Design and Synthesis of Acetohydrazide Derivatives: Enhancing Potency and Stability against Gram-negative Pathogens

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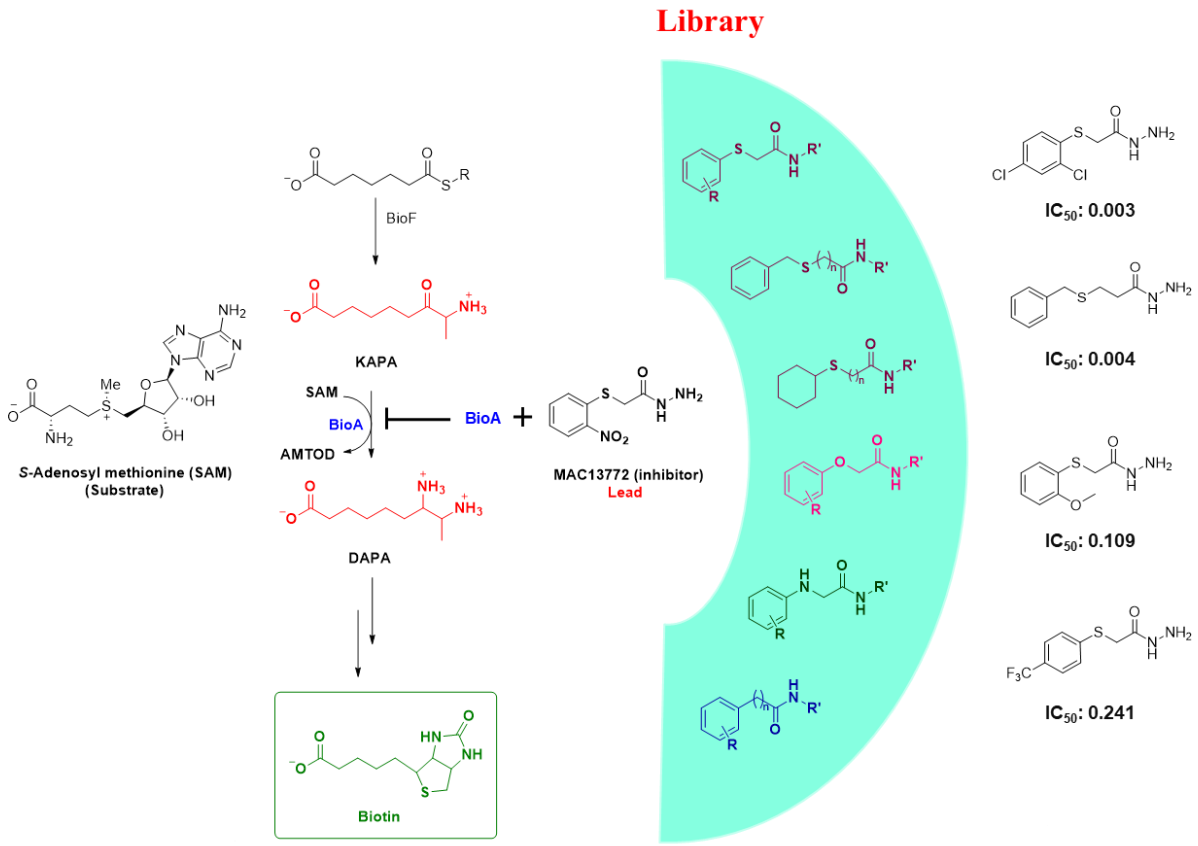
Abstract



The escalating challenge of antibiotic resistance in clinical settings underscores the urgent need for novel antimicrobial strategies. This thesis presents a concerted effort to address this crisis by targeting bacterial metabolism, specifically the biotin biosynthetic pathway. Biotin, a crucial vitamin for bacterial growth, is synthesized through a pathway involving the enzyme BioA. Our research focuses on MAC13772, a synthetic acetohydrazide identified as a potential inhibitor of BioA, which plays a pivotal role in this pathway. Originating from SAR studies at McMaster University, MAC13772 exhibited promising antibacterial properties, albeit with limitations in potency and potential toxicity that necessitated further optimization.

The primary objective of this study was to enhance the antibacterial efficacy of MAC13772 against Gram-negative pathogens through structural modification and optimization. A comprehensive series of analogs were synthesized, aiming to overcome the limitations of MAC13772 by improving its potency and metabolic stability. Notably, a trifluoromethyl derivative emerged as significantly more potent against *Acinetobacter baumannii*, achieving a four-fold increase in efficacy. Moreover, dichloro-substituted analogues demonstrated a remarkable improvement in inhibitory concentration values against BioA, with up to a 90-fold enhancement. Additionally, a benzimidazole derivative was identified for its superior metabolic stability in liver microsomes, indicating a potential for increased *in vivo* efficacy.

This research not only advances our understanding of the biotin biosynthetic pathway as a viable target for antibiotic intervention but also highlights the successful application of SAR studies in the development of more potent and stable antibacterial agents. By addressing the pharmacokinetic shortcomings of MAC13772 and its analogs, this work lays a foundational step towards the identification of new antibacterial drugs capable of combating the growing threat of drug-resistant bacteria.

Acknowledgements

On this scholarly journey, I have been fortunate to meet numerous individuals whose guidance, support, and inspiration have been crucial to my growth and success. At the forefront of this invaluable network is my supervisor, Prof. Michael Organ, whose mentorship has been nothing short of transformative. Dr. Organ, your high dedication to fostering a deep appreciation for chemistry has profoundly influenced my academic and personal development. Beyond academia, you have been a support in challenging times, for which I am eternally grateful.

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Preface to The Thesis

This thesis was adapted from the following manuscript in preparation:

Kumardip Sinha, Sepideh Sharif, Katerina Scotchburn, Rodion Gordzevich, Lindsey Carfrae, Monica Gill, Michael Organ and Eric D. Brown.

"Structure-activity relationship study and evaluation of an acetohydrazide Gram-negative antibiotic that targets biotin biosynthesis." (In preparation)

Synthetic chemistry experiments were performed by me, S. Sharif, K. Scotchburn and M. Gill with the following specifications:

- Molecules **4**, **7**, **12**, **20**, **21**, **24**, **32**, **33**, **46**, **53**, **54**, **55**, **56**, **69**, **72** were synthesized by me and other molecules were synthesized by S. Sharif, K. Scotchburn & M. Gill.
- All ^{13}C & ^{1}H NMR data was recorded by me.
- All HRMS data was recorded by Dr. Sharon Curtis.
- The Supplementary Information (SI) of above-mentioned manuscript was written by me under supervision of S. Sharif

All biology experiments were performed by Brown's group at McMaster University, Canada.

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List of Abbreviations

MRSA	Methicillin-Resistant *Staphylococcus aureus*
VRE	Vancomycin-resistant enterococci
VRSA	Vancomycin-resistant S. aureus
CRE	Carbapenem-resistant Enterobacteriaceae
WHO	World Health Organization
Boc	*tert*-Butyloxycarbonyl
CRE	Carbapenem-resistant Enterobacteriaceae
PABA	*Para*-aminobenzoic acid
˚C	Degree Celsius
KAPA	7-keto-8-aminopelargonic acid
DAPA	7,8-diaminopelargonic acid (DAPA)
SAR	Structure Activity Relationship
DNA	Deoxyribonucleic acid
MIC	Minimum Inhibitory Concentration
IC_{50}	Half maximal inhibitory concentration
K_2CO_3	Potassium carbonate K_2CO_3
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
PLP	Pyridoxal phosphate (coenzyme)
Lys	Lys Lysine, an amino acid
NMR	Nuclear Magnetic Resonance
EI	Electron ionization
Equiv.	Equivalents
ESI	Electrospray ionization
RLMs	Rat Liver Microsomes
LiCl	Lithium chloride
GC-MS	Gas Chromatography mass spectrometry detection
HRMS	High-resolution mass spectrometry
iPr	1,3-bis(2,6-diisopropylphenyl)imidazole-2-ylidine
iPr^{Cl}	1,3-bis(2,6-diisopropylphenyl)-4,5-dichloroimidazol-2-ylidine
iPr^{Me}	1,3-bis(2,6-diisopropylphenyl)-4,5-dimethylimidazol-2-ylidine
iPent	1,3-bis(2,6-di(3-pentyl)phenyl)imidazole-2-ylidine
$iPent^{Cl}$	1,3-bis(2,6-di(3-pentyl)phenyl)-4,5-dichloroimidazol-2-ylidine
$iPent^{Me}$	1,3-bis(2,6-di(3-pentyl)phenyl)-4,5-dimethylimidazol-2-ylidine
iHept	1,3-bis(2,6-di(4-hepty)phenyl)imidazole-2-ylidine
$iHept^{Cl}$	1,3-bis(2,6-di(4-hepty)phenyl)-4,5-dichloroimidazol-2-ylidine
$iHept^{Me}$	1,3-bis(2,6-di(4-hepty)phenyl)-4,5-dimethylimidazol-2-ylidine
Me	Methyl
$MgSO_4$	Magnesium sulfate
NMR	Nuclear magnetic resonance
NaH	Sodium hydride
PEPPSI	Pyridine-Enhanced Precatalyst Preparation Stabilization and Initiation.

SAM	*S*-Adenosyl methionine
TBAB	Tetrabutylammonium bromide
TMSCl	Trimethylsilyl chloride
TMEDA	Tetramethylethylenediamine
THF	Tetrahydrofuran

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CHAPTER 1: INTRODUCTION

1.1. Overview of Antibiotics

Antibiotics are substances that fight bacterial infections by either eradicating bacteria or preventing their growth. The word antibiotic is derived from the Greek words "anti" (against) and "bios" (life).[1,2] Their discovery was a critical turning point in medical history. Infections like pneumonia, tuberculosis, and even little scrapes and scratches could frequently be fatal before the development of medicines. But Sir Alexander Fleming's accidental discovery of penicillin in 1928 gave the medical profession a powerful weapon against such diseases.[2]

The popularity of penicillin made it easier to find and create other antibiotics. Different types of antibiotics have been introduced over the years, either derived from wild organisms or manufactured in labs. They have not only prevented many deaths, but they have also made risky procedures like chemotherapy, difficult surgeries, and organ transplants possible.[3]

It is impossible to overestimate the value of antibiotics in medicine. They are the foundation of contemporary therapies and have greatly improved both longevity and quality of life. Without antibiotics, the risk of infections would make many medical advancements futile because the susceptibility to bacterial infections during surgeries, chemotherapy, organ transplants, and even basic wound care would significantly increase.[3] In a fitting statement, the World Health Organization calls antibiotics "the cornerstone of modern medicine."[4]

Despite how important they have been, antibiotics are now a serious threat to global health because of their excessive and improper usage in both humans and animals.[5] It is necessary to continue focusing on the development of new medications and strategies in order to keep one

step ahead of these constantly adapting microorganisms due to the phenomena where bacteria evolve to resist the effects of antibiotics.[6]

1.2. Historical Evolution of Antibiotics

One of the most revolutionary chapters in medical history is the one about antibiotics. It marks the transition from a time when small illnesses might easily take lives to a time when medicine had a dependable defense against numerous bacterial diseases. A series of ground-breaking discoveries along the way have drastically changed the course of medicine. The history of antibiotics has been a fascinating story of human ingenuity, from the accidental discovery of penicillin through the purposeful search and development of new antibiotic classes.[7,8]

Discovery of the First Antibiotic: Penicillin

Few scientific advancements in medical history has been as impactful as the discovery of penicillin. The late summer of 1928 marked the beginning of our journey into the world of antibiotics. Sir Alexander Fleming, a Scottish bacteriologist working at St. Mary's Hospital in London, discovered something peculiar. Staphylococcus bacteria were being grown on one of the culture plates he had left on his lab work bench, but the plate had been contaminated with mold. It's interesting to note that the bacterial colonies were dissolving around this mold, which was later determined to be *Penicillium notatum*. Surprisingly, the mold appeared to inhibit the growth of bacteria. According to Fleming's early observations, the mold may have released a chemical that repelled the bacteria away. He named this substance "penicillin." Fleming famously remarked, "When I woke up just after dawn on September 28, 1928, I certainly didn't plan to revolutionize all medicine by discovering the world's first antibiotic, but I suppose that was exactly what I did."[9–12]

Even though the discovery was significant, it took some effort to turn this raw mold extract into a useful medicine. By accident, Fleming discovered penicillin, which he extracted in 1929. Although he recognized its potential as an antibacterial agent, it was Howard Florey, Ernst Boris Chain, and their team at the University of Oxford who later picked up his work during the World War II era, conducting further research and eventually leading to the mass production of penicillin by the 1940s. The Nobel Prize in Physiology or Medicine was awarded to them for their collaborative work on penicillin in 1945. Penicillin began to be mass-produced in the middle of the 1940s and its success in treating a variety of bacterial infections earned it the title of a "miracle drug."[2,10,11]

Major Milestones in Antibiotic Development

Following the discovery of penicillin, the 20th century witnessed a "golden era" of antibiotic discovery. The decades that followed saw a surge in the discovery and development of new antibiotics:

- **Streptomycin (1943):** The first antibiotic shown to be effective against tuberculosis was streptomycin, which Albert Schatz and Selman Waksman isolated from the bacteria *Streptomyces griseus*. Waksman, who coined the term "antibiotic," was awarded the Nobel Prize in Physiology or Medicine in 1952 for this discovery. The discovery of streptomycin prompted the start of a more extensive investigation into soil bacteria as a valuable source of new antibiotics.[13]

- **Tetracyclines (1945):** The antibiotics tetracyclines developed from research conducted by Benjamin Duggar on the soil bacterium *Streptomyces aureofaciens*. Because of their broad-spectrum action against both Gram-positive and Gram-negative bacteria,

tetracyclines have become widely used in the treatment of a variety of infections, including respiratory tract infections and acne.[14]

- **Chloramphenicol (1947):** Another product of soil bacteria, chloramphenicol was widely used in the 1950s and 1960s but later faced restrictions due to rare but severe side effects.[15]
- **Cephalosporins (1948):** Italian scientist Giuseppe Brotzu isolated this from the marine fungus *Cephalosporium acremonium*. Like penicillin, cephalosporins interfere with bacterial cell wall synthesis but are often resistant to bacterial enzymes that inactivate penicillin.[16]
- **Macrolides (1952):** Erythromycin, the first macrolide antibiotic, was introduced as a safer and more effective alternative to penicillin. It laid the foundation for other macrolides like azithromycin and clarithromycin.[17]
- **Quinolones (1962):** The discovery of nalidixic acid heralded the age of synthetic antibiotics. This class eventually led to the development of fluoroquinolones, powerful broad-spectrum antibiotics.[18,19]
- **Glycopeptides (1980s):** Vancomycin, a representative of this class, emerged as a potent weapon against MRSA (Methicillin-Resistant *Staphylococcus aureus*), a formidable superbug.[20]

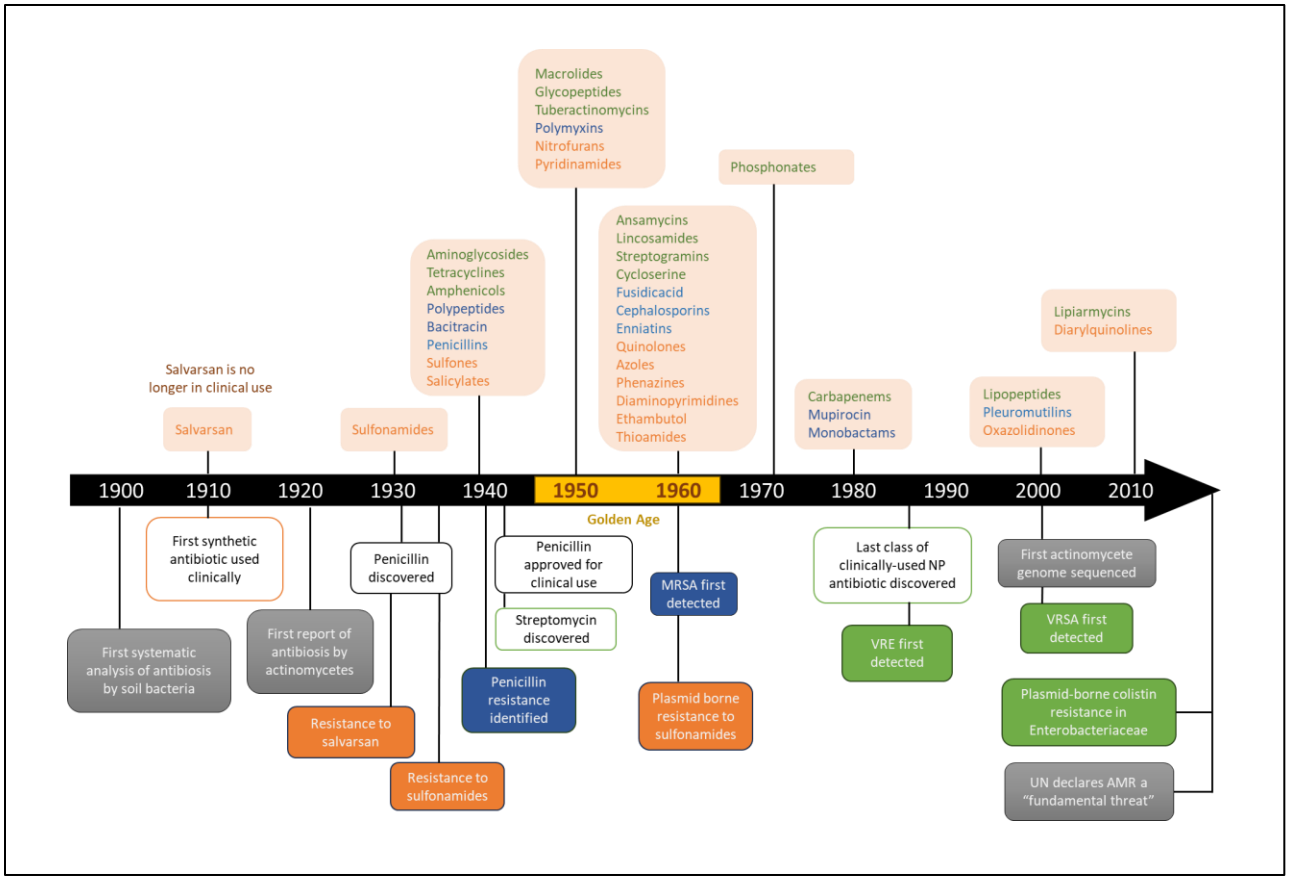


Figure 1-1. Timeline depicting when new classes of antibiotic reached the clinic. Antibiotics are colored per their source: green = actinomycetes, blue = other bacteria, purple = fungi and orange = synthetic. At the bottom of the timeline are key dates relating to antibiotic discovery and antimicrobial resistance, including the first reports of drug resistant strains methicillin-resistant S. aureus (MRSA), vancomycin-resistant enterococci (VRE), vancomycin-resistant S. aureus (VRSA) and plasmid-borne colistin resistance in Enterobacteriaceae. (Figure was adapted from Hutchings, M. I.; Truman, A. W.; Wilkinson, B. Antibiotics: Past, Present and Future. *Curr Opin Microbiol* **2019**, *51*, 72–80.)

