Mechanism of reduction of nitro group to amine.

Scaffold B was used as the building block for the synthesis of an exploratory library of C5-amino benzoisoxazolone derivatives 32a-n through N-alkylation with different N-(hetero)aryl substituted 2-bromoacetamides 63a-n under microwave heating condition in the presence of sodium bicarbonate in DMF in 40%-60% yield. Compounds 63a-n were prepared through the reaction of the aromatic and heteroaromatic amines Ar(Het)-NH₂ with bromoacetyl bromide as shown in Figure 37.
Concerning these $N$-alkylation reactions, as scaffold B has both an amide and a primary amine center in its structure, its reaction with the 2-bromoacetamides 63 could, in principle, take place at either (or both) the aniline or the amide nitrogen atoms. We also needed to take into consideration that $O$-alkylation of the amide oxygen could occur. Our belief was that, although an aniline-type nitrogen is weakly nucleophilic, the primary NH$_2$ group would be more reactive in nucleophilic substitution reactions. However, initial experiments showed that the amide nitrogen was $N$-alkylated. In fact, when K$_2$CO$_3$ or Cs$_2$CO$_3$ was used as the base under microwave heating conditions, alkylation of the amide nitrogen preferentially took place, giving compound 64a-e (Figure 38). In the $^1$H NMR spectra, the disappearance of the amide N-H peak in scaffold B was noted, and there was a new broad singlet around 4.5ppm, which corresponded to the NH$_2$ group in compound 64.
Base: K$_2$CO$_3$ or Cs$_2$CO$_3$

**Figure 38:** Synthesis of amide nitrogen alkylation compound 64-e.

To prove that this was the case, we purposely effected $N$-alkylation of the amide nitrogen in the 5-nitro intermediate 59 (Figure 39). Subsequent reduction of the nitro group in 65 provided the amide alkylation product 64c. Comparison of the $^1$H NMR data for 64c with that for the alkylation of scaffold B using bromomethyl acetamide 63c conclusively showed that the two molecules were identical.

**Figure 39:** An alternative route to synthesis amide nitrogen alkylation compound 64c.

It was apparent from these experiments that K$_2$CO$_3$ or Cs$_2$CO$_3$ was a strong enough base to efficiently deprotonate the amide nitrogen in scaffold B. To the extent that this occurred, the
derived amide anion will be the more reactive partner in the alkylation reactions. To prevent this from happening, we investigated the $N$-alkylation of scaffold $B$ using the weaker base NaHCO$_3$. Indeed, using NaHCO$_3$ as the base under microwave heating conditions, the primary amine $N$-alkylation products $32a-n$ were the major products formed (TLC control), being isolated in 40% to 60% yield. Note that, no competing $O$-alkylation was observed. This contrasts with the results observed for the corresponding alkylation of the benzisoxazolone nitrogen in scaffolds $D$ and $E$, which led to formation of both the $N$- and $O$-alkylation products (Figures 82 and 118).

To date, we have prepared 14 molecules corresponding to general formula $32$ (n=1) (Figure 37). The C5-amine benzoisoxazolone system and the CH$_2$CONH linker are the common features in this library of potential ROCK selective inhibitors. The different monocyclic and bicyclic aromatic amines and nitrogen containing hetero-aromatic amines corresponding to the Ar(Het) component were chosen as they contain polar and non-polar substituents offering different options for H-bonding to the DFG motif and interactions with the allosteric binding site in the ROCK1/2 type 2 kinase inhibitor binding pocket. Disappointingly, none of these compounds clearly displayed inhibitory activity in the limited “one point” ROCK assays that were performed (limited funding for the biological testing hindered further development of this project).

2.7.3 Synthesis of the C4-amino benzoisoxazolone scaffold $C$ in view of the construction of an isoform selective ROCK inhibitor library

Based on the strategy used to prepare scaffold $B$ (Figure 33), the logical approach to the construction of scaffold $C$ would be to use 2-hydroxy-6-nitro benzoic acid $66$ as the starting material (Figure 40). Indeed, the presence of the amino group at C4 in a protected/deactivated form (e.g., NO$_2$) in this molecule would be a convenient way to avoid competing reaction of the amino and phenolic hydroxyl group (see red box in Figure 40) during formation of the benzoisoxazolone ring. Unfortunately, compound $66$ is prohibitively
expensive, as it is only a minor component formed in the nitration of benzoic (salicylic) acid derivatives.

![Diagram](image)

**Figure 40:** Using 2-hydroxy-6-nitrobenzoic acid 66 or 2-chloro-6-nitrobenzoic acid 67 as the starting material to synthesis scaffold C.

The obvious alternative option at this point was to use commercial and inexpensive 2-chloro-6-nitrobenzoic acid 67 (Figure 41). The idea was to convert this compound to the hydroxamic acid intermediate 69 and to effect the intramolecular S$_N$Ar displacement of the chloro group by the CONHOH oxygen under base conditions to give the target 4-NO$_2$ substituted version of scaffold C (compound 68). In the experiment, reaction of acid 67 in neat thionyl chloride resulted in complete conversion to its acid chloride. After removal of the excess SOCl$_2$ *in vacuo*, this intermediate was taken up in DCM and reacted with hydroxylamine hydrochloride in the presence of N,N-diisopropylethylamine to give hydroxamic acid 69 in 82% yield.$^{115}$

![Diagram](image)

**Figure 41:** Proposed scheme to synthesis 68 from 2-Chloro-6-nitrobenzoic acid 67.

However, subsequent reaction of 69 with DBU$^{116}$ or potassium carbonate as base did not
result in formation of the cyclized product 68. Instead, there was exclusive formation of amine 70 in 81% yield (Figure 42), resulting from a competing (and unexpected) Lossen rearrangement\textsuperscript{117}.

Figure 42: Lossen rearrangement occurred when using DBU and K$_2$CO$_3$.

The mechanism of classic Lossen rearrangement is depicted in Figure 43\textsuperscript{118}. Hydroxamic acids are transformed to isocyanates 72 via the formation of an O-acyl intermediate 71. Isocyanates 72 can then be either trapped by reaction with amines to form ureas 73 or degrade to the respective amines 74 in the presence of water.

Figure 43: Mechanism of the classical Lossen rearrangement.

Pertinent to our work is the literature example wherein treatment of aromatic hydroxamic
acids with metal bases such as K$_2$CO$_3$, CH$_3$COOK and Zn(CH$_3$COO)$_2$ or tertiary amine bases such as DBU, TBD and DABCO, leads to formation of aniline-type products. The mechanism suggested for the metal base-assisted Lossen rearrangement reaction is shown in Figure 44. In this process, a metal complexe of the nitrogen-deprotonated hydroxamic acid 75 is formed, which rearranges to metal carbamates 77, which decompose to corresponding amine.

**Figure 44:** Metal-assisted Lossen rearrangement of hydroxamic acids.

In light of this competing reaction, a different route to scaffold C from 2-chloro-6-nitrobenzoic acid 67 was investigated. Drawing inspiration from the approach taken by Laura Bandy (a MSc student in our laboratory) to construct the related benzoisoxazol-3-amine system 81 through condensation of the potassium anion of benzophenone oxime 79 (a hydroxide ion equivalent) with 2-fluoro-5-nitrobenzonitrile 78 (Figure 45a), compound 67 was reacted with potassium anion of oxime 79, with the intention of generating compound 83 (Figure 45b). The mechanism was suggested in Figure 45b. Reaction between benzophenone 82 and hydroxylamine hydrochloride with pyridine in methanol heating at 60°C for 2 hours afforded oxime 79 in 83% yield. However, displacement of the C2-chloro group in 67 proved to be challenging. It only resulted in recovering of starting material acid 67 after several attempts. In an effort to understand why the reaction failed, the corresponding reaction of the potassium anion of benzophenone oxime 79 with 6-chloro-2-nitrobenzonitrile 84 was explored (Figure 46).
Figure 45: a) Two-step synthesis of 5-nitro-3-aminobenzisoxazole 81. b) Proposed scheme to synthesize nitro-compound 68 from acid 67 and the suggested mechanism.
Unexpectedly (once again), the reaction did not proceed as planned, as the only product isolated was compound 85, resulting from S_NAr displacement of the nitro rather than the chloro group. Indeed, it was observed that reaction of the corresponding 84 with the potassium anion of benzophenone oxime 79 resulted in preferred displacement of the nitro group to give 85.

![Chemical structure](image)

**Figure 46:** 6-Chloro-2-nitrobenzonitrile 84 was used instead of 2-chloro-6-nitrobenzoic acid 67. However, nitro group rather than chloro group in compound 84 was displaced.

Calculation of the electron densities (Spartan software)\textsuperscript{121} at the C2 and C6 positions in 84 indicated that the nitro substituted center would be more electrophilic, i.e. more susceptible to react with a nucleophile (Figure 47). Interestingly, the difference in the electron density on the C2 and C5 centers in 86 is smaller. This observation negated the use of 2-chloro-6-nitro carboxylic acid 67 as an alternate nitro benzoic acid starting material.

![Electron density calculation](image)

**Figure 47:** Electron density calculation of 2-chloro-6-nitrobenzonitrile 84 and 2-chloro-5-nitrobenzonitrile 86 results from Spartan software.

When taken together, these results suggest that the chloro acid 67 will not allow us to readily
access the OH compound 66 or our target benzoisoaxazolone scaffold C. Our attention was consequently directed toward two alternative strategies to obtain 2-amino-6-hydroxybenzoic acid 88: i) introduction of the carboxyl group via ortho-lithiation of N-pivaloyl protected 3-aminophenol 87, or ii) oxidation of the methyl group in readily available (inexpensive) 2-methyl-3-nitrophenol 89 to give the needed nitro-substituted phenol 66 (which can be subsequently reduced to 88) (Figure 48).

**Figure 48:** Synthesis of 2-amino-6-hydroxybenzoic acid 88 and 2-hydroxy-6-nitrobenzoic acid 66 from 3-aminophenol 87 and 2-methyl-3-nitrophenol 89, respectively. (DMG: directed metalation group)

### 2.7.3.1 Directed ortho-metalation strategy – Amino group protection/deactivation

The directed ortho-metalation reaction is a highly regioselectivity reaction whereby electrophiles can attach themselves exclusively to the ortho-position of a directing group on an aromatic ring. In our case, the objective was to place a carboxylic acid group between the oxygen and nitrogen atoms in 3-aminophenol 87. This reaction was favored by introducing ortho-directing groups onto both the phenolic OH and the aniline-type nitrogen. The choice of the appropriate ortho-directing groups was inspired from work by Gould et al\textsuperscript{122}. They observed (Figure 49) that for the 3-aminophenol derivative 90, where a O-MOM group and a N-Boc are present, the reaction of the anion produced upon treatment with t-BuLi at -78°C with methyl chloroformate, as the CO\textsubscript{2} source, led primarily to the unwanted 4-substituted compound 91\textsuperscript{122}. However, when the N-directing group was changed to a methyl carbamate, the desired compound 93 was predominantly formed (albeit in low yield (33%))\textsuperscript{122}. Similar yields were obtained when we repeated their work.
Figure 49: MOM and Boc as the direct metalation groups led to product 91; MEM and carbamate as the direct metalation groups led to product 93.

Further, in experiments where we replaced $t$-BuLi by the safer (easier to manipulate) and weaker base $n$-BuLi, the reaction at 0°C led exclusively to the formation of the addition product, pentanamide 95 (Figure 50). This result led us to abandon the use of carbamate as the direct metalation group in favor of the $N$-pivaloyl group, also well established as an ortho-directing motif.

Figure 50: MEM and carbamate as the direct metalation group and $n$-BuLi as the base led to product 95.
Gould *et al* also examined the use of this directing group to convert 3-aminophenol 87 to 2-hydroxy-6-pivalamidobenzoic acid 98 (Figure 51). Important to us was the observation that, for these authors, the reaction of O-MEM-N-pivaloyl protected 3-amino phenol 97 with n-BuLi at 0°C followed by CO₂, afforded the carboxylic acid derivative 98 in 82% yield. The key feature of this *ortho*-lithiation step is that it is a highly regioselective enabling the deprotonation of a C-H bond adjacent to the direct metatation groups and lead to site-specific addition of the electrophile. In our hands, compound 98 was reacted with hydroxylamine hydrochloride to yield the expected hydroxamic acid intermediate, which was readily converted to the benzoisoxazolone derivative 99 via the dehydrative cyclization in the presence of CDI in THF (overall yield for the two steps 86%).

![Chemical reaction diagram]

**Figure 51:** MEM and pivaloyl as the directed metatation groups to synthesis benzoisoxazolone derivative 99.

However, a caveat (i.e., inherent hidden limitation) to this approach is that the pivaloyl group is notoriously difficult to remove (Figure 52). Indeed, attempts to liberate the amino group in 99 via acid hydrolysis (acetic acid and hydrochloric acid at 45°C for 4 days) of the pivaloyl motif resulted in concomitant ring opening and recovery of 4-amino salicylic acid 88. Using HOAc-HBr for a shorter reaction time (90°C for 24 hours) resulted in both hydrolysis and decarboxylation, reverting our precious material back to 3-aminophenol 87.
**Figure 52:** Hydrolysis of pivaloyl group in 99 by heating with hydrochloric acid or hydrobromic acid.

It was clear from these experiments that the isoxazolone ring does not survive the harsh acid treatment necessary to remove the pivaloyl group. However, the Gould route did give us access to the 3-amino salicylic acid 88. At this juncture, we wanted to see whether the more acid labile \( N \)-acetyl (100), \( N \)-trifluoroacetyl (101) and \( N \)-Boc (102) derivatives of 88 (Figure 53) could be used to block competing formation of the pyrazolone ring during the crucial step where their hydroxamic acid intermediate undergoes dehydrative ring closure to 103-105. If this could be achieved, then simple acid hydrolytic removal of the blocking/protecting group would provide scaffold C.

**Figure 53:** Proposed scheme to synthesis scaffold C from \( N \)-acetyl, \( N \)-trifluoroacetyl and \( N \)-Boc derivatives of 100-102.

a) \( N \)-acetyl derivative 100

Initially, \( N \)-acetylation of 88 was attempted using acetic anhydride at 50°C for 5 hours\(^{125}\).
Under this condition, both the amino group and hydroxyl group in 88 were acetylated (Figure 54). Interestingly, when a literature procedure acetic acid alone was employed (120°C for 3 hours), selective N-acetylation was achieved, but decarboxylation also occurred, giving 107\textsuperscript{126}. Ultimately, treatment of 88 with a mixture of acetic anhydride and acetic acid at room temperature overnight gave compound 100 in 87% yield\textsuperscript{127}. However, in the subsequent step, the reaction of acid 100 with NH\textsubscript{2}OH/CDI did not result in formation of the hydroxamic acid intermediate 108. In fact, essentially all the starting material was recovered. The conversion of acid 100 to 108 was also studied using a two step/one flask procedure where the corresponding acid fluoride was generated in situ using TFFH and reacted with hydroxylamine. Again only the starting acid was recovered.

Conditions: a) CDI (1.5 equiv), NH\textsubscript{2}OH\cdot HCl in THF at room temperature for 24h. b) TFFH, DIEA, NH\textsubscript{2}OH\cdot HCl in CH\textsubscript{3}CN at room temperature for 24h.

**Figure 54:** Synthesis of hydroxamic acid 108 from N-acetyl protected compound 100.

Surprised by this result, the conversion of 100 to its acid fluoride derivative was checked by quenching the reaction with methanol (Figure 55). As expected, the corresponding methyl ester 109 was formed in quantitative yield (checked by \textsuperscript{1}H NMR and mass spectrometry). With methyl ester 109 in hand, it was subsequently reacted with hydroxylamine.
hydrochloride under the conditions used to access scaffold B. Once again, there was no conversion to compound 108.

Figure 55: Proposed scheme to synthesize hydroxamic acid 108 from methyl ester 109.

In light of this finding, the formation of the acid fluoride was further studied by $^{19}$F NMR. Typically, the chemical shift for $^{19}$F in acid fluorides are found in the range 20 to 60 ppm. In the $^{19}$F NMR spectrum of the acid fluoride derivative of acid 100 (Figure 56), the fluorine peak appears at -24 ppm which suggests that the reaction with TFFH did not generate the acid fluoride as we expected (story continued further on).

Figure 56: $^{19}$F NMR spectrum of acid fluoride derivative of acid 100.
b) **N-Trifluoroacetyl derivative 101**

When acid 88 was treated with trifluoroacetic anhydride at 0°C in the presence of sodium bicarbonate and the reaction was slowly warmed up to room temperature and quenched with water, the *N*-trifluoroacetylacetamide 101 was obtained. Alternatively, quenching the reaction with MeOH gave methyl ester 110 (Figure 57). Disappointingly, neither of these intermediates could be converted to hydroxyamic acid 111.

![Figure 57: Proposed scheme to synthesize hydroxamic acid 111 either from *N*-trifluoroacetyl acid 101 or methyl ester 110.](image)


c) **N-Boc derivative 102**

Treatment of carboxylic acid 88 with di-tert-butyl dicarbonate and triethylamine in anhydrous dichloromethane at room temperature for 1 day afforded the *N*-Boc derivative 102 in 96% yield (Figure 58). Again, the reaction with NH₂OH/CDI, or the acid fluoride derivative with hydroxylamine resulted only in the recovery of the starting acid 102.
Figure 58: Proposed scheme to synthesize hydroxamic acid 112 from N-Boc acid 102.

To summarize the above results, although the N-acetyl (100), N-trifluoroacetyl (101) and N-Boc (102) derivatives of 88 were readily prepared and definitively characterized, their conversion to the corresponding hydroxamic acid derivatives failed. These results pointed to the possibility that upon acid activation a competing intramolecular reaction occurs, leading to formation of the bicyclic 5-hydroxy-4H-benzo[d][1,3]oxazin-4-one derivative 113 (Figure 59).

Figure 59: Formation of benzoxazin-4-one ring from acid fluoride or acid imidazole intermediates.

Looking at the mechanism for this reaction (Figure 60), activation of the carboxylic acid 100 (formation of an acid imidazole or acid fluoride) to give 114 provides the opportunity for the amide carbonyl oxygen on the adjacent N-protected amine to react with formation of 115. Collapse of this intermediate with elimination of the activating group leads to formation of the benzoxazin-4-one ring system. Interestingly, in the reaction involving in situ generation of an acid fluoride (OAct=F), the cyclization step could lead to formation of a “stable” hemi-hydrate intermediate 115 (OAct=F). Formation of this species could account for the -24 ppm peak position of the fluorine signal in the $^{19}$F NMR spectrum in Figure 56. Indeed, it is
known that the presence of neighbouring fluorine atoms can favor the hydrated versus keto form of carbonyl compounds, and molecules containing a hemi hydrate motif, as found in our proposed intermediate 115, have been described.130

Figure 60: Proposed mechanism of the formation of benzoxazin-4-one ring 113.

So, given the distinct possibility that benzoxazin-4-one derivatives 113 were formed in the reaction of the N-protected compounds 100-102, the question became why these intermediates apparently did not react with the excess hydroxylamine present in the reaction medium to give the expected hydroxamic acid products. To understand this reaction better, the 2-methyl-4H-benzo[d][1,3]oxazin-4-one 116 was synthesized intentionally from anthranilic acid and reacted separately with NH₄OH, with water and with NH₂OH.HCl in water containing one equivalent of NaOH (Figure 61).131 Interestingly, whereas the reaction with NH₄OH gave the known product 2-acetamidobenzamide 117 in high yield, the reaction of 116 with both water and hydroxylamine in water led to complete conversion back to acid 118. These results are consistent with what we observed in the reaction between acid 100-102 and hydroxylamine, and suggest that the outcome of these reactions is particularly influenced to the steric environment around the reacting carbonyl center (rate of reaction...
with ammonia or water more rapid than with hydroxylamine). Further, it was concluded that due to the steric bulk of the N-pivaloyl group, compound 98 does not undergo competing intramolecular ring closure, and that there is direct reaction of the CDI activated acid function with NH₂OH.

![Chemical diagram]

**Figure 61**: Synthesis of 2-methyl-4H-benzo[d][1,3]oxazin-4-one 116 and the reaction of 116 with NH₄OH, with water and with NH₂OH.HCl, respectively.

*d)* Trityl group protection

Based on the notion that the steric bulk of the pivaloyl group governs reactivity, protection of the amino group in 88 using an acid labile trityl group should also favor formation of the desired hydroxamic acid. However, in the reaction of 88 with 1.5 equivalents of trityl chloride in DCM containing Et₃N (Figure 62), triphenylmethanol 119 was the only product that could be isolated (78% yield). Interestingly, when the amount of trityl chloride was increased to 4 equivalents, there was preferential formation of the trityl compound 120 in 65% yield.
Figure 62: Formation of compound of 119 and 120 with 1.5 equivalents of trityl chloride and 4 equivalents of trityl chloride, respectively.

e) Phthalimide group protection

Phthalimides can be synthesized by dehydrative condensation of primary amines with phthalic anhydride at high temperatures. Phthalimides can also be removed easily by treatment with hydrazine, generating a phthalhydrazides as by-product. The N-phthalimide protected compound 121 was readily prepared by reacting 88 with phthalic anhydride in refluxing acetonitrile for 2 hours (Figure 63)\textsuperscript{132}. However, subsequent reaction of 121 with CDI in anhydrous THF followed by the addition of hydroxylamine hydrochloride only served to cleave off the phthalimide protecting group and give back the starting acid 88.

Figure 63: Removal of phthalimide group with NH$_2$OH.HCl.

Phthalimide was removed by hydroxylamine in a similar way to hydrazine. The mechanism
for the cleavage of phthalimide group by hydroxylamine hydrochloride is depicted in Figure 64\textsuperscript{133}. The first step involves the nucleophilic addition at an amide carbonyl, followed by an intramolecular acyl transfer process to give the amine as the product.

![Figure 64: Mechanism of phthalimide deprotection by NH$_2$OH.HCl.](image)

\textit{f)} Protection of the primary amine as an imine derivative

Primary amines can be converted to imines (Schiff bases), via the reaction with aldehydes or ketones under a wide variety of conditions. Mechanistically the reaction proceeds via a nucleophilic addition giving a hemiaminal intermediate, followed by elimination of water to yield the imine derivative. Molecular sieves, or some other drying agent, are generally present in order to shift the equilibrium towards the formation of the imine product. In the experiment, reaction of benzophenone 82 with amine 88 in dry DCM, using TiCl$_4$ (1.5 equivalents) as a Lewis acid promoter and drying agent gave benzophenone imine 123, albeit in low (20\%) yield (Figure 65)\textsuperscript{134}. As the reaction did not produce the target imine in a synthetically significant yield, we did not investigate this procedure further.

![Figure 65: Synthesis of imine derivative 123.](image)

2.7.3.2 “Last chance” oxidation of the amino group of 2-amino-6-hydroxybenzoic acid 88
Instead of having amino group in compound 88 protected, another option would be to oxidize the amino group to obtain nitro compound 66. In the literature, various methods have been described for the oxidation of primary amines. Three different oxidizing agents were tried in our work to prepare the nitro compound (Figure 66).

In our first trial, mCPBA was used in 1,2-dichloroethane refluxing for 10 hours\textsuperscript{135}. This reaction did not produce the desired nitro compound 66. In the second trial, Oxone and EDTA were used in aqueous acetone to oxidize the amino group. From the literature, 5-aminosalicylic acid was readily oxidized to 5-nitrosalicylic acid in 74\% yield\textsuperscript{136}. In practice, oxidation of 6-aminosalicylic acid should proceed in a similar manner. To our surprise, in contrast to 5-aminosalicylic acid, this procedure failed due to formation of complex products, according to TLC, from which the desired nitro compound could not be isolated in a significant and isolable yield. In the third trial, hydrogen peroxide in trifluoroacetic acid has been used as an oxidizing agent due to the in situ formation of pertrifluoroacetic acid\textsuperscript{137}. This reagent has been found to oxidize aniline and substituted anilines to nitrobenzenes in excellent yield. However, the synthesis of this nitro compound 66 could not be achieved by any of the procedures followed.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure66.png}
\caption{Oxidation of amino group of intermediate 88.}
\end{figure}

Conditions: a) mCPBA in 1,2-dichloroethane refluxing for 10h. b) Oxone and EDTA in aqueous acetone at 0°C. c) Hydrogen peroxide in trifluoroacetic acid and at 50°C for 1h.

\textbf{Figure 66:} Oxidation of amino group of intermediate 88.

\textbf{2.7.3.3 Preparation of scaffold C: Starting from 2-methyl-3-nitrophenol 89}

As difficulties were encountered during acid hydrolysis of the ortho-directing amide protecting (pivaloyl) group, and oxidation of the amino group in compound 88 to the nitro
group level was unsuccessful, our attention was focused on the synthesis of 6-nitro salicylic acid 66 via oxidation of the methyl group in a suitable, commercially available/inexpensive, toluene derivative (recall Figure 48). It was expected that this intermediate could be efficiently converted in a limited number of steps to the corresponding C4-nitro substituted benzoisoxazolone intermediate 68, and that the Sn(II)/Sn(IV) condition described for the reduction of the nitro group to obtain the C5 amino functionalized scaffold B could be similarly be applied to obtain scaffold C.

![Chemical Reaction Diagram]

**Figure 67:** Synthesis of scaffold C from 2-Methyl-3-nitrophenol 89.

Our initial choice for the starting material was 2-methyl-3-nitrophenol 89. However, oxidation the methyl group in 89 through reaction with potassium permanganate and water for 6 hours\(^{138}\) or heating with urea hydrogen peroxide (UHP) at 150°C for 4 minutes\(^{139}\) proved problematic (presumably due to decarboxylation and ensuing reactions under the harsh conditions used)\(^{139}\). In contrast, the corresponding methoxy compound 124 (readily prepared from 89, but also commercially available/affordable) (Figure 67) was readily
oxidized to acid 125 using KMnO₄, and could be O-demethylated to give the nitrophenol acid 66 by using boron trifluoride diethyl etherate and sodium iodide in acetonitrile at room temperature for 12 hours. The overall yield for these two steps was > 60%.

The mechanism for the methyl aryl ether demethylation using BF₃·OEt₂ and sodium iodide is illustrated in Figure 68. This transformation is initiated by formation of an ether adduct between the boron center and the ether oxygen followed by the loss of fluoride ion. Free iodide ion in the reaction medium can act as a nucleophile, attacking the methyl group of the cationic intermediate cleaving the C–O bond and producing a difluoroborane ether-type derivative, which then undergoes hydrolysis upon aqueous work-up to produce phenol, boric acid, and hydrogen fluoride as by-products.

**Figure 68:** Mechanism of demethylation by BF₃ and NaI.

Having obtained compound 66, we were certain that the problem of synthesizing scaffold C was resolved. However, efforts to prepare hydroxamic acid 127 from this pivotal intermediate, using different acid activating agents (Table 1) failed. In fact, after extraction with EtOAc, the starting material was recovered in high yield from the organic layer, and the product (to the extent it was formed) remained in the aqueous phase, along with multiple other water soluble by-products.

In an effort to push the reaction of 66 with hydroxylamine toward the intended hydroxamic acid product, different carboxylic acid activating agents were studied (Table 1). However, reaction of carboxylic acid 66 with CDI and treatment of the derived acyl imidazole (activate acid derivative) with hydroxylamine hydrochloride, either at room temperature or at elevated temperature did not produce hydroxamic acid 127. The reaction of acid 66 with
hydroxylamine hydrochloride and DIEA in the presence of different coupling reagents (EDC/HOAt\textsuperscript{142}, EDC/DMAP\textsuperscript{143} or HBTU/HOAt\textsuperscript{144}) in anhydrous DMF at room temperature similarly failed, or did not advance to any significant extent. Treatment of acid 66 with ClCO\textsubscript {2}i\textsubscript{Bu} using NMM (N-methylmorpholine) as the base in anhydrous THF at room temperature again failed to give the hydroxamic acid product \textsuperscript{145}.

**Table 1:** Synthesis of hydroxamic acid 127 from acid 66 with different coupling agents.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Coupling agent</th>
<th>Base</th>
<th>Solvent</th>
<th>Temperature</th>
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<tbody>
<tr>
<td>1</td>
<td>CDI</td>
<td>NA</td>
<td>Anhydrous DMF</td>
<td>Room temperature</td>
</tr>
<tr>
<td>2</td>
<td>CDI</td>
<td>NA</td>
<td>Anhydrous DMF</td>
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<tr>
<td>4</td>
<td>EDC/DMAP</td>
<td>DIEA</td>
<td>Anhydrous DMF</td>
<td>Room temperature</td>
</tr>
<tr>
<td>5</td>
<td>HBTU/HOAt</td>
<td>DIEA</td>
<td>Anhydrous DMF</td>
<td>Room temperature</td>
</tr>
<tr>
<td>6</td>
<td>ClCO\textsubscript {2}i\textsubscript{Bu}</td>
<td>NMM</td>
<td>Anhydrous THF</td>
<td>Room temperature</td>
</tr>
</tbody>
</table>

In stark contrast, the acid chloride derivative of the O-Me substituted carboxylic acid 125 reacted efficiently with hydroxylamine, and was readily extracted from aqueous media using EtOAc (isolated in >80% yield!). However, the subsequent O-demethylation step, using the BF\textsubscript{3}-Et\textsubscript{2}O/NaI combination proved, again, to be complicated (Table 2).

This deprotection reaction releases one equivalent of HF and boric acid, which are weak acids. The ratio of compound 125/BF\textsubscript{3}-Et\textsubscript{2}O/NaI was 1:4:4 as there are 2 equivalents of BF\textsubscript{3}-Et\textsubscript{2}O/NaI supplied for the O-methyl group, another 2 equivalents for other “electron-donor” elements in the molecule\textsuperscript{146}. The reaction was monitored by TLC and was shown to advance. However, upon completion of the reaction, compound 127 could not be extracted to any significant extent from the water layer using EtOAc, or other organic solvents. Indeed, the product was too polar and could only be dissolved either in methanol or...
THF. Note that the small amount of material 127 that was isolated by extraction was characterized by $^1$H NMR. Therefore, we concentrated the aqueous phase and took up the residue in THF to remove most of the inorganic by-products. Unfortunately, however, sodium iodide exhibits high solubility in THF, and it is very hygroscopic. The product was obtained as a mixture with NaI and it absorbed atmospheric moisture quickly.

Table 2: O-demethylation step using different demethylation agents.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Demethylation agent</th>
<th>Equiv.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BF$_3$-Et$_2$O/NaI</td>
<td>4/4</td>
</tr>
<tr>
<td>2</td>
<td>AlCl$_3$/NaI</td>
<td>4/4</td>
</tr>
<tr>
<td>3</td>
<td>BF$_3$-Et$_2$O</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>BBr$_3$</td>
<td>4</td>
</tr>
</tbody>
</table>

Another literature procedure was also employed involving reaction with AlCl$_3$/NaI in acetonitrile$^{147}$. Again, demethylation was quantitative and formation of 127 was confirmed by $^1$H NMR, but sodium iodide remains in the product. In light of the difficulties encountered with sodium iodide, we decided to carry out this reaction with BF$_3$-Et$_2$O alone. Unfortunately, as fluoride is not as nucleophilic as iodide, the demethylation was not achieved.