1.3. Significance of Antibiotics in Modern Medicine

Modern medicine entered a new era with the discovery of antibiotics. Surgical procedures became far safer, and bacterial infections that were once fatal became treatable. Antibiotics have proven to be essential tools in the modern healthcare system because, in addition to their direct use in treating bacterial infections, they have facilitated breakthroughs in various fields of medicine.

Prevention and Treatment of Bacterial Infections: Before the discovery of antibiotics, bacterial infections such as syphilis, tuberculosis, or even a simple streptococcal throat infection could cause serious complications and in some cases result in death.[21] The introduction of antibiotics caused a significant change. Life-saving Treatment became possible; diseases such as pneumonia, which once had high mortality rates, became treatable. Antibiotics like penicillin could effectively combat the causative bacteria, drastically reducing fatalities.[22] Furthermore, diseases that once caused widespread outbreaks, such as diphtheria and whooping cough, became controllable, due to the curative and preventive powers of antibiotics.[23,24] Additionally, antibiotics have a preventive function in addition to a therapeutic one, particularly for patients with compromised immune systems or those susceptible to bacterial infections. This preventive measure is frequently used prior to certain surgeries, during dental procedures, and in the management of rheumatic fever.[25,26]

Role in Surgeries, Cancer Treatment, and Other Medical Procedures: The role of antibiotics goes well beyond just preventing infections. They play a crucial role in facilitating different medical procedures. In surgeries, especially those involving implants like joint replacements or heart valves, carry inherent infection risks. Antibiotics are administered before, during, and sometimes after surgeries to mitigate these risks, ensuring that the body's exposure to potential

bacterial contaminants is adequately encountered. This practice has significantly reduced post-operative infection rates, enhancing the success rate of surgeries.[27] In cancer Treatment, chemotherapy tends to weaken the immune system, leaving patients more vulnerable to infections. During the cancer treatment phase, antibiotics are essential in preventing opportunistic bacterial infections, which makes the therapy less stressful for the patient.[28] Transplant recipients, who take immunosuppressants to prevent organ rejection, have weakened immune systems that make them more susceptible to infections. Antibiotic administration is standard procedure to protect these patients from bacterial threats.[29,30] Additionally, antibiotics play a vital role in the treatment of secondary bacterial infections that can result from certain medical conditions or treatments. For example, viral diseases such as influenza may serve as a gateway for a bacterial infection to occur in the lungs, which could result in bacterial pneumonia. Antibiotics become vital for treating these kinds of secondary complications.[31]

1.4. Emergence of Antibiotic Resistance

Antibiotic resistance, an alarming global health concern, is the ability of bacteria to withstand the effects of antibiotics that were once effective in eliminating them. Our ability to cure common infections is compromised by this resistance, which raises the risk of extended illnesses, higher mortality rates, and a large increase in healthcare expenses. Comprehending the complexities of antibiotic resistance's emergence, history, and mechanisms is crucial for formulating effective strategies against it. [32–34]

Brief Overview of Resistance

Fundamentally, antibiotic resistance is a natural phenomenon. When bacteria are exposed to antibiotics, those that survive the drug's effects because of mutations or acquisition of resistance

genes propagate, leading to a population of antibiotic-resistant bacteria. Although this evolution might be inherent, human actions like as the overuse of antibiotics intensify and accelerate the process, creating serious problems and challenges for public health. [34]

Historical Emergence and Its Implications

The history of antibiotic resistance is nearly as old as the history of antibiotics themselves. In the 1940s, *Staphylococcus aureus* developed resistant strains shortly after penicillin was first introduced. This sudden rise in resistance served as an alarming indication of the fragile effectiveness of antibiotics.[35] Methicillin, introduced in 1960 as a solution to penicillin-resistant *S. aureus*, also quickly developed resistance, leading to the emergence of the notorious Methicillin-Resistant *Staphylococcus aureus* (MRSA).[36] In the decades following methicillin's introduction, a domino effect was witnessed, with resistance emerging for multiple classes of antibiotics, including cephalosporins, aminoglycosides, and fluoroquinolones.[37]

The implications of antibiotic resistance are severe and multifaced. Resistant infections often result in higher mortality rates. For example, patients with MRSA infections are estimated to be 64% more likely to die than those with a non-resistant form of the infection.[38] Furthermore, resistant infections lead to prolonged illnesses, extending hospital stays and necessitating the use of alternative, sometimes less effective, treatments. Additionally, resistant infections increase healthcare costs due to longer durations of illness, more intensive medical care, and the need for costlier medications.

Mechanisms of Antibiotic Resistance

Bacteria employ various strategies to evade the effects of antibiotics. Some bacteria produce enzymes that can modify or degrade the antibiotic, making them ineffective. Beta-lactamases, for

instance, break down penicillin and related drugs.[39] Additionally, bacteria might alter the structure of a protein targeted by an antibiotic, preventing the antibiotic from binding to the protein. A classic example is the modification of ribosomes to resist erythromycin and related antibiotics.[40] Bacteria can also decrease the intracellular concentration of an antibiotic by reducing its uptake or increasing its expulsion. Efflux pumps, present in many resistant bacteria, actively pump out antibiotics from the bacterial cell.[41] Furthermore, if an antibiotic targets a particular metabolic pathway, resistant bacteria might find an alternative pathway to carry out the same function, effectively bypassing the antibiotic's effects.[42] Antibiotic resistance is a prime example of the complex dance between human ingenuity and nature's adaptability. Understanding the origins and mechanisms of resistance becomes essential as we deal with its consequences.

1.5. Global Impact of Antibiotic Resistance

Antibiotic resistance, once a phenomenon studied primarily within the confines of microbiology labs, has spread its branches to impact economies, healthcare systems, and public health worldwide. With the world becoming increasingly interconnected, resistance effects are seen not only in isolated instances but also in an avalanche of difficulties that build up over time.[43] The impact on healthcare systems is evident. The emergence of multi-drug resistant organisms means many conventional & standard treatments no longer work. Hospitals struggle with a rise in healthcare-associated diseases, which were once easily curable but are now becoming fatal. Patients with infections resistant to antibiotics frequently need longer hospital stays as well as more complex, invasive operations, which puts further strain on the already overwhelmed healthcare systems. With first-line drugs becoming ineffective, there is a growing reliance on

second-line drugs. These alternatives might be less effective, have more side effects, and typically are more expensive.[44,45]

The economic consequences of antibiotic resistance are significant.[46,47] Resistant infections mean prolonged illnesses, leading to longer absences from work and school, translating to a tangible financial impact on communities and nations. Furthermore, the use of antibiotics in agriculture, especially livestock farming, has implications on food safety and trade. Resistant strains can enter the food chain, posing threats to food security and global trade dynamics.

Public health concerns include the global spread of resistant bacteria which can spread across borders and continents with alarming speed, making containment a significant challenge.[48,49] Resistance also threatens modern medicine's advances, such as chemotherapy and surgeries that rely on antibiotics to prevent infections, potentially reverting us to a time when even minor surgeries were risky.[43] Additionally, resistant infections heighten the risk of mortality. For instance, MRSA (Methicillin-resistant *Staphylococcus aureus*) is estimated to kill more people annually than several combined infectious conditions.[50]

Taken together, even though there is a greater need than ever for new antibiotics, the challenges are complicated and demand for a multifaceted, cooperative strategy to overcome them. One of the most important issues in modern medicine is balancing the urgency of the antibiotic crisis with the ethical, financial, and scientific challenges of drug development. This emphasizes how important it is to develop novel strategies, global collaboration, and innovative incentive models in order to boost antibiotic research and development.

1.6. Major Challenges in Antibiotic Development

The constant evolution of bacteria, coupled with a range of scientific, financial, and regulatory obstacles, has made the journey of antibiotic development increasingly difficult.[32] The complexity of bacterial systems presents significant challenges. Bacteria are highly adaptable and versatile organisms. They are resilient because of their ability for rapid mutation, horizontal gene transfer to acquire resistance genes, and adaptation to the hostile environment. This adaptive nature presents challenges in targeting them without inadvertently promoting resistance mechanisms.[51,52]

Many bacteria can form biofilms - complex, matrix-encased microbial communities. Bacteria are protected from antibiotics by these biofilm shields, which can increase their resistance to the drug 1,000 fold. One major challenge is developing antibiotics that can penetrate and act on these biofilm communities.[52,53] Furthermore, some bacteria, like *Mycobacterium tuberculosis*, can reside inside host cells, shielded from many antibiotics that cannot penetrate the host cell membrane. Targeting these intracellular pathogens without harming the host cells is an extremely challenging task.[54,55]

Financial and time investments add to the complexity. The financial model for antibiotic development is challenging. Unlike medications for chronic conditions consumed daily, antibiotics are typically short-course treatments. This means that even if a company develops a successful new antibiotic, the financial return might not justify the investment, especially given the push for cautious antibiotic use.[56] The development process, from discovery to market, an antibiotic can take over a decade of research and testing. This extended timeline demands significant investments, both in terms of time and resources. While the societal value of a new

antibiotic can be immense, the commercial rewards are often limited. This has led many major pharmaceutical companies to deprioritize or even exit antibiotic research and development.[56,57]

Clinical Trials and Regulatory Challenges further complicate development. Given the potential for widespread use, antibiotics undergo rigorous testing.[58] The gold standard of clinical testing, randomized control trials, raises ethical questions when it comes to testing antibiotics. Is it appropriate to not allow a control group access to a potentially life-saving medicine that could save their lives? Balancing ethical considerations with rigorous scientific testing is complex.[59]

Additionally, resistance can be developed even during the clinical trial phases, potentially making an antibiotic candidate less effective before it even reaches the market.[60] Even after approval, there is a need for continuous monitoring for resistance development. This post-market surveillance is resource-intensive and crucial to ensure the continued efficacy of the antibiotic.[45,60,61]

1.7. The Need for Novel Antibiotics

The search for novel antibiotics isn't just about discovering new compounds; it's about ensuring the continued efficacy and relevance of one of medicine's most potent tools. The rising resistance and a narrowing pipeline for antibiotic development pose a threat to the past successes of antibiotic therapy. The need for innovative antibiotics is greater than ever as the world struggles to address these issues.[22] Despite a plethora of antibiotics available, several infections remain challenging to treat. For instance, certain gram-negative bacterial infections, such as those caused by *Acinetobacter baumannii* or *Pseudomonas aeruginosa*, have limited treatment options due to multi-drug resistance.[62] While broad-spectrum antibiotics have been incredibly useful, they can disturb the normal microbial flora, leading to secondary infections and resistance

development. There's a pressing need for narrow-spectrum agents that precisely target pathogenic bacteria without causing broader microbial disturbances.[32,63] Some older antibiotics, while effective, come with significant toxicity and side effect profiles. The development of safer antibiotics with fewer adverse effects is a priority.[4,64]

The World Health Organization (WHO) has identified antibiotic resistance as one of the top ten global public health threats. Resistant strains can spread across borders and continents, making it a worldwide concern.[65] Organisms such as Methicillin-resistant *Staphylococcus aureus* (MRSA), Vancomycin-resistant *Enterococci* (VRE), and Carbapenem-resistant *Enterobacteriaceae* (CRE) have emerged as significant threats, with few, if any, effective treatment options.[66] The broad use of antibiotics, even when not essential, can cause "collateral damage," leading to the emergence of resistant organisms even in individuals not directly treated with the antibiotics.[67]

Biotechnological advancements are aiding research, offering hope for the future. The age of genomics has transformed antibiotic research. By sequencing bacterial genomes, scientists can identify potential drug targets more efficiently than ever before.[68,69] With the power of artificial intelligence and machine learning, researchers can look through vast libraries of compounds to identify potential antibiotic candidates, significantly speeding up the drug discovery process.[70,71] Only a fraction of natural microbial diversity has been explored for antibiotic potential. Advanced biotechnological tools now allow scientists to investigate previously inaccessible environments, like deep-sea vents or arid deserts, in search of novel antibiotic-producing organisms.[72,73] Custom-building organisms using synthetic biology can lead to entirely new classes of antibiotics. By utilizing the power of biology, scientists can design and produce targeted, potent antibiotics in laboratory settings.[74]

This is a crucial time in the history of antibiotic research because of the convergence of growing resistance, existing treatment gaps, and newly developed biotechnological innovations. There are many obstacles to overcome, but there is hope for the future because of the potential for innovative approaches in the fight against bacterial infections.

1.8. Natural vs. Synthetic Antibiotics

Antibiotics, potent weapons against bacterial infections, can be derived naturally from living organisms or synthesized in laboratories. The distinction between natural and synthetic antibiotics has implications in terms of their properties, advantages, applications, and limitations.

Natural antibiotics are derived from living organisms, typically microorganisms, that produce substances which can inhibit the growth of or kill bacteria. Penicillin (from the fungus *Penicillium*) and Vancomycin (from the bacterium *Streptomyces orientalis*) are examples of natural antibiotics. The advantages of natural antibiotics include a vast biodiversity offering a pool of bioactive molecules with antimicrobial activity, complex structures that may be challenging to reproduce synthetically, and evolutionary testing, indicating these antibiotics have been naturally optimized for their function. However, disadvantages include variable potency and quality, resource-intensive extraction processes, and potential side effects or allergenic properties, such as allergic reactions from echinacea in individuals sensitive to ragweed, heartburn and increased bleeding risk with garlic consumption, allergic reactions to honey, stomach upset from oil of oregano, skin irritation caused by tea tree oil, and digestive upset from goldenseal.[4,75–79]

Synthetic antibiotics are chemically manufactured in laboratories and aren't derived directly from natural sources. Fluoroquinolones (like Ciprofloxacin) and Sulfa drugs (like Sulfamethoxazole)

are examples of synthetic antibiotics. Their advantages include the ability to tailor-make these antibiotics to enhance efficacy or reduce side effects, consistency in potency and quality due to controlled manufacturing conditions, and scalability for large-scale production. Yet, they also face challenges such as being research-intensive to design, the inevitable development of bacterial resistance, and the high cost of research, development, and manufacturing processes.[80–82]

Both natural and synthetic antibiotics find applications across various fields of medicine, from treating infections to prophylactic use in surgeries. The choice between them depends on the infection, patient's health, potential side effects, and other factors.[4]

1.9. Mechanism of Action & Classification of Antibiotics

Antibiotics, as potent agents against bacterial infections, operate through various, complex mechanisms to either kill bacteria or inhibit their growth. The elucidation of these mechanisms has been essential for the development of new antibiotics and the improvement of existing ones. We can more effectively combat bacterial resistance, improve & optimize treatment plans, and direct future research initiatives by having a better understanding of how these agents function.[32,83]

Their diverse range of applications and functionality require a systematic structured classification to guide research, academic endeavors, and clinical decisions. The chemical structure, mechanism of action, and bacterial spectrum serve as the basis for several classification criteria. Each of these criteria will be discussed here in order to provide an understanding of antibiotic classes. A detailed classification was presented in **Table 1-1**.

Classification based on bacterial spectrum includes broad-spectrum antibiotics which are effective against a wide variety of both Gram-positive and Gram-negative bacteria. They are particularly useful when there's an urgent need to treat an infection, and the specific bacterium hasn't been identified. Ciprofloxacin (a fluoroquinolone) and Amoxicillin (a penicillin derivative) are examples of broad-spectrum antibiotics.[63,84,85] Narrow-spectrum antibiotics target specific types or groups of bacteria. Minimal disruption to non-targeted beneficial bacteria and less potential for resistance development. Glycopeptides like vancomycin target specific Gram-positive bacteria, and Polymyxins are particularly effective against Gram-negative bacteria.[63,86,87]

Classification based on chemical structure includes beta-lactams, characterized by a beta-lactam ring, crucial for their antibacterial activity. Examples of beta-lactams are Penicillin, cephalosporins, carbapenems, and monobactams.[39] Tetracycline contains four-ring structure with various side chains determining their specific properties. Doxycycline and minocycline are examples of tetracylines.[88] Macrolides consist of a large lactone ring coupled with sugars. Examples include Erythromycin, azithromycin.[89] Aminoglycosides are composed of amino-modified sugars linked in glycosidic bonds. Gentamicin, streptomycin are examples of aminoglycosides.[37] Quinolones and Fluoroquinolones bear central quinolone structure, with fluoroquinolones having a fluorine atom, imparting increased potency. Examples are Ciprofloxacin, levofloxacin are examples of this class.[84,90]

Antibiotics have been categorized according to a number of distinct characteristics in order to facilitate better understanding, prescription practices, and research projects. Understanding these classes is essential for efficient clinical application and to combat the impending threat of antibiotic resistance, regardless of the range of bacteria they target, the fundamental bacterial process they inhibit, or their inherent chemical structures.

In clinical decision-making, the difference between bacteriostatic and bactericidal antibiotics is crucial. The way that antibiotics work on bacteria determines how this classification is made.

Bactericidal antibiotics are agents that kill bacteria. They cause irreversible damage to vital bacterial components, leading to bacterial death. Examples include penicillin, cephalosporins, and aminoglycosides. For infections where the immune system is compromised or bacterial burden is high, such as in endocarditis or meningitis, bactericidal antibiotics are often preferred.[91,92]

On the other hand, bacteriostatic antibiotics work by inhibiting bacterial growth and replication without necessarily killing the bacteria. They primarily hinder metabolic processes essential for bacterial proliferation. Tetracyclines, sulfonamides, and macrolides are examples of bacteriostatic antibiotics. For infections where the immune system is robust enough to eliminate non-replicating bacteria, bacteriostatic antibiotics might be prescribed.[91–94] It's worth noting that the distinction is not absolute. In certain concentrations or conditions, bacteriostatic antibiotics can act as bactericidal and vice versa.[92]

Mode of Action of Antibiotics

Table 1-1. Antibiotics' classification by the mechanism of action. (This table was adapted from Pancu, D. F.; Scurtu, A.; Macasoi, I. G.; Marti, D.; Mioc, M.; Soica, C.; Coricovac, D.; Horhat, D.; Poenaru, M.; Dehelean, C. Antibiotics: Conventional Therapy and Natural Compounds with Antibacterial Activity-A Pharmaco-Toxicological Screening. *Antibiotics (Basel)* 2021, *10* **4**).

Antibiotic Class		**Target Site**	**Representatives**
β-lactams	Penicillins	Cell wall synthesis	Penicillin G and Penicillin V, Methicillin, Oxacillin, Cloxacillin, Dicloxacillin, Nafcillin, Ampicillin, Amoxicillin, Carbenicillin, Ticarcillin, Mezlocillin, Piperacillin, Azlocillin, Temocillin
	Cephalosporins	Cell wall synthesis	**1st generation**: Cephalothin, Cephapirin, Cephradine, Cephaloridine, Cefazolin **2nd generation**: Cefamandole, Cefuroxime, Cephalexin, Cefprozil, Cefaclor, Loracarbef, Cefoxitin, Cefmetazole **3rd generation**: Cefotaxime, Ceftizoxime, Ceftriaxone, Cefoperazone, Ceftazidime, Cefixime, Cefpodoxime, Ceftibuten, Cefdinir **4th generation**: Cefpirome, Cefepime **5th generation**: Ceftaroline, Ceftobiprole
	Carbapenems	Cell wall synthesis	Imipenem, Meropenem, Doripenem
	Monobactams	Cell wall synthesis	Aztreonam
Macrolides		Protein synthesis inhibitors—Inhibit 50 s subunit	Erythromycin, Azithromycin, Clarithromycin
Tetracyclines		Protein synthesis inhibitors—Inhibit 30 s subunit	Tetracycline, Chlortetracycline, Oxytetracycline, Demeclocycline, Minocycline, Methacycline, Doxycycline, Tigecycline

Antibiotic Class	Target Site	Representatives
Aminoglycosides	Protein synthesis inhibitors—Inhibit 30 s subunit	Streptomycin, Neomycin, Kanamycin, Paromomycin, Gentamicin, Tobramycin, Amikacin, Netilmicin, Spectinomycin, Sisomicin, Isepamicin
Sulfonamides	Folic acid synthesis inhibitors	Prontosil, Sulfonamide, Sulfanilamid, Para-Aminobenzoic Acid, Sulfadiazine, Sulfisoxazole, Sulfamethoxazole, Sulfathalidine
Quinolones	DNA synthesis inhibitors	Nalidixic Acid, Ciprofloxacin, Norfloxacin, Pefloxacin, Enoxacin, Ofloxacin, Levofloxacin, Sparfloxacin, Lomefloxacin, Fleroxacin
Izoniazid	Mycolic acid synthesis inhibitors	
Ansamycin	RNA synthesis inhibitors	Rifampin
Polymycins	Cytoplasmic membrane structure	
Daptomycin	Cytoplasmic membrane structure	

Understanding the diverse mechanisms by which antibiotics function is fundamental for pharmacology and medicinal chemistry. Broadly, these mechanisms can be categorized based on the primary cellular target they act upon.

Cell wall synthesis inhibition involves targeting the synthesis and cross-linking of the bacterial outer cell wall made of peptidoglycan, leading to osmotic lysis. Penicillins and cephalosporins inhibit enzymes responsible for cross-linking the peptidoglycan layers, while vancomycin hinders the elongation of peptidoglycan chains.[95,96]

Protein synthesis inhibition targets bacterial ribosomes, distinct from eukaryotic ribosomes, to prevent protein synthesis, a critical process for bacterial survival and proliferation.[97,98] Aminoglycosides interfere with the 30S ribosomal subunit, causing misreading of mRNA.[99] Tetracyclines prevent the attachment of aminoacyl-tRNA to the 30S subunit,[100] while macrolides and chloramphenicol act on the 50S ribosomal subunit, inhibiting peptide bond formation.[101,102]

Nucleic acid synthesis inhibition interferes with the replication and transcription processes of bacteria.[4,42] Fluoroquinolones like ciprofloxacin inhibit bacterial DNA gyrase and topoisomerase IV, while rifampicin hinders RNA synthesis by binding to the bacterial RNA polymerase.[42,103,104]

Metabolic pathway interference involves antibiotics mimicking bacterial substrates to inhibit critical metabolic pathways .[91,105] Sulfonamides, structural analogs of para-aminobenzoic acid (PABA), compete with PABA to inhibit dihydropteroate synthase, blocking folic acid synthesis and, consequently, DNA synthesis.[106,107]

Cell membrane disruption is achieved by some antibiotics integrating into bacterial cell membranes, creating pores that disrupt cellular homeostasis.[108] Polymyxins bind to the lipopolysaccharides and phospholipids in the bacterial outer membrane, leading to increased permeability and cell death.[109,110]

With the mounting challenge of antibiotic resistance, understanding these mechanisms in-depth becomes even more crucial. Such knowledge not only informs clinical decisions but also fuels the discovery and design of the next generation of antibiotics.

1.10. Biotin Biosynthesis Pathway and its Importance

Biotin, commonly known as Vitamin B7, is an essential water-soluble micronutrient required by all living organisms, although not all organisms can synthesize it. In humans, biotin plays a vital

role in cell growth, fatty acid synthesis, and the metabolism of carbohydrates, fats, and proteins. Bacterial biotin biosynthesis, in particular, has gained significant attention due to its role in the growth and survival of various bacterial species, and its potential as a target for antibacterial drug design.[111–114]

Biotin Biosynthesis Pathway in Bacteria

The biotin biosynthesis pathway in bacteria can be divided into two stages. The first stage culminates in the synthesis of pimeloyl-CoA, a seven-carbon dicarboxylic acid intermediate. In *Escherichia coli* (*E. coli*), a model organism for studying this pathway, pimeloyl-CoA is derived from malonyl-CoA through a series of enzymatic reactions. However, the exact origin of the pimeloyl moiety varies among bacteria and can sometimes be sourced from alternative pathways.[115–118]

The second stage of biotin biosynthesis involves the conversion of pimeloyl-CoA to biotin. This stage is universally conserved across biotin-producing organisms. The enzymes BioF, BioA, BioD, and BioB play pivotal roles in these conversion steps. BioF catalyzes the formation of 7-keto-8-aminopelargonic acid (KAPA) from pimeloyl-CoA. BioA then converts KAPA to 7,8-diaminopelargonic acid (DAPA), which is further acted upon by BioD to yield dethiobiotin. Finally, BioB introduces a sulfur atom into dethiobiotin to produce the final biotin molecule.[119,120]

The essentiality of biotin in numerous biological processes highlights its significance. In its active form, biotin is bound to proteins, creating biotinylated enzymes. These enzymes, called carboxylases, participate in various metabolic reactions, including gluconeogenesis, fatty acid synthesis, and amino acid catabolism. The carboxylation reactions catalyzed by biotinylated

enzymes are crucial to many organisms. For instance, Acetyl-CoA carboxylase, a biotinylated enzyme, is responsible for the committed step in fatty acid synthesis. Another enzyme, pyruvate carboxylase, is pivotal in gluconeogenesis.[121,122] Given the importance of the biotin biosynthesis pathway in bacteria and its absence in humans, it represents an attractive target for antibacterial drug discovery. Inhibitors designed against enzymes in this pathway, especially those that don't have human homologs, can be highly selective, potentially minimizing side effects.[113,123,124] The fact that many bacteria, especially pathogenic strains, rely on their biotin biosynthesis (as opposed to obtaining it from their environment) underscores the pathway's significance as a therapeutic target. Furthermore, the differences in the pathway among bacterial species and between bacteria and humans offer opportunities for designing species-specific or broad-spectrum inhibitors.[113]

Biotin biosynthesis, while a fundamental and conserved process, showcases variations across different organisms. Its central role in many metabolic reactions and the unique aspects of its synthesis in bacteria make the pathway an area of intense study. For researchers, especially those seeking novel antibacterial strategies, understanding this pathway offers promising solutions for therapeutic intervention.

Because biotin synthesis occurs exclusively in plants and microorganisms, enzymes involved in this pathway represent potential targets for creating antimicrobial drugs and herbicides. Inhibitors of these enzymes have been identified as either synthetic mechanism-based inhibitors or natural products. Notably, compounds from *Streptomyces* species that block DAPA synthase have been identified, with amiclenomycin being particularly potent against mycobacterial strains.[125–128]

1.11. Acetohydrazide Derivatives: A Novel Approach

The field of drug discovery presents both numerous possibilities and significant challenges. Researchers who explore the complex field of medicinal chemistry frequently look for compounds with both desirable pharmacokinetic and pharmacodynamic profiles and therapeutic benefits. Acetohydrazide derivatives, in particular MAC13772, have recently become molecules of great interest within this vast chemical space.[129]

Acetohydrazide derivatives belong to a class of organic compounds characterized by the presence of a hydrazide functional group attached to an acetyl moiety. This specific configuration lends these molecules certain properties that make them attractive candidates in drug design and synthesis. MAC13772 is one of the many compounds that belongs to the class of acetohydrazides.

Because of its biological activity, particularly against specific bacterial targets, this molecule has attracted immense attention. However, as with many compounds in early-stage research, MAC13772's potential is twinned with challenges that drive scientists to further refine and improve upon its design.[129]

MAC13772, while notable for its potency, is not without its limitations. Preliminary studies have showcased its activity against specific bacterial strains, likely by interfering with vital bacterial metabolic processes. Yet, as promising as these initial findings might be, MAC13772 does present some potential pitfalls. Some of these could relate to stability, solubility, or off-target effects. Moreover, like many drugs in their nascent stages, the pharmacokinetic properties of MAC13772 might not be ideal for therapeutic use. These challenges underscore the need to design analogs that maintain the desired activity while overcoming the molecule's inherent shortcomings.[129]

To optimize MAC13772, a comprehensive Structure-Activity Relationship (SAR) analysis was conducted. This approach, fundamental to medicinal chemistry, involves making systematic modifications to a molecule and then evaluating the biological activity of each modified version. For MAC13772, this study led to the synthesis of 24 distinct analogs. The goal was simple yet ambitious to identify an analog that surpassed the parent molecule in terms of efficacy, stability, and overall drug-likeness.[129]

However, the results of this SAR study were both enlightening and challenging. While the synthesis of 24 analogs provided a wealth of data and insights into the molecule's chemical behavior, it was found that none of the analogs were superior to MAC13772. The findings indicated that certain moieties, like the hydrazine group, were vital for the molecule's activity. Similarly, the aryl ring appeared to be crucial for target specificity.[129]

The SAR study's outcome, while not yielding a superior analog, provided critical insights that could shape further research. The absence of a superior analog underscores the delicate balance between potency, specificity, and drug-likeness. It also emphasizes the challenge that lies ahead: to either refine the existing analogs or consider a novel approach in the molecular design.[129]

Taken together, acetohydrazide derivatives, particularly MAC13772, represent a promising frontier in drug design. While the journey to optimizing this class of compounds is time consuming with challenges, there are also many potentials. Every SAR study, regardless of its immediate outcomes, advances our understanding of molecular interactions, guiding researchers closer to the next breakthrough in therapeutic design.

1.12. Conclusion of the Introduction

As science and technology advanced, researchers began using rational drug design, bioinformatics, and other novel methods to identify and develop new antibiotics. The evolving understanding of bacterial genomics and proteomics provided further insights, leading to targeted therapies and precision medicine approaches.[130–132] While the initial discoveries were serendipitous, they set the stage for systematic investigations, leading to a diverse range of antibiotics. However, with the emerging challenge of antibiotic resistance, it's evident that this journey is far from over. In addition to teaching us about our past successes, the advancement of antibiotics underscores the necessity of ongoing innovation and alertness in the field of infectious diseases.[5,6]

One strategy to address the crisis of antibiotic resistance is to target previously unexplored bacterial pathways or enzymes, which can diminish the likelihood of pre-existing resistance in bacterial populations. In this context, the enzyme 7,8-diaminopelargonic acid synthase (BioA) has emerged as a promising target. Its role in biotin biosynthesis, an essential vitamin for bacterial growth, offers a novel route for antibiotic development. Initial studies, as will be elaborated in this thesis, have pointed towards synthetic acetohydrazides like MAC-13772 as potent inhibitors of BioA. However, there are inherent challenges with every early endeavor. The pharmacokinetic limitations of MAC-13772, primarily its limited bioavailability *in vivo*, drives researchers for further exploration in search of more potent and stable analogs.

In order to shed light on novel acetohydrazide derivatives, this thesis explores the world of synthetic chemistry. These molecules, termed as MAC13772 analogs, have been synthesized with an aspiration to enhance their potency and stability, thus elevating their potential as effective antibacterial agents against Gram-negative pathogens. The following chapters will

unfold the journey of designing, synthesizing, and testing these analogs, revealing the challenges encountered, and the insights learned from the experiments. As we transition from understanding the grand challenge of antibacterial resistance to the microscopic world of molecules, it's a testimony to the interplay of biology and chemistry in our continuous dedication to medical advancement.

1.13. Plan of Study, Research Objectives & Organization of Thesis

Many current antibiotics are becoming less effective over time, highlighting the urgent need to discover new compounds to address this challenge. Targeting the role of biotin in bacterial growth presents a promising approach. Inhibiting its production can effectively curb bacterial growth. In this context, the synthetic acetohydrazide MAC-13772 has emerged as a potential candidate. However, its limitations, particularly, especially concerning its pharmacokinetic profile, potency, and stability.

My research focuses on the synthesis and structural optimization of MAC13772 analogs to enhance their antibacterial efficacy against Gram-negative pathogens. Guided by several hypotheses, my M.Sc. work seeks to:

1. **Structure-Activity Relationship of MAC13772 Analogs:** Explore specific structural modifications of MAC13772 to enhance its antibacterial efficacy. These include alterations in the aryl ring, replacement of the thioether group, and variations in the alkyl chain length between sulfur and acetohydrazide moieties. The goal is to identify analogs with improved potency and stability compared to MAC13772.
2. **Metabolic Stability Enhancement:** Improve the metabolic stability of MAC13772 analogs to enhance their *in vivo* efficacy and safety profile. By increasing the half-life in

biological systems, we aim for more sustained antibacterial activity and reduced dosing frequency. Focus will be on synthesizing analogs, like the benzimidazole derivative, that show increased resistance to metabolic degradation, especially in liver microsomes.

3. **Synergistic Interaction with Biological Targets:** Investigate how newly synthesized MAC13772 analogs interact synergistically with the BioA enzyme. This interaction is hypothesized to lead to more effective bacterial growth inhibition. The chemical structure of the analogs is anticipated to play a crucial role, potentially forming tighter complexes with cofactors like pyridoxal-5'-phosphate.

To validate these hypotheses, Structure-Activity Relationship (SAR) approaches were employed to synthesize MAC-13772 analogs. This synthetic strategy, based on the original MAC-13772 molecule, optimizes conditions, materials, and steps to create improved analogs. The focus is on modifications such as multiple simultaneous substitutions on the aryl ring, ring replacement, substitutions of the thioether, and variations in the alkyl chain length between sulfur and acetohydrazide moieties.

Our biologist collaborator conducted enzymatic assays to evaluate the efficacy of these new analogs. These assays target *E. coli* BioA (*Ec*BioA) and *A. baumannii* (*Ab*BioA), measuring potency through parameters like IC_{50} values. The study also includes whole-cell activity assessments through bacterial susceptibility testing in biotin-free and biotin-containing media, particularly focusing on wild-type *A. baumannii* and various *E. coli* strains.

Moreover, the metabolic stability of these compounds was examined with a particular focus on the benzimidazole analog. *In vitro* assays in Rat Liver Microsomes (RLMs) have provided insights into metabolic stability, revealing promising data about compound **60** and its improved

half-life. Further, *in vivo* assessments using a murine model have demonstrated the efficacy of these compounds in real-world scenarios, with compound **60** showing notable performance.

CHAPTER 2: SYNTHESIS OF ACETOHYDRAZIDE DERVATIVES

2.1. Review of MAC13722 Project:

2.1.1. Small molecule metabolites

Introduction to Small Molecules in Biological Systems

Small organic molecules play a crucial role in biological systems, often serving as the foundation for various cellular functions. These molecules are crucial in numerous biological processes, from acting as substrates and intermediates in metabolic pathways to functioning as signal transmitters or inhibitors in cellular communication. Their relatively small size and diverse structural capabilities make them versatile tools in modulating and probing biological systems.[133]

Role and Significance in Cellular Functions

Small molecules are fundamental in driving numerous biochemical reactions within cells. They often act as cofactors for enzymes, facilitating essential biochemical transformations. In metabolic pathways, they serve as crucial intermediates, carrying and transferring chemical groups between different enzymatic reactions. Beyond metabolism, small molecules like neurotransmitters play a key role in cell signaling, transferring information across synapses in the nervous system.[133]

Advantages as Biological System Perturbants

Small molecules' versatility makes them ideal candidates for probing and manipulating biological systems. Their ability to easily permeate cell membranes allows them to interact with intracellular targets. This characteristic is particularly advantageous in drug design, where the goal is to affect internal cellular processes. Moreover, the structural diversity of small molecules

provides a broad spectrum of activity, enabling the modulation of a wide range of biological targets, from enzymes to receptors.[134]

2.1.2. Usage of High-Throughput Screening for Antibacterial Drug Discovery: Targeting Bacterial Biosynthetic Pathways

The search for biologically active small molecules has been revolutionized by advancements in high-throughput screening (HTS) technologies. These techniques allow for the rapid testing of thousands to millions of compounds for biological activity against a specific target or in a particular cellular assay. HTS has become an invaluable tool in drug discovery, enabling the identification of potential drug candidates at an unprecedented pace. Coupled with computational methods like virtual screening and machine learning, HTS has vastly expanded the potential for discovering new small molecules with therapeutic benefits.[135,136]

Targeting bacterial vitamin biosynthetic pathways offers a promising direction in antibacterial therapy. These pathways are essential for all life forms, yet are uniquely carried out by microorganisms and plants, with most steps absent in human physiology. This distinction was first exploited in the development of the synthetic antibiotic sulfonamide, a *p*-aminobenzoic acid analog targeting folate biosynthesis. Combination therapies, such as sulfamethoxazole and trimethoprim, both inhibiting folate biosynthesis, remain effective against respiratory and urinary tract infections due to this targeted approach. Furthermore, bacteria impaired in biotin biosynthesis cannot be rescued by the human host, given the low biotin levels in human serum.