As isolation and purification were very challenging in the procedure with either BF$_3$-Et$_2$O/NaI or AlCl$_3$/NaI, we turned our attention toward the use of BBr$_3$.$^{148}$ The $^1$H NMR spectra of the crude product indicated that the starting material had been completely demethylated. However, after quenching the reaction with H$_2$O and extraction, the presence of acidic by-products (HBr and B(OH)$_3$), coupled with the solubility of 127 in water once again complicated the silica gel column chromatography and product isolation. The product 127 was obtained in a very low yield, as in the presence of the impurities it decomposed on silica gel.
To overcome the problem with the use of column chromatography in the BBr₃ procedure, the reaction was stopped by quenching with MeOH (Figure 69). The by-products MeBr and B(OCH₃)₃ could easily be removed under reduced pressure as their boiling points are, 4°C and 68°C, respectively. The solution was neutralized with Et₃N until pH >7. The solvents (acetonitrile and MeOH) were removed in vacuo, and the residues were redissolved in THF. The mixture was then filtered to remove the solid organic by-product (triethylamine hydrobromide). The organic layer was concentrated and it turned out that triethylamine hydrobromide salt contaminant was present in the concentrate. Note that the product yield for this procedure was greater than 100%. As the triethylamine hydrobromide salt is hard to get rid of by chromatography, the product 127 mixed with the Et₃N salt was carried forward to the next step. Reaction of 127 with CDI in THF was uneventful, leading to formation of cyclized product 68 (12% yield for these two steps). Note, in particular, that compound 68 could be extracted with EtOAc and that it was isolated as pure product with no contaminating Et₃N salt present. Subsequent reduction of the nitro group in 68 using the same procedure for reducing the nitro group in compound 59 gave the desired target compound, 4-aminobenzoisoxazolone (Scaffold C), in 54% yield (approx. 6% overall for the three steps).

Figure 69: Mechanism of demethylation by BBr₃ and the reaction media was quenched with MeOH.

These observations suggested that protection of the phenolic OH group would be necessary during formation of the hydroxamic acid intermediate 127. They also suggested that a more
readily removed $O$-protecting group must be employed in the synthesis. To counter the problems encountered, another strategy was developed (Figure 70), involving in the key operation conversion of the starting acid 66 to the cyclic ether-ester intermediate 128\(^{149}\). Acid 66 was diprotected in 89% yield by using dibromomethane as the alkylating agent and potassium phosphate tribasic as the base upon heating at 100°C in DMF for 8 hours. The next step was to react this cyclic ether-ester with NH$_2$OH.HCl/DIEA in dry THF. In the experiment, 2.5 equivalent of NH$_2$OH.HCl was used as formaldehyde generated from the reaction would consume one equivalent of NH$_2$OH.HCl. Note, when NaOH was used as the base, compound 128 was completely hydrolyzed back to starting acid 66 (note, water was generated in the reaction medium). Carrying out the reaction in dry THF and using dry DIEA, TLC monitoring revealed that varying quantities of the starting ester 128 remained in the product mixture. The reaction mixture was acidified with 1N HCl till pH<3. The remaining starting material 128 was removed by EtOAc extraction from the aqueous phase. The aqueous phase was concentrated and redissolved in THF. It was then filtered and the filtrate was concentrated. The residue contained product 127, mixed together with diisopropylethylamine hydrochloride salt. Treating the crude product mixtures with CDI in THF on heating at 70°C again led to the expected product 68. Overall, the average yield for this procedure to make 4-nitrobenzoisoaxazolone 68 from 128 (two steps) was about 15%.

**Figure 70:** Alternative route to synthesize key intermediate 128 and its conversion to scaffold C.
Despite the complications with reaction work-up, scaffold C was isolated as a pure product, and the reaction sequence permitted the synthesis of multi-gram quantities of this valuable material. The synthetic protocol still requires some further optimization but the results are meaningful, as prior to our work there was no report on the synthesis of scaffold C.

To date, we have not explored/exploited the reactivity of the C4 primary amine and the isoxazolone “amide” nitrogen to generate a library of Scaffold C functionalized compounds. This was, primarily, due to limited funding for the ROCK assay work. However, recent results from a collaborative project (Dr. Michel Roberge, Life Sciences Centre, UBC) to identify molecules that enhance aminoglycoside-promoted premature termination codon readthrough (PTCR)\textsuperscript{150} suggest strongly that compounds built upon scaffold C will provide an important opportunity to revive a pertinent therapeutic strategy to treat genetic diseases and certain cancers that result from “nonsense” DNA mutations. Indeed, it was known that aminoglycoside antibiotics such as G418 promote PTCR through incorporation of near cognate amino acids at positions where the premature codon stop are located\textsuperscript{151}. In this way full-length protein synthesis is restored. However, aminoglycoside toxicity was observed at concentrations needed for the therapeutic effect\textsuperscript{152}. The observation that small molecules in our compound collection/library enhance the action of aminoglycosides to promote PTCR at concentrations lower than the CC\textsubscript{50} threshold where toxicity is observed is actively being pursued.
Chapter 3

Benzoisoxazolone scaffold D: Development of inhibitors of HIV pre-mRNA alternative splicing

3.1 Introduction: HIV/AIDS

The Human Immunodeficiency Virus (HIV) is the causative agent of the acquired immunodeficiency syndrome (AIDS). Today, over 35 million people worldwide are infected with HIV, 72% of which are located in sub-Saharan Africa\textsuperscript{153}. Further, an estimated 2 million new infections occur annually, and approximately 1.2 million people die from AIDS each year\textsuperscript{153}. HIV infects cells in the human immune system (macrophages, dendritic cells and helper T cells (CD4\textsuperscript{+} T cells))\textsuperscript{154, 155}. When the number of CD4\textsuperscript{+}T cells has decreased to the critical point where the immune system is compromised, the patient is said to have AIDS. He or she becomes highly vulnerable to a wide range of opportunistic, and, if untreated, fatal infections.

The HIV replication cycle (Figure 71) begins with a HIV virion attaching itself to the cell surface protein CD4 and co-receptor (CCR5 or CXCR4)\textsuperscript{156}. This initial infective step can be blocked through treatment with co-receptor antagonists. Next, fusion of HIV envelope and host cell membrane allows the release of the HIV capsid into the cell, and the viral single-strand RNA genome is reverse transcribed into double-strand DNA. The process of reverse transcription is blocked using either/both nucleoside analogue reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs). The viral DNA is then integrated into the host genome by the viral enzyme integrase. Integrase strand transfer inhibitors (InSTIs) and allosteric integrase inhibitors (ALLINIs) are used to block this step. Transcription of the integrated proviral DNA then produces a pre-mRNA transcript, which is processed to give back copies of the viral RNA genome and a series of RNA templates for translation of crucial viral proteins. The new viral proteins and genomic RNA
then move to the surface of the cell and assemble at the cell membrane into immature HIV. The newly formed immature HIV virions bud from the host cell and become a mature virus through the action of viral protease. As only the mature virions are capable of infecting new cells, protease inhibitors which block cleavage of protein precursors also prevent viral replication\textsuperscript{157}.

\textbf{Figure 71:} Schematic overview of HIV replication cycle and the sites of action of the approved antiretroviral drugs: entry inhibitors; nucleoside and non nucleoside reverse transcriptase inhibitors; integrase and protease inhibitors. (Figure is adapted from reference 156.)

As the frequency of error (1/10,000) is high during transcription, point mutations are abundant in the key viral enzymes reverse transcriptase, integrase and protease. Consequently, the onset of drug resistance to monotherapy is rapid. However, the use of a combination of 3 or more anti-HIV drugs acting on different targets, referred to as ART (Anti-Retroviral Therapy), dramatically decrease mortality and morbidity rates for people who are infected with HIV\textsuperscript{158}. Indeed, the success of ART is such that HIV/AIDS is now considered a long-term chronic condition rather than a life threatening illness.
Despite the treatment success of the ART approach, the long term use of the current anti-HIV drugs is not a cure for HIV/AIDS. Further, issues of drug compliance, toxic side effects, drug resistance, and inability to access the “latent viral pool, using the current drugs remain”. To overcome these inherent limitations of ART, there is a continued need for new drugs, and in particular, those acting through new and yet unexplored mechanisms of action.

In this context, the goal of our research project is to design and synthesize “small molecule-based” drugs that perturb/block specific alternative splicing events crucial to HIV replication (see arrows in Figure 71). In a similar manner to the development of entry inhibitors, the novelty of this new anti-HIV strategy is that it targets host cell proteins involved in cellular splicing/alternative splicing. The challenge is to control toxicity. The advantage to this approach is that as cellular splicing factors are much less prone to mutation, the possibility exists that issues of long-term drug resistance will be greatly diminished or eliminated.

3.2 Splicing and alternative splicing

Splicing is the process whereby intervening sequences (introns) are excised in precursor messenger RNA (pre-mRNA) and expressed sequences (exons) are joined together to produce mature mRNAs (Figure 72). As up to 95% of eukaryotic genes contain introns, splicing is a crucial step in producing mRNAs that are translated into proteins. Alternative splicing is the process whereby exons in pre-mRNA are spliced in different arrangements to yield mRNAs that will produce protein isoforms and functionally different protein variants from the same gene. In this way a single gene codes for multiple proteins.
Figure 72: Alternative splicing of pre-mRNA can create different proteins. (Figure is adapted from “Genomic medicine-A primer”, 2002, The New England Journal of Medicine.)

Splicing/Alternative splicing is carried out in the cell by the spliceosome, a large complex consisting of more than 150 proteins and specialized RNAs. The major small nuclear RNAs (snRNA) that make up the spliceosome are U1, U2, U4, U5 and U6. A RNA-protein complex of small nuclear ribonucleoproteins (snRNP) is formed when snRNA’s are combined with the protein factors.

Spliceosome assembly is carried out at sites of transcription (Figure 73). This begins with the recognition of 5’ splice site on the pre-mRNA which binds U1 snRNP and 3’ splice site binds U2AF to form E complex (Figure 73A). The complex A forms when U2 snRNP is recruited and becomes tightly associated with the branch point sequence (BPS). In a subsequent reaction, the preassembled tri-snRNP U4/U6-U5 integrates in and forms complex B. After a series of rearrangements, complex B is activated (complex B*), U4 and U1 snRNPs are released. Complex B* then undergoes two transesterification steps producing a
post spliceosome complex, which contains the excised lariat intron and the two exons that have been joined together. Finally, the mRNA and lariat are released, and U2, U5 and U6 snRNPs are recycled for additional round of splicing\textsuperscript{169, 170}.

Figure 73: A) Initiation step of spliceosome assembly. B) Step-wise assembly of the spliceosome. Illustrated are formation of the lariat and joining of two exons. (Figure is adapted from reference 167.)

Due to the small size of the HIV genomic RNA, alternative splicing plays a crucial role in HIV replication. During HIV-1 replication the transcription of the integrated proviral DNA by RNA polymerase II generates a 9 kilobase (kb) polycistronic pre-mRNA that serves as
genomic RNA for progeny viruses and the mRNA that encodes the viral Gag and Gag-Pol proteins (Figure 74)\textsuperscript{166,171,172}. Successful infection and production of new infectious progeny viruses also requires the balanced expression of seven additional viral proteins (Tat, Rev, Nef, Vif, Vpr, Vpu, and Env).

**Figure 74:** Alternative splicing of HIV pre mRNA to produce key proteins Tat, Rev, Nef, Vif, Vpr, Vpu, and Env. (Figure is adapted from reference 171.)

To achieve this proteomic diversity, the primary transcript is alternatively and incompletely spliced and nuclear export of the unspliced (US) transcript is regulated\textsuperscript{173-175}. This highly orchestrated process involves at least four 5' splice sites (5'ss) D1-4 and eight 3' splice sites (3'ss) A1, 2, 3, 4c, 4a, 4b, 5 and 7, which enable alternative splicing to form more than 40 different mRNAs (Figure 74)\textsuperscript{175-177}. These mRNA variants can be divided into two classes:
multiply spliced (MS) (~ 2 kb) and singly spliced (SS) (~ 4 kb) mRNAs. In the early phase of HIV-1 gene expression, only completely spliced mRNAs are transported to the cytoplasm (Figure 75). These spliced mRNAs code for the Tat, Rev and Nef proteins. Tat is a regulatory protein that very significantly enhances the efficiency of viral transcription, and Nef modulates the physiological status of the host cell to suit the needs of the virus. As the Rev protein accumulates, it facilitates nuclear export of the SS and US mRNAs. The SS mRNAs express the Vif, Vpr, Vpu, Env proteins and the US RNAs express the Gag and Gag-Pol polyproteins. This requires Rev to mediate RNA export via the CRM1 pathway, through its interaction with the Rev response element (RRE) within the US and SS RNAs.

**Figure 75:** Control of HIV RNA export. In the early phase, only completely spliced mRNAs are exported to cytoplasm. Once Rev accumulates, it facilitates nuclear export of the SS and US mRNAs.

Cellular splicing is regulated by *cis*-acting splicing regulatory elements (SREs) and by *trans*-acting splicing factors (repressors and activators) in the pre-mRNA(Figure 76).
SREs can be classified as splicing enhances (exonic splicing enhancers (ESEs), intronic splicing enhancers (ISEs)) and silencers (exonic splicing silencers (ESSs), intronic splicing silencers (ISSs)). Most splicing activators are members of the SR (Arginine – Serine rich) protein family, which bind to ESE and ISE. Splicing repressors, on the other hand, are heterogeneous nuclear ribonucleoproteins (hnRNPs) that bind to ISS and ESS\textsuperscript{181}. These splicing factors that target the components of the spliceosome have effects on the function of U2 and U1 snRNPs in the initial step of splicesome assembly and can either activate or inhibit recognition of splice site\textsuperscript{165}.

![Diagram of splicing factors and their binding sites](image)

**Figure 76:** Regulation of alternative splicing. (Figure is adapted from reference 180.)

### 3.3 SR proteins and the discovery of IDC16

Through random screening of Institute Curie compound collection (collaboration with Dr. J. Tazi, U de Montpellier II, Fr), a fused tetracyclic indole compound “IDC16” (Figure 77) was identified as a molecule that blocks replication of HIV through inhibition of HIV-1 pre mRNA alternative splicing\textsuperscript{29}. Subsequently, *in vivo* studies suggested that IDC16 targets the general splicing factor SRSF1 (previously known as ASF1/SF2) in a concentration dependent manner\textsuperscript{29}. Indeed, the data led to the conclusion that IDC16 binds directly to SRSF1 and selectively interferes with the ESE-dependent splicing activity of this SR proteins,
thereby suppressing the production of key viral proteins Tat, Rev and Nef.

![Chemical Structure](image)

**Figure 77**: Structure of IDC16.

There are 12 different SR proteins (SRSF1 to SRSF12), each of which contains an N-terminal RNA recognition motif (RRM) and one or two C-terminal RS domains\(^{182}\). The RRM domain regulates the RNA interactions by controlling the recognition of ESE sequences, while RS domain mediates protein-protein interactions with multiple splicing components\(^{183}\). Two dominant features of SR proteins that enable them to “multi task” are that they are inherently disordered, i.e. conformationally mobile, and that they undergo selective phosphorylation/dephosphorylation at multiple serine sites in their structure in order to possess the appropriate arrangement of the \(O\)-phosphorylated serines required for the different protein-protein interactions they engage\(^{184}\).

Phosphorylation of SRSF1 can occur at 12 of the 22 serines in the RS domain. This is mediated by several kinases, including CLK1/2 and SRPK1/2\(^{185,186}\). In the cell, phosphorylated forms of SRSF1 associate with different protein partners regulate transportation of SR protein into the nucleus\(^{187}\). In contrast, phosphatase mediated removal of \(O\)-phosphate groups in SRSF1 after transcription allows export of spliced/matured mRNA’s out of the nucleus toward the translation machinery (Figure 78)\(^{184}\).
Figure 78: SR proteins are shuffled between different phosphorylated and dephosphorylated states that are important in splicing. (Figure is adapted from reference 184.)

Because SR proteins (bearing phosphate groups or not) are inherently disordered, there is essentially no information currently available concerning their global structure and the location of interaction/binding sites for protein-protein contacts. For this reason we have no insight as to how the relatively non-polar molecule IDC16 may block the function of SRSF1. A further complication to entertaining the idea of engaging in structure-activity relationship (SAR) studies on this molecule, are difficulties associated with the synthesis of IDC16 analogues. Indeed, very harsh reaction conditions are necessary to form the fused tetracyclic core of this compound. A final deterrent to drug development based on IDC16 is that, although it displays anti-HIV activity at a concentration inferior to the threshold of its cytotoxic effects, the selectivity index (ratio of the CC₅₀ and EC₅₀) is too low (SI=10) to seriously consider this molecule as a drug used for long term treatment of HIV infection (Figure 79a).
Figure 79: Evaluation of anti-HIV activity (column) and GXR-CEM cell viability (line) for IDC16 (a) and 1C8 (b) at concentration between 62.5nM and 16μM. (Figure is adapted from reference 31.)

Although IDC16 is an inherently cytotoxic molecule at micromolar concentration, its ability to block HIV replication through interference with HIV pre mRNA alternative splicing is a real phenomenon. Consequently, our laboratory initiated a compound library/parallel synthesis program to identify more conformationally mobile mimics of IDC16 that retain strong affinity for the anti-HIV SRSF1 protein target, but lose capacity to intercalate DNA (Figure 80). The strategy initially adopted was to design mimics of IDC16 in which the central B- and C-rings are replaced by an appropriate spacer/linker motif, and different alternatives of A- and D-rings project the binding interactions that beyond IDC16 and displace improved potency relative to IDC16\(^{31}\). The preliminary anti-HIV screening of structural mimics of IDC16 led to the identification of four active diheteroarylamide (DHA)-type compounds: 1C8, 2D3, 3C2 and 1E5. In the subsequent screens, the most potent compound 1C8 also inhibited the key HIV-1 drug-resistant strains\(^{31}\). Further comparison studies of 1C8 indicated that, the cytotoxic effect of 1C8 was dramatically decreased (Figure 79b). However, in contrast to the parent compound, 1C8 was showed to alter splicing regulation mediated by SRSF10\(^{188}\).
Figure 80: Strategy for the parallel synthesis of IDC16 mimics with retaining of A-and D-rings; four diheteroarylamide (DHA) compounds, 1C8, 2D3, 3C2 and 1E5, were identified as active in the preliminary anti-HIV screen and the discovery of the most active compound 1C8.

3.4 Research hypothesis and design strategy

Based upon the novel action of IDC16, our hypothesis was that through the synthesis and testing of a new series of “ring opened analogues” of IDC16, a novel class of anti-HIV/AIDS agents will be discovered, which specifically target SRSF1.

Toward this goal, we have developed a synthetic route to scaffold D, and its corresponding aldehyde derivative from 5-formyl salicylic acid 134 and 5-methyl salicylic acid 135, and have used these scaffolds to prepare a small exploratory library of five benzoisoxazolone-based IDC16 mimics (Figure 81a). The salient feature of these molecules are that the benzoisoxazolone ring system emulates the middle portion (B- and C-rings) of the fused tetracyclic indole compound (Figure 81a), and the pendant side chains can adopt a conformation such that the terminal nitrogen substituents overlap with the A- and D-ring
nitrogen atoms in IDC16 (which we believe play an important role in interactions with the human splicing factor SRSF1). This is illustrated in Figure 81b for the benzoisoxazolone derivatives 129 and 133, which can be superimposed on IDC16 in two different orientations. The ultimate objective in this research is to identify potential clinical candidates that can be transferred to the pharmaceutical industry for development of a new class of anti-HIV-AIDS.

![Chemical Structures and Diagrams]

**Figure 81**: a) Scaffold D and related C5-substituted benzoisoxazolones as precursors to the five molecules 129-133. b) Superposition of IDC16 and the benzoisoxazolone-based compounds 129 and 133.
3.5 Chemistry: Synthesis of benzoisoxazolone-based IDC16 mimics 129-133 from scaffold D and related C5-substituted benzoisoxazolones.

3.5.1 Synthesis of IDC16 mimics 129 and 130

To elaborate scaffold D from the 1,5-dicarboxy substituted phenol derivative 137 and then use it to prepare IDC16 mimics 129 and 130, it was first necessary to differentiate the C1 carboxylic acid function engaged in the construction of the isoxazolone ring from the C5 acid function that is reacted with the requisite amines. This was best achieved by the two-step protocol wherein 5-formylsalicylic acid 134 was reacted with MeOH in a few drops of conc. H₂SO₄ (reflux 12 h, 95% yield) and the derived methyl ester 136 was oxidized to 137 (74%) using Oxone in DMF (Figure 82). Subsequent reaction of ester-acid 137 with hydroxylamine hydrochloride proved to be efficient (92%), as did dehydrative cyclization to scaffold D in the presence of CDI at 70°C in THF. To activate scaffold D relative to conversion of the C5 acid function group to its amide derivative, it was reacted with CDI followed by quenching with a large excess of methanol giving methyl ester 139.
Figure 82: Scheme to synthesize scaffold D and IDC16 mimics 130 and 144.

The oxidizing agent Oxone is a component of a potassium triple salt with the formula 2KHSO₅·KHSO₄·K₂SO₄ and the active component is potassium peroxymonosulfate. The use of Oxone has increased rapidly due to its stability, the simple handling, non-toxic nature, nonpolluting by-products and low cost. The mechanism of Oxone mediated aldehyde oxidation is illustrated in Figure 83\textsuperscript{192}. Peroxyacetal intermediate 145 was generated by the
nucleophilic addition of KHSO$_5$ to the carbonyl carbon. The next step involves a hydride migration that reminiscent the Baeyer-Villager reaction, as well as the scission of the week O-O bond to give the final carboxylic acid product.

Figure 83: Mechanism of Oxone oxidation reaction.

The pivotal intermediate 139 was then reacted under microwave heating conditions with either 2-bromoethyl methyl ether or N-(2-Bromoethyl) phthalimide in the presence of K$_2$CO$_3$ as the base to give predominantly the N-alkylated compounds 141 (46%) and 143 (37%), respectively. The O-alkylation products 140 and 142 were also formed in these alkylation reactions and were separated from the desired N-alkylation products by flash column chromatography. In the final step, intermediates 141 and 143 were converted to the target amide derivatives 130 and 144, by reaction with ammonia (7N in methanol) at room temperature for 5 days$^{193}$.

3.5.2 Synthesis of IDC16 mimics 131

In the context of SAR studies, the amide motif found in the IDC16 mimic 129 was replaced by a nitrile function, as in compound 131. Compound 131 was prepared in four steps (Figure 84), starting with the preparation of oxime 146 by reaction of 5-formylsalicylic acid 134 with hydroxylamine hydrochloride in methanol-water at room temperature for 12 hours (92% yield)$^{194}$.

Attempted direct conversion of 146 to 5-cyano hydroxamic acid derivative 148 by sequential
reaction with 1 equivalent of CDI and hydroxylamine hydrochloride resulted in exclusive formation of the nitrile-acid 147. However, when an excess (3 equivalents) of CDI was employed, both the oxime and acid functions were activated, leading to formation of the 5-cyano substituted hydroxamic acid intermediate 148 in 25% isolated yield.

![Chemical reaction diagram](image)

**Figure 84:** Synthesis of IDC mimics 131.

These experiments revealed that reaction of the oxime OH with CDI, and subsequent abnormal Beckmann rearrangement occurred preferentially on contact of 146 with CDI (lower energy pathway). The Beckmann rearrangement (Figure 85) is an acid-catalyzed rearrangement of an oxime to an amide product through an alkyl migration and expulsion of the hydroxyl group followed by reaction of the transient nitrilium salt intermediate with water and tautomeric equilibration.
In contrast (Figure 86), the abnormal Beckmann reaction of oxime 146 proceeds by an E2 elimination pathway, involving simultaneous abstraction of the imine hydrogen in the \( O \)-acylated intermediate 151 by a base and elimination of the carboxylate anion to give the nitrile group as the stable product\(^{197}\).

In the following step, reaction of hydroxamic acid 148, with CDI (THF at 70\(^\circ\)C for 6 hours), induced ring closure and formation of benzoisoxazolone 149 (79\% yield). Looking back at the conversion of aldehyde 134 to intermediate 149, it seemed possible that all four transformations might be achieved in a “one pot” manner by adding hydroxylamine and CDI to the reaction medium. This idea will be explored in the future.

In the final step of the synthesis, the aliphatic chain was attached to the amide nitrogen in 45\%
yield via N-alkylation using 2-bromoethyl methyl ether and potassium carbonate as the base under microwave heating condition. \( O \)-alkylated compound 150 was also obtained as a minor product. IDC16 mimic 131 was obtained as sticky orange solid.

### 3.5.3 Synthesis of IDC16 mimics 132

Taking into account that the \( N \)-benzyl analogue of the IDC16 mimic 1C8 displayed anti-HIV activity (unpublished data), attention was directed to the construction of the \( O \)-benzyl oxime IDC16 mimic 132 (Figure 87a). Compound 132 was prepared in a similar fashion (Figure 87b) to compound 131, using \( O \)-benzylhydroxylamine hydrochloride instead of hydroxylamine hydrochloride to incorporate the C5 oxime motif. Note that, the \( O \)-benzyl group on the oxime oxygen atom prevented the abnormal Beckmann rearrangement from occurring, and afforded the desired oxime product 152 in 95% yield.

In the next step of the synthesis, \( O \)-Bn oxime derivative 152 was converted directly to the pivotal benzoisoxazolone intermediate 153 in a “one-pot” process involving addition of 2 equivalents of CDI, followed 2 hours later (once formation of \( CO_2 \) bubbles ceased) by addition of hydroxylamine hydrochloride. In this way, compound 153 was obtained in 25% yield. Reaction of intermediate 153 with 2-bromoethyl methyl ether in DMF in the presence of potassium carbonate under microwave heating at 60°C for 5 minutes afforded the \( N \)-alkylated \( O \)-benzyl oxime derivative 132 as white powder in 41% yield. \( O \)-alkylated compound 154 was obtained in less than 20% yield.
3.5.4 Synthesis of IDC16 mimic 133

The synthesis of IDC16 mimic 133 required elaboration of the aminomethyl motif at C5 of the benzoisoxazolone scaffold. It was anticipated that the amino group would occupy the same space as the D-ring nitrogen atom in IDC16, and consequently display the anticipated ability to bind to the SR protein target SRSF1 (see Figure 81b). Three different approaches were investigated in order to develop a synthetic route to 133.

3.5.4.1 Proposed scheme to synthesis IDC16 mimic 133 via acetal derivative 155

In the first approach, shown in Figure 88, our strategy was dependent upon making the key acetal intermediate 155 and finding a means for its conversion to hydroxamic acid 156. The benzoisoxazolone compound 157 could then be formed through a CDI induced cyclization.
reaction. Following introduction of the side chain onto the isoxazolone nitrogen, ketal deprotection and treatment of the liberated aldehyde with ammonia under reductive amination conditions would give 133.

**Figure 88**: Proposed scheme to synthesize IDC16 mimic 133 through key intermediate 155.

In practice, the methyl ester 136 derived from 5-formylsalicylic acid 134 (see Figure 88) was converted to acetal 155 by reaction with ethylene glycol and catalytic p-toluenesulfonic acid\(^{199}\) using a Dean-Stark apparatus. Initially the reaction did not proceed using either benzene or toluene as solvent, but by predistilling the ethylene glycol to eliminate water contamination and adding anhydrous CuSO\(_4\) to the mixture of reagents, complete conversion to the desired acetal (refluxing toluene, 6 h) was achieved (92% after column chromatography)\(^{199}\).

With acetal 155 in hand, it was reacted with hydroxylamine hydrochloride in dioxane and water in the presence of base at room temperature for 12 hours. Three different bases were tried: sodium carbonate, triethylamine and sodium hydride (as NaH is not compatible with water, dioxane was employed as the solvent). To our initial surprise, with these three bases, oxime 158 was formed as the major product (a small amount of hydroxamic acid 159 was observed). In the reaction, deprotection of the cyclic acetal group “formally” occurred to give back the aldehyde, which then reacted with hydroxylamine hydrochloride to afford the oxime (Figure 89a).
The mechanism for the reaction (Figure 89b) implicates a vinylogous (through double bond) E1cB-type elimination reaction with ring opening, following the deprotonation of the phenolic OH group in 155. In light of the difficulties to make compound 156, the strategy was abandoned and an alternative synthetic route was developed.

**Figure 89:** a) Synthesis of hydroxamic acid 158 with different bases; b) suggested mechanism of E1cB-elimination reaction.

### 3.5.4.2 Alternative scheme proposed to synthesis IDC16 mimic 133 via hydroxymethyl intermediate 160

To overcome the problems encountered in the previous approach, an alternative synthetic route was “briefly” investigated (Figure 90), involving preparation of the hydroxymethyl intermediate 160 in the first steps, and subsequently exchanging OH for NH₂, by reaction with azide ion (Mitsunobu reaction) and reduction. To set the stage, 5-formylsalicylic acid 134 was converted to ester 136 and the aldehyde function was reduced to give 160 using sodium borohydride in THF at 0°C for 5 hours. Ester derivative 160 was reacted with hydroxylamine hydrochloride, providing the hydroxamic acid intermediate 161 (52% yield).
**Figure 90:** Proposed scheme to synthesize 133 through hydroxymethyl intermediate 160.

In practice, although the hydroxamic acid 161 was successfully prepared, its dehydrative cyclization to benzoisoxazolone 162 failed. Once again, it appeared that a vinylogous E1cb elimination reaction occurred, leading to formation of the reactive quinidomethane intermediate 163, which “decomposed” (Figure 91).

**Figure 91:** Suggested mechanism of E1cB-elimination reaction of 161.

### Synthesis of IDC16 mimic 133 via bromomethyl intermediate 169

Since the benzyl OH group in 161 could not be easily used as a vehicle for introduction of the NH$_2$ group in IDC16 mimic 133, attention was directed to use 5-methyl salicylic acid 135 as the starting material (Figure 92). The idea was to first prepare the 5-methyl substituted benzoisoxazolone 167, and subsequently introduce the amino group through bromination of the methyl group and reaction of the derived benzyl bromide derivative 169 with an NH$_2$ equivalent.
This synthetic route involved conversion of 5-methyl salicylic acid 135 to its corresponding ester 164, followed by reaction with hydroxylamine hydrochloride to give hydroxamic acid 165 and subsequent cyclization to give the C5-methyl substituted benzoisoazolone 166 (56% yield for the two steps). N-alkylation of the amide nitrogen in 166 using 2-phthalimido bromoethane (DMF, microwave irradiation) then afforded intermediate 167 (34%) along with the O-alkylation product 168 in 27% yield\(^\text{201}\). Bromination of the methyl group in 167 using N-Bromosuccinimide (NBS)/AIBN provided compound 169 in 72% yield\(^\text{202}\). Subsequent reaction of benzyl bromide 169 with potassium phthalimide in DMF gave the diprotected diamine intermediate 170\(^\text{203}\). In the final step, the two phthalamido groups were removed (hydrazine, MeOH)\(^\text{204}\) to give the target compound 133 in 32% yield.