When cultured in minimal media, bacteria exhibit significant metabolic shifts to enable the de novo synthesis of amino acids, vitamins, and other cofactors. For instance, wild-type *E. coli*

requires only 303 genes for growth in rich medium, but an additional 119 genes become essential in nutrient-limited conditions (**Fig. 2-1**).

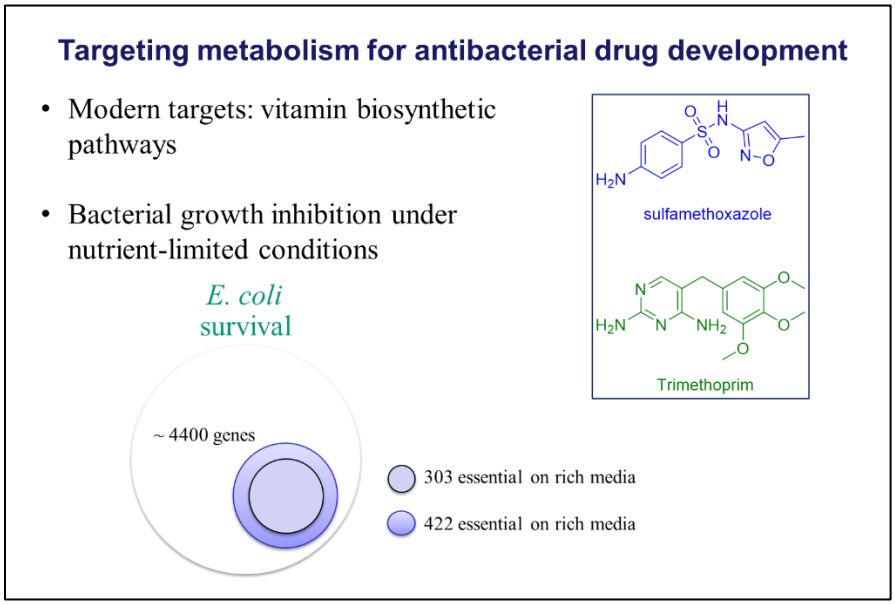


Figure 2-1. Genetic Requirements for *E. coli* Growth in Nutrient-Rich vs. Nutrient-Limited Media. (This figure was adapted from Zlitni, S., Ferrucio, L.F., Brown E.D. *Nature Chemical Biology* ***2013***, 9:796)

This highlights the potential of small molecules that target bacteria under nutritional stress as both mechanistic probes and potential leads for new antimicrobial agents. Such molecules could illuminate biological responses to nutritional stress while also offering pathways for novel therapeutic development.[129]

The Brown's group initiated their study with a high-throughput screening, aiming to identify compounds inhibiting *E. coli* MG1655 growth in nutrient-deficient media. They utilized a library of approximately 30,000 molecules, which included a diverse array of synthetic molecules, off-patent FDA-approved drugs, pharmacologically active compounds, and natural products. Their screening yielded 496 compounds that demonstrated growth inhibition in minimal media. After

excluding known antibiotics, 340 novel active compounds were subjected to dose-response studies in both minimal and supplemented minimal media. This step was crucial to pinpoint potent inhibitors with specificity towards bacterial growth in nutrient-limited conditions. Following this, 74 of these actives were selected for a more detailed analysis (**Fig. 2-2**).

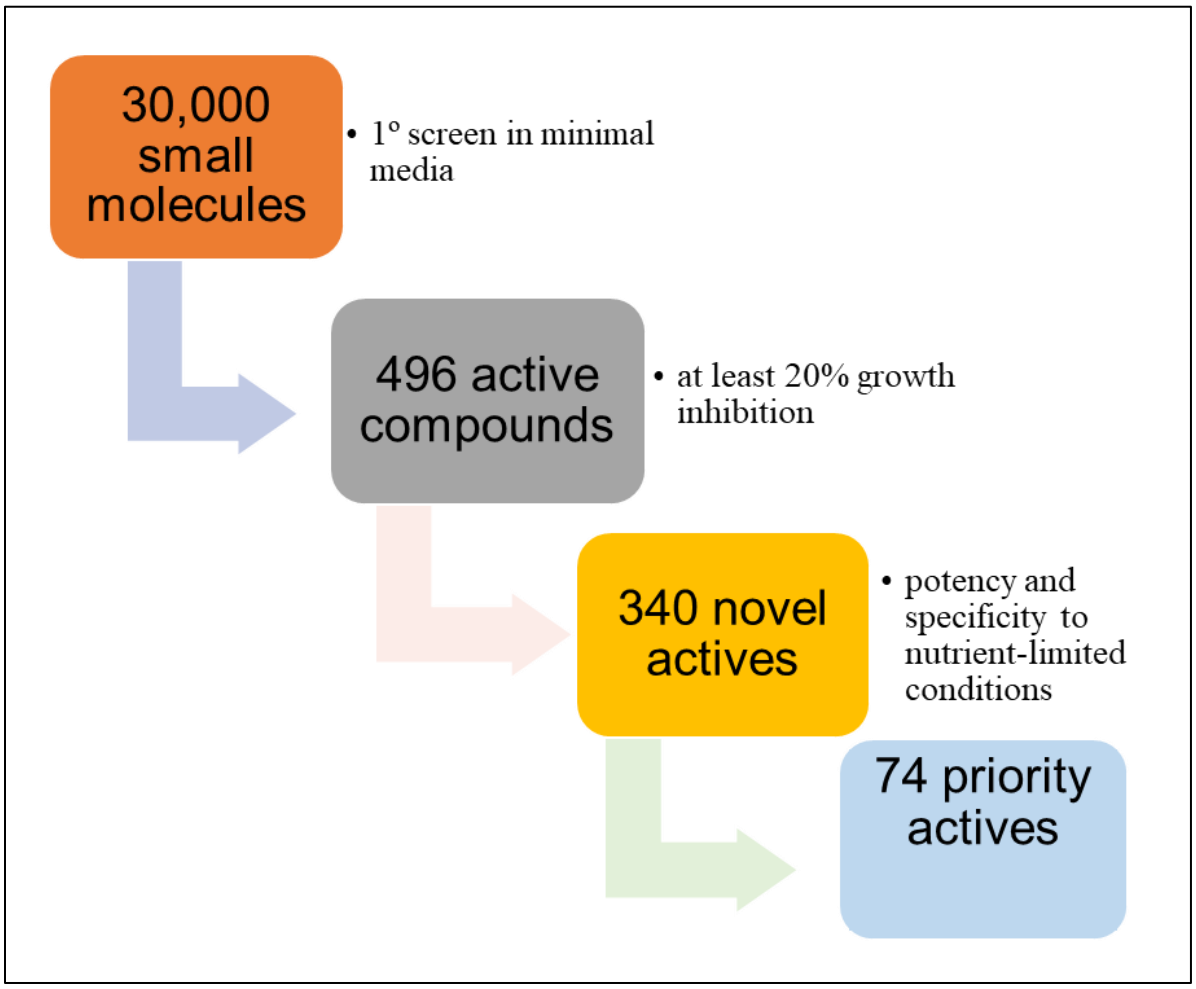


Figure 2-2. General approach to characterize inhibitors of bacterial physiology under nutrient-limited growth conditions. (This figure was adapted from Zlitni, S., Ferrucio, L.F., Brown E.D. *Nature Chemical Biology* ***2013***, 9:796)

They underwent metabolic suppression profiling against primary metabolites to ascertain the potential target pathways impacted by these inhibitors. This thorough approach helped narrow

down the search to compounds with specific antibacterial activity under defined conditions, facilitating the identification of promising candidates for further investigation.

In 2013, the research team under Prof. Brown announced the discovery of three novel antibacterial compounds, each targeting different metabolic pathways. These discoveries include MAC168425, found to disrupt glycine metabolism; MAC173979, identified as a time-dependent inhibitor of p-aminobenzoic acid (PABA) biosynthesis; and MAC13772, which acts as an inhibitor of the enzyme BioA (**Fig. 2-3**).

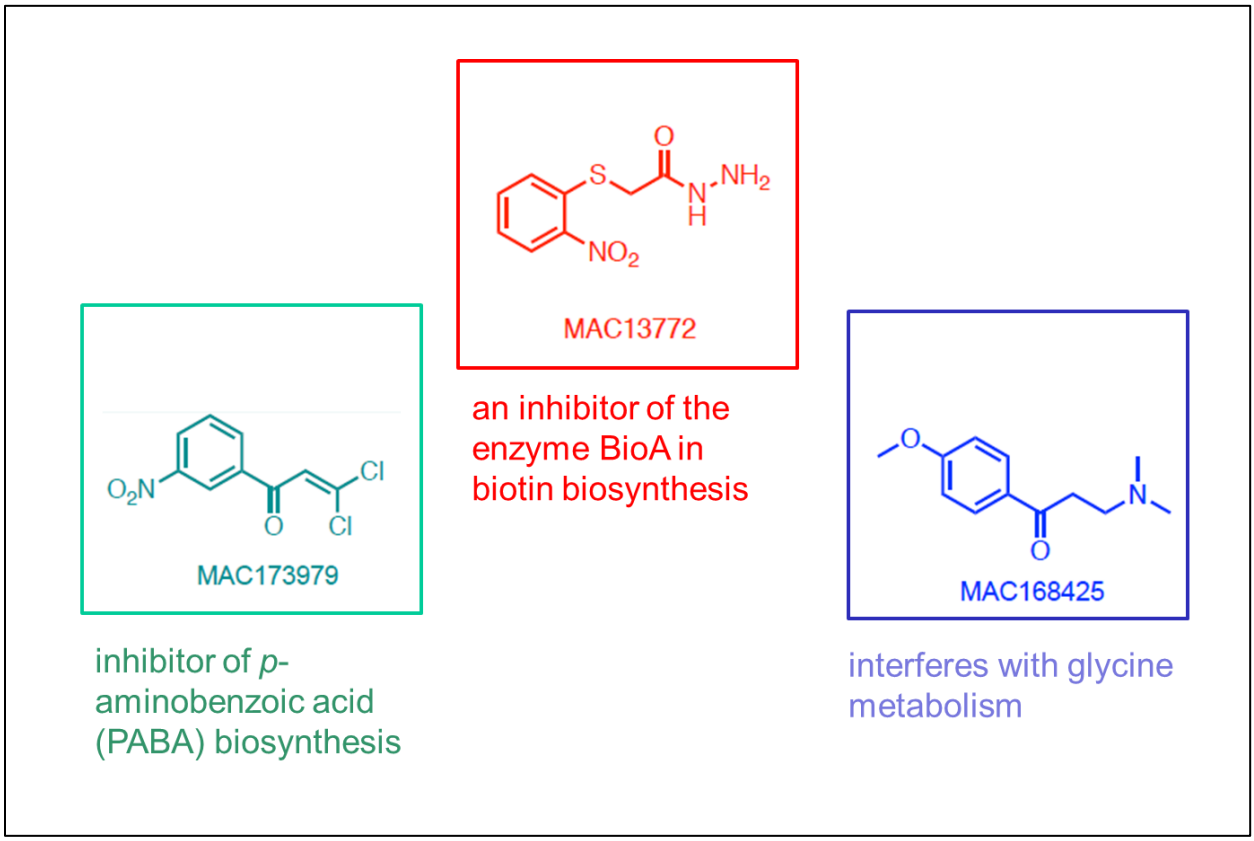


Figure 2-3. Novel Antibacterial Compounds Discovered by Prof. Brown's Research Team. (This figure was adapted from Zlitni, S., Ferrucio, L.F., Brown E.D. *Nature Chemical Biology* ***2013***, 9:796)

BioA is crucial in the antepenultimate step of biotin biosynthesis, a vital process in bacterial growth and survival. These compounds represent significant advancements in the field of antibacterial drug development, each offering unique mechanisms of action against bacterial metabolic processes.[129]

Brown's group elucidated the complex interaction between MAC13772 and BioA, pivotal for bacterial biotin biosynthesis, through detailed structural analysis. Utilizing ribbon diagrams, they visualized the dimeric structure of BioA, highlighting the identical monomers and the MAC13772-PLP adduct within each (**Fig. 2-4**).

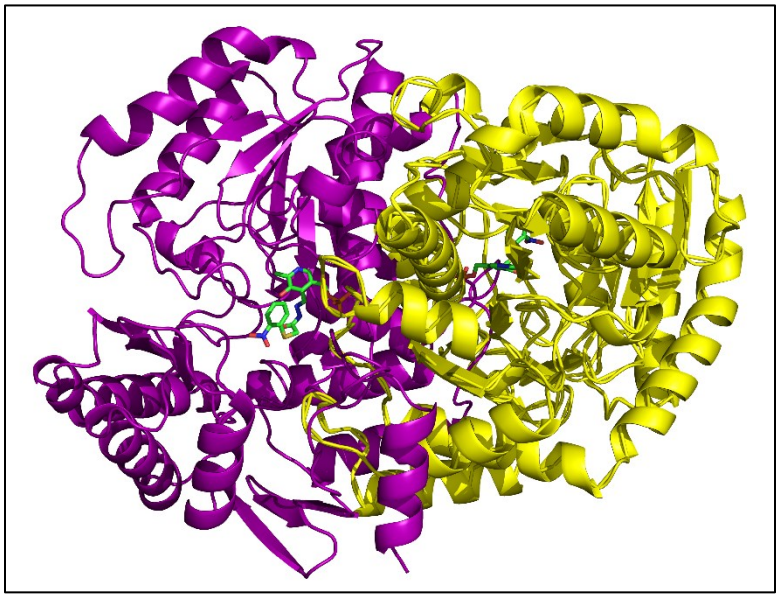


Figure 2-4. Structure of BioA bound to MAC13772. Ribbon diagram illustrating the BioA dimer formed by two identical monomers shown in yellow and purple; MAC13772-PLP is shown in stick form inside both monomers. (This figure was adapted from Carfrae, L.A., MacNair, Zlitni, S., C.R., Brown, C.M. *et al. Nat Microbiol 2020*, **5**, 93–101)

Comparative analysis with PLP-bound BioA, both in the absence and presence of MAC13772, revealed significant insights into active site architecture and the inhibitor's mechanism of action (**Fig. 2-5)**.

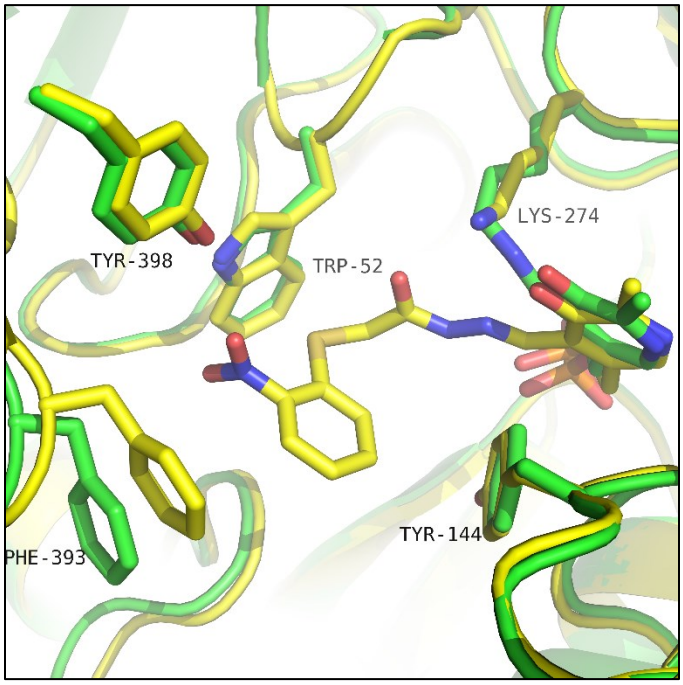


Figure 2-5. Active site region of BioA. Ribbon diagram showing the superimposition of PLP-bound BioA in the absence (green ribbon and stick; pdb code 1QJ5) and the presence of MAC13772 (yellow ribbon and stick). The MAC13772-PLP complex is shown in yellow stick. Residues close to the MAC13772-PLP adduct are annotated. (This figure was adapted from Carfrae, L.A., MacNair, Zlitni, S., C.R., Brown, C.M. *et al. Nat Microbiol 2020*, **5**, 93–101)

This examination underscored the detailed molecular interactions and the critical residues within proximity to the MAC13772-PLP complex, providing an understanding of its inhibitory effect (**Fig. 2-6)**.