Figure 92: Synthetic scheme to make IDC16 mimic 133 through bromomethyl intermediate 167.
3.6 Screening of the synthesized molecules for anti-HIV activity

So far, only compounds (130-133, 144) and the O-alkylation products (150 and 154) have been prepared and submitted for evaluation of their activity against wild-type HIV-1 (collaboration with Dr. Peter Cheung at the BC center for Excellence in HIV/AIDS). The initial screening experiments indicated that these molecules do not display significant anti-HIV activity up to 10µM concentration. The important question that was begging to be posed at this juncture was – what conclusions could be drawn from these negative results. The answer is “none”. Compounds 130 to 133 were synthesized primarily with the objective in mind to familiarize ourselves with the chemistry of scaffold D. The validation of our idea that conserving the central rings in IDC16 in the form of a benzoisoxazolone motif remains very much “a work in progress”. Indeed, having found a way to conveniently synthesize scaffold D from related C5-CHO compound 134 and CH$_2$NH$_2$ compound 133 from 135, it now remains to determine what substituents/functionalties can be attached onto C5 and N2 such that anti-HIV activity is found. If the discovery of the IDC16 mimic 1C8 can serve as an example, this will, most probably, require the synthesis of several hundred scaffold D analogues. Indeed, the diheteroarylamide compound 19, which most closely mimics the structure of IDC16 (as does compound 133) was inactive in the anti-HIV screen. It was necessary to replace the right side 2-chloropyridine subunit in 19 by a structurally unrelated 5-nitrobenzisothiazole motif, as in 1C8, before activity was found (Figure 93). This discovery was only made possible by decorating the 4-pyridinone-3-carboxamide scaffold with a wide variety of heterocyclic motifs using parallel synthesis of a compound library.
Figure 93: Replacement of the 2-chloropyridine subunit from the inactive IDC16 mimic 19 by 5-nitrobenzisothiazole motif led to the active molecule 1C8.
Chapter 4

Benzoisoxazolone scaffold E: Identify bioactive compounds targeting PRMTs

4.1 Protein Arginine Methyltransferases (PRMTs) overview

Arginine methylation is a post-translational modification found on both nuclear and cytoplasmic proteins, which is catalyzed by the PRMTs (protein arginine N-methyltransferase)\textsuperscript{205}. PRMTs are a family of proteins that transfer methyl groups from SAM (S-adenosyl methionine) in an S\textsubscript{N}2 reaction to the side chain of specific arginine residues to form a methylated amino acid residue and S-adenosyl-L-homocysteine (AdoHcy) as products\textsuperscript{206}. The PRMT family, which consists of at least nine members in mammals (PRMTs 1-9), can be classified into three types depending on their final product as it is shown in Figure 94\textsuperscript{207}. In the first step, by using AdoMet as the methyl source, all types of mammalian PRMTs transfer a methyl group from AdoMet to one of the terminal guanidine nitrogens of arginine to make \(\omega-N^G\)-monomethylarginine (MMA). In the second step, type I PRMTs (PRMT1, 2, 3, 4, 6, 8) catalyze the transfer of the second methyl group to the same guanidine nitrogen to form asymmetric \(\omega-N^G,N'^G\)-dimethylarginine (aDMA) whereas type II PRMTs (PRMT5, 9) transfer the methyl group to a different nitrogen and make symmetric \(\omega-N^G,N^G\)-dimethylarginine (sDMA)\textsuperscript{208, 209}. Type III PRMT (PRMT7) only transfer one methyl group and produce MMA\textsuperscript{210}.

To varying degrees, the nine human PRMTs are ubiquitously expressed throughout the body with the exception of PRMT8, which is only expressed in brain tissue\textsuperscript{211}. PRMTs are present both in the cytoplasm and nucleus and they methylate a number of cellular proteins\textsuperscript{212}. Proteins that contain glycine- and arginine-rich patches (GAR motifs) are often substrates of PRMTs\textsuperscript{213}. The methylation of arginine residues can affect a protein’s affinity for RNA, and affect the interaction of the substrate proteins with other proteins as well as their subcellular localization in yeast and mammalian cells\textsuperscript{214}. Arginine methylation is also involved in a
variety of biological processes such as gene expression, RNA splicing, DNA damage repair, signal transduction and protein trafficking.\textsuperscript{213, 215}

**Figure 94:** PRMT reactions. Type I PRMTs produce MMA and aDMA; Type II PRMTs produce MMA and sDMA; Type III PRMTs only produce MMA.

### 4.2 Overall Structure of PRMTs

The nine human PRMTs have a highly conserved catalytic core that is characterized by having a signature double E loop, a THW loop and a seven beta-strand Rossmann-like fold involved in AdoMet binding, as well as the unique domains that sit on either side of the conserved core region (Figure 95).\textsuperscript{216, 217} Crystal structures of PRMT1, PRMT3, PRMT 4 (also known as CARM1), PRMT 5 and PRMT6 revealed that PRMTs adopt a canonical
dimeric structure\textsuperscript{217, 218}. Monomeric structure of PRMTs can be divided into four parts (Figure 96a): N-terminal, AdoMet binding, \(\beta\)-barrel and dimerization arm\textsuperscript{219}. A superposition of PRMT1 and PRMT3 core (Figure 96b) shows that there is less than 1\(\text{Å}\) of root-mean-square deviation between them\textsuperscript{220}.

**Figure 95:** Conserved structure of human PRMT family members. All members harbor signature motif I, post-I, II and III and the conserved THW loop. (Figure is adapted from reference 216.)

The N-terminal domain contains a typical Rossmann-like fold which is also referred to as the AdoMet binding domain\textsuperscript{53}. The C-terminal domain of PRMT is barrel-like substrate binding domain\textsuperscript{220}. The AdoMet binding domain of the PRMT core has the consensus fold which is conserved in other AdoMet-dependent methyltransferases, whereas the \(\beta\) barrel is unique to the PRMT family\textsuperscript{220}. AdoHcy, the cofactor product, sits in a deep pocket on the carboxyl ends of the parallel strands and is surrounded by residues that are highly conserved in the PRMT family\textsuperscript{221}. PRMTs form a ring-like homodimer and the dimerization arm is stabilized
by the hydrophobic interaction with the outer surface of the AdoMet-binding site. Sequences beyond the conserved region of PRMTs are believed to be also important for the enzymatic activity. For example, the SH3 domain in PRMT2, a Zn finger in PRMT3 and the myristoylation motif in PRMT8 are implicated in protein-protein interactions and subcellular localization. Two invariant glutamic acid residues in the double E loop are considered to coordinate the redistribution of the positive charge of the substrate arginine guanidine group and deprotonation of the guanidine nitrogen to attack the methyl group of AdoMet.

**Figure 96:** a) Conserved catalytic core structure of PRMTs. b) Superposition of catalytic site of PRMT1 and PRMT3. (Figure is adapted from reference 220.)

### 4.3 PRMTs and disease

The dysregulation of PRMTs is manifested in a number of human conditions including cancer. PRMT1 is the predominant arginine methyltransferase with approximately 85% cellular activity associated with it, and the abrogation of its expression has been shown to suppress growth of bladder and lung cancer cells. The first concrete evidence showing the essential function of PRMTs in oncogenesis came from the study that knockdown of
PRMT1 or Sam68 (a bridge molecule in the complex for PRMT1 interaction) expression suppressed mixed lineage leukaemia (MLL) mediated transformation\textsuperscript{229}.

PRMT1, PRMT4 and PRMT6 are transcriptional co-activators for nuclear receptors such as androgen receptors (ARs) and estrogen receptors (ERs) and therefore they have emerged as a new drug target for hormone-dependent cancers\textsuperscript{230, 231}. Over-expression of PRMT4 has been observed in both prostate and breast cancers. Studies revealed that the level of PRMT4 is increased in castration-resistant prostate cancer\textsuperscript{54}. Androgen stimulation led to PRMT4 recruitment which catalyzed methylation of histone H3\textsuperscript{54}. Interestingly, SiRNA mediated knockdown of PRMT4 had effects on decreasing androgen-induced gene expression that led to diminished cell proliferation and increased apoptosis\textsuperscript{54}. Meanwhile, PRMT4 is a positive regulator of estrogen receptor α (ERα) that mediates breast cancer cell proliferation\textsuperscript{232}. PRMT4 is essential for estrogen-induced cell cycle progression in the MCF-7 breast cancer cell line\textsuperscript{233}. Dimethylation on histone H3 arginine 17 by PRMT4 has been reported to control the estrogen-induced expression of key cell cycle transcriptional regulator E2F1\textsuperscript{233}. The silencing of PRMT4 impedes estrogen-simulated breast cancer growth. Thus, PRMT4 has been identified as a plausible target in the treatment of both prostate and breast cancer.

PRMT6 functions as a transcriptional coactivator in gene expression that can regulate alternative splicing\textsuperscript{230}. PRMT6 coactivates estrogen receptors in a hormone-dependent manner and reduced PRMT6 expression has been shown to disrupt estrogen-stimulated transcription and MCF-7 breast cancer cell line proliferation\textsuperscript{234}. PRMT5 protein levels are elevated in lymphoma and leukemia cells\textsuperscript{235}. Studies have shown that methylation of histone H3R8 and H4R3 by PRMT5 can downregulate the suppressor tumorigenicity 7 (ST7) in lymphoid cancer cells, which leads to the growth of transformed lymphoid cells\textsuperscript{236}. Furthermore, reduction of PRMT5 expression via siRNA-mediated knockdown in lymphoid cancer cell lines inhibited cell proliferation, supporting the notion that PRMT5 might be a potential therapeutic target for treating cancers like lymphoma and leukemia\textsuperscript{237}.
4.4 Current PRMTs inhibitors

There are several sites at the PRMT surface that can be targeted for inhibition: the AdoMet binding site, the peptide-binding site and the allosteric binding site. Since dimerization is essential for enzymatic activity, the PRMT dimerization surface can also be targeted for inhibition. PRMT inhibitors can potentially act at any one of these possible sites and result in different patterns of inhibition.

Peptide and AdoMet substrates analogues: these types of structures were developed in an effort to mimic methyl transfer and to take advantage of the inherent selectivity of PRMTs toward arginine-containing peptides (Figure 97). The development of AdoMet analogues is limited as these types of inhibitors can target other AdoMet dependent methyltransferases to varying degrees. Chloroacetamidine 171 and N⁷-substituted arginine 172 were designed to target the arginine-binding pocket. A selectivity for arginine methyltransferases over other AdoMet-dependent methyltransferase enzymes can be achieved by targeting the arginine rather than the AdoMet binding pocket. However, no significant specificity within the PRMTs family was obtained. The bi-substrate analogue AdoMet mustard congener 173 and AzaAdoMet analogue 174 can occupy both the arginine and the AdoMet binding pockets. Indeed, bi-substrate inhibitors have more affinity than other myethyltransferase enzymes as they target both the arginine and the AdoMet binding pockets. However, these inhibitors still lack selectivity within the PRMT family.

![Figure 97: Rational designed PRMT inhibitors that are AdoMet and/or arginine analogues.](image-url)
Screening of structurally diverse compound libraries: A valid approach to identify PRMT inhibitors is to screen both existing compound libraries and virtual libraries. For example, a high throughput screening of 9000 synthetic molecules has been conducted by Bedford and his coworker and 9 compounds (including the cell permeable compound AMI-1 shown in Figure 98) have been identified to inhibit arginine methylation in vitro with IC$_{50}$ values ranged from 0.15μM to 16μM$^{243}$. A PRMT3-selective inhibitor BTD1, which was identified by the Structural Genomics Consortium, contains a benzothiadiazole group in the molecule and binds to PRMT3 at the allosteric site (binding mode is shown in Figure 99d)$^{212}$. By using virtual screening approach, SGC2085 has been identified recently as a potent inhibitor of PRMT4 with an IC$_{50}$ of 50nM and more than hundred-fold selectivity over other PRMTs (binding mode was revealed in Figure 99a)$^{244}$. CMPD1 and CMPD2 are the most potent PRMT inhibitors known so far with IC$_{50}$ values of 30nM and 27nM, respectively$^{245-247}$. Although they were not active in cell-based assays, they help solve crystal structure of PRMT4 bound to sinefungin and CMPD1, and AdoHcy with CMPD2. Almost all of these inhibitors shown in Figure 98 showed low cellular permeability and stability or they have affinity for other PRMTs and so they cannot be used as drug candidates$^{243,248}$.

![Figure 98: PRMT inhibitors identified from compound library screening or virtual screening.](image-url)
The predicted binding mode of SGC2085 with PRMT4 (Figure 99a and 99b) revealed interactions in the substrate-binding pocket that the terminal alanine-amide tail is directed toward the bottom of the arginine-binding cavity and formed hydrogen bonds with E258, E267 and H415. The 3-methyl group binds into the pocket formed by M163, F153 and Q159 and favorable hydrophobic interactions with these residues are formed. Figure 99c and 99d showed that a PRMT3-selective inhibitor BTD1 binds outside the enzyme active site (referred to the allosteric site). In the absence of the inhibitor (Figure 99c), R396 occupied the binding pocket. BTD1 binding induced flipping of R396 out of the pocket accompanied by the conformational rearrangements of helix α-X segment (Figure 99d). The benzothiadiazole moiety is deeply buried in the allosteric pocket and is hydrogen bonded to the side chain of T466. The middle urea moiety is located at the entrance of the cavity and forms hydrogen bonds with R396 and E422. The cyclohexenylethyl group extends out of the allosteric pocket and makes hydrophobic interactions with the nonpolar surface.

**Figure 99:** a) & b) Prediction binding mode of SGC2085 with PRMT4 derived from the molecular dynamics simulation. c) The structure of PRMT3 catalytic core in the absence of...
inhibitor. d) Binding interactions of BTD1 with PRMT3 induced conformational rearrangement. (Figure is adapted from reference 244 and 249.)

4.5 Virtual screening of compounds on PRMT4 crystal structure 2Y1X

The crystal structure (2Y1X) of the human PRMT4 catalytic domain (residues 135-482, downloaded from the RCSB Protein Data Bank), which co-crystallized with SAH and CMPD-2, was used as a docking template for downstream screening (collaboration with Shona Sedlock and Jennifer Brown in Dr. Adam Frankel’s group using Accelrys Discovery Suite 4.5® software in Dr. Tara Leah Klassen group). In silico modeling of our GPS compound library of 384 drug-like molecules (conducted by Shona Sedlock) suggested that compound GPS383, an intermediate in a project to synthesize MAOB inhibitors to bind selectively to PRMT4 250 (Figure 100a).

With the help of Jennifer Brown (a PhD student from Dr. Adam Frankel’s group) by preparing the protein and generating the model, compound GPS383 was drawn in Accelrys Draw 4.2 and converted into .mol files and in silico docking was performed. Docking experiments showed that the methylglycine tail of GPS383 was directed towards the bottom of the arginine-binding cavity (Figure 100c), where the terminal OH group and carbonyl oxygen interacts with Glu258 and Glu267, respectively (Figure 100d). Subsequent evaluation of compound GPS383 in an in vitro assay determined that the molecule showed little to no inhibitory activity even at 200μM 250. Understanding that this is most likely the consequence of rapid on-off kinetics (single acid OH does not provide sufficiently strong H-binding to the enzyme), the analogue “35” in which the terminal COOH group was replaced by a urea group (Figure 101) was evaluated in silico and found to have enhanced binding affinity with PRMT4.
Figure 100: a) Canonical tautomer of GPS383 was generated using the virtual screening software. b) The orientation of GPS383 at the arginine-binding pocket of PRMT4. c) Interactions of GPS383 with PRMT4 in the arginine-binding cavity as docked using CDOCKER. Figures were generated using Accelrys Discovery Suite 4.5®.

Analogue “35” was built on scaffold E which has an amino group on C7 and allows attaching the urea group on it. The modeling/docking experiments showed that there is a deep cavity (arginine-binding cavity) in the PRMT4 catalytic domain which extends towards the site of methyl transfer. As shown in Figure 101&102, the terminal urea group which mimics the guanidino moiety in arginine is directed towards the bottom of the arginine-binding cavity and formed intensive hydrogen bonds with PRMT4 residues E258, H415, E267 and Y154. Outside of the arginine-binding cavity, the benzoisoxazolone ring system is positioned at the mouth of the cavity and allows additional hydrophilic interactions with the residues lining the pocket. The two benzyl moiety sitting above will make extensive hydrophobic interactions with residues lining at the mouth of the cavity.
Figure 101: a) Canonical tautomer of 35 (n=1) was generated using the virtual screening software. b) The orientation of 35 (n=1) at the arginine-binding pocket of PRMT4. Figures were generated using Accelrys Discovery Suite 4.5®.

Figure 102: a) Conformation of 35 (n=1) with PRMT4 from the crystal structure (2Y1X). b) Interactions of 35 (n=1) with PRMT4 in the arginine-binding cavity using CDOCKER. Figures were created using Accelrys Discovery Suite 4.5®.
According to the literature, SGC2085 is the first report on a virtual screening campaign producing potent PRMT4 inhibitors. Docking software and algorithms were not mentioned by the author. Here, for ease of comparison, docking experiment of SGC2085 on PRMT4 binding site was performed using the same virtual screening protocol as we conducted for 35 (n=1) (different docking condition may result in slightly different binding interactions). The key binding interactions within the PRMT4 active site shown in Figure 103 maximum match those described in the literature. The binding mode generated of SGC2085 explains that GPS383, 35 (n=1) and SGC2085 all followed the same binding pattern and formed hydrogen-bonds with the three key residues E258, E267 and H415.

**Figure 103:** a) Conformation of SGC2085 with PRMT4 from the crystal structure (2Y1X). b) Interactions of SGC2085 with PRMT4 in the arginine-binding cleft as docked using CDOCKER. Figures were produced using Accelrys Discovery Suite 4.5®.

By deploying two different scoring algorithms, LibDock and CDOCKER Accelrys, docking studies on SGC2085, GPS383 and 35 (n=1) showed that 35 (n=1) displayed the highest scores among these three molecules (Figure 104). This gives the general impression that 35 (n=1) may exploit best interactions within the arginine-binding cavity and also the region outside of the arginine-binding cavity. Therefore, the synthesis of the corresponding
benzoisoxazolone compound \(35\) \((n=1)\) was thus investigated in order to discover novel inhibitors of PRMT4.

<table>
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<tr>
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<th>SGC2085</th>
<th>GPS383</th>
<th>35</th>
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<tbody>
<tr>
<td>Libdock</td>
<td>99.177</td>
<td>137.001</td>
<td>153.22</td>
</tr>
<tr>
<td>CDOCKER</td>
<td>47.857</td>
<td>52.333</td>
<td>57.95</td>
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**Figure 104**: Docking scores of SGC2085, GPS383 and \(35\) \((n=1)\) for PRMT4 by using LibDock and CDOCKER screening algorithms.

### 4.6 Chemistry

Two different screening algorithms LibDock and CDOCKER Accelrys, docking studies on these two types of molecules showed that the *in silico* binding interaction of \(35\) \((n=1)\) is stronger. In light of this result, the synthesis of compounds \(35\) \((n=1)\) and \(35\) \((n=2)\) using scaffold \(E\) was undertaken. The description of the synthesis of these molecules has been divided into three stages: i) preparation of scaffold \(E\) and selective C5 monoamidation ii) conversion of intermediate \(183\) to the di-\(N\)-benzyl amide \(187\) and nitro group reduction, and iii) assembly of the urea containing side arms found in compounds \(35\) \((n = 1 \text{ and } 2)\).

**Part I: Synthesis of the carboxylic acid intermediate 178 and its conversion to Nitro-scaffold E and selective monoamidation to intermediate 183**

The synthetic route initially followed to access compound \(178\) involved nitration of 5-formylsalicylic acid \(134\) with sulfuric acid/nitric acid conditions to give \(175\) and attempted diprotection of the acid/phenol groups in this intermediate through reaction with dibromomethane to obtain intermediate \(177\) (Figure 105). However, the di-alkylation reaction could not be achieved, presumably due to the strong electron-withdrawing effect produced by the nitro group. This problem was resolved by reversing the order for the two steps, i.e.
compound 134 was first di-protected (CH$_2$Br$_2$, DMF, K$_3$PO$_4$, 100°C, 10 hours)$^{149}$, and compound 176 was subsequently converted to the target compound 177$^{251}$. In this way, compound 177 was obtained in 60% overall yield. Note that, whereas the cyclic protecting group in compound 176 is labile in mild basic media (see Figure 106, from 178 to 179), it is astoundingly stable in sulfuric acid. Oxidation of compound 177 was achieved in 79% yield by using Oxone in DMF at room temperature for 12 hours$^{252}$.

**Figure 105:** Scheme of the synthesis of carboxylic acid derivative 178.

With carboxylic acid intermediate 178 in hand, we were interested to see whether its acid function would react selectively with benzyl amine using peptide coupling reagents. However, due to the extreme liability of the 1,3-dioxin-4-one motif to even mild amine base treatment, it was not surprising that reaction of 178 with benzyl amine and HOAt/EDC/DIEA in DMF at room temperature for 12 hours led to exclusive formation (TLC monitoring) of the dibenzylamide compound 179 (isolated in 48% yield after chromatography) (Figure 106).

**Figure 106:** Reaction between 178 and benzylamine afforded dibenzylamide product 179.
This result indicated to us that treatment of intermediate 178 with hydroxylamine hydrochloride and base would probably result uniquely in hydrolysis to diacid 184. For this reason, benzo-1,3-dioxin-4-one 178 was treated with K$_2$CO$_3$ in methanol at room temperature (over the weekend) to give the methyl ester 180 in 97% yield$^{253}$ (Figure 107). In this way, the lower reactivity of the methyl ester function was used to drive selective generation of the hydroxamic acid intermediate 181 (91% yield). Dehydrative cyclization of hydroxamic acid 181 in the presence of CDI at 70°C in THF for 6 hours then gave 182 (nitro scaffold E) (65% overall yield for the two steps). Reaction of benzoisoxazolone 182 with benzyl amine using CDI-benzyl amine in THF conditions then gave the monoamide 183 as the only product in 67% yield.

![Chemical structures](image.png)

**Figure 107:** Completion of the synthesis of scaffold E and its conversion to monoamide product 183.

When DMF was used as the solvent instead of THF in the reaction to make benzyl amide 183, the methyl ester 185 was formed as the major product (Figure 108a). DMF is typically used as catalyst in the synthesis of carboxylic acid chlorides$^{254}$. However, it was used as the solvent in this reaction to form the activated acid intermediate. The mechanism proposed for the formation of 185 is illustrated in Figure 108b. To recover ester derivative 185, it was
hydrolyzed back to acid 182 in high yield with lithium hydroxide\textsuperscript{255}.

\begin{figure}
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\includegraphics[width=\textwidth]{figure108.png}
\caption{a) Formation of 185 when DMF was used as the solvent; b) Proposed mechanism for the formation of the methyl ester.}
\end{figure}

**Figure 108:** a) Formation of 185 when DMF was used as the solvent; b) Proposed mechanism for the formation of the methyl ester.

**Part II: N- versus O-alkylation of intermediate 183**

Alkylation of compound 183 with benzyl bromide (Figure 109) using K\textsubscript{2}CO\textsubscript{3} as the base under microwave heating conditions resulted in formation of both the O- and N-alkylation products 186/187 (in 37\% and 43\% yield, respectively). Chromatographic separation of these two intermediates and nitro group reduction using the SnCl\textsubscript{2}/SnCl\textsubscript{4} mixture in concentrated HCl at 0°C for 4 hours afforded intermediates 188 (59\% yield) and 189 (61\% yield), respectively.
Figure 109: Synthesis of pivotal amine intermediates 189 and the corresponding O-alkylated product 188.

Part III: Synthesis of target compounds 35 (n=1 and n=2)

a) Synthesis of PRMT inhibitor 35 (n=1)

As shown in Figure 110, the synthesis of target compound 35 (n=1) from amine derivative 189 was planned to involve 4 steps. In the first step, the reaction between amine derivative 189 and bromoacetyl bromide in DCM containing triethylamine as the base gave intermediate 190 in 72% yield\textsuperscript{256}. At this point, the idea was to react 190 with ammonia in methanol to produce the “glycinamide” derivative 191\textsuperscript{257}. The subsequent steps to 35 (n=1) would involve the reaction of acetamide derivative 191\textsuperscript{258} with N-TMS isocyanate and N-desilylation. However, according to TLC, the reaction between 190 with ammonia in methanol at room temperature resulted in the formation of multiple products, from which the desired product could not be isolated.
Figure 110: Proposed scheme to synthesize target compound 35 (n=1) through acetamide derivative 191.

As shown in Figure 111, an alternative was to react amine 189 with Boc-Gly-OH using peptide coupling conditions\textsuperscript{259}, followed by cleavage of the N-Boc protecting group to give compound 191\textsuperscript{260}. In this way, the side-chain amino group would be introduced directly. However, reaction of 189 with Boc-Gly-OH in the presence of HOAt/EDC/DIEA in DMF at room temperature or 60°C for 24h did not result in the formation of amide 193\textsuperscript{261}. This amide coupling was also attempted using the acid-fluoride-TMS-amine protocol developed in our laboratory (described in Chapter 1, Figure 8). Once again, the starting amine was recovered unchanged. A potential complication to this reaction was the competing intramolecular reaction of Boc-Gly-OH to give the oxazolidine-2,5-dione 194\textsuperscript{262}, but one might have expected this cyclic anhydride to react with 189 with formation of the desired product 191\textsuperscript{263}.
Condition: a) HOAt/EDC/DIEA in DMF at room temperature or 60°C for 24h. b) TFFH and CsF in CH₃CN at 50°C for 24h (amine 183 was silylated with TMSCN beforehand).

**Figure 111:** Proposed scheme to synthesize intermediate 191 using Boc-Gly-OH. Note competing formation of oxazolidinone 194.

In light of this result, and the fact that the reaction with 190 with NH₃ was complicated by the formation of multiple alkylation products, the obvious/classical way to circumvent this situation would be to react 190 with azide ion²⁶⁴ and then selectively reduce the azido group to the corresponding amine²⁶⁵ (Figure 112). As time did not permit, this approach will be a priority for future work on the development of synthesis using scaffold E as a building block to make PRMT inhibitors.

**Figure 112:** Alternative approach to introduce amino side chain by using sodium azide.

*b) Synthesis of PRMT inhibitor 35 (n=2)*

The synthesis of compound 35 (n=2) was approached from a similar direction, i.e. the
reaction of Boc-β-Ala-OH\textsuperscript{266} with amine 189 (Figure 113). However, this reaction was attempted numerous times with different peptide coupling reagents and TLC monitoring revealed that the starting amine 189 did not react.

**Figure 113:** Proposed scheme to synthesize target compound 35 (n=2).

The coupling reaction of intermediate 189 with 3-ureidopropanoic acid 199 (commercially available), which would have allowed introduction of the urea side chain in compound 35 in a single step\textsuperscript{267}, also failed. These two results indicated to us that the Boc-β-Ala-OH and the 3-ureidopropanoic acid 199 components in the reaction underwent competing intramolecular reaction, leading to the cyclized 1,3-oxazinane-2,6-dione 198\textsuperscript{268} and dihydropyrimidine 200\textsuperscript{269} by-products, respectively. Once again, this problem could potentially be circumvented by converting compound 189 to the acrylamide intermediate 201 through the reaction with acryloyl chloride\textsuperscript{270}, which could undergo 1,4-addition of a nitrogen nucleophile\textsuperscript{271}, such as the N-silyl urea 202, to give the target compound 35 (n = 2). Completion of the preparation of compound 35 (n = 2) is also a major priority for future work on the synthetic applications of scaffold E.
Figure 114: Proposed alternative scheme to synthesize target compound 35 (n=2).

As our current objective with scaffold E is to generate a wide variety of library molecules, the lack of chemoselectivity in the N- versus O-alkylation of the scaffold works towards our purpose. However, in cases where bioactive compounds are identified, it may prove necessary to selectively N- or O-alkylate the C(=O)NH motif in the isoxazolone ring. Selective formation of the N- or O-alkylation product can be achieved in many ways. For example, reaction of a benzoic acid derivative with N-alkyl hydroxylamines (NHROH instead of NH₂OH) (Figure 115a) would lead to formation of 203. Cyclization of this intermediate via an intramolecular S_N_Ar reaction would, in principle, lead specifically to formation of the N2 alkyl substituted benzoisoxazolone 204 (without competing Lossen rearrangement). Further, amidoalkylation of the N2 nitrogen in the benzoisoxazolone ring with diverse and readily accessible aldehydes is anticipated to generate compounds 205, which could be reduced under mild conditions to also give N-alkylated products (Figure 115b). Conversely, to obtain O-alkylated benzoisoxazolones 208, one could investigate POCl₃ promoted formation of a 3-chlorobenzoxazole intermediate 207 and S_N_Ar reaction with different alkoxide (or even amine) nucleophiles (Figure 115c). These and other techniques will be used to generate a rich library of molecules based on scaffolds B to E.
Figure 115: Proposed schemes towards $N$- versus $O$-alkylation.
Chapter 5: Conclusions and Future Directions

The value of privileged scaffolds is that they enable the synthesis of highly diverse compound libraries based around a common core structure; whose members may display activity against distinct targets of therapeutic interest. At the heart of this compound library approach is the search for novel scaffolds, which are used to assemble bioactive molecules containing unexplored architectures. This research project focused on the synthesis of the four benzoisoxazolone-based scaffolds B to E (Figure 15), as privileged scaffolds for the synthesis of diversity driven small molecule libraries of potential isoform selective inhibitors of Rho-kinase (ROCK), anti-HIV agents, and PRMT inhibitors. The scant attention previously directed toward the four benzoisoxazolone scaffolds B to E was clearly reflected by the limited number of SciFinder hits associated with their structure and use. This strongly suggested that there is still much room for research that can be done with these novel scaffolds.