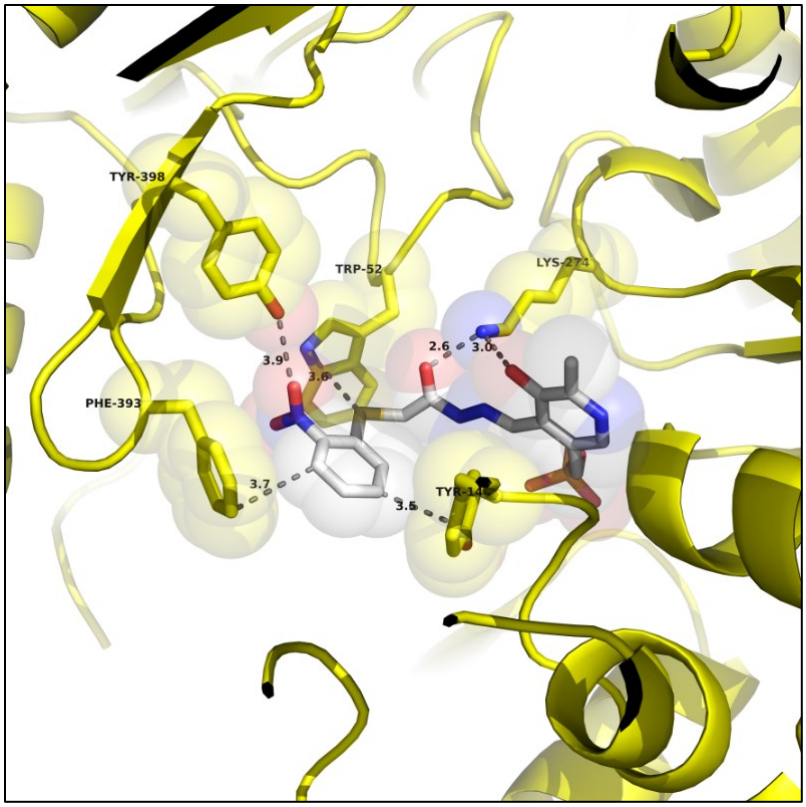


Figure 2-6. Structure of the active site region of BioA bound to MAC13772. Active site of a BioA monomer showing the MAC13772-PLP adduct (grey stick). Residues within ~4 Å radius are shown in yellow stick and their interactions with the PLP-inhibitor adduct are shown in dashed lines. (This figure was adapted from Carfrae, L.A., MacNair, Zlitni, S., C.R., Brown, C.M. *et al. Nat Microbiol 2020*, **5**, 93–101)

In this study by the Brown's group, the role of MAC13772 as an inhibitor of the bacterial enzyme BioA was meticulously investigated. The team focused on understanding the interaction between MAC13772 and BioA by utilizing various biochemical and structural approaches. Their hypothesis centered around the hydrazine moiety of MAC13772 interacting with the pyridoxal-5'-phosphate (PLP) in the enzyme's active site, forming a complex that renders BioA inactive (**Fig. 2-7**).

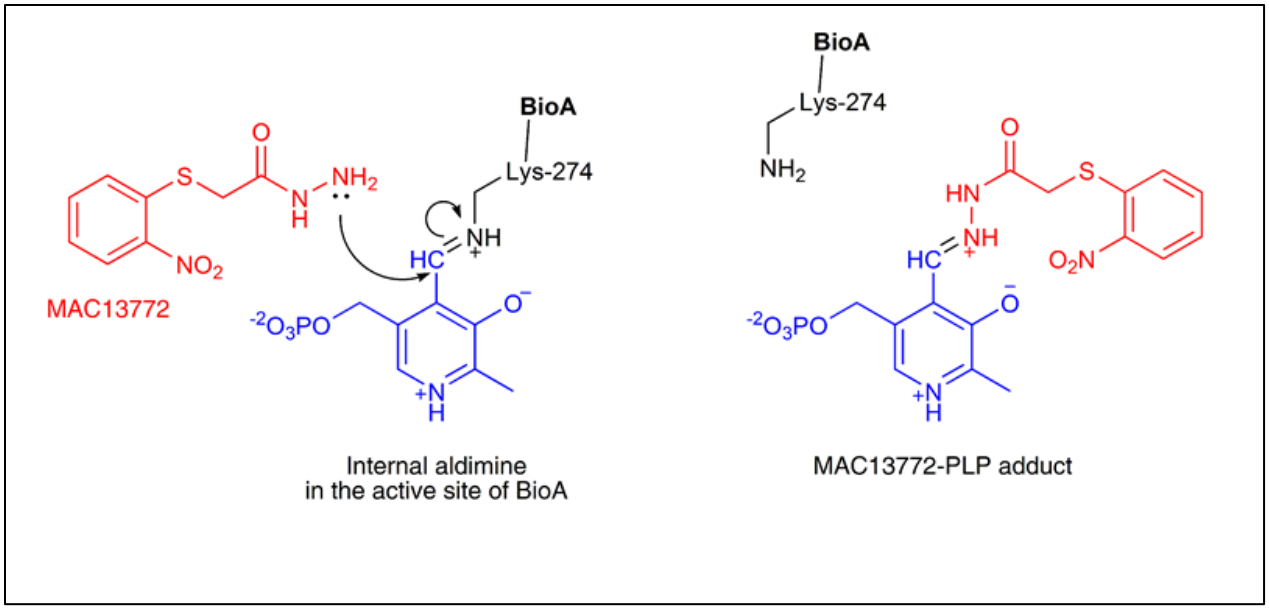


Figure 2-7. Interaction of MAC13772 with BioA: Model for BioA-MAC13772 interaction. The primary amine of the hydrazine group in MAC13772 displaces the Schiff base between Lys-274 and PLP (internal aldimine) in the active site of BioA and reacts with the cofactor. (This figure was adapted from Zlitni, S., Ferrucio, L.F., Brown E.D. *Nature Chemical Biology 2013*, 9:796)

Brown's group conducted experiments to determine the effect of MAC13772 on *E. coli*'s biotin biosynthesis intermediates, such as KAPA and DAPA. This was pivotal in identifying BioA as the target enzyme for inhibition. BioA was co-crystallized with MAC13772, and the 3D structure of this complex was elucidated. The structure revealed the formation of a covalent adduct between MAC13772 and PLP, which inactivates the enzyme (**Fig. 2-7**). This finding was crucial in confirming the proposed inhibition model. The structural analysis showed key residues within BioA that interact with MAC13772, mainly through hydrophobic interactions and hydrogen bonding (**Fig. 2-5 & Fig. 2-6**). This detailed mapping of the active site provided a deeper understanding of how MAC13772 inhibits BioA. Comparing the structure of BioA when bound

to MAC13772 with its native state (PLP-bound BioA) shed light on the conformational changes occurring upon inhibitor binding, specifically involving Lys274 and PLP (**Fig. 2-5**). Through spectral studies, the Brown group proposed a mechanism where MAC13772's primary amine displaces the internal aldimine between Lys274 and PLP, forming a tight complex with the cofactor (**Fig. 2-7**). This exploration by the Brown's group shed light on the mechanism of BioA inhibition by MAC13772, offering valuable insights into the design of novel antibacterial agents targeting metabolic biosynthetic pathways. Their work highlights the importance of structure-based drug design in understanding and developing new therapeutic agents.

2.1.3. Initial Structure-activity relationship (SAR) studies & Activity of MAC13772 and analogs against mycobacterial species

Brown's group, following their identification of the biochemical interaction between BioA and MAC13772, initiated a Structure-Activity Relationship (SAR) analysis of MAC13772, which initially involved assessing the antibacterial and biochemical effects of 24 analogs (**Fig. 2-8**).

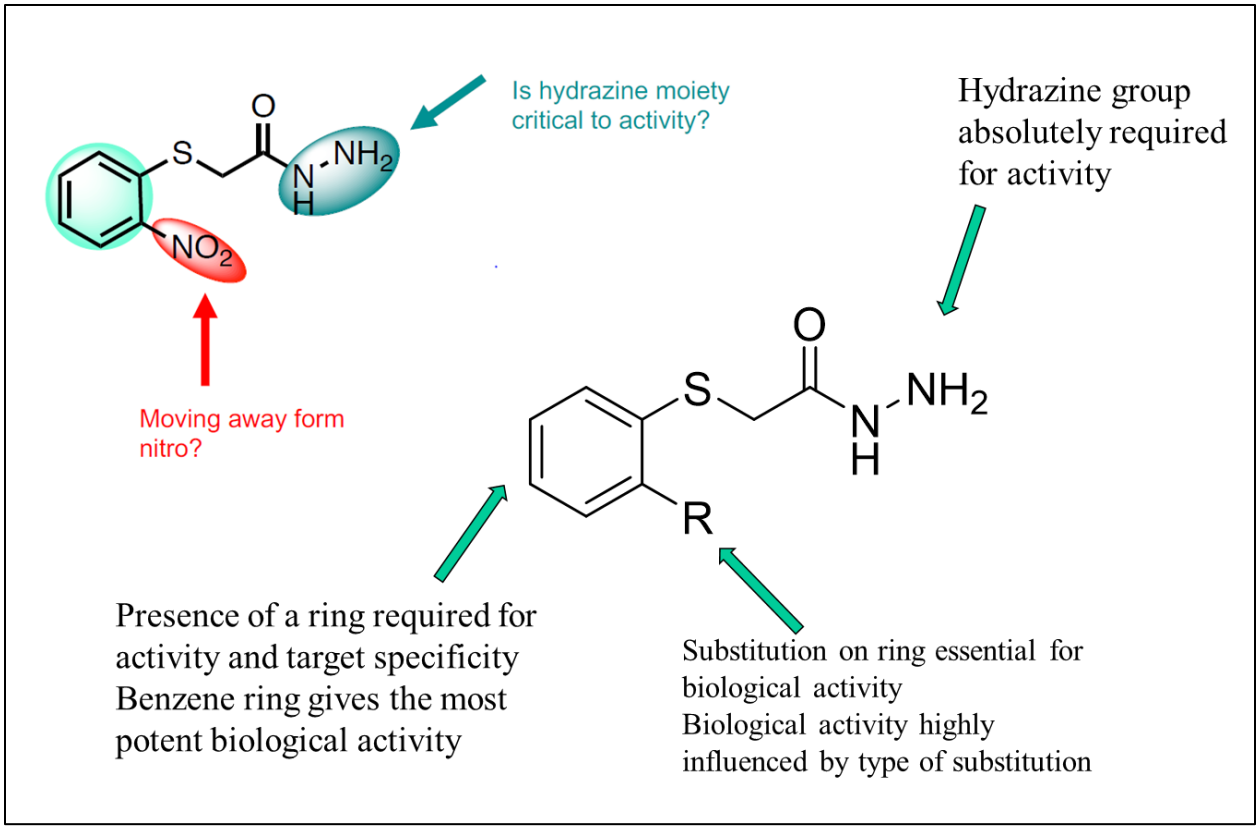
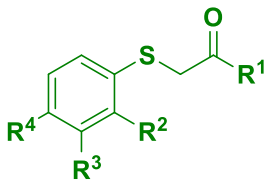


Figure 2-8. Summary of structure-activity relationship investigation of MAC13772 and analogs: This schematic summarizes the main findings from the SAR study into the biological and biochemical activity of MAC13772 and 24 analogs.

Their approach included varying the substituents on the phenyl ring of the parent molecule, particularly focusing on their position relative to the thioacetohydrazine chain (analogs **1a** through **1i**, **Table 2-1**). This subset of compounds, although less potent in inhibiting BioA compared to MAC13772, demonstrated varying degrees of antibacterial activity against *E. coli*. The study revealed that the position of the nitro group on the phenyl ring significantly influences biological activity (analogs **1a**, **1b** and **1c**). Further exploration into different substituents, like *chloro* and methyl groups, showed that these alterations did not significantly affect antibacterial activity (analogs **1e** and **1h**). They also examined the necessity of the hydrazine moiety and

found that analogs lacking this group were inactive in both antibacterial and biochemical assays (analogs **1j** through **1n**). This SAR analysis provided crucial insights into the structural features essential for the activity of MAC13772 and its analogs.

Table 2-1. Structure-activity relationships of MAC13772 and analogs against *E. coli*. (Part A) (This table was adapted from Zlitni, S., Ferrucio, L.F., Brown E.D. *Nature Chemical Biology 2013*, 9:796)



1

Compound	R^1	R^2	R^3	R^4	MIC (µg/ml)[a]		% Inhibition[b]	
					-bio	+bio	1µM	10 µM
MAC13772	$NHNH_2$	NO_2	H	H	8	>256	100	100
***Effect of Substitution on Benzene ring in Series 1**								
1a	$NHNH_2$	H	NO_2	H	256	>256	42	84
1b	$NHNH_2$	H	H	NO_2	64	>256	66	100
1c	$NHNH_2$	H	H	H	64	>256	49	100
1d	$NHNH_2$	F	H	H	32	>256	36	100
1e	$NHNH_2$	Cl	H	H	16	>256	63	100
1f	$NHNH_2$	OH	H	H	256	256	32	70
1g	$NHNH_2$	NH_2	H	H	256	>256	58	100
1h	$NHNH_2$	CH_3	H	H	16	>256	81	100
1i	$NHNH_2$	OCH_3	H	H	128	>256	65	100

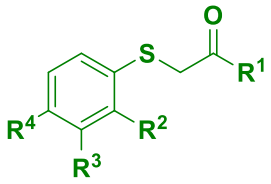
***Effect of changing hydrazine functionality on R^1 in Series 1**								
1j	NHNHAc	H	H	NO_2	>256	>256	0	0
1k	CH_2CH_3	NO_2	H	H	>256	>256	0	0
1l	NH_2	NO_2	H	H	>256	>256	0	0
1m	CH_3	NO_2	H	H	>256	>256	0	0
1n	OH	NO_2	H	H	>256	>256	0	0

[a] MICs were determined against *E. coli* MG1655 in absence and presence of 2 nM of biotin

[b] The biochemical activity of analogs is determined against recombinant *E. coli* BioA through a feeding assay of a bioA auxotroph at 1 and 10 μM and expressed as a percentage (%) of the respective DMSO control

Brown's group evaluated the efficacy of MAC13772 and 24 analogs against mycobacterial strains- the BSL-1 *M. smegmatis* and the BSL-2 *M. tuberculosis* mc^2 6020. In mycobacterial minimal media, MAC13772 showed MIC values of 4 μg/mL and 2 μg/mL against *M. smegmatis* and *M. tuberculosis* mc^2 6020, respectively. Modifications to the phenyl ring, specifically in relation to the thioacetohydrazine chain (analogs **1a-1i**), were found to be more effective for both strains compared to their activity against *E. coli* (**Table 2-2**). This series of analogs was particularly potent against *M. smegmatis*. However, analogs **1j-1n**, where the hydrazine group was altered, exhibited no activity against either mycobacterial strain, underscoring the critical role of the hydrazine moiety in MAC13772's antibacterial function.

Table 2-2. Structure-activity relationships of MAC13772 and analogs against *Mycobacterium smegmatis* & *Mycobacterium tuberculosis* mc^2 6020. (Part A) (This table was adapted from Zlitni, S., Ferrucio, L.F., Brown E.D. *Nature Chemical Biology 2013*, 9:796)



1

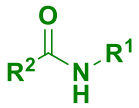
Compound	R^1	R^2	R^3	R^4	MIC (µg/ml)[a]		MIC (µg/ml)[b]	
					-bio	+bio	-bio	+bio
MAC13772	$NHNH_2$	NO_2	H	H	4	>256	2	>256
***Effect of Substitution on Benzene ring in Series 1**								
1a	$NHNH_2$	H	NO_2	H	16	>256	16	>256
1b	$NHNH_2$	H	H	NO_2	4	>256	16	>256
1c	$NHNH_2$	H	H	H	4	>256	16	128
1d	$NHNH_2$	F	H	H	4	>256	16	256
1e	$NHNH_2$	Cl	H	H	4	>256	4	256
1f	$NHNH_2$	OH	H	H	32	256	128	256
1g	$NHNH_2$	NH_2	H	H	32	>256	64	128
1h	$NHNH_2$	CH_3	H	H	4	>256	4	256
1i	$NHNH_2$	OCH_3	H	H	16	>256	16	256
***Effect of changing hydrazine functionality on R^1 in Series 1**								
1j	NHNHAc	H	H	NO_2	>256	>256	>256	>256
1k	CH_2CH_3	NO_2	H	H	>256	>256	>256	>256
1l	NH_2	NO_2	H	H	128	>256	>256	>256
1m	CH_3	NO_2	H	H	>256	>256	>256	>256
1n	OH	NO_2	H	H	>256	>256	>256	>256

[a] MICs are determined against *M. smegmatis* in Sauton minimal media in the absence and presence of 2 nM of biotin

[b] MICs are determined against *M. tuberculosis* mc^2 6020 (BSL-2) in a modified Middlebrook 7H9 media in the absence and presence of 2 nM of biotin

In exploring MAC13772's structure-activity relationship, Brown's group tested analogs with varied hydrazine-containing side chains but without the aryl ring (analogs **2a** to **2e, Table 2-3**). These analogs displayed only modest *in vitro* inhibition of BioA and lacked significant antibacterial activity. Notably, compounds **2a** and **2e** showed reduced antibacterial activity, but their growth inhibition wasn't affected, even with biotin present. Further experiments replacing the aryl ring with different rings (analogs **2f** to **2j**) resulted in decreased antibacterial effectiveness. These findings underline the importance of the aryl ring for both the specificity and potency of MAC13772.

Table 2-3. Structure-activity relationships of MAC13772 and analogs against *E. coli*. (Part B) (This table was adapted from Zlitni, S., Ferrucio, L.F., Brown E.D. *Nature Chemical Biology 2013*, 9:796)



2

Compound	R^1	R^2	R^3	R^4	MIC (µg/ml)[a]		% Inhibition[b]	
					-bio	+bio	1µM	10 µM
MAC13772	NH_2	$C_7H_6NO_2S$	-	-	4	>256	2	>256
***Activity of the side chain of the parent molecule in Series b**								
2a	NH_2	CH_2SCH_3	-	-	64	64	50	64
2b	NH_2	CH_2SH	-	-	256	256	20	34
2c	NH_2	$CH_2CH_2CH_3$	-	-	>256	>256	1	40
2d	NH_2	CH_2CH_3	-	-	>256	>256	0	8
2e	NH_2	CH_3	-	-	128	128	7	19

***Effect of different ring substituents in R^2 in Series b[c]**								
2f	NH_2	Benzothiophen	-	-	>256	>256	15	69
2g	NH_2	CH_2SCH_2Ph	-	-	64	>256	60	100
2h	NH_2	CH_2SNaph	-	-	>256	>256	71	100
2i	NH_2	CH_2SPyr	-	-	64	>256	32	77
2j	NH_2	Nitrobenzyl	-	-	>256	>256	0	29

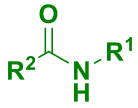
[a] MICs are determined against *E. coli* MG1655 in absence and presence of 2 nM of biotin

[b] The biochemical activity of analogs is determined against recombinant *E. coli* BioA through a feeding assay of a *bioA* auxotroph at 1 and 10 μM and expressed as a percentage (%) of the respective DMSO control

[c] Abbreviations: Ph: phenyl; Naph: naphthalenyl; Pyr: pyridine

The evaluation of hydrazine-containing side chains without the aryl ring, specifically analogs **2a** through **2e** (**Table 2-4**), demonstrated moderate antibacterial activity against both tested strains. However, intriguingly, the presence of biotin did not reverse the growth inhibition caused by these compounds, suggesting they may employ a different mode of inhibition. When the aryl ring in the parent compound was substituted with alternative ring structures (analogs **2f** through **2j**, **Table 2-4**), the changes were generally not well-tolerated, except for analogs **2g** and **2i**, which maintained reasonable antimycobacterial activity. This suggests the aryl ring's critical role in the compound's effectiveness.