Our initial objective was to generate libraries of compounds that are based on C5 and C4-amino benzoisoxazolone scaffolds B and C to identify isoform selective Rho-kinase (ROCK1/2) inhibitors. Such molecules have potential for the treatment of diabetic cardiovascular disease and different cancers. The strategy adopted to build the benzoisoxazolone ring was well established in Chapter 2 by making the pivotal hydroxamic acid intermediate from corresponding 5-nitro carboxylic acid precursors first, which was then reacted with activating agents (CDI was used in our case) to effect ring closure reaction. The last step to build C5-amino benzoisoxazolone scaffold B involved selectively reduction of the 5-nitro group using a SnCl₄/SnCl₂ mixture in concentrated HCl, without cleaving the N-O bond of the isoxazolone ring. Scaffold B was used as the building block for the synthesis of C5-amino benzoisoxazolone derivatives 32 through N-alkylation with different N-(hetero)aryl substituted 2-bromoacetamides 63 under microwave heating condition. To date, 14 C5-amino benzoisoxazolone molecules have been prepared corresponding to general
formula 32. Subsequently, the scope of our project was enlarged to also synthesize C4-amino benzoisoxazalone scaffold C based compounds as potential ROCK inhibitors. All compounds tested were inactive in the single point in vitro screening assay performed. However, recent results from our laboratory strongly suggest an alternative application of these compounds to investigate enhancement of aminoglycoside promoted PTC readthrough as an approach to treat a number of genetic diseases and cancers.

In continued work to design “ring opened analogues” of IDC16 which blocks HIV-1 pre-mRNA alternative splicing through inhibition of SRSF1, we engaged in the synthesis of a library of 5-carboxy benzoisoxazolone scaffold D-based compounds as a new class of anti-HIV agents. As seen from the 2D structure of the benzoisoxazalone ring in Figure 35, it superimposed with the middle portion (B and C-rings) of IDC16. Different A/D-ring nitrogen and/or oxygen functionalities were connected to this ring system in order to target human splicing factor SRSF1. A total of 5 non-fused IDC16 mimics were obtained by this strategy possessing different shapes and composition relative to IDC16. Subsequent preliminary screening of these compounds indicated that they don’t show improved anti-HIV activity compared to the active molecule IDC16 mimic 1C8. Inspired from the example that diheteroarylamide compound 19, which most closely mimics the structure of IDC16 (as does compound 133), did not display any activity. However, of the 256 mimics (made by parallel thesis) tested, the most active compound 1C8 bears a structurally unrelated 5-nitrobenzisothiazole motif. Therefore, it is strongly suggested that active compound will be found by decorating our scaffold with a wide variety of different motifs using parallel synthesis methodology.

On the basis of the virtual screening/docking experiments experiment on PRMT4, it was observed that terminal urea group of 35 mimics the guanidine moiety in arginine, which is directed towards the bottom of the arginine-binding cavity and formed intensive hydrogen bonds with PRMT4 residues E258, H415, E267 and Y154. The two benzyl groups introduced
at N2 and C5 positions make extensive hydrophobic interactions with residues lining at the mouth of the arginine-binding cavity. In order to synthesize the urea compound 35 (n=1) and (n=2), scaffold E and selective C5 monoamidation was first prepared. Conversion of this monoamide to the di-N-benzyl amide and selective nitro group reduction afforded the pivotal amine derivative 189 in overall good yield. However, assembling the urea containing side arm was not completed. In light of the observations we have made, an attractive strategy to complete the synthesis of 35 (n=1) would be to replace the bromo substituent in intermediate 190 by an azido group, and to subsequently reduce it to the corresponding amine, which on reaction with N-TMS isocyanate would introduce the needed urea motif (Figure 112). Alternatively, completion of the synthesis of PRMT inhibitor 35 (n=2) is envisaged to involve, in the key operation, the conjugate addition of a nitrogen nucleophile to intermediate 201 (Figure 114).
Experimental

Materials and methods: All chemicals and solvents were purchased from commercial sources and they were used without further purification unless mentioned. DMF (anhydrous) and acetonitrile (anhydrous) were purchased from Sigma Aldrich. THF was pre-dried by refluxing over sodium/benzophenone under nitrogen atmosphere and distilling prior to use. Reactions were monitored by TLC (pre-coated silica gel 60 F\textsubscript{254} plates). Flash chromatography was performed using silica gel (Silicycle, Silicaflash, F60, 40-63 μm, 230-400 mash) or on a Biotage Isolera purification system using pre-packed silica gel columns. Microwave reactions were performed on a Biotage Initiator Robot Eight System. Low resolution mass spectra were measured on a AB Sciex QTRAP 5500-Agilent 1290. High resolution mass spectra were recorded using a Thermo Scientific Q Exactive Orbitrap High Resolution Mass Spectrometer. \textsuperscript{1}H, \textsuperscript{13}C and \textsuperscript{19}F NMR were recorded (at 400MHz, 100MHz and 376.5 MHz respectively) on a Bruker AC 400 Ultrashield 10 spectrophotometer. Chemical shifts are expressed in ppm (δ scale). Coupling constant are reported in Hertz (Hz). The splitting patterns (multiplicities) were reported as follows: s (singlet), bs (broad singlet), m (multiplet), dd (doublet of doublet), t (triple), q (quartet), dt (doublet of triplet), ddd (doublet of doublet of doublets). In silico docking were performed using the “Accelrys Discovery Suite 4.5” software.

Note: The experiments are presented in the order in which they appear in the reaction schemes in Chapters 2-4.

Methyl 2-hydroxy-5-nitrobenzoatemethyl 60:

\[
\begin{array}{c}
\text{O}_2\text{N} \\
\text{HO} \\
\text{\textbullet} \\
\text{CH}_3
\end{array}
\]

To a solution of 2-hydroxy-5-nitrosalicylic acid 58 (5.0 g, 27.3 mmol) in absolute methanol
(125 mL) was added concentrated sulfuric acid (4 mL). The reaction mixture was heated to reflux for 15 h. After cooling to room temperature, a lime coloured solid started to crystallize out. The mixture was filtered and the solid was washed with cold methanol. Recrystallization from methanol gave 60 as white crystals (4.4 g, 82%).

$^1$H-NMR (400 MHz, CDCl$_3$): δ 11.42 (s, 1H), 8.76 (s, 1H), 8.32 (d, J=8.7 Hz, 1H), 7.08 (d, J=8.7 Hz, 1H), 4.04 (s, 3H).

MS ESI (m/z): 196.1 [M-H]$^-$.

2-Hydroxy-5-nitrobenzhydroxamic acid 61:

Following the literature procedure$^{105}$, to a solution of hydroxylamine hydrochloride (2.08 g, 30 mmol) in water (2.4 mL) was added NaOH (2.82 g in 2.4 mL H$_2$O), with stirring under a N$_2$-atmosphere. To this solution 5-nitro methyl salicylate 60 (3 g, 15.18 mmol) dissolved in dioxane (7.5 mL), was added dropwise, followed by H$_2$O (30 mL). The mixture was stirred at room temperature for 12h under nitrogen. The solvent was then removed under reduced pressure at 50°C. The residue was cooled and acidified with 12N HCl. The mixture was stirred for another 30 min at room temperature. The resulting precipitate was filtered and washed with ice-water and dried under vacuum to give product 61 (2.9 g, 96%) as a white solid.

$^1$H-NMR (400 MHz, DMSO-d$_6$): δ 13.20 (bs, 1H), 11.59 (s, 1H), 9.52 (bs, 1H), 8.64 (d, J=2.8 Hz, 1H), 8.24 (dd, J=9.1 Hz, 2.8 Hz, 1H), 7.12 (d, J=9.1 Hz, 1H).

MS ESI (m/z): 197.0 [M-H]$^-$.

5-Nitrobenzo[d]isoxazol-3(2H)-one 59:

O$_2$N

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| O | N | H | O
| O |  |  | O

Following the literature procedure$^{105}$, to a solution of hydroxylamine hydrochloride (2.08 g, 30 mmol) in water (2.4 mL) was added NaOH (2.82 g in 2.4 mL H$_2$O), with stirring under a N$_2$-atmosphere. To this solution 5-nitro methyl salicylate 60 (3 g, 15.18 mmol) dissolved in dioxane (7.5 mL), was added dropwise, followed by H$_2$O (30 mL). The mixture was stirred at room temperature for 12h under nitrogen. The solvent was then removed under reduced pressure at 50°C. The residue was cooled and acidified with 12N HCl. The mixture was stirred for another 30 min at room temperature. The resulting precipitate was filtered and washed with ice-water and dried under vacuum to give product 61 (2.9 g, 96%) as a white solid.

$^1$H-NMR (400 MHz, DMSO-d$_6$): δ 13.20 (bs, 1H), 11.59 (s, 1H), 9.52 (bs, 1H), 8.64 (d, J=2.8 Hz, 1H), 8.24 (dd, J=9.1 Hz, 2.8 Hz, 1H), 7.12 (d, J=9.1 Hz, 1H).

MS ESI (m/z): 197.0 [M-H]$^-$. 

5-Nitrobenzo[d]isoxazol-3(2H)-one 59:

O$_2$N

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123
Following the literature procedure\textsuperscript{105}, a solution of CDI (3.03 g, 18.7 mmol) in THF (45 mL) was added over 30 min to a solution of \textbf{61} (1.87 g, 9.5 mmol) in THF (25mL) heated to reflux. The reaction mixture was stirred for another 2h at 60°C, then cooled and the solvent was removed. The residue was taken-up in water (15 mL) and acidified with 12N HCl to pH 2. The mixture was stirred at room temperature for 30 min and the resulting precipitate was filtered, washed with ice-water and dried under vacuum to give product \textbf{59} (1.55 g, 91%) as a white solid.

\textbf{1H-NMR (400 MHz, DMSO-d6):} \(\delta 8.58 \ (d, J=2.3 \text{ Hz}, 1H), 8.44 \ (dd, J=9.2 \text{ Hz}, 2.3 \text{ Hz}, 1H), 7.61 \ (d, J=9.2 \text{ Hz}, 1H)\).

\textbf{13C-NMR (100 MHz, DMSO-d6):} \(\delta 166.4, 166.0, 144.1, 126.4, 119.3, 115.7, 111.8\).

\textbf{MS ESI (m/z):} 178.9 [M-H]⁻.

\textbf{5-Amino-2-hydroxybenzamide 62:}

\[
\begin{array}{c}
\text{H}_2\text{N} \\
\text{O} \\
\text{NH}_2 \\
\text{OH}
\end{array}
\]

According to reference 108, to a solution of nitro compound \textbf{59} (86.5 mg, 0.48 mmol) in EtOH (1 mL) and H₂O (0.5 mL), was added iron powder (139 mg, 2.5 mmol) and glacial acetic acid (1 mL). The mixture was stirred under ultrasonic irradiation at 30°C for 1 h. The reaction mixture was then filtered to remove residual iron powder, and the filtrate was taken up in 10% KOH (30 mL) and extracted with EtOAc (three times 30 mL). The combined organic phases were dried over Na₂SO₄, concentrated, dry loaded onto silica gel and column chromatographed (Hexane/EtOAc, 1:2) to afforded compound \textbf{62} as a white solid (55.4 mg, 76%).

\textbf{1H-NMR (400 MHz, DMSO-d6):} \(\delta 11.79 \ (s, 1H), 8.13 \ (s, 1H), 7.63 \ (s, 1H), 7.01 \ (d, J=2.7 \text{ Hz}, 1H), 6.74 \ (dd, J=8.6 \text{ Hz}, 2.7 \text{ Hz}, 1H), 6.64 \ (d, J=8.6 \text{ Hz}, 1H), 5.13 \ (s, 2H)\).

\textbf{MS ESI (m/z):} 151.0 [M-H]⁻.

\textbf{5-Aminobenzo[d]isoxazol-3(2H)-one B:}
1) Following the literature procedure\textsuperscript{104}, to compound 59 (900 mg, 5 mmol) in concentrated HCl (4.5 mL) was added a solution of SnCl\textsubscript{2}.2H\textsubscript{2}O (6.54 g, 29 mmol) in concentrated HCl (5.5 mL). The reaction mixture was stirred at room temperature for 7 days. The precipitate that formed was collected by filtration and washed with water. The white solid was recrystallized from water to give scaffold B as its HCl salt. (486.3 mg, 54%).

2) Following the literature procedure\textsuperscript{113}, stannic chloride (1.75 mL, 1.0 M in methylene chloride) and concentrated HCl (0.9 mL) were mixed and cooled to 0°C. Nitro compound 59 (180 mg, 1 mmol) was added to this mixture, followed by dropwise addition over 30 min. of a solution of stannous chloride (682 mg, 3.6 mmol) in concentrated HCl (0.5 mL). After stirring for 4 h, the mixture was filtered and the white solid was collected. The solid was dissolved in EtOAc and extracted with saturated sodium bicarbonate. The organic phase was separated, dried over Na\textsubscript{2}SO\textsubscript{4}, and concentrated. The residue was dry loaded onto silica gel and column chromatography (DCM/EtOAc, 9:1) to afford scaffold B as a white solid. (93 mg, 61%).

\textsuperscript{1}H-NMR (400 MHz, DMSO-d\textsubscript{6}): δ 10.68 (bs, 3H), 7.90 (d, J=1.7 Hz, 1H), 7.70 (d, J=8.9 Hz, 1H), 7.61 (dd, J=8.9 Hz, 1.7 Hz, 1H).

\textsuperscript{13}C-NMR (100 MHz, DMSO-d\textsubscript{6}): δ 165.6, 162.5, 127.9, 126.6, 116.6, 115.5, 112.0.

MS ESI (m/z): 149.0 [M-H]\textsuperscript{-}.

\textbf{2-Bromo-N-phenylacetamide 63a}:

To a stirred solution of aniline (93 mg, 1 mmol) in DCM (3 mL) at 0°C, triethylamine (0.17 mL, 1.2 mmol) and bromoacetyl bromide (0.1 mL, 1.2 mmol) were sequentially added dropwise. The reaction mixture was slowly warmed up to room temperature, stirred for an
additional 12 hours and concentrated. The residue was silica chromatographed (DCM/EtOAc, 95:5) to afford 63a as a yellow solid (153 mg, 72%).

$^1$H-NMR (400 MHz, CDCl$_3$): δ 8.11 (s, 1H), 7.53 (d, J=8.0 Hz, 2H), 7.37 (t, J=8.0 Hz, 2H), 7.17 (t, J=7.4 Hz, 1H), 4.03(s, 2H).

**MS ESI (m/z):** 211.8 [M-H]$^+$.

**N-Benzyl-2-bromoacetamide 63b:**

Following the procedure for 63a, benzylamine (107 mg, 1 mmol) was reacted with Et$_3$N (0.17 mL, 1.2 mmol) and bromoacetyl bromide (0.1 mL, 1.2 mmol). The crude product mixture was separated by silica flash column chromatography (DCM/EtOAc, 95:5) to afford 63b as a white solid (209 mg, 92%).

$^1$H-NMR (400 MHz, CDCl$_3$): δ 7.36 (m, 2H), 7.30 (m, 3H), 6.74 (bs, 1H), 4.49 (d, J=5.7 Hz, 2H), 3.94 (s, 2H).

**MS ESI (m/z):** 226.0 [M-H]$^+$.

**2-Bromo-N-(2,3-dimethylphenyl)acetamide 63c:**

Following the procedure for 63a, 2,3-dimethylaniline (121 mg, 1 mmol) was reacted with Et$_3$N (0.17 mL, 1.2 mmol) and bromoacetyl bromide (0.1 mL, 1.2 mmol). The crude product mixture was separated by silica flash column chromatography (DCM/EtOAc, 95:5) to afford 63c as a white solid (202 mg, 83%).

$^1$H-NMR (400 MHz, CDCl$_3$): δ 8.13 (s, 1H), 7.55 (d, J=7.9 Hz, 1H), 7.16 (t, J=7.8 Hz, 1H), 7.08 (d, J=7.5 Hz, 1H), 4.10 (s, 2H), 2.34 (s, 3H), 2.21 (s, 3H).

**MS ESI (m/z):** 242.0 [M-H]$^+$.
2-Bromo-N-(3-phenoxyphenyl)acetamide 63d:

Following the procedure for 63a, 3-phenoxyaniline (185 mg, 1 mmol) was reacted with Et₃N (0.17 mL, 1.2 mmol) and bromoacetyl bromide (0.1 mL, 1.2 mmol). The crude product mixture was separated by silica flash column chromatography (DCM/EtOAc, 95:5) to afford 63d as a yellow solid (216 mg, 71%).

\[ ^1H-NMR\ (400\ MHz,\ CDCl_3): \delta \ 8.08\ (s,\ 1H),\ 7.35\ (m,\ 2H),\ 7.29\ (d,\ J=8.6\ Hz,\ 1H),\ 7.27\ (m,\ 1H),\ 7.25\ (d,\ J=1.6\ Hz,\ 1H),\ 7.13\ (d,\ J=7.0\ Hz,\ 1H),\ 6.81\ (d,\ J=7.9\ Hz),\ 7.03\ (m,\ 2H),\ 4.00\ (s,\ 2H).\]

MS ESI (m/z): 304.1 [M-H]⁻.

2-Bromo-N-(4-fluorophenyl)acetamide 63e:

Following the procedure for 63a, 4-fluoroaniline (111 mg, 1 mmol) was reacted with Et₃N (0.17 mL, 1.2 mmol) and bromoacetyl bromide (0.1 mL, 1.2 mmol). The crude product mixture was separated by silica flash column chromatography (DCM/EtOAc, 95:5) to afford 63e as a yellow solid (210 mg, 90%).

\[ ^1H-NMR\ (400\ MHz,\ CDCl_3): \delta \ 8.11\ (s,\ 1H),\ 7.49\ (m,\ 2H),\ 7.05\ (m,\ 2H),\ 4.02\ (s,\ 2H).\]

MS ESI (m/z): 231.9 [M-H]⁻.

N-([1,1'-Biphenyl]-2-yl)-2-bromoacetamide 63f:
Following the procedure for 63a, [1,1’-biphenyl]-2-amine (169 mg, 1 mmol) was reacted with Et₃N (0.17 mL, 1.2 mmol) and bromoacetyl bromide (0.1 mL, 1.2 mmol). The crude product mixture was separated by silica flash column chromatography (DCM/EtOAc, 95:5) to afford 63f as a yellow solid (211 mg, 73%).

H-NMR (400 MHz, CDCl₃): δ 8.32 (d, J=8.3 Hz, 1H), 8.29 (s, 1H), 7.50 (m, 2H), 7.46-7.36 (m, 4H), 7.29 (dd, J=7.6 Hz, 1.5 Hz, 1H), 7.22 (t, J=7.4 Hz, 1H), 3.90 (s, 2H).

MS ESI (m/z): 288.1 [M-H].

2-Bromo-N-(4-chlorophenyl)acetamide 63g:

Following the procedure for 63a, 4-chloroaniline (127 mg, 1 mmol) was reacted with Et₃N (0.17 mL, 1.2 mmol) and bromoacetyl bromide (0.1 mL, 1.2 mmol). The crude product mixture was separated by silica flash column chromatography (DCM/EtOAc, 95:5) to afford 63g as a white solid (220 mg, 89%).

H-NMR (400 MHz, CDCl₃): δ 8.14 (s, 1H), 7.52 (m, 2H), 7.16 (m, 2H), 4.05 (s, 2H).

MS ESI (m/z): 245.9 [M-H].

2-Bromo-N-(p-tolyl)acetamide 63h:

Following the procedure for 63a, p-toluidine (107 mg, 1 mmol) was reacted with Et₃N (0.17 mL, 1.2 mmol) and bromoacetyl bromide (0.1 mL, 1.2 mmol). The crude product mixture was separated by silica flash column chromatography (DCM/EtOAc, 95:5) to afford 63h as a yellow solid (208 mg, 92%).

H-NMR (400 MHz, CDCl₃): δ 8.11 (s, 1H) 7.43 (m, 2H), 7.18 (m, 2H), 4.05 (s, 2H), 2.35 (s, 3H).
MS ESI (m/z): 225.9 [M-H].

*N-(Benzo[d][1,3]dioxol-5-yl)-2-bromoacetamide 63i:*

Following the procedure for 63a, benzo[d][1,3]dioxol-5-amine (137 mg, 1 mmol) was reacted with Et$_3$N (0.17 mL, 1.2 mmol) and bromoacetyl bromide (0.1 mL, 1.2 mmol). The crude product mixture was separated by silica flash column chromatography (DCM/EtOAc, 95:5) to afford 63i as a white solid (156 mg, 61%).

$^1$H-NMR (400 MHz, CDCl$_3$): $\delta$ 8.06 (s, 1H), 7.24 (d, J=2.0 Hz, 1H), 6.85 (dd, J=8.3 Hz, 2.0 Hz, 1H), 6.79 (d, J=8.3 Hz, 1H), 5.99 (s, 2H), 4.03 (s, 2H).

MS ESI (m/z): 279.8 [M+Na].

2-Bromo-N-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)acetamide 63j:

Following the procedure for 63a, 2,3-dihydrobenzo[b][1,4]dioxin-6-amine (151 mg, 1 mmol) was reacted with Et$_3$N (0.17 mL, 1.2 mmol) and bromoacetyl bromide (0.1 mL, 1.2 mmol). The crude product mixture was separated by silica flash column chromatography (DCM/EtOAc, 95:5) to afford 63j as a pale yellow solid (220 mg, 81%).

$^1$H-NMR (400 MHz, CDCl$_3$): $\delta$ 7.98 (s, 1H), 7.16 (d, J=2.4 Hz, 1H), 6.92 (dd, J=8.7 Hz, 2.4 Hz, 1H), 6.86 (d, J=8.7 Hz, 1H), 4.25 (s, 4H), 4.00 (s, 2H).

MS ESI (m/z): 293.5 [M+Na].

2-Bromo-N-(3-methoxyphenyl)acetamide 63k:

Following the procedure for 63a, 3-methoxyaniline (123 mg, 1 mmol) was reacted with Et$_3$N
(0.17 mL, 1.2 mmol) and bromoacetyl bromide (0.1 mL, 1.2 mmol). The crude product mixture was separated by silica flash column chromatography (DCM/EtOAc, 95:5) to afford 63k as a yellow solid (213 mg, 87%).

\(^1\)H-NMR (400 MHz, CDCl\(_3\)): \(\delta 8.11\) (s, 1H), 7.29 (d, J=1.9 Hz, 1H), 7.27 (d, J=8.2 Hz, 1H) 7.04 (dd, J=8.2 Hz, 1.3 Hz, 1H), 6.75 (dd, J=8.2 Hz, 2.2 Hz, 1H), 4.05 (s, 2H), 3.84 (s, 3H).

**MS ESI (m/z):** 244.0 [M-H]*.

2-Bromo-N-(3,4,5-trimethoxyphenyl)acetamide 63l:

Following the procedure for 63a, 3,4,5-trimethoxyaniline (183 mg, 1 mmol) was reacted with Et\(_3\)N (0.17 mL, 1.2 mmol) and bromoacetyl bromide (0.1 mL, 1.2 mmol). The crude product mixture was separated by silica flash column chromatography (DCM/EtOAc, 95:5) to afford 63l as a light yellow solid (270 mg, 89%).

\(^1\)H-NMR (400 MHz, CDCl\(_3\)): \(\delta 8.09\) (s, 1H), 6.84 (s, 2H), 4.03 (s, 2H), 3.88 (s, 6H), 3.84 (s, 3H).

**MS ESI (m/z):** 327.9 [M+Na].

2-Bromo-N-(4-methoxyphenyl)acetamide 63m:

Following the procedure for 63a, 4-methoxyaniline (123 mg, 1 mmol) was reacted with Et\(_3\)N (0.17 mL, 1.2 mmol) and bromoacetyl bromide (0.1 mL, 1.2 mmol). The crude product mixture was separated by silica flash column chromatography (DCM/EtOAc, 95:5) to afford 63m as a light orange solid (221 mg, 91%).

\(^1\)H-NMR (400 MHz, CDCl\(_3\)): \(\delta 8.04\) (s, 1H), 7.43 (m, 2H), 6.89 (m, 2H), 4.02 (s, 2H), 3.81
(s, 3H).

**MS ESI (m/z):** 265.6 [M+Na].

2-Bromo-N-(naphthalen-1-yl)acetamide 63n:

![Chemical Structure](image)

Following the procedure for 63a, naphthalen-1-amine (143 mg, 1 mmol) was reacted with Et$_3$N (0.17 mL, 1.2 mmol) and bromoacetyl bromide (0.1 mL, 1.2 mmol). The crude product mixture was separated by silica flash column chromatography (DCM/EtOAc, 95:5) to afford 63n as a grey solid (234 mg, 89%).

**$^1$H-NMR (400 MHz, CDCl$_3$):** δ 10.35 (s, 1H), 8.08 (m, 1H), 7.97 (dd, J=2.4 Hz, J=6.9 Hz, 1H), 7.82 (d, J=8.2 Hz, 1H), 7.69 (d, J=7.3 Hz, 1H), 7.56-7.62 (m, 2H), 7.52 (m, 1H), 4.23 (s, 2H).

**MS ESI (m/z):** 262.0 [M-H].

2-((3-Oxo-2,3-dihydrobenzo[d]isoxazol-5-yl)amino)-N-phenylacetamide 32a:

![Chemical Structure](image)

To a solution of scaffold B (182 mg, 1 mmol) and 2-bromo-N-phenylacetamide 63a (255 mg, 1.2 mmol) in DMF (1.5 mL) was added NaHCO$_3$ (168 mg, 2 mmol). The mixture was heated under microwave condition at 60°C for 10 min. On completion of the reaction, as followed by TLC, the solvent was removed under reduced pressure. The residue was taken up in water (20 mL), acidified with 12N HCl to pH 4, and extracted with EtOAc (3 times 30 mL). The combined organic phases were dried over Na$_2$SO$_4$ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc; 3:1) to afford 32a as a brown solid (119 mg, 42%).

**$^1$H-NMR (400 MHz, DMSO-d$_6$):** δ 11.97 (bs, 1H), 10.02 (s, 1H), 7.62 (m, 2H), 7.31 (m,
3H), 7.07 (d, J=2.3 Hz, 1H), 7.05 (m, 1H), 6.62 (d, J=2.3 Hz, 1H), 6.18 (t, J=6.0 Hz, 1H), 3.90 (d, J=6.0 Hz, 2H).

$^{13}$C-NMR (100 MHz, DMSO-d6): δ 169.1, 165.0, 156.9, 144.9, 138.8, 128.8 (2CH), 123.3, 119.6, 119.2 (2CH), 114.6, 110.4, 98.7, 47.8.

MS ESI (m/z): 282.2 [M-H], HRMS: m/z calculated for C15H13N3O3 [M-H]: 282.08841, found: 282.08862.

*N*-Benzyl-2-((3-oxo-2,3-dihydrobenzo[d]isoxazol-5-yl)amino)acetamide 32b:

Following the procedure for 32a, scaffold B (182 mg, 1 mmol) was reacted with *N*-benzyl-2-bromoacetamide 63b (272 mg, 1.2 mmol) and NaHCO$_3$ (168 mg, 2 mmol) under microwave condition for 10 min. The residue was separated by silica flash column chromatography (DCM/EtOAc; 3:1) to afford 32b as a white solid (113 mg, 38%).

$^1$H-NMR (400 MHz, DMSO-d6): δ 11.98 (bs, 1H), 8.47 (t, J=6.1 Hz, 1H), 7.31 (d, 1H, J=9.0 Hz), 7.28-7.18 (m, 5H), 7.02 (dd, J=9.0 Hz, 2.1 Hz, 1H), 6.62 (d, J=2.1 Hz, 1H), 6.17 (t, J=5.9 Hz, 1H), 4.32 (d, J=6.1 Hz, 2H), 3.75 (d, J=5.8 Hz, 2H).

$^{13}$C-NMR (100 MHz, DMSO-d6): δ 170.7, 165.6, 157.4, 145.3, 140.0, 128.6 (2CH), 127.5 (2CH), 127.1, 120.1, 115.1, 110.7, 99.1, 48.0, 42.4.

MS ESI (m/z): 296.2 [M-H], HRMS: m/z calculated for C16H15N3O3 [M-H]: 296.10406, found: 296.10410.

*N*-(2,3-Dimethylphenyl)-2-((3-oxo-2,3-dihydrobenzo[d]isoxazol-5-yl)amino)-
acetamide 32c:

Following the procedure for 32a, scaffold B (182 mg, 1 mmol) was reacted with
2-bromo-N-(2,3-dimethylphenyl)acetamide 63c (290 mg, 1.2 mmol) and NaHCO₃ (168 mg, 2 mmol) under microwave condition for 15 min. The residue was separated by silica flash column chromatography (DCM/EtOAc; 3:1) to afford 32c as a pale yellow solid (137 mg, 44%).

¹H-NMR (400 MHz, DMSO-d₆): δ 11.99 (bs, 1H), 9.42 (s, 1H), 7.34 (d, J=9.0 Hz, 1H), 7.21 (d, J=7.6 Hz, 1H), 7.08 (dd, J=9.0 Hz, 2.3Hz, 1H), 7.04 (d, J=7.6 Hz, 1H), 6.99 (d, J=7.2 Hz, 1H), 6.68 (d, J=2.3 Hz, 1H), 6.30 (t, J=5.8 Hz, 1H), 3.90 (d, J=5.7 Hz, 2H), 2.22 (s, 3H), 1.97 (s, 3H).

¹³C-NMR (100 MHz, DMSO-d₆): δ 169.1, 165.1, 157.6, 157.4, 156.8, 145.3, 140.8, 130.6, 126.8, 125.2, 123.1, 119.7, 114.6, 110.3, 99.0, 47.7, 20.2, 13.4.

MS ESI (m/z): 310.1 [M-H]⁻, HRMS: m/z calculated for C₁₇H₁₇N₃O₃ [M-H]: 310.11971, found: 310.12012.

2-((3-Oxo-2,3-dihydrobenzo[d]isoxazol-5-yl)amino)-N-(3-phenoxyphenyl)acetamide 32d:

Following the procedure for 32a, scaffold B (182 mg, 1 mmol) was reacted with 2-bromo-N-(3-phenoxyphenyl)acetamide 63d (366 mg, 1.2 mmol) and NaHCO₃ (168 mg, 2 mmol) under microwave condition for 12 min. The residue was separated by silica flash column chromatography (DCM/EtOAc; 3:1) to afford 32d as a dark brown solid (139 mg, 37%).

¹H-NMR (400 MHz, DMSO-d₆): δ 11.97 (bs, 1H), 10.13 (s, 1H), 7.43-7.34 (m, 4H), 7.33-7.28 (m, 2H), 7.15 (m, 1H), 7.06-7.01 (m, 3H), 6.72 (ddd, J=7.9 Hz, J=2.4 Hz, J=1.2 Hz, 1H), 6.60 (d, J=2.1 Hz, 1H), 6.15 (t, J=6.1 Hz, 1H), 3.88 (d, J=6.0 Hz, 2H).

¹³C-NMR (100 MHz, DMSO-d₆): δ 169.8, 165.5, 157.6, 157.4, 156.8, 145.3, 140.8, 130.6, 130.5 (2CH), 124.1, 120.1, 119.4 (2CH), 115.1, 114.4, 113.7, 110.8, 109.4, 99.2, 48.2.

Following the procedure for 32a, scaffold B (182 mg, 1 mmol) was reacted with 2-bromo-\(N\)-(4-fluorophenyl)acetamide 63e (280 mg, 1.2 mmol) and NaHCO\(_3\) (168 mg, 2 mmol) under microwave condition for 10 min. The residue was separated by silica flash column chromatography (DCM/EtOAc; 3:1) to afford 32e as a brown solid (132 mg, 44%).

**\(^1H\)-NMR (400 MHz, DMSO-d6):** \(\delta\) 11.97 (bs, 1H), 10.09 (s, 1H), 7.64 (m, 2H), 7.32 (d, J=9.0 Hz, 1H), 7.16 (m, 2H), 7.06 (dd, J=9.0 Hz, 2.1Hz, 1H), 6.62 (d, J=2.1 Hz, 1H), 6.19 (t, J=6.0 Hz, 1H), 3.89 (d, J=6.0 Hz, 2H).

**\(^{13}C\)-NMR (100 MHz, DMSO-d6):** \(\delta\) 169.1, 165.1, 159.3, 157.0, 156.8, 144.9, 135.3, 121.1, 121.0, 119.6, 115.4, 115.2, 114.6, 110.4, 98.7, 47.8.

**MS ESI (m/z):** 300.2 [M-H], **HRMS:** m/z calculated for C15H12FN3O3 [M-H]: 300.07899, found: 300.07925.

Following the procedure for 32a, scaffold B (182 mg, 1 mmol) was reacted with \(N\)-([1,1'-biphenyl]-2-yl)-2-bromoacetamide 63f (347 mg, 1.2 mmol) and NaHCO\(_3\) (168 mg, 2 mmol) under microwave condition for 10 min. The residue was separated by silica flash column chromatography (DCM/EtOAc; 3:1) to afford 32f as a white solid (183 mg, 51%).

**\(^1H\)-NMR (400 MHz, DMSO-d6):** \(\delta\) 11.97 (bs, 1H), 9.18 (s, 1H), 8.11 (d, J=8.1 Hz, 1H), 7.38 (m, 1H), 7.33 (d, J=9.0 Hz, 1H), 7.24 (m, 3H), 7.16 (m, 4H), 6.88 (dd, J=9.0 Hz, 2.3 Hz,
1H), 6.54 (d, J=2.3 Hz, 1H), 6.29 (t, J=5.5 Hz, 1H), 3.72 (d, J=5.5 Hz, 2H).

$^{13}$C-NMR (100 MHz, DMSO-d6): δ 169.8, 165.6, 157.8, 144.8, 134.9, 133.7, 130.7, 129.1 (2CH), 129.0 (2CH), 128.5, 127.9, 125.2, 122.8, 120.1, 115.1, 110.0, 99.9, 49.0.

MS ESI (m/z): 358.0 [M-H]$, HRMS$: m/z calculated for C21H17N3O3 [M-H]: 358.11971, found: 358.12006.

$N$-(4-Chlorophenyl)-2-((3-oxo-2,3-dihydrobenzo[d]isoxazol-5-yl)amino)acetamide 32g:

Following the procedure for 32a, scaffold B (182 mg, 1 mmol) was reacted with 2-bromo-$N$-(4-chlorophenyl)acetamide 63g (196 mg, 1.2 mmol) and NaHCO$_3$ (168 mg, 2 mmol) under microwave condition for 10 min. The residue was separated by silica flash column chromatography (DCM/EtOAc; 3:1) to afford 32g as a yellow solid (158 mg, 50%).

$^1$H-NMR (400 MHz, DMSO-d6): δ 11.97 (bs, 1H), 10.17 (s, 1H), 7.67 (m, 1H), 7.65 (m, 1H), 7.38 (m, 1H), 7.35 (m, 1H), 7.32 (d, J=9.0 Hz, 1H), 7.05 (dd, J=9.0 Hz, 2.4Hz, 1H), 6.62 (d, 1H, J=2.4 Hz), 6.19 (t, J=6.0 Hz, 1H), 3.91 (d, J=6.0 Hz, 2H).

$^{13}$C-NMR (100 MHz, DMSO-d6): δ 169.9, 165.7, 157.4, 145.7, 138.2, 129.1 (2CH), 127.4, 121.5 (2CH), 120.2, 115.1, 110.8, 99.1, 48.3.

MS ESI (m/z): 316.1 [M-H], HRMS$: m/z calculated for C15H12ClN3O3 [M-H]: 316.04944, found: 316.04950.

2-((3-Oxo-2,3-dihydrobenzo[d]isoxazol-5-yl)amino)-$N$-(p-tolyl)acetamide 32h:

Following the procedure for 32a, scaffold B (182 mg, 1 mmol) was reacted with 2-bromo-$N$-phenylacetamide 63h (255 mg, 1.2 mmol) and NaHCO$_3$ (168 mg, 2 mmol) under microwave condition for 10 min. The residue was separated by silica flash column
chromatography (DCM/EtOAc; 3:1) to afford 32h as a yellow solid (122 mg, 41%).

$^1$H-NMR (400 MHz, DMSO-d$_6$): δ 12.01 (bs, 1H), 9.93 (s, 1H), 7.50 (d, J=8.4 Hz, 2H), 7.31 (d, J=9.0 Hz, 1H), 7.11 (d, J=8.4 Hz, 2H), 7.06 (dd, J=9.0 Hz, 2.3 Hz, 1H), 6.63 (d, J=2.3 Hz, 1H), 6.16 (t, J=6.0 Hz, 1H), 3.88 (d, J=6.0 Hz, 2H), 2.24 (s, 3H).

$^{13}$C-NMR (100 MHz, DMSO-d$_6$): δ168.8, 165.2, 157.1, 144.8, 136.3, 132.3, 129.3 (2CH), 119.6, 119.3 (2CH), 114.7, 110.3, 98.5, 47.5, 20.3.

MS ESI (m/z): 296.2 [M-H], HRMS: m/z calculated for C$_{16}$H$_{15}$N$_3$O$_3$ [M-H]: 296.10406, found: 296.10406.

N-(Benzo[d][1,3]dioxol-5-yl)-2-((3-oxo-2,3-dihydrobenzo[d]isoxazol-5-yl)amino)acetamide 32i:

Following the procedure for 32a, scaffold B (182 mg, 1 mmol) was reacted with N-(benzo[d][1,3]dioxol-5-yl)-2-bromoacetamide 63i (337 mg, 1.2 mmol) and NaHCO$_3$ (168 mg, 2 mmol) under microwave condition for 12 min. The residue was separated by silica flash column chromatography (DCM/EtOAc; 3:1) to afford 32i as a brown solid (163 mg, 50%).

$^1$H-NMR (400 MHz, DMSO-d$_6$): δ 11.97 (bs, 1H), 9.93 (s, 1H), 7.32 (m, 2H), 7.05 (dd, J=9.0 Hz, 2.4 Hz, 1H), 7.00 (dd, J=8.4 Hz, 2.1 Hz, 1H), 6.85 (d, J=8.4 Hz, 1H), 6.61 (d, J=2.1 Hz, 1H), 6.16 (t, J=6.0 Hz, 1H), 5.98 (s, 2H), 3.86 (d, J=5.9 Hz, 1H).

$^{13}$C-NMR (100 MHz, DMSO-d$_6$): δ 169.2, 165.5, 157.5, 147.5, 145.5, 143.5, 133.7, 120.0, 115.1, 112.6, 110.8, 108.4, 101.8, 101.4, 99.3, 48.1.

MS ESI (m/z): 326.1 [M-H], HRMS: m/z calculated for C$_{16}$H$_{13}$N$_3$O$_5$ [M-H]: 326.07824, found: 326.07870.

N-(2,3-Dihydrobenzo[b][1,4]dioxin-6-yl)-2-((3-oxo-2,3-dihydrobenzo[d]isoxazol-5-yl)amino)acetamide 32j:
Following the procedure for 32a, scaffold B (182 mg, 1 mmol) was reacted with 2-bromo-N-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)acetamide 63j (325 mg, 1.2 mmol) and NaHCO$_3$ (168 mg, 2 mmol) under microwave condition for 15 min. The residue was separated by silica flash column chromatography (DCM/EtOAc; 3:1) to afford 32j as a brown solid (194 mg, 57%).

$^1$H-NMR (400 MHz, DMSO-d$_6$): $\delta$ 12.00 (bs, 1H), 9.85 (s, 1H), 7.31 (d, J=9.0 Hz, 1H), 7.26 (d, J=2.4 Hz, 1H), 7.05 (dd, J=9.0 Hz, 2.1 Hz, 1H), 7.00 (dd, J=8.7 Hz, 2.4 Hz, 1H), 6.78 (d, J=8.7 Hz, 1H), 6.60 (d, J=2.1 Hz, 1H), 6.15 (t, J=6.0 Hz, 1H), 4.20 (m, 4H), 3.85 (d, J=5.9 Hz, 2H).

$^{13}$C-NMR (100 MHz, DMSO-d$_6$): $\delta$ 168.7, 165.1, 156.9, 144.9, 143.0, 139.5, 132.4, 119.6, 116.8, 114.6, 112.7, 110.4, 108.4, 98.7, 64.2, 63.9, 47.8.

MS ESI (m/z): 340.1 [M-H], HRMS: m/z calculated for C$_{17}$H$_{15}$N$_3$O$_5$ [M-H]$^-$: 340.09389, found: 340.09390.

$N$-(3-Methoxyphenyl)-2-((3-oxo-2,3-dihydrobenzo[d]isoxazol-5-yl)amino)acetamide 32k:

Following the procedure for 32a, scaffold B (182 mg, 1 mmol) was reacted with 2-bromo-$N$-(3-methoxyphenyl)acetamide 63k (294 mg, 1.2 mmol) and NaHCO$_3$ (168 mg, 2 mmol) under microwave condition for 15 min. The residue was separated by silica flash column chromatography (DCM/EtOAc; 3:1) to afford 32k as a yellow solid (128 mg, 41%).

$^1$H-NMR (400 MHz, DMSO-d$_6$): $\delta$ 11.99 (bs, 1H), 10.01 (s, 1H), 7.33 (m, 2H), 7.21 (t, J=7.9 Hz, 1H), 7.17 (td, J=8.1 Hz, 1.4 Hz, 1H), 7.06 (dd, J=9.0 Hz, 2.3 Hz, 1H), 6.64 (m, 2H), 6.17 (t, J=6.0 Hz, 1H), 3.90 (d, J=6.0 Hz, 2H), 3.72 (s, 3H).

$^{13}$C-NMR (100 MHz, DMSO-d$_6$): $\delta$ 169.7, 165.5, 160.1, 157.4, 145.4, 140.5, 130.1, 120.2,
2-((3-Oxo-2,3-dihydrobenzo[d]isoxazol-5-yl)amino)-N-(3,4,5-trimethoxyphenyl)-acetamide 32l:

Following the procedure for 32a, scaffold B (182 mg, 1 mmol) was reacted with 2-bromo-N-(3,4,5-trimethoxyphenyl)acetamide 63l (363 mg, 1.2 mmol) and NaHCO₃ (168 mg, 2 mmol) under microwave condition for 15 min. The residue was separated by silica flash column chromatography (DCM/EtOAc; 3:1) to afford 32l as a brown solid (198 mg, 53%).

1H-NMR (400 MHz, DMSO-d6): δ 12.05 (bs, 1H), 9.99 (s, 1H), 7.38 (d, J=9.0 Hz, 1H), 7.10 (m, 3H), 6.67 (d, J=2.1 Hz, 1H), 6.24 (t, J=6.0 Hz, 1H), 3.92 (d, J=6.0 Hz, 2H), 3.79 (s, 6H), 3.66 (s, 3H).

13C-NMR (100 MHz, DMSO-d6): δ 169.2, 165.0, 156.9, 152.7 (2C), 145.0, 135.0, 133.4, 119.6, 114.6, 110.4, 98.8, 96.9 (2CH), 60.1, 55.7 (2CH3), 47.9.

MS ESI (m/z): 372.2 [M-H], HRMS: m/z calculated for C18H19N3O6 [M-H]: 372.12011, found: 372.12021.

N-(3-Methoxyphenyl)-2-((3-oxo-2,3-dihydrobenzo[d]isoxazol-5-yl)amino)acetamide 32m:

Following the procedure for 32a, scaffold B (182 mg, 1 mmol) was reacted with 2-bromo-N-(3-methoxyphenyl)acetamide 63m (294 mg, 1.2 mmol) and NaHCO₃ (168 mg, 2 mmol) under microwave condition for 15 min. The residue was separated by silica flash column chromatography (DCM/EtOAc; 3:1) to afford 32m as a brown solid (198 mg, 53%).

1H-NMR (400 MHz, DMSO-d6): δ 12.05 (bs, 1H), 9.99 (s, 1H), 7.38 (d, J=9.0 Hz, 1H), 7.10 (m, 3H), 6.67 (d, J=2.1 Hz, 1H), 6.24 (t, J=6.0 Hz, 1H), 3.92 (d, J=6.0 Hz, 2H), 3.79 (s, 6H), 3.66 (s, 3H).

13C-NMR (100 MHz, DMSO-d6): δ 169.2, 165.0, 156.9, 152.7 (2C), 145.0, 135.0, 133.4, 119.6, 114.6, 110.4, 98.8, 96.9 (2CH), 60.1, 55.7 (2CH3), 47.9.

MS ESI (m/z): 372.2 [M-H], HRMS: m/z calculated for C18H19N3O6 [M-H]: 372.12011, found: 372.12021.
mmol) under microwave condition for 15 min. The residue was separated by silica flash column chromatography (DCM/EtOAc; 3:1) to afford 32m as a yellow solid (128 mg, 41%).

$^1$H-NMR (400 MHz, DMSO-d$_6$): $\delta$ 11.96 (s, 1H), 9.86 (s, 1H), 7.52 (m, 2H), 7.34 (d, J=9.0 Hz, 1H), 7.06 (dd, J=2.3 Hz, 9.0Hz, 1H), 6.88 (m, 2H), 6.63 (d, J=2.1 Hz, 1H), 6.15 (t, J=5.9 Hz, 1H), 3.86 (d, J=5.8 Hz, 2H), 3.72 (s, 3H).

$^{13}$C-NMR (100 MHz, DMSO-d$_6$): $\delta$ 168.8, 165.0, 157.0, 155.4, 144.9, 131.9, 120.8, 119.6, 114.6, 113.8, 110.4, 98.9, 54.9, 47.4.

MS ESI (m/z): 312.2 [M-H]$, HRMS$: m/z calculated for C$_{16}$H$_{15}$N$_3$O$_4$ [M-H]: 312.09898, found: 312.09937.

$N$-(Naphthalen-1-yl)-2-((3-oxo-2,3-dihydrobenzo[d]isoxazol-5-yl)amino)acetamide 32n:

Following the procedure for 32a, scaffold B (182 mg, 1 mmol) was reacted with 2-bromo-$N$-(naphthalen-1-yl)acetamide 63n (315 mg, 1.2 mmol) and NaHCO$_3$ (168 mg, 2 mmol) under microwave condition for 10 min. The residue was separated by silica flash column chromatography (DCM/EtOAc; 3:1) to afford 32n as a brown solid (137 mg, 41%).

$^1$H-NMR (400 MHz, DMSO-d$_6$): $\delta$ 12.04 (bs, 1H), 10.05 (s, 1H), 7.94 (m, 2H), 7.78 (d, J=8.2 Hz, 1H), 7.69 (d, J=6.9 Hz, 1H), 7.51 (m, 3H), 7.36 (d, J=9.0 Hz, 1H), 7.13 (dd, J=9.0 Hz, 2.3 Hz, 1H), 6.79 (d, J=2.3 Hz, 1H), 6.34 (t, J=5.9 Hz, 1H), 4.08 (d, J=6.0 Hz, 2H).

$^{13}$C-NMR (100 MHz, DMSO-d$_6$): $\delta$ 169.9, 165.2, 157.1, 144.9, 133.6, 133.2, 128.1, 127.9, 126.0, 125.8, 125.6, 125.4, 122.5, 121.7, 119.8, 114.7, 110.5, 98.9, 47.9.

MS ESI (m/z): 332.1 [M-H]$, HRMS$: m/z calculated for C$_{19}$H$_{15}$N$_3$O$_3$ [M-H]: 332.09896, found: 332.10006.

2-(5-Amino-3-oxobenzo[d]isoxazol-2(3H)-yl)-$N$-phenylacetamide 64a:
To a solution of scaffold B (182 mg, 1 mmol) and 2-bromo-N-phenylacetamide 63a (255 mg, 1.2 mmol) in DMF (1.5 mL) was added K$_2$CO$_3$ (276 mg, 2 mmol). The reaction mixture was heated under microwave condition at 60°C for 10 min. On completion of the reaction, as followed by TLC, the solvent was removed under reduced pressure. The residue was taken up in water (20 mL) and extracted with EtOAc (3 times 30 mL). The combined organic phases were dried over Na$_2$SO$_4$ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc; 3:1) to afford 64a as a brown solid (136 mg, 48%).

$^1$H-NMR (400 MHz, CDCl$_3$): δ 8.06 (s, 1H), 7.51 (d, J=7.7 Hz, 2H), 7.28 (t, J=8.0 Hz, 2H), 7.19 (d, J=1.3 Hz, 1H), 7.09 (t, J=8.9 Hz, 1H), 6.89 (dd, J=8.9 Hz, 2.3Hz, 1H), 6.80 (d, J=2.3 Hz, 1H), 4.96 (s, 2H), 3.71 (bs, 2H).

MS ESI (m/z): 284.0 [M+H]$^+$. 

2-(5-Amino-3-oxobenzo[d]isoxazol-2(3H)-yl)-N-benzylacetamide 64b:

Following the procedure for 64a, scaffold B (182 mg, 1 mmol) was reacted with 2-bromo-N-(3,4,5-trimethoxyphenyl)acetamide 63b (363 mg, 1.2 mmol) and K$_2$CO$_3$ (276 mg, 2 mmol) under microwave condition for 10 min. The residue was separated by silica flash column chromatography (DCM/EtOAc; 3:1) to afford 64b as a white solid (157.4 mg, 53%).

$^1$H-NMR (400 MHz, DMSO-d$_6$): δ 8.78 (t, J=6.2 Hz, 1H), 7.19-7.37 (m, 6H), 6.96 (d, J=8.4 Hz, 1H), 6.79 (s, 1H), 5.24 (bs, 2H), 4.89 (s, 2H), 4.37 (d, J=5.8 Hz, 2H).

MS ESI (m/z): 298.1 [M+H]$^+$. 

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2-(5-Amino-3-oxobenzo[d]isoxazol-2(3H)-yl)-N-(2,3-dimethylphenyl)acetamide 64c:

Stannic chloride (1.75 mL, 1.0 M in DCM) and concentrated HCl (0.9 mL) were mixed and cooled to 0°C. Nitro compound 65 (340 mg, 1 mmol) was added to this mixture, followed by dropwise addition over 30 min of a solution of stannous chloride (682 mg, 3.6 mmol) in concentrated HCl (0.5 mL). After stirring for 4h, the mixture was filtered and the white solid was collected. The solid was dissolved in EtOAc and extracted with saturated sodium bicarbonate. The organic phase was separated, dried over Na₂SO₄, and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 8:2) to afforded 64c as a white solid (168 mg, 54%).

¹H-NMR (400 MHz, DMSO-d₆): δ 9.74 (s, 1H) ppm 7.32 (d, J=8.9 Hz, 1H), 7.15 (m, 1H) 7.07 (m, 2H), 6.98 (dd, 1H, J=8.9 Hz, 2.2Hz), 6.82 (d, J=2.2 Hz, 1H), 5.33 (bs, 2H), 5.05 (s, 2H), 2.27 (s, 3H), 2.09 (s, 3H).

MS ESI (m/z): 312.3 [M+H]⁺.

2-(5-Amino-3-oxobenzo[d]isoxazol-2(3H)-yl)-N-(4-phenoxyphenyl)acetamide 64d:

Following the procedure for 64a, scaffold B (182 mg, 1 mmol) was reacted with 2-bromo-N-(3,4,5-trimethoxyphenyl)acetamide 63d (363 mg, 1.2 mmol and K₂CO₃ (276 mg, 2 mmol) under microwave condition for 10 min. The residue was separated by silica flash column chromatography (DCM/EtOAc, 8:2) to afford 64d as a white solid (191.7 mg, 51%).
**1H-NMR (400 MHz, CDCl3):** δ 8.09 (s, 1H), 7.29-7.22 (m, 4H), 7.19 (t, J=8.8 Hz, 2H), 7.03 (m, 1H), 6.94 (dd, J=8.6 Hz, 1.0 Hz, 2H), 6.87 (dd, J=8.9 Hz, 2.3 Hz, 1H), 6.77 (d, J=1.8 Hz, 1H), 6.72 (ddd, J=7.8 Hz, J=2.3 Hz, J=1.3 Hz, 1H), 4.90 (s, 2H), 3.70 (bs, 2H).

**MS ESI (m/z):** 376.3 [M+H]+.

2-((5-Amino-3-oxobenzo[d]isoxazol-2(3H)-yl)-N-(4-fluorophenyl)acetamide 64e:

![Chemical Structure]

Following the procedure for 64a, scaffold B (182 mg, 1 mmol) was reacted with 2-bromo-N-(3,4,5-trimethoxyphenyl)acetamide 63e (363 mg, 1.2 mmol) and K₂CO₃ (276 mg, 2 mmol) under microwave condition for 10 min. The residue was separated by silica flash column chromatography (DCM/EtOAc, 8:2) to afford 64e as a white solid (171.6 mg, 57%).

**1H-NMR (400 MHz, DMSO-d6):** δ 10.32 (s, 1H), 7.63 (m, 2H), 7.30 (d, J=8.9 Hz, 1H), 7.18 (m, 2H), 6.96 (dd, J=2.3 Hz, J=8.9 Hz, 1H), 6.78 (d, 1H, J=2.3 Hz), 5.23 (s, 2H), 5.01 (s, 2H).

**MS ESI (m/z):** 302.0 [M+H]+.

N-(2,3-Dimethylphenyl)-2-((5-nitro-3-oxobenzo[d]isoxazol-2(3H)-yl)acetamide 65:

![Chemical Structure]

To a solution of 5-nitro-benzo[d]isoxazol-3-ol 59 (181 mg, 1 mmol) and 2-bromo-N-(2,3-dimethylphenyl)acetamide 63c (292 mg, 1.2 mmol) in DMF (0.5 mL) was added K₂CO₃ (268 mg, 2 mmol). The reaction mixture was heated under microwave condition at 60°C for 20 min. On completion of the reaction, as followed by TLC, the solvent was
removed under reduced pressure. The residue was taken up in water (20 mL) and acidified with 12N HCl to pH 4 and extracted with EtOAc (3 times 30 mL). The combined organic phases were dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc; 3:1) to afford 65 as a white solid (85 mg, 25%).

\(^1\)H-NMR (400 MHz, DMSO-d6): \(\delta 9.88 \text{ (s, 1H)}, 8.88 \text{ (d, J=2.1 Hz, 1H)}, 8.55 \text{ (dd, J=9.2 Hz, 2.1 Hz, 1H)}, 7.95 \text{ (d, J=9.2 Hz, 1H)}, 7.12 \text{ (m, 3H)}, 5.16 \text{ (s, 2H)}, 2.28 \text{ (s, 3H)}, 2.10 \text{ (s, 3H)}.\)

**MS ESI (m/z):** 340.4 [M-H].

2-Chloro-N-hydroxy-6-nitrobenzamide 69:

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

Nitrobenzoic acid 67 (403 mg, 2 mmol) and thionyl chloride (5 mL) were mixed together and heated at 70°C for 6 hours. The excess thionyl chloride was then evaporated and the solid residue was dissolved in anhydrous DCM (5 mL) at room temperature. Hydroxylamine hydrochloride (280 mg, 4 mmol) and DIEA (1.4 mL, 8 mmol) were added to this solution and the mixture was stirred at room temperature overnight under a nitrogen atmosphere. The solvent was then removed under reduced pressure, and the residue was acidified with 1N HCl (45 mL) and was extracted with EtOAc (3 times 25 mL). The combined organic extracts were washed with saturated brine, dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc; 4:1) to afford 69 as a yellow solid (355.1 mg, 82%).

\(^1\)H-NMR (400 MHz, CD₃OD): \(\delta 8.20 \text{ (d, J=8.2 Hz, 1H)}, 7.91 \text{ (d, J=7.8 Hz, 1H)}, 7.71 \text{ (t, J=8.2 Hz, 1H)}.\)

\(^{13}\)C-NMR (100 MHz, CD₃OD): \(\delta 163.4, 148.8, 136.5, 135.4, 132.7, 130.5, 124.4.\)

**MS ESI (m/z):** 214.6 [M-H], **HRMS:** m/z calculated for C7H5ClN2O4 [M-H]: 214.98651, found: 214.97446.
2-Chloro-6-nitroaniline 70:

To a solution of hydroxamic acid 69 (90 mg, 0.5 mmol) in DMF (2 mL), K$_2$CO$_3$ (140 mg, 1 mmol) or DBU (0.7 mL, 0.5 mol) was added and the mixture was stirred at 120°C for 2 hours. The solvent was then removed under reduced pressure at 50°C, and the residue was taken-up in water (30 mL) and extracted with EtOAc (3 times 20 mL). The combined organic layers were washed with brine, dried with Na$_2$SO$_4$ and concentrated. The residue was flash column chromatographed on silica gel (DCM/EtOAc, 9:1) to give 70 as a yellow powder (70 mg, 81%).

$^1$H-NMR (400 MHz, CD$_3$OD): $\delta$ 8.07 (d, J=8.7 Hz, 1H), 7.49 (d, J=7.7 Hz, 1H), 6.57 (t, J=8.1 Hz, 1H).

$^{13}$C-NMR (100 MHz, CD$_3$OD): $\delta$ 143.3, 136.5, 134.3, 126.3, 122.9, 116.5.

MS ESI (m/z): 170.9 [M-H]$^-$, HRMS: m/z calculated for C$_6$H$_5$ClN$_2$O$_2$ [M-H]$^-$: 170.99668, found: 170.99535.

Diphenylmethanone oxime 79:

Pyridine (5 mL, 62 mmol) was added to a solution of benzophenone 82 (1 g, 5.5 mmol) and hydroxylamine hydrochloride (1 g, 14.2 mmol) in MeOH (5 mL) and the reaction was stirred at 60°C for 2 hours. The mixture was then cooled to room temperature, acidified with 1N HCl to pH<3, and extracted with DCM (3 times 60 mL). The combined organic layers were dried over Na$_2$SO$_4$ and concentrated. Oxime 79 was obtained in pure form as a white solid (900 mg, 83%).

$^1$H-NMR (400 MHz, CDCl$_3$): $\delta$ 8.98 (s, 1H), 7.53-7.32 (m, 10H).

MS ESI (m/z): 198.1 [M+H]$^+$.
2-Chloro-6-(((diphenylmethylene)amino)oxy)benzonitrile **85**: 

Oxime **79** (40 mg, 2 mmol) and potassium *tert*-butoxide (246 mg, 2.2 mmol) were dissolved in THF (25 mL) and the mixture was stirred for 30 minutes, prior to addition of 6-chloro-2-nirobenzonitrile **84** (362 mg, 2 mmol). The reaction mixture was stirred at 55°C for 24 hours, and then quenched by addition of 1N HCl (60 mL). The aqueous layer was extracted with EtOAc (3 times 45 mL), and the combined organic extracts were washed with saturated brine, dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 3:1), affording **85** as a white solid (589 mg, 89%).

**¹H-NMR (400 MHz, CDCl₃)**: δ 7.72-7.63 (m, 3H), 7.59-7.40 (m, 9H), 7.14 (d, J=8.4 Hz, 1H).

**MS ESI (m/z):** 333.2 [M+H]⁺.

Methyl (3-((2-methoxyethoxy)methoxy)phenyl)carbamate **94**: 

According to Gould procedure¹²², methyl chloroformate (3.89 mL, 50.5 mmol) and 8% of aqueous sodium bicarbonate (150 mL) were added to a solution of aminophenol **87** (5.0 g, 45.9 mmol) in EtOAc (75 mL). The resultant mixture was stirred at room temperature overnight. The two phases were then separated, and the organic phase was washed with 1N
HCl (100 mL), water (100 mL) and brine. It was dried over Na₂SO₄, and concentrated. The residue (a brown oil) was crystallized from DCM/Hexane (1:1) to give methyl 3-hydroxyphenylcarbamate as white crystals (5.9 g, 77.0%). The obtained carbamate (247.4 mg, 1.48 mmol) was dissolved in THF (8 mL) containing NaH (137 mg, 5.7 mmol) and the mixture was stirred at 0°C under nitrogen for 1 hour. MEMCl (221.2 mg, 1.78 mmol) was then added and stirring was continued for a further 12 hours at room temperature. EtOAc (25 mL) was added to the mixture and it was washed with 5% NaOH (20 mL), water (20 mL) and brine. The organic layer was dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 9:1), affording 94 as a white solid (284 mg, 75.8%).

**1H-NMR (400 MHz, (CD₃)₂CO):** δ 8.66 (s, 1H) 7.36 (d, J=1.9 Hz, 1H) 7.22 (m, 1H) 7.18 (m, 1H) 6.74 (td, J=2.3 Hz, J=7.3 Hz, 1H) 5.27 (s, 2H) 3.80 (m, 2H) 3.71 (s, 3H) 3.54 (m, 2H) 3.30 (s, 3H).

**MS ESI (m/z):** 278.4 [M+Na].

**N-(3-((2-Methoxyethoxy)methoxy)phenyl)pentanamide 95:**

Carbamate 94 (406 mg, 1.6 mmol) was dissolved in dry THF (20 mL) and cooled to 0°C under N₂. n-BuLi (1.3 mL, 2.5 M in hexane) was added dropwise and the mixture was stirred for 2 hours. Dry CO₂ was then bubbled continuously through the mixture for 2 hours. The reaction was stopped by addition of 5% NaOH (100 mL) and EtOAc (100 mL). The aqueous layer was acidified to pH1 with 1N HCl, separated and further extracted with EtOAc (3 times 30 mL). The combined organic layers were washed with water and saturated brine, then dried over Na₂SO₄ and concentrated. The residue (a red-brown oil) was silica gel column chromatographed (DCM/EtOAc, 9:1), affording 95 as yellow crystals (318 mg, 70.6%).

**1H-NMR (400 MHz, DMSO-d6):** δ 9.86 (s, 1H), 7.35 (t, J=1.7 Hz, 1H), 7.20 (m, 2H), 6.70
(m, 1H), 5.22 (s, 2H), 3.72 (m, 2H), 3.47 (m, 2H), 3.23 (s, 3H), 2.30 (t, J=7.4 Hz, 2H), 1.57 (m, 2H), 1.32 (m, 2H), 0.90 (t, J=7.3 Hz, 3H).