Table 2-4. Structure-activity relationships of MAC13772 and analogs against *Mycobacterium smegmatis* & *Mycobacterium tuberculosis* mc^2 6020. (Part B) (This table was adapted from Zlitni, S., Ferrucio, L.F., Brown E.D. *Nature Chemical Biology 2013*, 9:796)



2

Compound	R^1	R^2	R^3	R^4	MIC (µg/ml)[a]		MIC (µg/ml)[b]	
					-bio	+bio	-bio	+bio
MAC13772	NH_2	$C_7H_6NO_2S$	-	-	4	>256	2	>256
***Activity of the side chain of the parent molecule in Series b**								
2a	NH_2	CH_2SCH_3	-	-	32	32	32	32
2b	NH_2	CH_2SH	-	-	32	32	>256	>256
2c	NH_2	$CH_2CH_2CH_3$	-	-	64	64	32	32
2d	NH_2	CH_2CH_3	-	-	64	64	128	>256
2e	NH_2	CH_3	-	-	>256	>256	128	128
***Effect of different ring substituents in R^2 in Series b[c]**								
2f	NH_2	Benzothiophene	-	-	64	>256	64	128
2g	NH_2	CH_2SCH_2Ph	-	-	8	>256	16	128
2h	NH_2	CH_2SNaph	-	-	32	>256	>256	>256
2i	NH_2	CH_2SPyr	-	-	8	128	16	>256
2j	NH_2	Nitrobenzyl	-	-	256	>256	128	>256

[a] MICs are determined against *M. smegmatis* in Sauton minimal media in the absence and presence of 2 nM of biotin
[b] MICs are determined against *M. tuberculosis* mc^2 6020 (BSL-2) in a modified Middlebrook 7H9 media in the absence and presence of 2 nM of biotin
[c] Abbreviations: Ph: phenyl; Naph: naphthalenyl; Pyr: pyridine

2.2. Exploration of New Scaffold Modifications

So far, we have discussed that the original study performed by Brown's group already included a SAR analysis involving the synthesis of 24 analogs. This research highlighted the crucial role of hydrazine moiety for activity and the benzyl ring for target specificity. Although substitutions on the phenyl ring were possible, they generally resulted in reduced potency. Specifically, modifications like *chloro* and methyl substitutions at the 2-*nitro* group doubled the MIC, indicating a minor impact on activity. Given the susceptibility of nitroaromatic compounds to metabolic reduction to aniline[137], maintaining potency while replacing the nitro group could enhance metabolic stability. Overall, this work served as a valuable starting point for a fuller SAR analysis of MAC13772 leading to the requirement for new synthetic strategies.

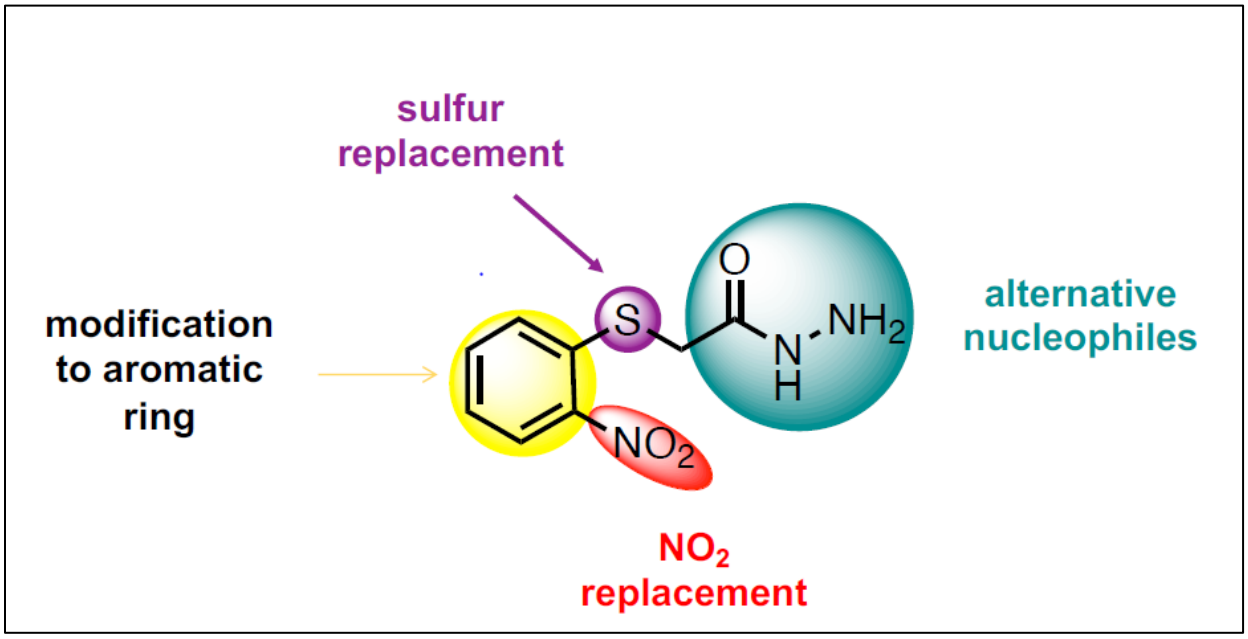


Figure 2-9. Scope of Modifications in Lead Molecule MAC13772.

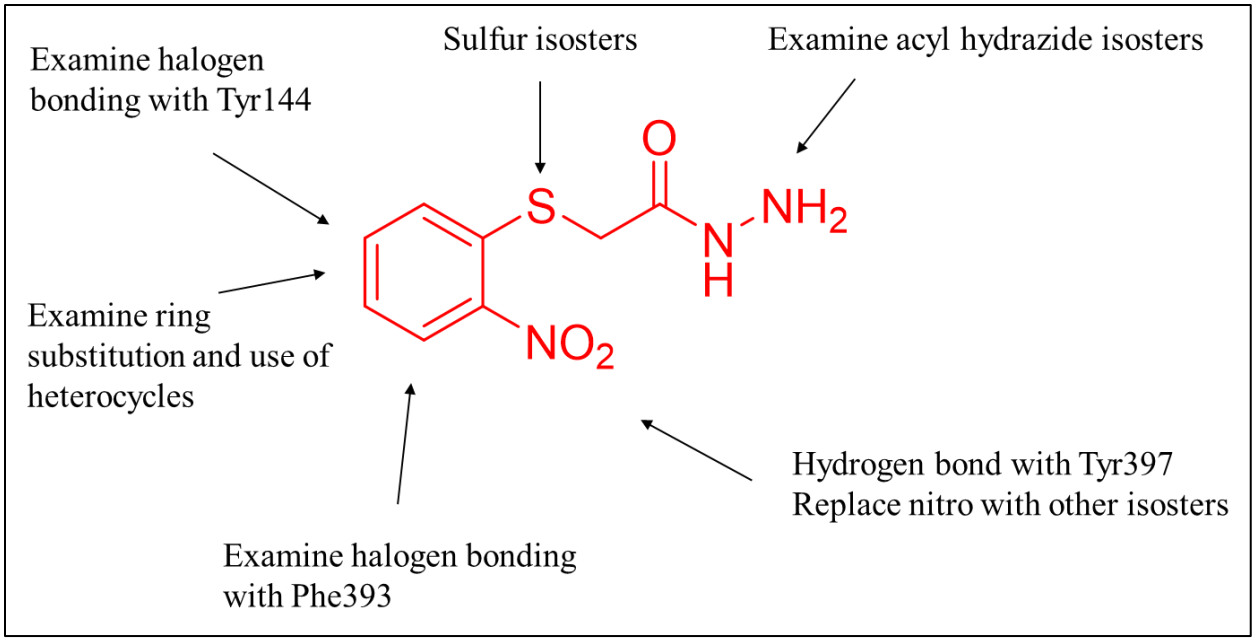


Figure 2-10. Exploration of New Scaffold Modifications.

Several types of scaffold modifications were not sufficiently explored in the previous study (**Fig. 2-9 & 2-10)**. These include multiple simultaneous substitutions on the aryl ring (M1, orange), ring replacement (M2, red) by heterocycles or biphenyl or fused aromatic rings, substitutions of the thioether (M3, green), and varying the alkyl chain length between the sulfur and the acetohydrazide moieties (M4, blue) (**Fig. 2-11**).

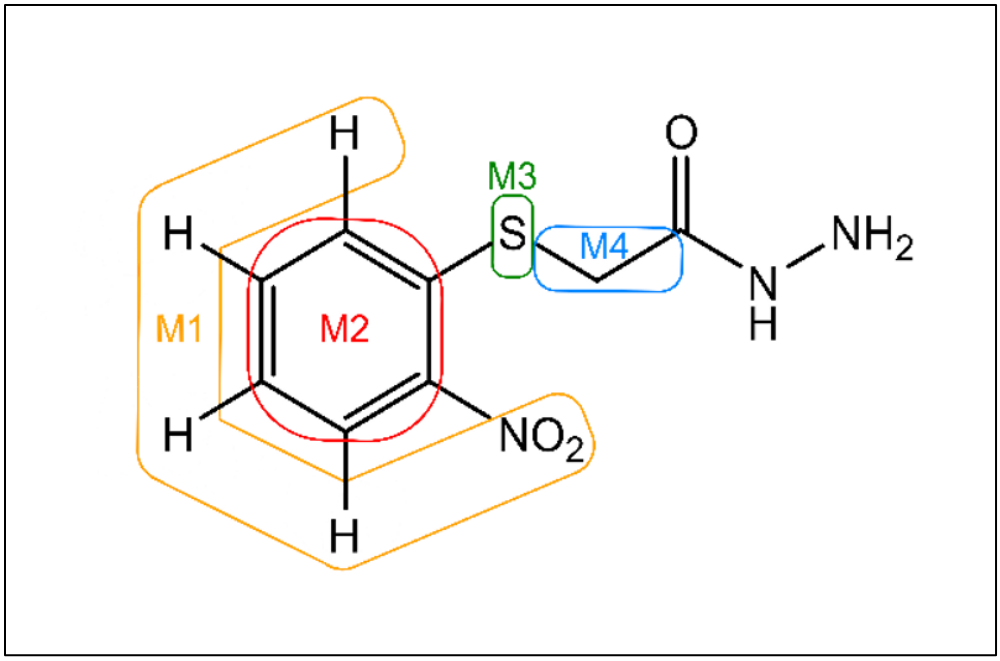


Figure 2-11. Overview of structural modifications applied to the MAC-13772 scaffold for SAR analysis, with color-coded highlights indicating the areas of modification.

Accordingly, we explored the impact of these modifications, both individually and in combination on the potency towards *E. coli* BioA (*Ec*BioA). Fourteen analogs exhibited lower IC_{50} values against *Ec*BioA than the parent molecule (**1**, 0.280 µM). Ten of these analogs involved M1 modifications – four with dichloro substitutions (**10-13**, 0.003-0.088 µM), four with alkyl substitutions (**19**, **22**, **26**, **28**; 0.023-0.223 µM), one with a 2-methoxy (**24**, 0.241 µM), and one with a 4-CF_3 (**32**, 0.109 µM) substitution (**Table 2-5**). Two analogs featured M2 modifications – one with a 4-pyridine replacement (**40**, 0.097 µM) and another with a 2,4-pyrimidine replacement (**41**, 0.193 µM) of the phenyl ring. One analog combined M1 and M2 modifications, incorporating a 4-CF_3 substitution and 2-pyridine replacement (**43**, 0.074 µM) (**Table 2-6)**. Another analog combined M1 and M3 modifications, featuring 4-PhF and S→O substitutions (**14**, 0.252 µM). M3 modifications were only tolerated (i.e., resulted in a ≤2-fold increase in IC_{50}) when combined with M1 modifications, as seen in analogs with 2,4-Cl with

S→O (**7**, 0.580 μM) and 4-Me with S→CH_2 (**20**, 0.362 μM) substitutions. In summary, dichloro substitutions led to the most significant increase in potency, enhancing inhibition of *Ec*BioA by up to 90-fold compared to the lead molecule MAC-13772. Any higher-order M1, ring replacement in M2 and all M4 modifications were found to be detrimental to the enzyme inhibition.

After establishing the most potent inhibitors of *Ec*BioA, we investigated their effectiveness against *A. baumannii* (*Ab*BioA). MAC-13772 exhibited a similar IC_{50} against the BioA enzyme (0.269 vs. 0.280 μM). Compounds **13**, **28** and **32** all had 4-7-fold lower IC_{50} against the *Ab*BioA (0.009, 0.018, 0.022 μM, respectively) compared to *Ec*BioA (0.063, 0.064, 0.109 μM). The preservation of potency was anticipated since the catalytic residues in the active sites of both enzymes are conserved and shown to be a good predictor of activity.[138] Overall, our synthetic chemistry campaign revealed that the MAC-13772 scaffold is more amenable to alternations than previously thought.

After identifying potent enzyme inhibitors, we sought to evaluate their whole-cell activity. Bacterial susceptibility testing of MAC-13772 and its analogs was performed in biotin-free and biotin-containing media. Supplementation with biotin was required as a control for non-specific activity, which was observed with previously synthesized hydrazides lacking an aromatic moiety.[129] The minimum inhibitory concentrations (MICs) were determined using a standard strain of *A. baumannii* (ATCC 17978) and four strains of *E. coli* (BW25113): the wild-type (WT), an efflux-deficient strain (Δ*tolC*), a hyperpermeable strain, and a hyperpermeable efflux-deficient strain (Δ*tolC*-pore). It is noteworthy that the hyperpermeable strain constitutively expresses a truncated version of the FhuA pore (WT-pore),[139–141] a channel in the bacterial outer membrane that facilitates the entry of molecules, thereby making the bacteria more susceptible to

inhibitors. *A. baumannii* ATCC 17978 serves as a model organism in our systemic infection murine model, which is designed to evalutate inhibitors of biotin biosynthesis.[138] The choice of hyperpermeable and efflux-deficient strains aimed to investigate if the effectiveness of the new analogs was reduced by barriers to entry into the bacterial cell or by being pumped out by the bacteria's natural defense mechanism, known as the AcrAB-TolC efflux pump.

All synthesized analogs had either ≥4-fold poorer MICs (≥64 μg/mL) or, with the majority having no activity against *E. coli* WT in comparison to MIC value of the lead molecule, MAC-13772(**Table 2-5 to Table 2-8**). *E. coli* Δ*tolC* was susceptible to a broader spectrum of analogs. However, their potency was either equivalent to or lower than the parent compound. In total, 19 compounds had MICs of ≤4-fold (16-64 μg/mL) compared to that of MAC-13772. Eight of those were among the most potent enzymatic inhibitors. *E. coli* with the FhuA pore (referred to simply as 'pore') and Δ*tolC*-pore showed no enhancement in susceptibility compared to *E. coli* Δ*tolC*. Compound **46** showed non-specific activity, evident by no change in MIC with biotin supplementation. Strikingly, *A. baumannii* was susceptible to 15 analogs that possessed an MIC of ≤4-fold (≤32 μg/mL) relative to that of MAC-13772. Compound **43**, the 4-CF_3-2-pyridine derivative, was the most potent compound with an MIC of 2 μg/mL. Nine analogs were among the most potent *Ec*BioA inhibitors as well. Despite identifying multiple potent enzymatic inhibitors of *Ec*BioA, their activities were lost in a whole-cell assay against the WT strain. Efflux deficiency improved the activities of analogs, but, unfortunately, the MICs did not surpass those of MAC-13772. In contrast, *A. baumannii* demonstrated increased susceptibility to the analogs, particularly compound **43**, which exhibited a potency four-times greater than MAC-13772.

Minimum inhibitory concentrations (MICs) are shown in μg/mL in **Table 2-5 to Table 2-8**. The red gradient indicates lower MIC, while the blue gradient indicates lower IC_{50}. This visual

representation allows for an immediate understanding of the compounds' potency and enzyme inhibition capabilities. All values presented are based on at least two replicates, confirming the consistency of the findings. "n.d." stands for no detectable enzyme inhibition, indicating that some compounds did not exhibit activity in the assays conducted.

Table 2-5. Antibacterial activities and IC_{50} values of MAC-13772 and its analogs (Part A).