**MS ESI (m/z):** 280.1 [M-H]⁻.

**N-(3-Hydroxyphenyl)pivalamide 96:**

![N-(3-Hydroxyphenyl)pivalamide](image)

To a solution of 3-aminophenol 87 (3.982 g, 36.53 mmol) in EtOAc (125 mL) was added a solution of NaHCO₃ (9.2 g, 109.5 mmol) in water (150 mL) at room temperature. Trimethylacetyl chloride (4.72 mL, 38.35 mmol) was then added, and the two-phased solution was stirred for 2 h at room temperature. The two phases were then separated and the organic layer was washed with 1N HCl (125 mL), H₂O (125 mL), and saturated brine (125 mL). The organic phase was dried over Na₂SO₄, and concentrated. The solid residue was recrystallized from DCM/hexane (1:9) to give 96 as a white solid (6.32 g, 90%).

**¹H-NMR (400 MHz, DMSO-d6):** δ 9.29 (s, 1H), 9.03 (s, 1H), 7.21 (t, J=1.9 Hz, 1H), 7.05 (m, 1H), 7.02 (m, 2H), 6.44 (m, 1H), 1.21 (s, 9H).

**MS ESI (m/z):** 191.6 [M-H]⁻.

**N-(3-((2-Methoxyethoxy)methoxy)phenyl)pivalamide 97:**

![N-(3-((2-Methoxyethoxy)methoxy)phenyl)pivalamide](image)

To a suspension of NaH (698 mg, 17.46 mmol) in dry THF (50 mL) under N₂ at 0°C was added phenol 96 (3.07 g, 15.89 mmol). The mixture was stirred at 0°C for 3h, and the phenoxide anion formed was treated with methoxyethoxymethyl chloride (2.2 g, 17.8 mmol). The mixture was allowed to warm to room temperature overnight, then diluted with Et₂O (125 mL) and extracted with 5% NaOH (125 mL). The organic layer was washed with water.
(125 mL) and saturated brine (125 mL), then dried over Na$_2$SO$_4$ and concentrated. The residue (a yellow oil) was silica gel column chromatographed (DCM/EtOAc, 9:1), affording 97 as a white solid (3.7 g, 82%).

$^1$H-NMR (400 MHz, DMSO-d$_6$): $\delta$ 9.17 (s, 1H), 7.42 (t, 1H, J=2.2 Hz), 7.31 (m, 1H), 7.19 (t, 1H, J=8.1 Hz), 6.70 (dd, 1H, J=2.0 Hz, J=7.7 Hz), 5.22 (s, 2H), 3.72 (m, 2H), 3.47 (m, 2H), 3.23 (s, 3H), 1.22 (s, 9H).

MS ESI (m/z): 280.1 [M-H]$^-$. 

2-Hydroxy-6-pivalamidobenzoic acid 98:

\[
\begin{align*}
\text{OH} & \quad \text{COOH} \\
& \quad \text{NHCOC(CH$_3$)$_3$}
\end{align*}
\]

According to reference 123, n-BuLi (2.2 mL, 5.6 mmol) was added dropwise over 30 min to a cooled (0°C) solution of pivalamide 97 (488 mg, 1.7 mmol) in dry THF (20 mL), and the mixture was stirred under N$_2$ for 2 hours. Dry CO$_2$ was then bubbled continuously through the reaction for an additional 2 hours. The resultant mixture was diluted with 5% NaOH (100 mL) and EtOAc (100 mL). The layers were separated and the aqueous layer was acidified to pH1 with 1N HCl, and extracted with EtOAc (2 times 100 mL). The combined organic extracts were washed with water (100 mL) and saturated brine (100 mL), then dried over Na$_2$SO$_4$ and concentrated. The residue (a red-brown oil) was silica gel and column chromatographed (DCM/MeOH, 9:1), affording 98 as yellow crystals (334.1 mg, 82%).

$^1$H-NMR (400 MHz, DMSO-d$_6$): $\delta$ 13.67 (s, 1H), 7.92 (dd, J=8.2 Hz, 1.1 Hz, 1H), 7.07 (t, J=8.2 Hz, 1H), 6.36 (dd, J=8.2 Hz, 1.1 Hz, 1H), 1.21 (s, 9H).

MS ESI (m/z): 236.3 [M-H]$^-$. 

N-(3-Oxo-2,3-dihydrobenzo[d]isoxazol-4-yl)pivalamide 99:

\[
\begin{align*}
& \quad \text{O} \quad \text{NH} \\
& \quad \text{NHCOC(CH$_3$)$_3$}
\end{align*}
\]
Adapting the literature procedure\textsuperscript{195}, CDI (150 mg, 1.5 mmol) was added to a solution of carboxylic acid 98 (237.2 mg, 1 mmol) in dry THF (1 mL). The reaction mixture was stirred for 1 hour under N\textsubscript{2}-atmosphere and hydroxylamine hydrochloride (80 mg, 2 mmol) was then added. The resulting mixture was stirred overnight at room temperature. The mixture was diluted with 5% aq. KHSO\textsubscript{4} (20 mL) and extracted with EtOAc (3 times 20 mL). The combined organic phase was washed with brine and dried over Na\textsubscript{2}SO\textsubscript{4}. The ethyl acetate layer was then concentrated \textit{in vacuo} and the residue was loaded onto silica gel and column chromatography (DCM/MeOH, 9:1) to afford 99 as a white product (201 mg, 86\%).

\textbf{1H-NMR (400 MHz, DMSO-d6):} $\delta$ 11.33 (s, 1H), 7.94 (dd, $J$=8.3 Hz, 1.1 Hz, 1H), 7.32 (t, $J$=8.3 Hz, 1H), 6.59 (dd, $J$=8.3 Hz, 1.1 Hz, 1H), 1.22 (s, 9H).

\textbf{MS ESI (m/z):} 234.7 [M+H]\textsuperscript{+}.

3-Aminophenol 87:

\begin{figure}[h]
\centering
\includegraphics[width=0.2\textwidth]{3-aminophenol.png}
\end{figure}

According to reference 122, to a solution of HBr (0.6 mL) and acetic acid (0.6 mL) was added compound 99 (112 mg, 0.5 mmol). The reaction mixture was stirred at 90\textdegree C for 24 h, then cooled to 0\textdegree C, and the precipitate formed was collected via vacuum filtration. The precipitate was recrystallized from MeOH and Et\textsubscript{2}O to afford 87 as colorless crystals (24.9 mg, 46\%).

\textbf{1H-NMR (400 MHz, DMSO-d6):} $\delta$ 8.85 (s, 1H), 6.79 (t, $J$=8.2 Hz, 1H), 6.01 (m, 2H), 5.95 (m, 1H), 4.88 (s, 2H).

\textbf{MS ESI (m/z):} 108.1 [M-H]\textsuperscript{−}.

2-Amino-6-hydroxybenzoic acid 88:

\begin{figure}[h]
\centering
\includegraphics[width=0.2\textwidth]{2-amino-6-hydroxybenzoic_acid.png}
\end{figure}
According to reference 124, to a solution of HCl (1.2 mL) and acetic acid (0.6 mL) was added compound 99 (150 mg, 0.7 mmol). The reaction mixture was stirred at 45°C for 4 days, then cooled to 0°C, and the precipitate formed was collected via vacuum filtration. The precipitate was recrystallized (MeOH /Et₂O, 1:9) to afford 88 as its HCl salt (66.4 mg, 52%).

**1H-NMR (400 MHz, DMSO-d6):** δ 8.20 (bs, 4H), 7.11 (t, J=8.1 Hz, 1H), 6.31 (dd, J=8.1 Hz, 0.9 Hz, 1H), 6.14 (dd, J=8.1 Hz, 0.9 Hz, 1H).

**MS ESI (m/z):** 151.8 [M-H]⁻.

2-Acetamido-6-acetoxybenzoic acid 106:

According to reference 126, triethylamine (0.5 mL) was added to a solution of 6-aminosalicylic acid hydrochloride salt (189 mg, 1 mmol) in acetic anhydride (5 mL), and the mixture was stirred at 50°C for 5 hours. An aqueous 5% solution of KHSO₄ (30 mL) was then added and the aqueous solution was extracted with EtOAc (3 times 25 mL). The combined organic layers were dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 9:1) to afford 106 as a white solid (171 mg, 71%).

**1H-NMR (400 MHz, DMSO-d6):** δ 13.40 (s, 1H), 10.00 (s, 1H), 7.65 (d, J=8.1 Hz, 1H), 7.49 (t, J=8.2 Hz, 1H), 6.95 (d, J=7.5 Hz, 1H), 2.22 (s, 3H), 2.04 (s, 3H).

**MS ESI (m/z):** 239.8 [M-H]⁻.

N-(3-Hydroxyphenyl)acetamide 107:

According to reference 127, triethylamine (0.5 mL) was added to a solution of
6-aminosalicylic acid hydrochloride salt (189 mg, 1 mmol) in acetic acid (1 mL), and the mixture was refluxing at 120°C for 3 hours. An aqueous 5% solution of KHSO₄ (30 mL) was then added and the aqueous phase was extracted with EtOAc (3 times 25 mL). The combined organic layers were dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 9:1) to afford **107** as a white solid (103 mg, 68%).

**¹H-NMR (400 MHz, DMSO-d₆):** δ 9.79 (s, 1H), 9.34 (s, 1H), 7.21 (t, J=2.1 Hz, 1H), 7.07 (t, J=8.1 Hz, 1H), 6.94 (d, J=8.1 Hz 1H), 6.45 (dd, J=8.5 Hz, 2.7 Hz, 1H), 2.01 (s, 3H).

**MS ESI (m/z):** 149.8 [M-H]⁻.

2-Acetamido-6-hydroxybenzoic acid **100**:

![Chemical Structure](image)

According to reference 128, sodium acetate (164 mg, 2 mmol) was added to a solution of 6-aminosalicylic acid hydrochloride salt (98 mg, 0.5 mmol) in acetic acid (1 mL) and acetic anhydride (0.06 mL, 0.06 mmol), and the mixture was stirred at room temperature for 12 hours. An aqueous 5% solution of KHSO₄ (30 mL) was then added and the aqueous phase was extracted with EtOAc (3 times 25 mL). The combined organic layers were dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 9:1) to afford **100** as a white solid (85 mg, 87.4%).

**¹H-NMR (400 MHz, DMSO-d₆):** δ 10.53 (s, 1H), 8.70 (bs, 1H), 7.58 (d, J=8.2 Hz, 1H), 7.31 (t, J=8.2 Hz, 1H), 6.63 (dd, J=8.2 Hz, 1.0Hz, 1H), 2.06 (s, 3H).

**MS ESI (m/z):** 193.7 [M-H]⁻.

Methyl 2-acetamido-6-hydroxybenzoate **109**:
To a solution of acid 100 (195 mg, 1 mmol) in anhydrous acetonitrile (5 mL) was added TFFH (264 mg, 1 mmol) and DIEA (260 mg, 2 mmol) at room temperature, and the mixture was stirred at room temperature under a N₂-atmosphere for 2 hours. The reaction was then quenched by addition of MeOH (30 mL) and it was stirred for another 12 hours. On completion of the reaction (monitored by TLC), the solvent was evaporated in vacuo and the residue was redissolved in EtOAc and washed with water and brine. The organic layer was dried over Na₂SO₄ and concentrated under reduced pressure. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 9:1) to afford 109 as a white solid (140 mg, 67%).

**1H-NMR (400 MHz, DMSO-d₆):** δ 10.01 (s, 1H), 9.68 (s, 1H), 7.31 (t, J=8.1 Hz, 1H), 7.08 (d, J=8.1 Hz, 1H), 6.76 (d, J=8.1 Hz, 1H), 3.82 (s, 3H), 2.05 (s, 3H).

**MS ESI (m/z):** 208.0 [M-H]⁻.

2-Hydroxy-6-(2,2,2-trifluoroacetamido)benzoic acid 101:

Triethylamine (0.07 mL, 0.5 mmol) was added to a cooled (0°C) solution of 6-aminosalicylic acid hydrochloride salt (116.5 mg, 0.5 mmol) and TFFA (168 mg, 0.8 mmol) in THF (4 mL). The reaction mixture was slowly warmed to room temperature and stirred for 30 minutes. H₂O (20 mL) was then added and stirring was continued for 2 days. At the end of this period, the solvent was evaporated and the residue was then redissolved in EtOAc (30 mL), washed with water and brine, and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 9:1) to afford 101 as a white solid (103 mg, 83%).
$^1$H-NMR (400 MHz, DMSO-d$_6$): $\delta$ 13.17 (s, 1H), 8.01 (bs, 2H), 7.50 (dd, $J$=8.2 Hz, 1.0 Hz, 1H), 7.37 (t, $J$=8.2 Hz, 1H), 6.77 (dd, $J$=8.2 Hz, 1.1 Hz, 1H).

MS ESI (m/z): 248.0 [M-H]$^-$.

Methyl 2-hydroxy-6-(2,2,2-trifluoroacetamido)benzoate 110:

To a solution of 6-aminosalicylic acid hydrochloride salt (116.5 mg, 0.5 mmol) and TFFA (168 mg, 0.8 mmol) in THF (4 mL) at 0°C was added sodium bicarbonate (42 mg, 0.5 mmol). The reaction mixture was slowly warmed to room temperature and stirred for 30 minutes, followed by addition of MeOH (20 mL) and continued stirring for 2 days. The solvent was then evaporated and the residue was dissolved in EtOAc, washed with water and brine and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 9:1) to afford 110 as a white solid (91 mg, 69%).

$^1$H-NMR (400 MHz, DMSO-d$_6$): $\delta$ 11.23 (s, 1H), 10.25 (s, 1H), 7.22 (t, $J$=8.1 Hz, 1H), 6.94 (s, 1H), 6.92 (s, 1H), 3.75 (s, 3H).

MS ESI (m/z): 262.6 [M-H]$^-$.

2-((tert-Butoxycarbonyl)amino)-6-hydroxybenzoic acid 102:

To a solution of 6-aminosalicylic acid hydrochloride salt (226 mg, 1.2 mmol) and triethylamine (0.85 mL, 6 mmol) in MeOH (5 mL) at 0°C was added di-tert-butyl dicarbonate (785.7 mg, 3.6 mmol). The reaction mixture was slowly warmed up to room temperature and stirred for 24 hours. The solvent was then evaporated and the residue was taken up in 5% aqueous KHSO$_4$ (30 mL) and extracted with EtOAc (3 times 25 mL). The
combined organic layers were washed with brine and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 9:1) to afford 102 as a white solid (292 mg, 96%).

**1H-NMR (400 MHz, DMSO-d6):** δ 10.07 (s, 1H), 7.61 (d, J=8.3 Hz, 1H), 7.32 (t, J=8.3 Hz, 1H), 6.56 (d, J=8.2 Hz, 1H), 1.46 (s, 9H).

**MS ESI (m/z):** 252.2 [M-H]-.

**2-Methyl-4H-benzo[d][1,3]oxazin-4-one 116:**

![2-Methyl-4H-benzo[d][1,3]oxazin-4-one 116](image)

According to reference 132, anthranilic acid (2 g, 14.6 mmol) was dissolved in acetic anhydride (4 mL) and the mixture was heated to 130°C with stirring for 6 hours. The solvent was then removed *in vacuo* and the product was dried under high vacuum. Compound 116 was obtained as a brown solid (2.1 g, 90%) and was used for next step without any purification.

**1H-NMR (400 MHz, CDCl3):** δ 8.19 (dd, J=7.4 Hz, 1.5 Hz, 1H), 7.80 (ddd, J=1.5 Hz, J=7.4 Hz, J=8.2 Hz, 1H), 7.55 (d, J=8.2 Hz, 1H), 7.51 (ddd, J=1.5 Hz, J=7.4 Hz, J=8.2 Hz, 1H), 2.48 (s, 3H).

**13C-NMR (100 MHz, CDCl3):** δ 160.4, 159.8, 146.5, 136.7, 128.5, 128.3, 126.5, 116.7, 21.7.

**MS ESI (m/z):** 162.2 [M+H]+, **HRMS:** m/z calculated for C9H7NO2 [M+H]+: 162.05496, found: 162.05505.

**2-Acetamidobenzoic acid 118:**

![2-Acetamidobenzoic acid 118](image)
A mixture of compound 116 (137 mg, 1 mmol), hydroxylamine hydrochloride (420 mg, 6 mmol) and NaOH (240 mg, 6 mmol) in water (3 mL) and dioxane (3 mL) was stirred at room temperature under N₂-atmosphere for 12 hours. 1N HCl (60 mL) was then added and the aqueous layer was extracted with EtOAc (3 times 45 mL). The combined organic extracts were washed with saturated brine, dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 3:1) to afford 118 as a white solid (164 mg, 92%).

**1H-NMR (400 MHz, DMSO-d6):** δ 13.59 (s, 1H), 11.07 (s, 1H), 8.46 (d, J=8.4 Hz, 1H), 7.97 (dd, 1H, J=1.6 Hz, J=7.9 Hz), 7.58 (ddd, J=1.6 Hz, J=7.9 Hz, J=8.4 Hz, 1H), 7.14 (ddd, J=1.6 Hz, J=7.9 Hz, J=8.4 Hz, 1H), 2.14 (s, 3H).

**13C-NMR (100 MHz, DMSO-d6):** δ 169.9, 168.9, 141.3, 134.4, 131.5, 123.0, 120.3, 117.0, 25.6.

**MS ESI (m/z):** 177.3 [M-H], **HRMS:** m/z calculated for C9H9NO3 [M-H]: 178.05097, found: 178.04997.

**2-Acetamidobenzamide 117:**

A solution of compound 116 (137 mg, 1 mmol) in ammonia hydroxide (10 mL) was stirred at room temperature for 12 hours. 1N HCl (60 mL) was added and the aqueous layer was extracted with EtOAc (3 times 45 mL). The combined organic extracts were washed with saturated brine, dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 3:1) to afford 117 as a white solid (157 mg, 88%).

**1H-NMR (400 MHz, DMSO-d6):** δ 12.21 (s, 1H), 8.07 (dd, J=7.9 Hz, 1.2 Hz, 1H), 7.77 (ddd, J=1.2 Hz, J=7.9 Hz, J=8.5 Hz, 1H), 7.57 (d, J=7.9 Hz, 1H), 7.45 (ddd, J=1.2 Hz, J=7.9 Hz, J=8.5 Hz, 1H), 2.35 (s, 3H).
**13C-NMR (100 MHz, DMSO-d6):** δ 162.2, 154.8, 149.4, 134.7, 127.0, 126.3, 126.1, 121.5, 21.6.

**MS ESI (m/z):** 177.7 [M-H].

**Triphenylmethanol 119:**

![Triphenylmethanol](insert_image)

To a solution of 6-aminosalicylic acid hydrochloride salt (113 mg, 0.6 mmol) and triethylamine (0.34 mL, 2.4 mmol) in DCM (4 mL) was added trityl chloride (251 mg, 0.9 mmol). The reaction mixture was stirred at room temperature overnight. The solvent was then evaporated and the residue was taken-up in 5% aqueous KHSO₄ (45 mL) and extracted with EtOAc (3 times 45 mL). The combined organic extracts were washed with saturated brine, dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 9:1) to afford 119 as a white solid (182 mg, 78%).

**1H-NMR (400 MHz, DMSO-d6):** δ 7.33-7.19 (m, 15H), 6.46 (s, 1H).

**MS ESI (m/z):** 261.0 [M+H].

**6-Amino-2-hydroxy-3-tritylbenzoic acid 120:**

![6-Amino-2-hydroxy-3-tritylbenzoic acid](insert_image)

To a solution of 6-aminosalicylic acid hydrochloride salt (226 mg, 1.2 mmol) and triethylamine (0.85 mL, 6 mmol) in THF (10 mL) was added trityl chloride (1.34g, 4.8 mmol). The reaction mixture was stirred at room temperature overnight. The solvent was then evaporated and the residue was taken-up in 5% KHSO₄ (45 mL) and extracted with
EtOAc (3 times 45 mL). The combined organic extracts were washed with saturated brine, dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 2:1) to afford 120 as a white solid (308 mg, 65%).

**1H-NMR (400 MHz, DMSO-d6):** δ 12.97 (s, 1H), 9.24 (bs, 2H), 7.26-7.06 (m, 15H), 6.93 (d, J=8.8 Hz, 1H), 6.21 (d, J=8.8 Hz, 1H).

**MS ESI (m/z):** 393.8 [M-H]⁻.

2-(1,3-Dioxoisoindolin-2-yl)-6-hydroxybenzoic acid 121:

![Structure of 2-(1,3-Dioxoisoindolin-2-yl)-6-hydroxybenzoic acid 121](image)

To a solution of 6-aminosalicylic acid hydrochloride salt (150 mg, 0.8 mmol) and phthalic anhydride (118 mg, 0.8 mmol) in acetonitrile (5 mL) was added sodium bicarbonate (134.4 mg, 1.6 mmol) and the mixture was stirred at 90°C for 2 hours. The reaction mixture was cooled to room temperature and 20% aqueous KHSO₄ (50 mL) was added. The aqueous mixture was extracted with EtOAc (3 times 45 mL) and the combined organic extracts were washed with saturated brine, dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 4:1) to afford 121 as a white solid (122 mg, 54%).

**1H-NMR (400 MHz, DMSO-d6):** δ 11.83 (s, 1H), 7.83 (m, 2H), 7.62 (m, 3H), 7.34 (t, J=8.2 Hz, 1H), 6.63 (dd, J=8.3 Hz, 1.0Hz, 1H).

**MS ESI (m/z):** 282.0 [M-H]⁻.

**1H-Benzod[d][1,2]oxazine-1,4(3H)-dione 122:**
Acid 121 (50 mg, 0.18 mmol) and CDI (114 mg, 0.7 mmol) were dissolved in anhydrous THF (5 mL) and stirred for 2 hours at room temperature under N₂. Hydroxylamine hydrochloride (25 mg, 0.36 mmol) was then added and stirring was continued for another 12 hours. The solvent was removed and the residue was taken-up in water (30 mL) and acidified with 12N HCl to pH 2. The aqueous layer was extracted with EtOAc (3 times 45 mL) and the combined organic extracts were washed with saturated brine, dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 5:1) to afford 122 as a white solid (22 mg, 75%).

\[ {^1}H\text{-NMR (400 MHz, DMSO-d}_6\text{)}: \delta 10.81 \text{ (s, 1H), 7.84 (s, 4H).} \]

\[ \text{MS ESI (m/z): 161.7 [M-H]}. \]

2-((Diphenylmethylene)amino)-6-hydroxybenzoic acid 123:

To a stirred solution of 6-aminosalicylic acid hydrochloride salt (96 mg, 0.5 mmol) and benzophenone (546 mg, 3 mmol) in DCM (4 mL) containing pyridine (0.04 mL, 0.5 mmol) was slowly added TiCl₄ solution (1.0 M in DCM) (0.6 mL, 0.6 mmol). The mixture was stirred at room temperature overnight. Water (30 mL) was then added and the aqueous mixture was extracted with EtOAc (3 times 25 mL). The combined organic phases were dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 9:1) to afford 123 as a white solid (19 mg, 12%).

\[ {^1}H\text{-NMR (400 MHz, DMSO-d}_6\text{)}: \delta 9.27 \text{ (s, 1H), 7.69 (m, 2H), 7.58 (td, J=2.0 Hz, J=2.8 Hz,} \]

\[ \text{158} \]
1H), 7.52 (t, J=7.9 Hz, 2H), 7.39 (dd, J=1.7 Hz, J=5.0 Hz, 2H), 7.20 (dd, J=3.2 Hz, J=6.4 Hz, 2H), 6.96 (t, J=7.9 Hz, 1H), 6.35 (m, 1H), 6.15 (m, 2H).

1-Methoxy-2-methyl-3-nitrobenzene 124:

Methyl iodide (0.3 mL, 4.5 mmol) was added dropwise to a cooled (0°C) mixture of nitro compound 89 (459 mg, 3 mmol) and potassium carbonate (827 mg, 6 mmol) in DMF (3.5 mL). The reaction mixture was slowly warmed up to room temperature and stirred overnight. The solvent was then evaporated and the residue was redissolved in EtOAc, washed with water and brine and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 9:1) to afford 124 as a white solid (476 mg, 95%).

^1H-NMR (400 MHz, CDCl\textsubscript{3}): \(\delta\) 7.42 (d, J=8.2 Hz, 1H), 7.29 (t, J=8.2 Hz, 1H), 7.06 (d, J=8.2 Hz, 1H), 3.91 (s, 3H), 2.38 (s, 3H).

MS ESI (m/z): 190.1 [M+Na].

2-Methoxy-6-nitrobenzoic acid 125:

KMnO\textsubscript{4} (1.1 g, 7 mmol) was added in small portions at room temperature to a solution of nitro compound 124 (167 mg, 1 mmol) and NaOH (132 mg, 3.3 mmol) in H\textsubscript{2}O (5 mL). The mixture was heated to 90°C with stirring for 6 hours. The reaction mixture was then filtered and the solid material was washed with water. The filtrate was acidified with 1N HCl to pH 3 and extracted with EtOAc (3 times 25 mL). The combined organic layers were dried over Na\textsubscript{2}SO\textsubscript{4} and concentrated. The residue was dry loaded onto silica gel and column
chromatographed (DCM/EtOAc, 4:1) to afford 125 as a yellow solid (95 mg, 62%).

$^1$H-NMR (400 MHz, CD$_3$OD): $\delta$ 7.74 (d, J=8.2 Hz, 1H), 7.60 (t, J=8.3 Hz, 1H), 7.48 (d, J=8.4 Hz, 1H), 3.93 (s, 3H).

$^{13}$C-NMR (100 MHz, CD$_3$OD): $\delta$ 168.1, 158.3, 147.4, 132.0, 121.8, 118.5, 116.7, 57.4.

MS ESI (m/z): 195.9 [M-H], HRMS: m/z calculated for C$_8$H$_7$NO$_5$ [M-H]: 196.02515, found: 196.02438.

2-Hydroxy-6-nitrobenzoic acid 66:

Following the literature procedure$^{140}$, boron trifluoride etherate (0.27 mL, 1 mmol) was added slowly to a stirred mixture of O-methoxy compound 125 (98 mg, 0.5 mmol) and sodium iodide (150 mg, 1 mmol) in anhydrous DCM (5 mL) at $0^\circ$C. The reaction mixture was slowly warmed up to room temperature and stirred for 12h. It was then quenched with water (30 mL) and extracted with EtOAc (3 times 20 mL). The combined organic layers were dried over Na$_2$SO$_4$, concentrated in vacuo, affording 66 as a brown solid (88 mg, 98%). This brown solid was taken forward to the next step without any further purification.

$^1$H-NMR (400 MHz, CD$_3$OD): $\delta$ 7.51 (t, J=8.0 Hz, 1H) 7.46 (d, J=8.0 Hz, 1H) 7.24 (d, J=8.0 Hz, 1H).

$^{13}$C-NMR (100 MHz, CD$_3$OD): $\delta$ 169.1, 158.7, 149.5, 133.0, 122.3, 115.8, 115.4.

MS ESI (m/z): 182.0 [M-H], HRMS: m/z calculated for C$_7$H$_5$NO$_5$ [M-H]: 182.00950, found: 182.00839.

$N$-Hydroxy-2-methoxy-6-nitrobenzamide 126:
Nitrobenzoic acid 125 (197 mg, 1 mmol) and thionyl chloride (2 mL) were mixed together and heated at 80°C for 6 hours. The excess thionyl chloride was removed in vacuo and the residue was redissolved in anhydrous DCM (5 mL) at room temperature. Hydroxylamine hydrochloride (140 mg, 2 mmol) and triethylamine (636 mg, 4 mmol) were then added and the mixture was stirred at room temperature overnight under a N₂-atmosphere. The solvent was removed under reduced pressure and 1N HCl (30 mL) was added. The aqueous layer was extracted with EtOAc (3 times 30 mL) and the combined organic extracts were washed with saturated brine, dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 3:1) to afford 126 as a yellow solid (173.8 mg, 82%).

\[ ^{1}H\text{-NMR (400 MHz, CD}_{3}\text{OD): } \delta 7.77 (d, J=8.2 \text{ Hz}, 1\text{H}), 7.66 (t, J=7.9 \text{ Hz}, 1\text{H}), 7.50 (d, J=8.2 \text{ Hz}, 1\text{H}), 3.95 (s, 3\text{H}). \]

\[ ^{13}C\text{-NMR (100 MHz, CD}_{3}\text{OD): } \delta 162.9, 158.1, 147.3, 131.3, 118.9, 116.9, 115.7, 55.9. \]

\[ \text{MS ESI (m/z): 211.1 [M-H]}^{-}, \text{ HRMS: m/z calculated for C}_{8}\text{H}_{8}\text{N}_{2}\text{O}_{5} [M-H]}^{-}: 211.03604, \text{ found: 211.03546}. \]

**N,2-Dihydroxy-6-nitrobenzamide 127:**

![N,2-Dihydroxy-6-nitrobenzamide](image)

Hydroxylamine hydrochloride (175 mg, 2.5 mmol) and DIEA (517 mg, 4 mmol) were dissolved in anhydrous THF (10 mL) at room temperature. The reaction mixture was stirred under a N₂-atmosphere for 1 hour, after which time the ether-ester intermediate 128 (195 mg, 1 mmol) was slowly added, and the mixture was stirred for another 24 hours. The reaction mixture was then acidified with 1N HCl to pH 3 and extracted with EtOAc (30 mL) to remove the unreacted staring ester 128. The aqueous phase was concentrated and redissolved in THF. It was then filtered and the filtrate was concentrated. The hydroxamic acid product 127 was thereby obtained in a mixture with diisopropylethylamine hydrochloride salt.
1H-NMR (400 MHz, DMSO-d6): δ 9.50 (s, 2H), 7.36 (t, J=8.1 Hz, 1H), 7.08 (m, 2H).

MS ESI (m/z): 197.0 [M-H]', HRMS: m/z calculated for C7H6N2O5 [M-H]': 197.02039, found: 197.01961.

4-Nitrobenzo[d]isoxazol-3(2H)-one 68:

![Chemical structure](image)

CDI (243 mg, 1.5 mmol) was added to a solution of the hydroxamic acid 127 (198 mg, 1 mmol) in anhydrous THF (5 mL), and the mixture was heated to 70°C with stirring for 4 h. The reaction was then cooled to room temperature and the solvent was removed. The residue was taken up in water (50 mL) and extracted with EtOAc (3 times 30 mL). The combined organic layers were dried over Na2SO4, and concentrated in vacuo. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 4:1) to afford 68 as a white solid.

1H-NMR (400 MHz, CD3OD): δ 8.01 (d, J=7.4 Hz, 1H), 7.87 (d, J=8.3 Hz, 1H), 7.76 (t, J=8.0 Hz, 1H).