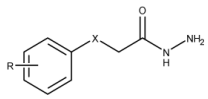
Compounds	*A. baumannii* ATCC 17978 WT		*E. coli* BW25113 WT		*E. coli* BW25113 pore		*E. coli* BW25113 Δ*tolC*		*E. coli* BW25113 Δ*tolC*- pore		*E. coli* BioA IC_{50}	*A.baumannii* BioA IC_{50}
	-biotin	+biotin	-biotin	+biotin	-biotin	+biotin	-biotin	+biotin	-biotin	+biotin	μM	
												
1: R = 2-NO_2, X = S	8	>256	16	>256	16	>256	16	>256	16	>256	0.28	0.269
2: R = 2-NO_2, X = O	256	>256	>256	>256	>256	>256	128	>256	>256	>256	5.2	
3: R = 2-NO_2, X = NH	128	>256	256	>256	256	>256	256	>256	256	>256	3	
4: R = 2-NO_2, X = CH_2	256	>256	>256	>256	>256	>256	256	256	>256	>256	71.29	
5: R = 4-Br, X = O	256	>256	>256	>256	>256	>256	256	>256	>256	>256	5	
6: R = 2-Cl, X = O	256	>256	>256	>256	>256	>256	>256	>256	>256	>256	9.67	
7: R = 2,4-Cl, X = O	64	>256	>256	>256	>256	>256	256	>256	>256	>256	0.58	0.3
8: R = 2,3,4,5,6-Cl, X = S	>128	>128	>128	>128	>128	>128	64	>128	128	>128	1.9	
9: R = 2,6-Cl, X = S	256	>256	>256	>256	>256	>256	128	>256	256	>256	0.486	
10: R = 2,5-Cl, X = S	16	>256	>256	>256	>256	>256	128	256	128	256	0.088	
11: R = 3,5-Cl, X = S	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	0.025	
12: R = 2,4-Cl, X = S	8	>256	128	>256	64	>256	32	256	16	256	0.003	
13: R = 2,3-Cl, X = S	64	>256	128	>256	128	>256	32	128	16	128	0.063	0.009
14: R = 4-PhF, X = O	>128	>128	>128	>128	>128	>128	64	128	32	128	0.252	
15: R = 2,3,5,6-F, X = S	256	>256	256	>256	256	>256	64	>256	256	>256	2.53	
16: R = 3,4-F, X = S	128	>256	256	>256	>256	>256	128	>256	128	>256	0.485	
17: R = 2-Me, 4-F; X = O	256	>256	>256	>256	>256	>256	>256	>256	>256	>256	12.12	
18: R = 3-F, 4-OMe; X = CH_2	>256	>256	>256	>256	>256	>256	128	>256	>256	>256	0.637	
19: R = 2-iPr, X = S	128	>256	256	>256	>256	>256	32	>256	32	>128	0.223	
20: R = 4-Me; X = CH_2	128	>256	256	>256	256	>256	128	>256	128	>256	0.362	
21: R = 2-Me, X = NH	256	>256	256	>256	>256	>256	256	>256	>256	>256	148.3	
22: R = 2-Me, X = S	16	>256	256	>256	128	>256	32	128	64	>256	0.023	
23: R = 2-Me, X = O	256	>256	>256	>256	>256	>256	256	>256	>256	>256	4.4	
24: R = 2-OMe, X = S	64	>256	>256	>256	256	>256	>256	>256	256	>256	0.241	
25: R = 2-OMe, X = SO	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	13.93	
26: R = 2,4-Me, X = S	8	>256	128	>256	128	>256	16	128	16	>256	0.064	0.018
27: R = 2,5-Me, X = S	32	>256	>256	>256	>256	>256	128	>256	128	256	0.169	
28: R = 2,6-Me, X = S	256	>256	256	>256	>256	>256	>256	>256	>256	>256	0.326	
29: R = 2,4,6-Me, X = S	>256	>256	>256	>256	>256	>256	64	256	256	256	0.873	
30: R = 2-Me, 4-Boc-Piperazine; X = S	>256	>256	>256	>256	>256	>256	32	>256	32	256	0.321	
31: R = 2-Me, 4-Me-Piperazine; X = S	>256	>256	>256	>256	256	>256	32	>256	32	256	0.687	
32: R = 4-CF_3, X = S	16	>256	>256	>256	>256	>256	32	>256	64	>256	0.109	0.022
33: R = 2-CF_3, X = S	32	>256	128	>256	128	>256	32	>256	32	>256	0.46	0.095
34: R = 2-CF_3, X = SO_2	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	13.36	
35: R = 2-CF_3, X = SO	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	1000	
36: R = 2-CF_3, X = O	>256	>256	>256	>256	>256	>256	256	>256	>256	>256	10.65	
37: R = 2-CF_3, X = NH	256	>256	>256	>256	>256	>256	256	>256	256	>256	1.7	

Table 2-6. Antibacterial activities and IC_{50} values of MAC-13772 and its analogs (Part B).

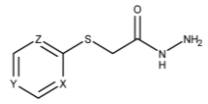
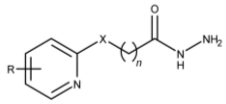
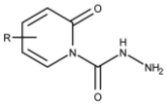
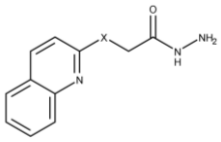
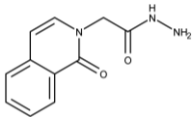
	A. baumannii ATCC 17978 WT		*E. coli* BW25113 WT		*E. coli* BW25113 pore		*E. coli* BW25113 Δ*tolC*		*E. coli* BW25113 Δ*tolC*- pore		*E. coli* BioA IC_{50}	*A.baumannii* BioA IC_{50}
	-biotin	+biotin	-biotin	+biotin	-biotin	+biotin	-biotin	+biotin	-biotin	+biotin	μM	
												
39: X = N, Y = CH, Z = CH	8	>256	64	>256	128	>256	128	>256	128	>256	0.578	0.342
40: X = CH, Y = N, Z = CH	32	>256	256	>256	256	>256	64	>256	128	>256	0.097	
41: X = N, Y = CH, Z = N	32	>256	256	>256	>256	>256	>256	>256	>256	>256	0.193	
												
42: R = 4-F, X = O, *n* = 1	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	4.752	
43: R = 4-CF_3, X = S, *n* = 1	2	>256	>256	>256	>256	>256	>256	>256	>256	>256	0.074	
44: R = 2-CF_3, X = S, *n* = 1	16	>256	>256	>256			16	>256			0.629	
45: R = 3-CF_3, X = O, *n* = 1	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	19.96	
46: R = 4-CF_3, X = CH_2, *n* = 2	128	>256	256	256	64	64	256	256	64	64	9.73	
												
47: R = 4-CF_3	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	106.8	
48: R = 2-CF_3	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	4250	
												
49: X = S	32	>256	>256	>256	>256	>256	32	256	32	>256	0.712	
50: X = O	128	>256	>256	>256	>256	>256	64	128	128	128	3.212	
												
51	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	125.5	

Table 2-7. Antibacterial activities and IC_{50} values of MAC-13772 and its analogs (Part C).

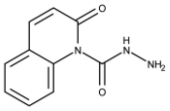
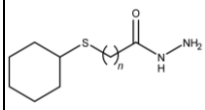
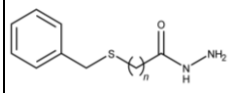
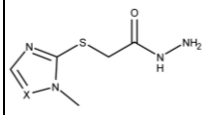
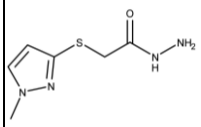
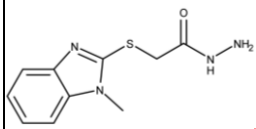
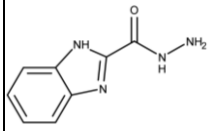
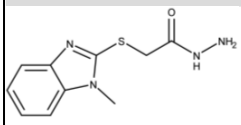
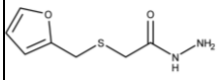
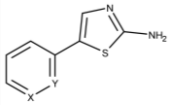
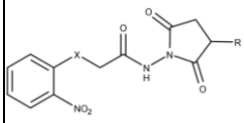
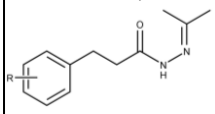
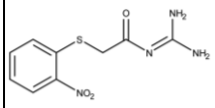
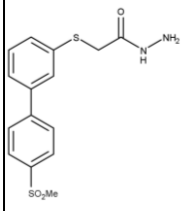
Compounds	*A. baumannii* ATCC 17978 WT		*E. coli* BW25113 WT		*E. coli* BW25113 pore		*E. coli* BW25113 Δ*tolC*		*E. coli* BW25113 Δ*tolC*- pore		*E. coli* BioA IC_{50}	*A.baumannii* BioA IC_{50}
	-biotin	+biotin	-biotin	+biotin	-biotin	+biotin	-biotin	+biotin	-biotin	+biotin		μM
												
52	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	96.3	
												
53: n = 1	128	>256	>256	>256	>256	>256	128	>256	128	>256	13.92	
54: n = 2	>256	>256	>256	>256	>256	>256	128	>256	128	>256	10.53	
												
55: n = 1	64	>256	256	>256	256	>256	64	>256	64	128	7.591	
56: n = 2	32	>256	>256	>256	>256	>256	64	>256	32	>256	0.0039	
												
57: X = CH	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	9.485	
58: X = N	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	271.8	
												
59	>256	>256	>256	>256							2.285	
												
60	8	>256	256	>256	256	256	256	256	128	128	1.49	
												
61	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	218.3	

Table 2-8. Antibacterial activities and IC_{50} values of MAC-13772 and its analogs (Part D).

Compounds	*A. baumannii* ATCC 17978 WT		*E. coli* BW25113 WT		*E. coli* BW25113 pore		*E. coli* BW25113 Δ*tolC*		*E. coli* BW25113 Δ*tolC*- pore		*E. coli* BioA IC_{50}	*A.baumannii* BioA IC_{50}
	-biotin	+biotin	-biotin	+biotin	-biotin	+biotin	-biotin	+biotin	-biotin	+biotin	μM	
 **62**	>256	>256	>256	>256	>256	>256	256	256	256	256	2.804	
 **63**	256	>256	>256	>256	>256	>256	256	>256	>256	>256	3.994	
 **64:** X = CH_2, Y = CH_2	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	n.d.	
65: X = N, Y = CH_2	128	>256	>256	>256	>256	>256	256	>256	256	>256	n.d.	
66: X = CH_2, Y = N	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	n.d.	
 **67:** R = H, X = O	>256	>256	>256	>256	>256	>256	>256	>256	>512	>256		
68: R = H, X = S	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	n.d.	
69: R = Me, X = S	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	n.d.	
 **70**: R = 2-F	256	>256	256	>256	256	>256	256	>256	256	>256	19.43	
71: R = 4-NO_2	128	>256	256	>256	>256	>256	128	>256	>256	>256	97.71	
 **72**	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	n.d.	
 **73**	>256	>256	>256	>256	>256	>256	128	>256	64	>256	0.791	

*n.d: not determined

In total, nine compounds (**10**, **12**, **22**, **26**, **32**, **39**, **43**, **44** and **60**) were active against *A. baumannii* with an MIC of ≤ 2-fold compared to the parent compound (**Table 2-5 to Table 2-8**). Brown's group selected those for an *in vitro* metabolic stability assay in rat liver microsomes (RLMs). All tested analogs demonstrated half-life ($t_{½}$) values equivalent to, or lower than MAC-13772, except for compound **60** (**Table 2-9**). It exhibited a $t_{½}$ of 2.3 h (approx. 138 min), corresponding to a 76% improvement in the metabolic stability over the parent compound. It was noticed that Benzimidazole Analog Exhibits Greater Metabolic Stability In Vitro. Compound **60** incorporates a methyl benzimidazole group in place of the phenyl ring, a modification that removes the *nitro* group prone to metabolic breakdown, potentially accounting for the enhanced $t_{½}$ of the analog. Additionally, it exhibited increased water solubility, a favorable trait for *in vivo* testing. Encouraged by this result, compound **60** was further evaluated in the animal model. Unfortunately, the compound was not efficacious *in vivo*, likely due to rapid renal clearance of the analog (data not shown).

Table 2-9. *In vitro* metabolic stability of MAC-13772 and its analogs in rat liver microsomes.

Compounds	*A. baumannii* MIC (μg/ML)	*in vitro* $t_{½}$ (h)
MAC-13772	8	1.30
43	2	1.46
12	8	0.34
26	8	0.82
39	8	1.37
60	8	2.29
10	16	0.39
22	16	0.92
32	16	0.62
44	16	1.19

The brown gradient represents a longer $t_{½}$.

2.3. Synthetic Rationale

S-Adenosyl methionine (SAM) serves as the substrate for the BioA enzyme, which is integral to the biotin biosynthesis pathway. MAC13772, the lead compound in our study, effectively inhibits this pathway by targeting SAM. To investigate the further structure-activity relationships (SAR) of MAC13772 analogs, we analyzed the structure of both SAM and MAC13772 (**Fig. 2-12**) and adopted a general synthetic approach that involved synthesizing sulfur, nitrogen, oxygen, and carbon analogs (**Table 2-10 & Table 2-12**).

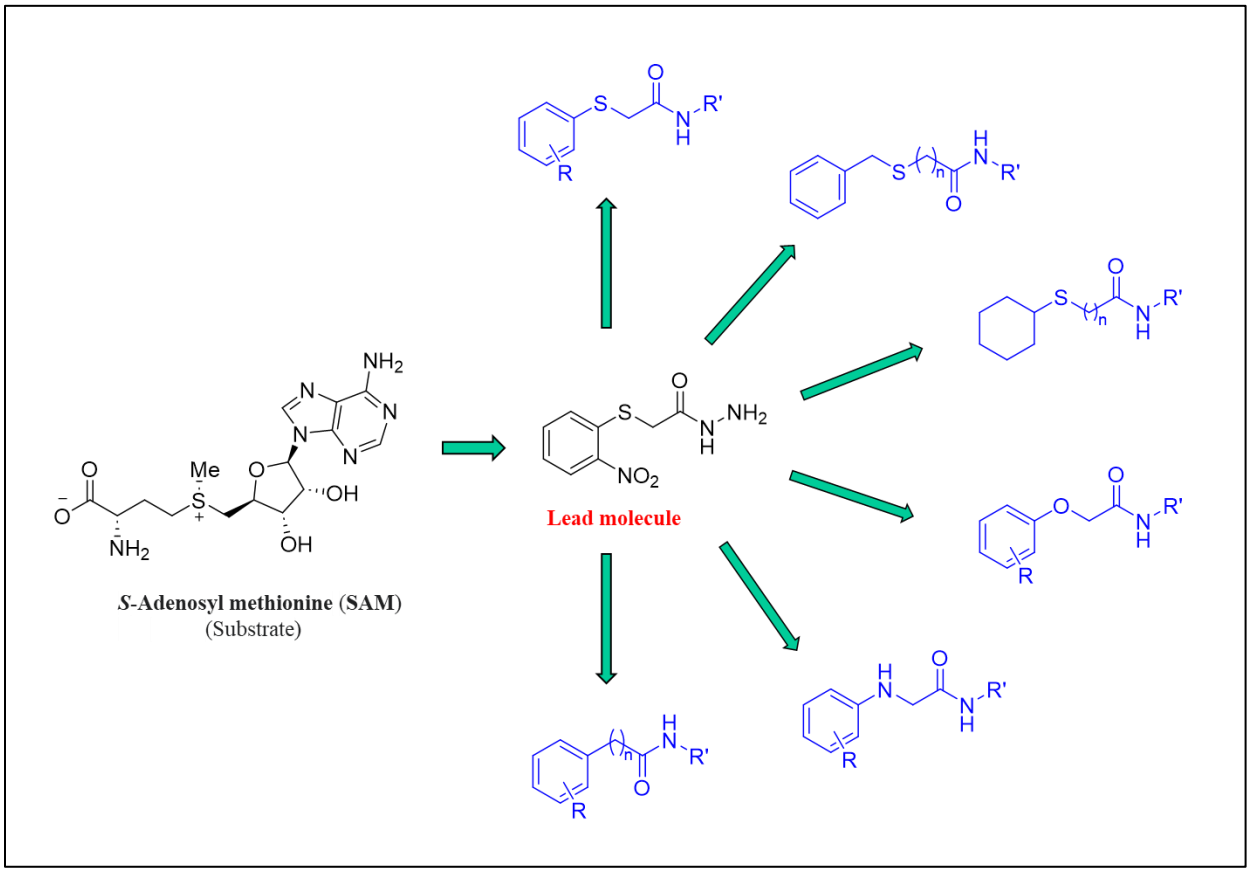
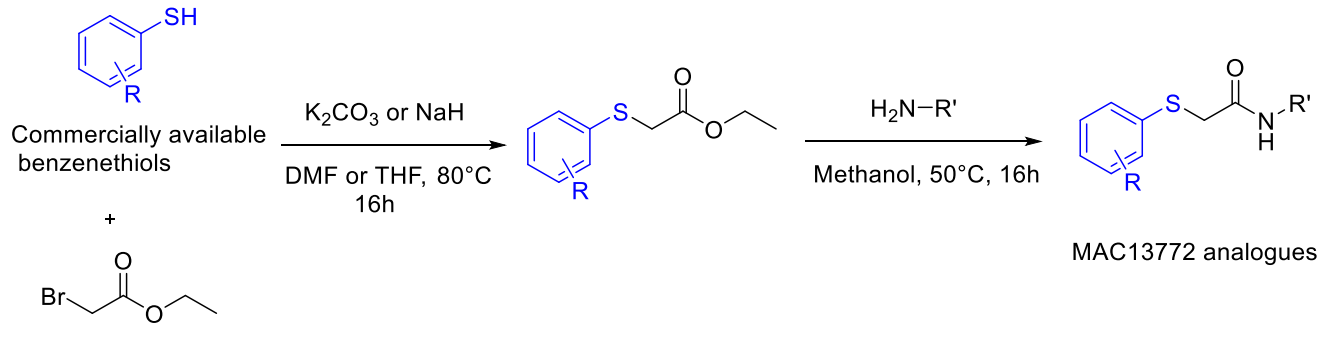
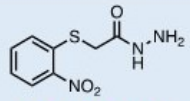
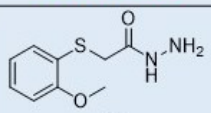
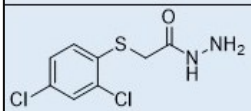
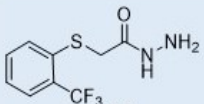
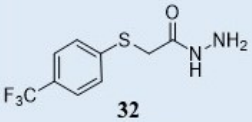
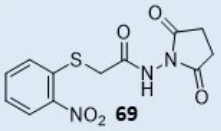
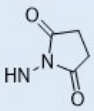


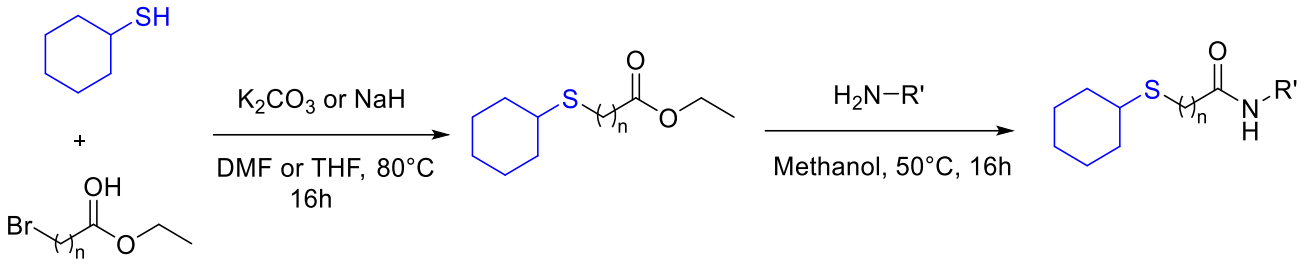
Figure 2-12. Outlining the progression from the substrate SAM to the lead compound MAC13772 and its derived analogs, demonstrating the strategic modifications undertaken to enhance biotin biosynthesis inhibition.