MS ESI (m/z): 178.9 [M-H]', HRMS: m/z calculated for C7H4N2O4 [M-H]': 179.00983, found: 179.00839.

4-Aminobenzo[d]isoxazol-3(2H)-one scaffold C:

![Chemical structure](image)

Stannic chloride (1.75 mL, 1.0 M in DCM) and concentrated HCl (0.9 mL) were mixed and cooled to 0°C. Nitro compound 46 (180 mg, 1 mmol) was added to this mixture, followed by dropwise addition over 30 min of a solution of stannous chloride (682 mg, 3.6 mmol) in concentrated HCl (0.5 mL). After stirring for 4h, the mixture was filtered and the white solid was collected. The white solid was crystallized from hot water to give scaffold C as its HCl
salt (103 mg, 54%).

\(^1\)H-NMR (400 MHz, DMSO-d\textsubscript{6}): \(\delta\) 8.42 (bs, 4H), 7.38 (t, J=8.0 Hz, 1H), 6.94 (d, J=8.3 Hz, 1H), 6.75 (d, J=7.7 Hz, 1H).

\(^{13}\)C-NMR (100 MHz, DMSO-d\textsubscript{6}): \(\delta\) 165.6, 164.5, 137.4, 132.7, 110.9, 104.3, 102.4.

MS ESI (m/z): 148.9 [M-H], HRMS: m/z calculated for C\textsubscript{7}H\textsubscript{6}N\textsubscript{2}O\textsubscript{2} [M-H]: 149.03565, found: 149.03426.

5-Nitro-4H-benzo[d][1,3]dioxin-4-one 128:

\[
\begin{array}{c}
\text{NO}_2 \\
\text{O} \\
\text{O}
\end{array}
\]

According to reference 150, dibromomethane (2 mL) was added to a stirred mixture of acid 66 (1.83 g, 10 mmol) and potassium phosphate tribasic (4.25 g, 20 mmol) in anhydrous DMF (10 mL). The mixture was heated to 100°C with stirring for 8 h, then cooled to room temperature and EtOAc (30 mL) was added. The organic phase was washed with 1N HCl (30 mL), brine, dried over Na\textsubscript{2}SO\textsubscript{4}, and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 9:1) to afford 128 as a yellow solid (1.73 g, 89%).

\(^1\)H-NMR (400 MHz, CDCl\textsubscript{3}): \(\delta\) 7.72 (t, J=8.0 Hz, 1H), 7.43 (d, J=7.8 Hz, 1H), 7.34 (d, J=8.4 Hz, 1H), 5.76 (s, 2H).

\(^{13}\)C-NMR (100 MHz, CDCl\textsubscript{3}): \(\delta\) 159.4, 156.5, 151.6, 136.3, 120.5, 118.8, 108.2, 91.0.

MS ESI (m/z): 217.7 [M+Na], HRMS: m/z calculated for C\textsubscript{8}H\textsubscript{5}NO\textsubscript{5} [M+H]\textsuperscript{+}: 196.02405, found: 196.02446.

Methyl 5-formyl-2-hydroxybenzoate 136:

\[
\begin{array}{c}
\text{COOCH}_3 \\
\text{OH}
\end{array}
\]

5-Formylsalicylic acid 134 (10 g, 60 mmol) was dissolved in MeOH (110 mL) and concentrated sulfuric acid (12.5 mL) was slowly added. The mixture was stirred at 90°C for
12 h. The solvent was then evaporated and the residue was taken up in EtOAc (125 mL) and water (125 mL). The organic phase was washed with brine, dried over Na$_2$SO$_4$ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 9:1) to afford 136 as a white powder (10.3 g, 95%).

$^1$H-NMR (400 MHz, CDCl$_3$): $\delta$ 11.29 (s, 1H), 9.81 (s, 1H), 8.31 (s, 1H), 7.93 (d, $J$=8.6 Hz, 1H), 7.03 (d, $J$=8.6 Hz, 1H), 3.94 (s, 1H).

MS ESI (m/z): 178.9 [M-H]$^-$.  

4-Hydroxy-3-(methoxycarbonyl)benzoic acid 137:

\[
\text{HOOC}-\text{COOCH}_3
\]

According to the reference 192, ester-aldehyde 136 (162 mg, 0.9 mmol) and Oxone (338 mg, 1.1 mmol) were dissolved in DMF (6 mL), and the mixture was stirred at room temperature for 24 h. The solvent was then evaporated under reduced pressure at 50°C and the residue was taken up in H$_2$O (30 mL) and extracted with EtOAc (3 times 30 mL). The combined organic layers were washed with brine, dried over Na$_2$SO$_4$ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 5:1) to afford 137 as a white solid (131 mg, 74%).

$^1$H-NMR (400 MHz, DMSO-d6): $\delta$ 12.93 (s, 1H), 11.0 (s, 1H), 8.36 (d, $J$=2.0 Hz, 1H), 8.03 (dd, $J$=8.6 Hz, 2.3Hz, 1H), 7.08 (d, $J$=8.6 Hz, 1H), 3.91 (s, 3H).

$^{13}$C-NMR (100 MHz, DMSO-d6): $\delta$ 168.4, 166.9, 163.3, 136.8, 132.9, 122.3, 118.3, 114.0, 53.0.

MS ESI (m/z): 194.8 [M-H]$^-$.  

4-Hydroxy-3-(hydroxycarbamoyl)benzoic acid 138:

\[
\text{HOOC}-\text{N}^+\text{H}_2\text{O}
\]

Hydroxylamine hydrochloride (238 mg, 3.4 mmol) and NaOH (476 mg, 11.9 mmol) were
dissolved in a mixture water (5 mL) and dioxane (3 mL) at room temperature, and the mixture was stirred under a N₂-atmosphere for 1 hour prior to addition of methyl ester 137 (335 mg, 1.7 mmol). The mixture was stirred at room temperature for 12 h. On completion of the reaction, as followed by TLC, the solvent was removed under reduced pressure. The residue was taken up in water (20 mL), acidified with 1N HCl to pH 3 and extracted with EtOAc (3 times 30mL). The combined organic phases were dried over Na₂SO₄ and concentrated. This afforded 138 as a white solid (308 mg, 92%). The compound was used for next step without any further purification.

**1H-NMR (400 MHz, DMSO-d6):** δ 12.78 (s, 1H), 12.74 (s, 1H), 11.54 (s, 1H), 9.38 (s, 1H), 8.35 (d, J=2.0 Hz, 1H), 7.93 (dd, J=8.6 Hz, 2.1 Hz, 1H), 6.99 (d, J=8.6 Hz, 1H).

**13C-NMR (100 MHz, DMSO-d6):** δ 167.1, 165.7, 163.0, 134.7, 130.2, 121.8, 117.8, 115.1.

**MS ESI (m/z):** 195.9 [M-H]⁻.

3-Oxo-2,3-dihydrobenzo[d]isoxazole-5-carboxylic acid scaffold D:

CDI (486 mg, 3 mmol) was added to a solution of hydroxamic acid 138 (197 mg, 1 mmol) in anhydrous THF (5 mL) and the mixture was stirred at 70°C for 8h. The solvent was then removed in vacuo. The residue was taken up in EtOAc (25 mL) and washed with 1N HCl (30 mL) and brine, dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 3:1) to afford scaffold D as a white solid (138 mg, 77%).

**1H-NMR (400 MHz, DMSO-d6):** δ 12.93 (s, 1H), 8.36 (d, J=1.3 Hz, 1H), 8.16 (dd, J=8.8 Hz, 0.6 Hz, 1H), 7.66 (d, J=8.8 Hz, 1H).

**13C-NMR (100 MHz, DMSO-d6):** δ 167.0, 166.1, 165.7, 132.0, 126.5, 124.2, 115.4, 110.8.

**MS ESI (m/z):** 177.7 [M-H]⁻.
Methyl 3-oxo-2,3-dihydrobenzo[d]isoxazole-5-carboxylate 139:

Scaffold D (179 mg, 1 mmol) and CDI (324 mg, 2 mmol) was dissolved in THF (5 mL) and the solution was stirred at room temperature under N₂-atmosphere for 2h. MeOH (20 mL) was then added and the mixture was stirred for another 12h. The solvents were evaporated in vacuo and the residue was taken up in EtOAc followed by washing with 1N HCl (45 mL) and water (30 mL). The extract was dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 5:1) to afford ester 139 as a white solid (168 mg, 87%).

¹H-NMR (400 MHz, DMSO-d₆): δ 12.71 (s, 1H), 8.38 (s, 1H), 8.15 (dd, J=1.7 Hz, J=8.8 Hz, 1H), 7.68 (d, J=9.4 Hz, 1H), 3.89 (s, 3H).

¹³C-NMR (100 MHz, DMSO-d₆): δ 166.1, 165.9, 165.7, 131.9, 125.3, 124.4, 115.3, 111.0, 52.8.

MS ESI (m/z): 191.6 [M-H]⁻.

Methyl 3-((2-methoxyethoxy)benzo[d]isoxazole-5-carboxylate 140 and Methyl 2-((2-methoxyethyl)-3-oxo-2,3-dihydrobenzo[d]isoxazole-5-carboxylate 141:

To a solution of ester 139 (130 mg, 0.67 mmol) and 2-bromoethyl methyl ether (0.1 mL, 1 mmol) in DMF (1 mL) was added K₂CO₃ (143 mg, 1.3 mmol). The reaction mixture was heated under microwave condition at 60°C for 5 min. On completion of the reaction, as followed by TLC, the solvent was removed under reduced pressure. The residue was taken up in water (20 mL), acidified with 12N HCl to pH 4 and extracted with EtOAc (3 times 30
mL). The combined organic phases were dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 4:1). The least polar compound eluted corresponded to 140 (white solid; 62.4 mg, 37%), and the more polar compound eluted corresponded to 141 (white solid; 77 mg, 46%).

**Compound 140:** ¹H-NMR (400 MHz, DMSO-d₆): δ 8.21 (dd, J=0.7 Hz, J=1.7 Hz, 1H), 8.19 (dd, J=1.7 Hz, J=8.8 Hz, 1H), 7.75 (dd, J=0.7 Hz, J=8.8 Hz, 1H), 4.55 (m, 2H), 3.89 (s, 3H), 3.78 (m, 2H), 3.35 (s, 3H).

¹³C-NMR (100 MHz, DMSO-d₆): δ 166.6, 165.9, 165.7, 132.3, 126.1, 123.6, 114.4, 111.3, 70.4, 70.2, 58.7, 52.8.

**MS ESI (m/z):** 252.0 [M+H]+.

**Compound 141:** ¹H-NMR (400 MHz, DMSO-d₆): δ 8.29 (dd, J=3.1 Hz, 1.9 Hz, 1H), 8.27 (d, J=1.8 Hz, 1H), 7.66 (d, J=9.3 Hz, 1H), 4.20 (t, J=5.2 Hz, 2H), 3.90 (s, 3H), 3.67 (t, J=5.2 Hz, 2H), 3.24 (s, 3H).

¹³C-NMR (100 MHz, DMSO-d₆): δ 165.6, 162.5, 162.1, 135.0, 126.1, 125.9, 116.8, 111.5, 68.1, 58.1, 52.8, 46.2.

**MS ESI (m/z):** 252.0 [M+H]+.

2-(2-Methoxyethyl)-3-oxo-2,3-dihydrobenzo[d]isoxazole-5-carboxamide 130:

Ester 141 (251 mg, 1 mmol) was dissolved in a MeOH solution of NH₃ (7 N in methanol) (10 mL) and the mixture was stirred at room temperature for 5 days. On completion of the reaction (TLC monitoring), the solvent was evaporated under reduced pressure and the residue was dry-loaded onto silica gel and column chromatographed (DCM/EtOAc, 4:1) to afford 130 as a white solid (110 mg, 47%).

¹H-NMR (400 MHz, DMSO-d₆): δ 8.36 (d, J=1.3 Hz, 1H), 8.24 (dd, J=8.8 Hz, 1.9 Hz, 1H),
8.16 (s, 1H), 7.59 (d, J=8.8 Hz, 1H), 7.51 (s, 1H), 4.19 (t, J=5.2 Hz, 2H), 3.66 (t, J=5.2 Hz, 2H), 3.24 (s, 3H).

\(^{13}\)C-NMR (100 MHz, DMSO-d6): \(\delta\) 166.9, 162.7, 161.8, 134.0, 130.6, 124.2, 116.4, 110.8, 68.3, 57.9, 46.2.

HRMS: m/z calculated for C11H12N2O4 \([M+H]^+\), 237.08698, found: 237.08664.

Methyl-3-(2-(1,3-dioxoisindolin-2-yl)ethoxy)-2,3-dihydrobenzo[d]isoxazole-5-carboxylate \(142\) and
Methyl-2-(2-(1,3-dioxoisindolin-2-yl)ethyl)-3-oxo-2,3-dihydrobenzo[d]isoxazole-5-carboxylate \(143\):

To a solution of ester \(139\) (193 mg, 1 mmol) and \(N\)-(2-bromoethyl)phthalimide (508 mg, 2 mmol) in DMF (0.5 mL) was added \(Na_2CO_3\) (213 mg, 2 mmol). The reaction mixture was heated under microwave condition at 95°C for 25 minutes. On completion of the reaction, as followed by TLC, the solvent was removed under reduced pressure. The residue was taken-up in water (20 mL), acidified with 12N HCl to pH 4, and extracted with EtOAc (3 times 30 mL). The combined organic phases were dried over \(Na_2SO_4\) and concentrated. The residue was dry-loaded onto silica gel and column chromatographed (DCM/EtOAc, 4:1). The least polar compound eluted corresponded to \(142\) (white solid; 116.7 mg, 32%), and the more polar compound eluted corresponded to \(143\) (white solid; 135 mg, 37%).

**Compound 142:** \(^1\)H-NMR (400 MHz, DMSO-d6): \(\delta\) 8.17 (dd, J=1.7 Hz, J=8.9 Hz, 1H), 8.09 (d, J=1.0 Hz, 1H), 7.85 (m, 4H), 7.72 (d, J=8.9 Hz, 1H), 4.67 (t, J=5.1 Hz, 2H), 4.11 (t, J=5.1 Hz, 2H), 3.88 (s, 3H).

\(^{13}\)C-NMR (100 MHz, DMSO-d6): \(\delta\) 168.4 (2C), 166.4, 165.8, 165.7, 135.0 (2C), 132.3,
132.0 (2C), 126.0, 123.6 (2C), 123.2, 114.6, 111.4, 68.5, 52.9, 37.2.

**MS ESI (m/z):** 389.0 [M+Na].

**Compound 143:** $^1$H-NMR (400 MHz, DMSO-d6): $\delta$ 8.27 (dd, J=8.8 Hz, 1.8 Hz, 1H), 8.15 (d, J=1.3 Hz, 1H), 7.83 (s, 4H), 7.63 (d, J=9.2 Hz, 1H), 4.34 (t, J=5.4 Hz, 2H), 3.95 (t, J=5.4 Hz, 2H), 3.88 (s, 3H).

**$^{13}$C-NMR (100 MHz, DMSO-d6):** $\delta$ 168.0 (2C), 165.5, 162.6, 161.9, 135.2, 135.0 (2C), 131.9 (2C), 126.1, 125.8, 123.6 (2C), 116.6, 111.6, 53.0, 44.9, 35.8.

**MS ESI (m/z):** 389.1 [M+Na].

Ester 143 (366 mg, 1 mmol) was dissolved in a MeOH solution of NH$_3$ (7 N in methanol) (10 mL) and the solution was stirred at room temperature for 5 days. On completion of the reaction, the solvent was evaporated under reduced pressure and the residue was dry-loaded onto silica gel and column chromatographed (DCM/EtOAc, 2:1) to afford 144 as a white solid (73 mg, 21%).

**$^1$H-NMR (400 MHz, DMSO-d6):** $\delta$ 8.22 (m, 2H), 8.12 (s, 1H), 7.83 (s, 1H), 7.55 (dd, J=8.3 Hz, 1.3 Hz, 1H), 7.48 (s, 1H), 4.34 (t, J=5.4 Hz, 2H), 3.94 (t, J=5.4 Hz, 2H).

**$^{13}$C-NMR (100 MHz, DMSO-d6):** $\delta$ 168.0 (2C), 166.8, 162.2, 161.6, 135.0 (2C), 134.2, 131.9 (2C), 130.5, 123.7, 123.6 (2C), 115.9, 110.8, 44.5, 35.8.

**HRMS:** m/z calculated for C$_{18}$H$_{13}$N$_3$O$_5$ [M-H]: 350.07824, found: 350.07874.

**$^3$E)-2-Hydroxy-5-((hydroxyimino)methyl)benzoic acid 146:**
A solution of Na$_2$CO$_3$ (3.49 g, 33 mmol) in water (20 mL) was slowly added to a solution of 5-formylsalicylic acid 134 (4.98 g, 30 mmol) and hydroxylamine hydrochloride (2.29 g, 33 mmol) in 1:1 H$_2$O/MeOH (40 mL), and the mixture was stirred overnight at room temperature. The solvents were then evaporated in vacuo and the residue was acidified with 1N HCl till pH 3. The aqueous phase was extracted with EtOAc (3 times 125 mL) and the combined organic phases were dried over Na$_2$SO$_4$ and concentrated. Compound 146 was obtained as a yellow powder (5 g, 92%).

**1H-NMR (400 MHz, CD$_3$OD):** δ 8.03 (s, 2H), 7.76 (d, J=9.0 Hz, 1H), 6.94 (d, J=8.1 Hz).

**MS ESI (m/z):** 180.0 [M-H]$^-$.

**5-Cyano-2-hydroxybenzoic acid 147:**

A mixture of oxime 146 (2.0 g, 11 mmol) and CDI (5.36 g, 11 mmol) in anhydrous DMF (4 mL) was stirred at room temperature under a N$_2$-atmosphere for 2 hours. Hydroxylamine hydrochloride (1.53 g, 22 mmol) was then added and stirring was continued overnight. The mixture was subsequently taken up in 20% aq. citric acid (250 mL) and extracted with EtOAc (3 times 100 mL). The combined organic phases were dried over Na$_2$SO$_4$ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 3:1) to afford 147 as a white powder (1.34 g, 75%).

**1H-NMR (400 MHz, CD$_3$OD):** δ 8.21 (s, 1H), 7.79 (d, J=9.0 Hz, 1H), 7.08 (d, J=9.0 Hz, 1H).

**MS ESI (m/z):** 162.0 [M-H]$^-$.

5-Cyano-N,2-dihydroxybenzamide 148:
A mixture of oxime 146 (2 g, 11 mmol) and CDI (5.36 g, 33 mmol) in anhydrous DMF (4 mL) was stirred at room temperature under nitrogen for 2 hours. Hydroxylamine hydrochloride (1.53 g, 22 mmol) was then added, and stirring was continued overnight. The mixture was subsequently taken up in 20% aq. citric acid (250 mL) and extracted with EtOAc (3 times 100 mL). The combined organic phases were dried over Na$_2$SO$_4$, concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 2:1) to afford 148 as a yellow powder (492 mg, 25%).

$^1$H-NMR (400 MHz, CD$_3$OD): δ 8.07 (s, 1H), 7.72 (d, J=8.5 Hz, 1H), 7.07 (d, J=9.1 Hz).

MS ESI (m/z): 177.0 [M-H]$^-$.

3-Oxo-2,3-dihydrobenzo[d]isoxazole-5-carbonitrile 149:

A mixture of hydroxamic acid 148 (200 mg, 1.12 mmol) and CDI (272 mg, 1.68 mmol) in anhydrous THF (10 mL) was stirred at 70°C for 6h, and then concentrated in vacuo. The residue was taken-up in EtOAc (30 mL) and washed with water and saturated brine, dried over Na$_2$SO$_4$, and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 4:1) to afford 149 as a white solid (142 mg, 79%).

$^1$H-NMR (400 MHz, CD$_3$OD): δ 8.07 (s, 1H), 7.79 (d, J=8.5 Hz, 1H), 7.54 (d, J=8.5 Hz, 1H).

MS ESI (m/z): 158.9 [M-H]$^-$, HRMS: m/z calculated for C$_8$H$_4$N$_2$O$_2$ [M-H]$^-$: 159.02000, found: 159.01881.

2-(2-Methoxyethyl)-3-oxo-2,3-dihydrobenzo[d]isoxazole-5-carbonitrile 131, and 3-(2-methoxyethoxy)-2,3-dihydrobenzo[d]isoxazole-5-carbonitrile 150:
To a solution of 3-oxo-2,3-dihydrobenzo[d]isoxazole-5-carbonitrile 149 (95.4 mg, 0.6 mmol) and 2-bromoethyl methyl ether (0.1 mL) in DMF (1 mL) was added Cs₂CO₃ (169 mg, 1.2 mmol). The reaction mixture was heated under microwave condition at 60°C for 5 min. The solvent was then removed under reduced pressure, and the residue was taken-up in water (20 mL), acidified with 12N HCl to pH 4 and extracted with EtOAc (3 times 30 mL). The combined organic phases were dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 4:1). The least polar compound eluted corresponded to 150, and the more polar compound eluted corresponded to 131 (sticky orange solid (60 mg, 45%).

**Compound 131:**

\( ^1H\)-NMR (400 MHz, CDCl₃): \( \delta \) 8.10 (s, 1H), 7.79 (d, J=8.9 Hz, 1H), 7.31 (d, J=8.9 Hz, 1H), 4.19 (t, J=5.5 Hz, 2H), 3.67 (t, J=5.5 Hz, 2H), 3.30 (s, 3H).

\( ^{13}C\)-NMR (100 MHz, CDCl₃): \( \delta \) 161.5, 160.9, 136.3, 129.8, 117.7, 117.6, 111.6, 107.9, 68.3, 58.5, 45.8,


**Compound 150:**

\( ^1H\)-NMR (400 MHz, CDCl₃): \( \delta \) 8.00 (s, 1H), 7.71 (d, J=8.7 Hz, 1H), 7.47 (d, J=8.7 Hz, 1H), 4.54 (m, 2H), 3.77 (m, 2H), 3.39 (s, 3H).

MS ESI (m/z): 241.1 [M+Na].

\((E)\)-5-(((Benzyloxy)imino)methyl)-2-hydroxybenzoic acid 152:

\[ \text{Na}_2\text{CO}_3 \text{ (702 mg, 6.6 mmol) in water (8 mL) was slowly added to a solution of} \]
5-formylsalicylic acid 134 (1.0 g, 6 mmol) and O-benzylhydroxylamine hydrochloride (1.1 g, 6.6 mmol) dissolved in 1:1 H₂O/MeOH (8 mL), and the mixture was stirred overnight at room temperature. The solvents were then evaporated in vacuo and the residue was taken up in H₂O (150 mL) and acidified with 1N HCl till pH 3. The aqueous phase was extracted with EtOAc (3 times 125 mL) and the combined organic phases were dried over Na₂SO₄ and concentrated. Compound 152 was obtained as a white powder (1.6 g, 95% yield).

**^1H-NMR (400 MHz, CD₃OD):** δ 8.13 (s, 1H), 8.06 (s, 1H), 7.78 (d, J=8.4 Hz, 1H), 7.42-7.30 (m, 5H), 6.96 (d, J=8.4 Hz, 1H), 5.17 (s, 2H).

**MS ESI (m/z):** 270.1 [M-H].

(E)-3-Oxo-2,3-dihydrobenzo[d]isoxazole-5-carbaldehyde O-benzyl oxime 153:

A mixture of oxime 152 (2.0 g, 7.3 mmol) and CDI (2.98 g, 18.5 mmol) in anhydrous THF (60 mL) was stirred at 60°C for 2h. Hydroxylamine hydrochloride (1.02 g, 14.8 mmol) was then added, and stirring was continued overnight at 60°C. The reaction mixture was subsequently acidified with 20% aq. citric acid (250 mL) and extracted with EtOAc (3 times 100 mL). The combined organic phases were dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 3:1) to afford 153 was obtained as a white powder. (495 mg, 25% yield).

**^1H-NMR (400 MHz, CD₃OD):** δ 8.03 (s, 1H), 7.31-7.14 (m, 7H), 7.07 (d, J=8.5 Hz, 1H), 5.05 (s, 2H).

**MS ESI (m/z):** 267.2 [M-H], HRMS: m/z calculated for C15H12N2O3 [M-H]: 267.07752, found: 267.07764.

(E)-2-(2-Methoxyethyl)-3-oxo-2,3-dihydrobenzo[d]isoxazole-5-carbaldehyde O-benzyl oxime 132 and (E)-3-(2-methoxyethoxy)-2,3-dihydrobenzo[d]isoxazole-5-carbaldehyde
O-benzyl oxime 154:

To a solution of benzoisoxazolone 153 (95.4 mg, 0.4 mmol) and 2-bromoethyl methyl ether (0.1 mL) in DMF (0.5 mL) was added K$_2$CO$_3$ (96.6 mg, 0.7 mmol). The reaction mixture was heated under microwave condition at 60°C for 5 min. On completion of the reaction, as followed by TLC, the solvent was removed under reduced pressure. The residue was taken-up in water (20 mL) and acidified with 12N HCl to pH 4 and extracted with EtOAc (3 times 30 mL). The combined organic phases were dried over Na$_2$SO$_4$ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 4:1). The least polar compound eluted corresponded to 154, and the more polar compound eluted corresponded to 132 (white powder; 52 mg, 41%).

**Compound 132:** $^1$H-NMR (400 MHz, CDCl$_3$): δ 8.16 (s, 1H), 7.1–7.6 (m, 6H), 7.26 (d, J=9.0 Hz, 1H), 7.18 (d, J=9.0 Hz, 1H), 5.25 (s, 2H), 4.03 (t, J=4.9 Hz, 2H), 3.73 (t, J=4.9 Hz, 2H), 3.36 (s, 3H).

$^{13}$C-NMR (100 MHz, CDCl$_3$): δ 154.6, 148.5, 143.8, 137.5, 132.2, 128.5 (2C), 128.4 (2C), 128.0, 122.7, 109.9, 106.7, 76.5, 69.8, 58.8, 42.6.

**MS ESI (m/z):** 327.3 [M+H]$^+$, HRMS: m/z calculated for C18H18N2O4 [M+H]$^+$: 327.1339, found: 327.13391.

**Compound 154:** $^1$H-NMR (400 MHz, CDCl$_3$): δ 10.82 (s, 1H), 8.00 (s, 1H), 7.90 (s, 1H), 7.71 (d, J=8.7 Hz, 1H), 7.11–7.37 (m, 7H), 6.90 (d, J=8.7 Hz, 1H), 5.11 (s, 2H), 4.44 (m, 2H), 3.66 (m, 2H), 3.35 (s, 3H).

**MS ESI (m/z):** 349.1 [M+Na].

Methyl 5-(1,3-dioxolan-2-yl)-2-hydroxybenzoate 155:
Pre distilled ethylene glycol (1.1 mL, 20 mmol) and anhydrous CuSO₄ (1.2 g) were heated at 155°C overnight, using a Dean-Stark apparatus. Aldehyde 136 (360 mg, 2 mmol) and p-toluenesulfonic acid (20 mg, 0.1 mmol) in toluene (10 mL) were then added and the mixture was heated at 140°C for additional 6 hours. After cooling to room temperature water (150 mL) was added and the aqueous layer was extracted with EtOAc (3 times 125 mL). The combined organic extracts were washed with saturated brine, dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 5:1) to afford 155 as a white solid (414 mg, 92.4%).

**1H-NMR (400 MHz, CD₃OD):** δ 7.95 (s, 1H), 7.59 (d, J=8.6 Hz, 1H), 6.98 (d, J=8.5 Hz, 1H), 5.69 (s, 1H), 4.11 (t, J=6.0 Hz, 2H), 4.01 (t, J=6.0 Hz, 2H), 3.97 (s, 3H).

**13C-NMR (100 MHz, CD₃OD):** δ 170.1, 161.8, 133.8, 129.3, 128.3, 117.2, 111.9, 103.0, 64.9, 51.5 (2CH2).

**MS ESI (m/z):** 223.6 [M-H]⁻.

Methyl (E)-2-hydroxy-5-((hydroxyimino)methyl)benzoate 158:

Compound 158 was prepared following the procedure to make compound 159 from methyl ester 155. Compound 158 was obtained as a white solid (296 mg 76%).

**1H-NMR (400 MHz, CD₃OD):** δ 8.02 (s, 2H), 7.76 (d, J=8.5 Hz, 1H), 6.96 (d, J=8.5 Hz, 1H), 3.96, (s, 3H).

**13C-NMR (100 MHz, CD₃OD):** δ 170.1, 162.1, 147.6, 132.9, 128.5, 124.9, 117.4, 112.3, 51.3.

**MS ESI (m/z):** 194.0 [M-H]⁻.
**(E)-N,2-Dihydroxy-5-((hydroxylimino)methyl)benzamide 159:**

\[
\text{HO-N=CH(OH)CH(OH)NH}
\]

Hydroxylamine hydrochloride (280 mg, 4 mmol) and Na₂CO₃ (636 mg, 6 mmol) were dissolved in a mixture of water (3 mL) and dioxane (5 mL) at room temperature. After stirring under a N₂-atmosphere for 1 hour, methyl ester **155** (443 mg, 2 mmol) was added. The mixture was stirred for another 12 hours. The solvents were then evaporated *in vacuo*, and the residue was taken up in water, acidified with 1N HCl to pH 3. The aqueous layer was extracted with EtOAc (3 times 25 mL) and the combined organic extracts were washed with saturated brine, dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 1:2) to afford **159** as a white solid (20 mg, 5%).

**1H-NMR (400 MHz, DMSO-d₆):** δ 12.24 (s, 1H), 11.40 (s, 1H), 11.05 (s, 1H), 8.02 (s, 1H), 7.88 (d, J=1.9 Hz, 1H), 7.63 (dd, J=8.6 Hz, 1.9 Hz, 1H), 6.95 (d, J=9.0 Hz, 1H).

**MS ESI (m/z):** 195.0 [M-H]⁻.

Methyl 2-hydroxy-5-(hydroxymethyl)benzoate **160:**

\[
\text{HOOC-CH(OH)CH(OH)CH(OH)CO}\
\]

Sodium borohydride (189 mg, 5 mmol) was slowly added at 0°C to a solution of aldehyde **136** (900 mg, 5 mmol) in anhydrous MeOH (10 mL). The reaction mixture was stirred at 0°C for 5 hours and the solvent was concentrated. Saturated ammonia chloride solution (100 mL) was then added and the product was extracted with EtOAc (3 times 45 mL). The combined organic extracts were washed with saturated brine, dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 3:1) to afford **160** as a white powder (646 mg, 71%).

**1H-NMR (400 MHz, CD₃OD):** δ 7.83 (s, 1H), 7.48 (d, J=8.5 Hz, 1H), 6.93 (d, J=8.4 Hz,
1H), 4.53 (s, 2H), 3.95 (s, 3H).

\(^{13}\)C-NMR (100 MHz, CD\(_3\)OD): \(\delta\) 170.8, 161.0, 134.8, 132.3, 128.0, 117.0, 111.9, 62.8, 51.3.

**MS ESI (m/z):** 180.9 [M-H]-.

\(N,2\)-Dihydroxy-5-(hydroxymethyl)benzamide 161:

\(\text{HO} \begin{array}{c} \text{O} \\ \text{N} \end{array} \text{OH} \text{OH}

Hydroxylamine hydrochloride (280 mg, 4 mmol) and NaOH (400 mg, 10 mmol) were dissolved in a mixture of water (3 mL) and dioxane (3 mL) at room temperature. The reaction mixture was stirred under N\(_2\)-atmosphere for 1 hour and methyl ester (370 mg, 2 mmol) 160 were then added. The mixture was stirred for another 12 hours, before removal of the solvents \textit{in vacuo}. The residue was taken up in water and acidified with 1N HCl to pH 3. The aqueous layer was extracted with EtOAc (3 times 25 mL) and the combined organic extracts were washed with saturated brine, dried over Na\(_2\)SO\(_4\) and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 1:1) to afford 161 as a brown powder (190 mg, 52%).

\(^1\)H-NMR (400 MHz, DMSO-d\(_6\)):
\(\delta\) 12.07 (s, 1H), 11.38 (s, 1H), 9.28 (s, 1H), 7.65 (d, J=1.9 Hz, 1H), 7.33 (dd, J=8.4 Hz, 1.9Hz, 1H), 6.87 (d, J=8.4 Hz, 1H), 5.11 (t, J=5.5 Hz, 1H), 4.39 (d, J=5.2 Hz, 2H).

\(^{13}\)C-NMR (100 MHz, DMSO-d\(_6\)):
\(\delta\) 166.7, 158.4, 133.2, 132.6, 126.0, 117.4, 114.1, 63.0.

**MS ESI (m/z):** 181.7 [M-H]-.

**Methyl 2-hydroxy-5-methylbenzoate 164:**

\(\text{HO} \begin{array}{c} \text{O} \\ \text{OH} \end{array}

5-methyl salicylic acid 135 (5 g, 33 mmol) was dissolved in MeOH (125 mL) and concentrated sulfuric acid (4 mL) was slowly added. The mixture was stirred at 85°C for 16h.
The MeOH was then removed *in vacuo* and the residue was taken up in EtOAc (125 mL) and water (125 mL). The organic phase was washed with brine, dried over Na$_2$SO$_4$ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 9:1) to afford 164 as a white powder (5.0 g, 91.6%).

$^1$H-NMR (400 MHz, CDCl$_3$): $\delta$ 10.59 (s, 1H), 7.65 (d, J=2.0 Hz, 1H), 7.29 (dd, J=8.4 Hz, 2.3 Hz, 1H), 6.91 (d, J=8.5 Hz, 1H), 3.96 (s, 3H), 2.31 (s, 3H).

$^{13}$C-NMR (100 MHz, CDCl$_3$): $\delta$ 170.8, 159.7, 137.0, 129.7, 128.3, 117.4, 111.9, 52.2, 20.1.

MS ESI (m/z): 189.2 [M+Na].

N, 2-Dihydroxy-5-methylbenzamide 165:

Hydroxylamine hydrochloride (4.2 g, 60 mmol) and NaOH (4.8 g, 120 mmol) were dissolved in a mixture of water (20 mL) and dioxane (10 mL) at room temperature. The reaction mixture was stirred under N$_2$-atmosphere for 1 hour and methyl ester 164 (5.0 g, 30 mmol) was added. The mixture was stirred for another 12 hours. The solvent was then removed under reduced pressure, and the residue was taken-up in water (200 mL) and acidified with 1N HCl to pH 3 and extracted with EtOAc (3 times 150 mL). The combined organic phases were dried over Na$_2$SO$_4$ and concentrated. Compound 165 was obtained as a white solid (4.4 g, 89%).

$^1$H-NMR (400 MHz, DMSO-d$_6$): $\delta$ 11.97 (s, 1H), 11.36 (s, 1H), 9.31 (s, 1H), 7.51 (d, J=1.6 Hz, 1H), 7.19 (dd, J=8.4 Hz, 1.9 Hz, 1H), 6.80 (d, J=8.4 Hz, 1H), 2.22 (s, 3H).

$^{13}$C-NMR (100 MHz, DMSO-d$_6$): $\delta$ 166.7, 157.8, 134.4, 127.7, 127.4, 117.5, 114.1, 20.7.

MS ESI (m/z): 166.0 [M-H$^-$].

5-Methylbenzo[d]isoxazol-3(2H)-one 166:
A solution of the hydroxamic acid 165 (4.6 g, 27.5 mmol) and CDI (8.8 g, 55 mmol) in anhydrous THF (30 mL) was stirred at 70°C for 6h. The solvent was then removed in vacuo, and the residue was taken-up in EtOAc (150 mL), washed with water and saturated brine, dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 4:1) to afford 166 as a white solid (2.6 g, 63%).

\[ ^1H\text{-NMR (400 MHz, DMSO-d6): } \delta 12.25 (s, 1H), 7.51 (m, 1H), 7.42 (m, 2H), 2.41 (s, 3H). \]

\[ ^{13}C\text{-NMR (100 MHz, DMSO-d6): } \delta 165.8, 162.3, 132.7, 132.4, 120.9, 114.9, 110.1, 21.1. \]

MS ESI (m/z): 147.9 [M-H]⁻.

2-(2-(5-Methyl-3-oxobenzo[dl]isoxazol-2(3H)-yl)ethyl)isoindoline-1,3-dione 167 and 2-(2-((5-Methyl-2,3-dihydrobenzo[dl]isoxazol-3-yl)oxy)ethyl)isoindoline-1,3-dione 168:

To a solution of benzoisoxazolone 166 (596 mg, 4 mmol) and N-(2-bromoethyl) phthalimide (1.01 g, 8 mmol) in DMF (2 mL) was added Na₂CO₃ (426 mg, 8 mmol), and the reaction mixture was heated under microwave condition at 100°C for 30 min. On completion of the reaction, as followed by TLC, the solvent was removed under reduced pressure. The residue was taken-up in water (60 mL), acidified with 12N HCl to pH 4 and extracted with EtOAc (3 times 60 mL). The combined organic phases were dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 3:1). The least polar compound eluted corresponded to 168 (white powder, 348 mg, 27%), and the more polar compound eluted corresponded to 167 (white powder; 438 mg, 34%).
**Compound 167:** \(^1\)H-NMR (400 MHz, DMSO-d6): \(\delta\) 7.89 (m, 4H), 7.67 (d, J=1.5 Hz, 1H), 7.59 (dd, J=8.5 Hz, 2. 2Hz, 1H), 7.28 (d, J=8.4 Hz, 1H), 4.38 (t, J=5.3 Hz, 2H), 3.95 (t, J=5.3 Hz, 2H), 2.35 (s, 3H).

\(^{13}\)C-NMR (100 MHz, DMSO-d6): \(\delta\) 168.4, 157.8, 150.2, 146.1, 137.2, 135.4, 134.8 (2H), 132.2, 127.1, 123.5(2H), 116.8, 115.2, 73.4, 55.1, 36.4, 20.3.

**MS ESI (m/z):** 345.1 [M+Na].

**Compound 168:** \(^1\)H-NMR (400 MHz, DMSO-d6): \(\delta\) 7.82-7.89 (m, 4H), 7.47 (d, J=8.7 Hz, 1H), 7.43 (dd, J=1.4 Hz, J=8.7 Hz, 1H), 7.34 (m, 1H), 4.64 (t, J=5.3 Hz, 2H), 4.10 (t, J=5.3 Hz, 2H), 2.38 (s, 3H).

\(^{13}\)C-NMR (100 MHz, DMSO-d6): \(\delta\) 168.4 (2C), 165.9, 162.3, 134.9 (2C), 133.5, 133.0, 132.0, 123.6 (2C), 120.0, 114.0, 110.2, 67.9, 55.4 (2C), 37.3 (2C), 20.7 (3C).

**MS ESI (m/z):** 345.1 [M+Na].

2-(2-(5-(Bromomethyl)-3-oxobenzo[di]oxazol-2(3H)-yl)ethyl)isoindoline-1,3-dione 169:

![Image of 169](image)

According to reference 204, \(N\)-bromosuccinimide (213.6 mg, 1.2 mmol) and AIBN (18.6 mg, 0.1 mol) were slowly added to a solution of benzoisoxazolone 167 (322.2 mg, 1 mmol) in CCl\(_4\) (10 mL), and the reaction mixture was heated to 90°C for 2 days. On completion of the reaction, check by TLC, the solvent was evaporated in vacuo. Water (30 mL) was added and the aqueous phase was extracted by EtOAc (3 times 25 mL). The combined organic extracts were washed with saturated brine, dried over Na\(_2\)SO\(_4\) and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 5:1) to afford 169 as a yellow solid (288 mg, 72%).

\(^1\)H-NMR (400 MHz, DMSO-d6): \(\delta\) 7.82 (s, 4H), 7.79 (dd, J=8.7 Hz, 1.2Hz, 1H), 7.77 (d, J=1.2 Hz, 1H), 7.47 (d, J=8.7 Hz, 1H), 4.81 (s, 2H), 4.30 (t, J=5.4 Hz, 2H), 3.93 (t, J=5.4 Hz,
MS ESI (m/z): 423.1 [M+Na].

2-((2-(2-(1,3-Dioxoisoindolin-2-yl)ethyl)-3-oxo-2,3-dihydrobenzo[d]isoxazol-5-yl)methyl)isoindoline-1,3-dione 170:

Potassium phthalimide (278 mg, 1.5 mmol) was added to a solution of bromomethyl compound 169 (400 mg, 1 mmol) in DMF (3 mL), and the reaction mixture was stirred at room temperature for 24 hours. On completion of the reaction, check by TLC, the solvent was evaporated in vacuo. Water (60 mL) was added and the aqueous phase was extracted by EtOAc (3 times 45 mL). The combined organic extracts were washed with saturated brine, dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 5:1) to afford 170 as a white solid (285 mg, 61%).

^1^H-NMR (400 MHz, DMSO-d₆): δ 7.87 (m, 4H), 7.80 (s, 4H), 7.68 (dd, J=8.7 Hz, 1.2 Hz, 1H), 7.57 (d, J=1.2 Hz, 1H), 7.44 (d, J=8.7 Hz, 1H), 4.84 (s, 2H), 4.28 (t, J=5.4 Hz, 1H), 3.91 (t, 1H, J=5.4 Hz).

^1^3^C-NMR (100 MHz, DMSO-d₆): δ 168.2 (2C), 167.9 (2C), 162.2, 159.6, 135.0 (2C), 135.0 (2C), 134.2, 133.0, 132.1 (2C), 131.9 (2C), 123.7 (2C), 123.6 (2C), 122.9, 116.0, 111.0, 44.5 (2C), 40.7 (2C), 35.9 (2C).

MS ESI (m/z): 490.3 [M+Na].

2-(2-Aminoethyl)-5-(aminomethyl)benzo[d]isoxazol-3(2H)-one 133:

Hydrazine hydrate (96 mg, 3 mmol) was added to a solution of compound 170 (467 mg, 1
mmol) in MeOH (5 mL), and the reaction mixture was stirred at 90°C for 2 hours. On completion of the reaction, check by TLC, the solvent was evaporated in vacuo. The residue was taken-up in EtOAc (50 mL) and washed with water and brine. The organic layer was dried over Na₂SO₄ and concentrated under reduced pressure. The residue was dry loaded onto silica gel and column chromatographed (DCM/MeOH, 5:1) to afford 133 as a sticky brown solid (66.3 mg, 32%).

**1H-NMR (400 MHz, DMSO-d6):** δ 7.73 (d, J=1.8 Hz, 1H), 7.67 (dd, J=1.8 Hz, J=8.6 Hz, 1H), 7.41 (d, J=8.6 Hz, 1H), 3.96 (t, J=6.4 Hz, 2H), 3.79 (s, 2H), 2.87 (t, J=6.4 Hz, 2H).

**13C-NMR (100 MHz, DMSO-d6):** δ 163.0, 159.1, 140.8, 133.8, 121.7, 116.1, 110.2, 49.4, 45.4, 39.8.

**MS ESI (m/z):** 230.0 [M+Na].

5-Formyl-2-hydroxy-3-nitrobenzoic acid 175:

![5-Formyl-2-hydroxy-3-nitrobenzoic acid 175](image)

Aldehyde 134 (1 g, 6 mmol) and sulfuric acid (2.5 mL) were mixed in ice-cold bath. A mixture of sulfuric acid (0.5 mL) and nitric acid (0.5 mL) was slowly added over 30 minutes. The reaction mixture was stirred at 0°C for a further 3 hours and poured onto crushed ice. The precipitate was filtered and washed with cold water, and dried under high vacuum to afford 175 as a yellow solid (1.3 g, 91%).

**1H-NMR (400 MHz, DMSO-d6):** δ 9.78 (s, 1H), 8.46 (d, J=2.3 Hz, 1H), 8.43 (d, J=2.3 Hz, 1H).

4-Oxo-4H-benzo[d][1,3]dioxine-6-carbaldehyde 176:

![4-Oxo-4H-benzo[d][1,3]dioxine-6-carbaldehyde 176](image)

To a solution of 5-formylsalicylic acid 134 (1.7 g, 10 mmol) in anhydrous DMF (10 mL)
containing Potassium phosphate tribasic (4.3 g, 20 mmol) was added dibromomethane (2 mL). The mixture was stirred at 100°C for 10h. The solvent was then evaporated *in vacuo* and the residue was taken-up in EtOAc (250 mL) and washed with 1N HCl (200 mL). The organic layer further washed with water and brine, then dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 9.5:0.5) to afford 176 as a white solid (1.5 g, 83%).

**1H-NMR (400 MHz, CDCl₃)**: δ 9.92 (s, 1H), 8.44 (s, 1H), 8.07 (d, J=8.6 Hz, 1H), 7.17 (d, J=8.6 Hz, 1H), 5.69 (s, 2H).

**13C-NMR (100 MHz, CDCl₃)**: δ 189.7, 162.5, 160.1, 135.7, 133.9, 132.2, 118.3, 115.1, 91.1.

**MS ESI (m/z)**: 178.9 [M+H]+.

8-Nitro-4-oxo-4H-benzo[d][1,3]dioxine-6-carbaldehyde 177:

![Image of compound structure]

Aldehyde 176 (500 mg, 2.8 mmol) was dissolved in concentrated H₂SO₄ (1.25 mL) and cooled to 0°C. A mixture of concentrated H₂SO₄ (0.25 mL) and HNO₃ (0.25 mL) was added dropwise over 30 min and the reaction was stirred for a further 3h at 0°C. The mixture was then poured onto crushed ice, and a white solid precipitated out. The resulting precipitate was collected by filtration, washed with ice-water, and dried *in vacuo* to afford product 177 (450 mg, 72%) as a yellow solid.

**1H-NMR (400 MHz, CDCl₃)**: δ 9.99 (s, 1H), 8.73 (s, 1H), 8.71 (s, 1H), 5.84 (s, 2H).

**13C-NMR (100 MHz, CDCl₃)**: δ 187.6, 158.0, 155.7, 138.7, 136.8, 131.5, 131.0, 117.8, 91.7.

**MS ESI (m/z)**: 223.6 [M+H]+.

8-Nitro-4-oxo-4H-benzo[d][1,3]dioxine-6-carboxylic acid 178:
Nitro derivative 177 (200 mg, 0.9 mmol) and Oxone (338 mg, 1.1 mmol) were dissolved in DMF (6 mL) and stirred at room temperature overnight. The solvent was evaporated in vacuo and the residue was taken up in water, acidified with 1N HCl till pH 3, and extracted with EtOAc (3 times 25 mL). The combined organic extracts were washed with saturated brine, dried over Na$_2$SO$_4$ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/MeOH, 9:1) to afford 178 as a yellow solid (170 mg, 79%).

$^1$H-NMR (400 MHz, DMSO-d$_6$): $\delta$ 13.91 (s, 1H), 8.74 (d, J=2.1 Hz, 1H), 8.61 (d, J=2.1 Hz, 1H), 6.09 (s, 2H).

$^{13}$C-NMR (100 MHz, CD$_3$OD): $\delta$ 165.0, 159.3, 155.0, 137.8, 135.9, 132.1, 125.9, 117.4, 92.1.

MS ESI (m/z): 237.8 [M-H]$^-$. 

N1, N3-Dibenzyl-4-hydroxy-5-nitroisophthalamide 179:

Acid 178 (160 mg, 0.67 mmol), HOAt (109 mg, 0.8 mmol), EDCI (154 mg, 0.8 mmol) were mixed and dissolved in DMF (5 mL). Benzylamine (85.7 mg, 0.8 mmol) and DIEA (0.3 mL, 1.7 mmol) were added and the mixture was stirred at room temperature under a N$_2$-atmosphere overnight. The solvent was evaporated in vacuo and the residue was taken-up in EtOAc (30 mL). The organic phase was washed with 1N HCl (30 mL), followed by water and brine, dried over Na$_2$SO$_4$ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/MeOH, 9:1) to afford 179 as a yellow solid (131 mg, 48%).
\[ ^1H-\text{NMR (400 MHz, DMSO-d6): } \delta 10.09 (s, 1H), 9.16 (t, J=5.8 \text{ Hz}, 1H), 8.78 (d, J=2.1 \text{ Hz}, 1H), 8.66 (d, J=2.1 \text{ Hz}, 1H), 7.37-7.23 (m, 10H), 4.55 (d, J=5.8 \text{ Hz}, 2H), 4.50 (d, J=5.8 \text{ Hz}, 2H). \]

**MS ESI (m/z):** 404.8 [M-H].

4-Hydroxy-3-(methoxycarbonyl)-5-nitrobenzoic acid 180:

\[
\text{HOOC} \quad \text{O} \quad \text{NO}_2 \\
\text{HO} \\
\text{OH}
\]

Acid 178 (239 mg, 1 mmol) was dissolved in dry MeOH (5 mL) and K$_2$CO$_3$ (552 mg, 4 mmol) was added. The reaction mixture was stirred at room temperature for 2 days. The solvent was then evaporated in vacuo and the residue was taken-up in 1N HCl (100 mL) and extracted with EtOAc (3 times 45 mL). The combined organic extracts were dried over Na$_2$SO$_4$ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/MeOH, 9:1) to afford 180 as a lightly yellow solid (233 mg, 97%).

\[ ^1H-\text{NMR (400 MHz, DMSO-d6): } \delta 13.55 (s, 1H), 8.58 (d, J=2.2 \text{ Hz}, 1H), 8.53 (d, J=2.2 \text{ Hz}, 1H), 3.96 (s, 3H). \]

\[ ^{13}C-\text{NMR (100 MHz, DMSO-d6): } \delta 167.5, 165.3, 156.3, 139.0, 136.0, 131.2, 121.9, 118.0, 53.9. \]

**MS ESI (m/z):** 239.8 [M-H], **HRMS:** m/z calculated for C9H7NO7 [M-H]: 240.01497, found: 240.01596.

4-Hydroxy-3-(hydroxycarbamoyl)-5-nitrobenzoic acid 181:

\[
\text{HOOC} \quad \text{O} \quad \text{NH} \quad \text{OH} \\
\text{NO}_2 \\
\text{OH}
\]

Hydroxylamine hydrochloride (1.4 g, 20 mmol) and NaOH (2.4 g, 60 mmol) were dissolved in a mixture of water (20 mL) and dioxane (10 mL) at room temperature. The reaction
mixture was stirred under N₂-atmosphere for 1 hour and methyl ester 180 (2.4 g, 10 mmol) was then added. The mixture was stirred for an additional 24 hours. On completion of the reaction, as followed by TLC, the solvent was removed under reduced pressure. The residue was taken-up in water (100 mL), acidified with 1N HCl to pH 3 and extracted with EtOAc (3 times 50 mL). The combined organic phases were dried over Na₂SO₄ and concentrated. Compound 181 was obtained as a white solid (2.2 g, 91%).

**1H-NMR (400 MHz, DMSO-d₆):** δ 12.30 (s, 1H), 8.60 (d, J=2.0 Hz, 1H), 8.50 (d, J=2.0 Hz, 1H).

**MS ESI (m/z):** 241.0 [M-H]-.

7-Nitro-3-oxo-2,3-dihydrobenzo[d]isoxazole-5-carboxylic acid 182:

![Chemical Structure](image)

A solution of the hydroxamic acid 181 (242 mg, 1 mmol) and CDI (405 mg, 2.5 mmol) in anhydrous THF (5 mL) was stirred at 70°C for 6h. The solvent was then removed in vacuo, and the residue was taken-up in EtOAc (50 mL) and washed with water followed by saturated brine, dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/MeOH, 9:1) to afford 182 as a white solid (159 mg, 71%).

**1H-NMR (400 MHz, DMSO-d₆):** δ 8.78 (d, J=1.6 Hz, 1H), 8.68 (d, J=1.6 Hz, 1H).

**13C-NMR (100 MHz, DMSO-d₆):** δ 166.4, 165.4, 157.7, 132.8, 130.6, 127.6, 127.3, 120.5.

**MS ESI (m/z):** 223.0 [M-H]-; **HRMS:** m/z calculated for C₈H₄N₂O₆ [M-H]-: 222.99966, found: 222.99881.

**N-Benzyl-7-nitro-3-oxo-2,3-dihydrobenzo[d]isoxazole-5-carboxamide 183:**

186
A solution of acid 182 (150 mg, 0.7 mmol) and CDI (215 mg, 1.3 mmol) in anhydrous THF (5 mL) was stirred at room temperature under a N₂-atmosphere for 2 hours. Benzyamine (85.7 mg, 0.8 mmol) was then added and the reaction mixture was stirred for an additional 12 h. On completion of the reaction (monitored by TLC), the solvent was evaporated and the residue was taken-up in EtOAc (30 mL) and washed with water and brine. The organic layer was dried over Na₂SO₄ and concentrated under reduced pressure. The residue was dry loaded onto silica gel and column chromatographed (DCM/MeOH, 9:1) to afford 183 as a yellow solid (141 mg, 67%).

¹H-NMR (400 MHz, DMSO-d6): δ 9.50 (t, J=5.7 Hz, 1H), 8.90 (d, J=1.8 Hz, 1H), 8.73 (d, J=1.8 Hz, 1H), 7.37-7.24 (m, 5H), 4.54 (d, J=5.8 Hz, 2H)

MS ESI (m/z): 312.0 [M-H], HRMS: m/z calculated for C₁₅H₁₁N₃O₅ [M-H]: 312.06259, found: 312.06277.

Methyl 7-nitro-3-oxo-2,3-dihydrobenzo[d]isoxazole-5-carboxylate 185:

A solution of acid 182 (150 mg, 0.7 mmol) and CDI (215 mg, 1.3 mmol) in anhydrous DMF (5 mL) was stirred at room temperature under a N₂-atmosphere for 2 hours. Benzyamine (85.7 mg, 0.8 mmol) was added and the reaction mixture was stirred for another 12 h. On completion of the reaction (monitored by TLC), the solvent was evaporated and the residue was taken-up in EtOAc (30 mL) and washed with water and brine. The organic layer was dried over Na₂SO₄ and concentrated under reduced pressure. The residue was dry loaded
onto silica gel and column chromatographed (DCM/EtOAc, 1:1) providing METHYL ESTER 185 as a light yellow solid (131 mg, 82%).

**1H-NMR (400 MHz, DMSO-d6):** \(\delta 13.40 (s, 1H), 8.77 (d, J=1.5 \text{ Hz}, 1H), 8.69 (d, J=1.5 \text{ Hz}, 1H), 3.96 (s, 3H).\)

**MS ESI (m/z):** 236.6 [M-H]⁻.

*N,2-Dibenzyl-7-nitro-3-oxo-2,3-dihydrobenzo[d]isoxazole-5-carboxamide 187*, and

*N-Benzyl-3-(Benzyloxy)-7-nitrobenzo[d]isoxazole-5-carboxamide 186:*

![Chemical Structures](image)

To a solution of benzoisoxazolone 183 (940 mg, 3 mmol) and benzyl bromide (770 mg, 4.5 mmol) in DMF (2 mL) was added Na₂CO₃ (636 mg, 6 mmol), and the mixture was heated under microwave condition at 60°C for 10 min. On completion of the reaction, as followed by TLC, the solvent was removed under reduced pressure. The residue was taken-up in water (60 mL), acidified with 12N HCl to pH 4 and extracted with EtOAc (3 times 60 mL). The combined organic phases were dried over Na₂SO₄ and concentrated. The residue was dry loaded onto silica gel and column chromatographed (DCM/EtOAc, 3:1). The least polar compound eluted corresponded to 186 (white solid; 448 mg, 37%), and the more polar compound eluted corresponded to 187 (white solid; 520 mg, 43%).

**Compound 187: ****1H-NMR (400 MHz, DMSO-d6):** \(\delta 9.56 (t, J=5.8 \text{ Hz}, 1H), 8.98 (d, J=1.7 \text{ Hz}, 1H), 8.84 (d, J=1.7 \text{ Hz}, 1H), 7.41 (m, 4H), 7.36 (m, 5H), 7.27 (m, 2H), 5.36 (s, 2H), 4.54 (d, J=5.9 \text{ Hz}, 2H).\)

**13C-NMR (100 MHz, DMSO-d6):** \(\delta 163.3, 160.6, 154.1, 139.5, 135.1, 132.9, 130.7, 130.1, 129.3 (2C), 129.2, 128.8 (2C), 128.6, 128.3 (2C), 127.9 (2C), 127.4, 120.7, 50.0, 43.5.\)

**MS ESI (m/z):** 401.9 [M-H]⁻, **HRMS:** m/z calculated for C₂₂H₁₇N₃O₅ [M-H]⁻: 402.10954,
Compound 186: $^1$H-NMR (400 MHz, DMSO-d6): $\delta$ 9.51 (s, 1H), 8.96 (d, J=1.5 Hz, 1H), 8.79 (d, J=1.5 Hz, 1H), 7.62 (m, 2H), 7.45 (m, 3H), 7.30 (m, 5H), 5.56 (s, 2H), 4.53 (d, J=5.9 Hz, 2H).

$^{13}$C-NMR (100 MHz, DMSO-d6): $\delta$ 166.6, 163.3, 157.2, 139.4, 135.4, 132.9, 131.0, 129.4 (2CH), 129.1 (2CH), 128.8 (2CH), 127.9 (2CH), 127.6, 127.4, 127.1, 119.1, 73.2, 43.5.


7-Amino-N,2-dibenzyl-3-oxo-2,3-dihydrobenzo[d]isoxazole-5-carboxamide 189:

Stannic chloride (1.75 mL, 1.0 M in DCM) and concentrated HCl (0.9 mL) were mixed and cooled to 0°C. Nitro compound 187 (403 mg, 1 mmol) was added to this mixture followed by dropwise addition over 30 min of a solution of stannous chloride (682 mg, 3.6 mmol) in concentrated HCl (0.5 mL). After stirring for 4 hours, the mixture was filtered and the white solid was collected. The solid was dissolved in EtOAc and extracted with saturated sodium bicarbonate. The organic phase was separated, dried over Na$_2$SO$_4$, and concentrated. The residue was loaded onto silica gel and column chromatographed (DCM/EtOAc, 1:1) to afford 189 as a white solid (228 mg, 61%).

$^1$H-NMR (400 MHz, DMSO-d6): $\delta$ 9.07 (t, J=6.1 Hz, 1H), 7.58 (d, J=1.7 Hz, 1H), 7.47 (d, J=1.7 Hz), 7.39 (m, 1H), 7.37 (m, 1H), 7.35 (m, 2H), 7.32 (m, 5H), 7.26 (m, 1H), 5.79 (s, 2H), 5.24 (s, 2H), 4.46 (d, J=6.1 Hz, 2H).

$^{13}$C-NMR (100 MHz, DMSO-d6): $\delta$ 166.1, 163.5, 150.7, 140.2, 135.8, 133.3, 131.8, 129.2 (2C), 128.7 (2C), 128.4, 128.0 (2C), 127.7 (2C), 127.2, 116.7, 116.2, 108.9, 49.9, 43.1.

MS ESI (m/z): 374.3 [M+H]$^+$. HRMS: m/z calculated for C22H19N3O3 [M-H]$^-$: 372.13536,
found: 372.13565.

7-Amino-N-benzyl-3-(benzyloxy)benzo[d]isoxazole-5-carboxamide 188:

Prepared according to the procedure for compound 189, nitro compound 186 was reduced to afford 188 as a white powder (220mg, 59%).

$^1$H-NMR (400 MHz, DMSO-d$_6$): $\delta$ 9.01 (t, $J$=5.4 Hz), 7.58 (m, 2H), 7.48 (d, $J$=1.5 Hz, 1H), 7.43 (m, 3H), 7.39 (d, $J$=1.5 Hz, 1H), 7.31 (m, 4H), 7.23 (m, 1H), 5.91 (s, 2H), 5.47 (s, 2H), 4.45 (d, $J$=5.7 Hz, 2H).

$^{13}$C-NMR (100 MHz, DMSO-d$_6$): $\delta$ 167.1, 166.3, 154.6, 140.2, 136.0, 133.3, 132.0, 129.2 (2C), 129.1, 129.0 (2C), 128.7 (2C), 127.7 (2C), 127.1, 114.3, 113.3, 106.0, 72.2, 43.1.

MS ESI (m/z): 374.3 [M+H]$^+$. HRMS m/z calculated for C$_{22}$H$_{19}$N$_3$O$_3$ [M-H]: 372.13536, found: 372.13565.

N,2-Dibenzyl-7-(2-bromoacetamido)-3-oxo-2,3-dihydrobenzo[d]isoxazole-5-carboxamide 190:

To a solution of amine 189 (373 mg, 1 mmol) in DCM (3mL) and triethylamine (0.17 mL, 1.2 mmol) at 0°C was added dropwise bromoacetyl bromide (0.1 mL, 1.2 mmol). The reaction mixture was slowly warmed up to room temperature and stirred for a further 12 h.
The mixture was concentrated and the residue was loaded to silica gel chromatography to be purified and afforded 190 as a white solid (355.6 mg, 72%).

$^{1}$H-NMR (400 MHz, CDCl$_3$): $\delta$ 10.65 (s, 1H), 9.28 (t, $J$=5.9 Hz, 1H), 8.65 (d, $J$=1.6 Hz, 1H), 8.24 (d, $J$=1.6 Hz, 1H), 7.33 (m, 10H), 5.29 (s, 2H), 4.50 (d, $J$=5.9 Hz, 2H), 4.16 (s, 2H).

**MS ESI (m/z):** 491.9 [M-H]$^-$.
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