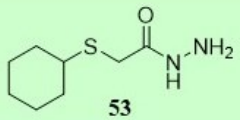
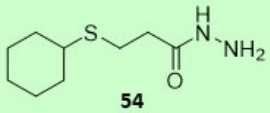
These analogs were synthesized through substitution reactions between substituted aryl thiols or alcohols and bromoesters under basic conditions, or via cross-coupling of substituted aryl halides with glycine ethyl ester hydrochloride (primary amine) or organozinc compounds, using palladium catalysis. This cross-coupling method was pivotal, serving as the main strategy for diversification. In the first step of our synthesis, these reactions produced ester derivatives through coupling, which were then transformed into acetohydrazide derivatives by treating with hydrazine hydrate, thus yielding our target analogs.

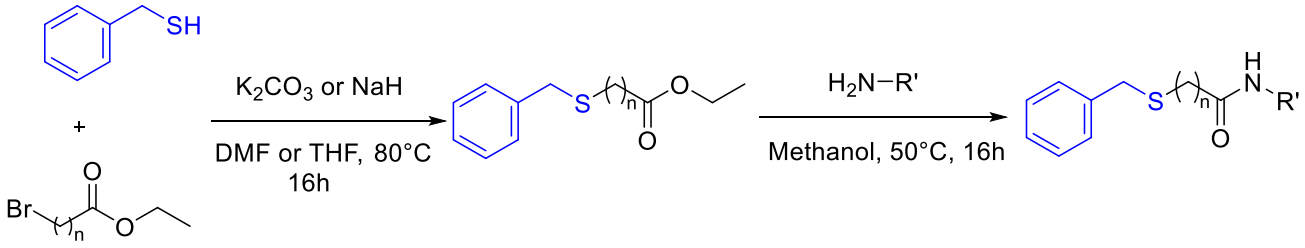
Table 2-10. General Synthetic Schemes for MAC13772 Sulphur and Oxygen Analogs with Listed Synthesized Molecules.

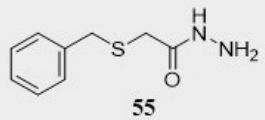
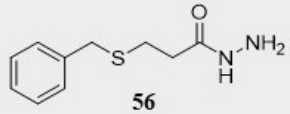


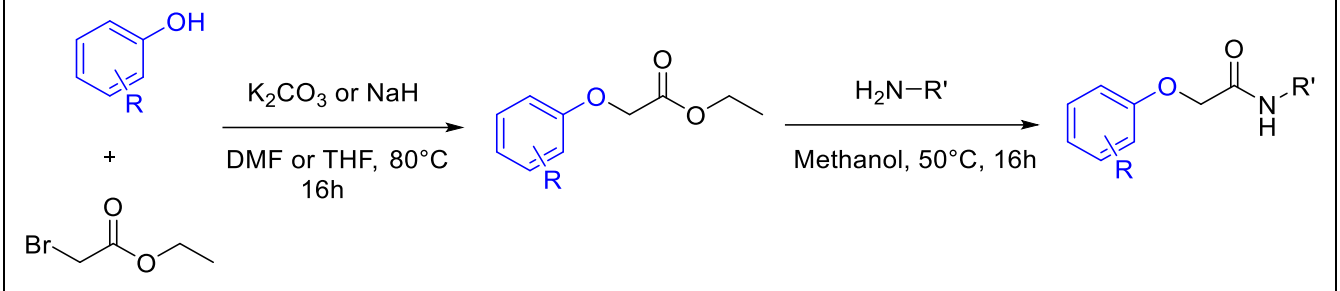
Compounds	R'	R	%Yield	*A. baumannii* MIC (μg/mL)	*E. Coli* MIC (μg/mL)	IC_{50} *E. coli* BioA (μM)
 Lead Molecule	$NHNH_2$	2-NO_2	64	8	16	0.28±0.02
 **24**	$NHNH_2$	2-OMe	77	64	>256	0.241
 **12**	$NHNH_2$	2, 4-Cl	42	8	128	0.003
 **33**	$NHNH_2$	2-CF_3	83	32	128	0.46
 **32**	$NHNH_2$	4-CF_3	69	16	>256	0.109
 **69**		1	53	256	256	n.d



Compounds	R'	n	%Yield	*A. baumannii* MIC (µg/mL)	*E. Coli* MIC (µg/mL)	IC_{50} *E. coli* BioA (µM)
 **53**	$NHNH_2$	1	71	128	256	13.92
 **54**	$NHNH_2$	2	73	>256	>256	10.53



Compounds	R'	n	%Yield	*A. baumannii* MIC (µg/mL)	*E. Coli* MIC (µg/mL)	IC_{50} *E. coli* BioA (µM)
 **55**	$NHNH_2$	1	76	64	256	7.591
 **56**	$NHNH_2$	2	63	32	>256	0.004



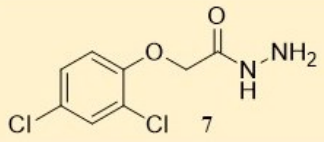
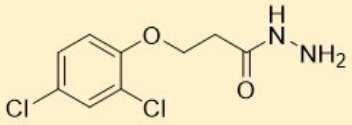
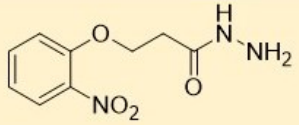
Compounds	R'	R	%Yield	*A. baumannii* MIC (μg/mL)	*E. Coli* MIC (μg/mL)	IC_{50} *E. coli* BioA (μM)
 7	$NHNH_2$	2, 4-Cl	88	64	>256	0.58
	$NHNH_2$	2, 4-Cl	trace, not isolated	n.d	n.d	n.d
	$NHNH_2$	2-NO_2	trace, not isolated	n.d	n.d	n.d

Table 2-10 outlines the general synthetic schemes for the generation of sulfur and oxygen analogs of the lead compound MAC13772. The table lists analogs with specific substituents on the aryl or cyclohexyl ring, such as trifluoromethyl, nitro, and methoxy groups and alkyl chains of varying lengths, each with corresponding yields under each synthetic scheme. The first type of sulfur analog synthesis began with commercially available benzenethiols which were subjected to a nucleophilic aromatic substitution reaction with bromoacetate esters. Similar approaches were employed for oxygen analogs and other types of sulfur analogs as mentioned in subsequent schemes. The choice of base for this reaction was either potassium carbonate (K_2CO_3) or sodium

hydride (NaH), and the reaction medium was either dimethylformamide (DMF) or tetrahydrofuran (THF), conducted at 80°C for 16h. This step was likely aimed at forming a thioether linkage in the case of sulfur analogs, or an ether linkage for oxygen analogs, representing a key structural divergence from the parent molecule. The resultant intermediates were then further reacted with various hydrazine derivatives in methanol at 50°C for 16h to introduce the hydrazine moiety, a critical pharmacophoric element retained from MAC13772. This synthetic approach was designed to explore the impact of sulfur and oxygen substitutions on the biological activity and pharmacokinetic properties of the resulting analogs, aiming to potentially improve upon the lead compound's efficacy and stability.

For carbon and nitrogen analogs the key synthetic step was a cross-coupling to unite fragments 2 and 3 that for which we utilized our *Pd-PEPPSI* pre-catalysts (**Fig. 2-13**), which were commercially available and used by pharmaceutical companies worldwide. The connection point between the aryl ring and the alkyl chain, denoted as 'X', could be a nitrogen, or carbon motif. These motifs can be efficiently assembled using Organ's catalysts (**Table 2-11**).

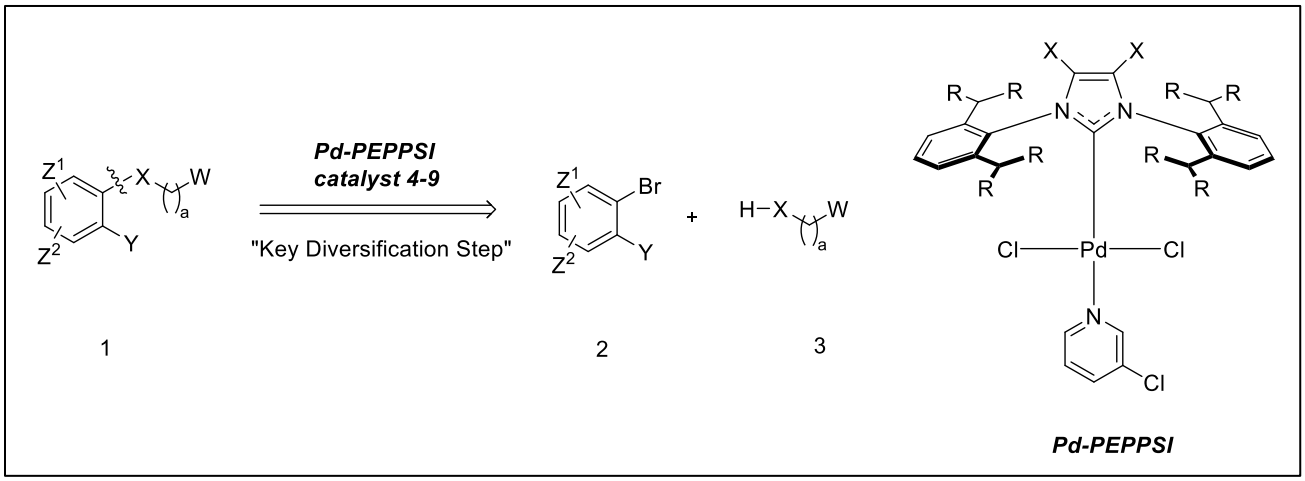
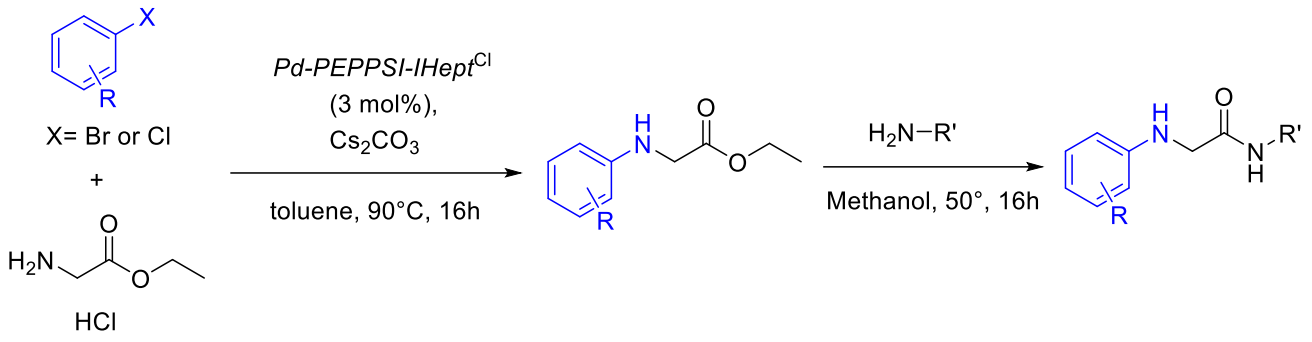


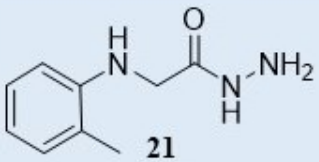
Figure 2-13. Cross-Coupling of Fragments 2 and 3 Using *Pd-PEPPSI* with Variable 'X' Connection Points.

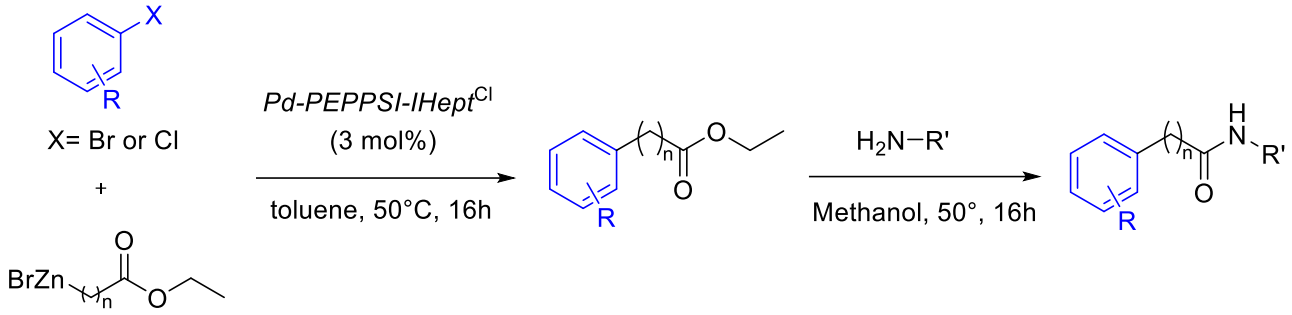
Table 2-11. Variants of *Pd-PEPPSI* Catalysts.

	catalyst	X	R
4.	*Pd-PEPPSI-IPr*	H	Me
5.	*Pd-PEPPSI-IPr^{Cl}*	Cl	Me
6.	*Pd-PEPPSI-IPent*	H	Et
7.	*Pd-PEPPSI-Pent^{Cl}*	Cl	Et
8.	*Pd-PEPPSI-IHept*	H	nPr
9.	*Pd-PEPPSI-IHept^{Cl}*	Cl	nPr

Table 2-12. General Synthetic Schemes for MAC13772 Nitrogen and Carbon Analogs with Listed Synthesized Molecules.



Compound	R'	R	%Yield	*A. baumannii* MIC (µg/mL)	*E. Coli* MIC (µg/mL)	IC_{50} *E. coli* BioA (µM)
 **21**	$NHNH_2$	2-Me	88	256	>256	148.3



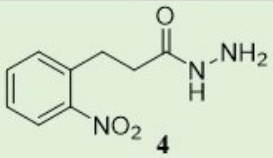
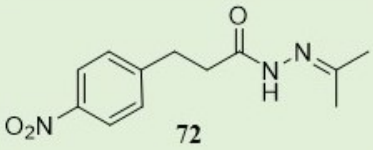
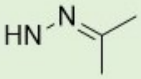
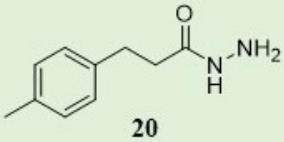
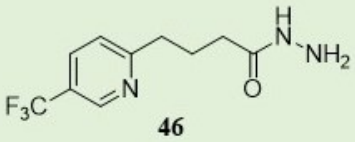
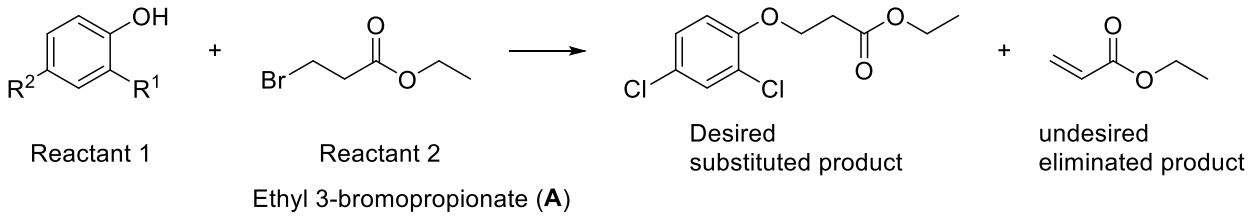
Compounds	R'	R	n	%Yield	*A. baumannii* MIC (µg/mL)	*E. Coli* MIC (µg/mL)	IC_{50} *E. coli* BioA (µM)
 **4**	$NHNH_2$	2-NO_2	2	68	256	>256	71.29
 **72**		4-NO_2	2	73	128	>256	n.d
 **20**	$NHNH_2$	4-Me	2	63	128	>256	0.362
 **46**	$NHNH_2$	4-CF_3	3	73	128	>256	9.73

Table 2-12 provides an overview of the synthetic strategies employed for the generation of nitrogen and carbon analogs of MAC13772. This table outlines the reaction conditions and lists the synthesized nitrogen and carbon analogs derived using these methods.

The compounds synthesized, such as numbers **21**, **4**, **72**, **20,** and **46**, feature distinct substitutions on the aryl ring, such as trifluoromethyl, nitro, and methoxy groups, reflecting a targeted

approach to modulate the electronic and steric characteristics of the molecules. These modifications are designed to probe the influence of different electron-withdrawing or electron-donating groups on the biological activity of the analogs. The strategic modifications to the core structure of MAC13772 highlight an effort to optimize the pharmacological properties of the lead compound, with an emphasis on enhancing potency and specificity through meticulous structural refinement.

Table 2-13. Systematic Optimization Attempts for the Synthesis of Extended Ether Chain Oxygen Analogs of MAC13772 (Part A).



Experiment No.	Reactant 1	Reactant 2	Reagents & Solvent	Temperature (°C)	Outcome
1	R^1= Cl R^2= Cl (1.0 equiv.)	A (1.2 equiv.)	K_2CO_3 (1.5 equiv.), NaI DMF, 16h	80°C	Elimination Product
2	R^1= NO_2 R^2= H (1.0 equiv.)	A (1.2 equiv.)	K_2CO_3 (1.5 equiv.), NaI DMF, 16h	80°C	Elimination Product
3	R^1= Cl R^2= Cl (1.0 equiv.)	A (1.2 equiv.)	NaH (1.5 equiv.), DMF, 16h	80°C	Elimination Product

4	$R^1 = NO_2$ $R^2 = H$ (1.0 equiv.)	A (1.2 equiv.)	NaH (1.5 equiv.), DMF, 16h	80°C	Elimination Product
5	$R^1 = NO_2$ $R^2 = H$ (1.0 equiv.)	A (0.95 equiv.)	n-BuLi (1 equiv.), TMEDA (1.1 equiv.), THF, 16h	25°C	No Product formed
Experiment No.	**Reactant 1**	**Reactant 2**	**Reagents & Solvent**	**Temperature (°C)**	**Outcome**
6	$R^1 = NO_2$ $R^2 = H$ (1.0 equiv.)	A (5.0 equiv.)	Tetrabutylammonium bromide (1.0 equiv.) K_2CO_3 (15.0 equiv.), $H_2O/CHCl_3$, 16h	25°C	Elimination Product
7	$R^1 = Cl$ $R^2 = Cl$ (1.0 equiv.)	A (large excess)	K_2CO_3 (excess) THF, 16h	70°C	Desired Substituted Product
8	$R^1 = Cl$ $R^2 = Cl$ (1.0 equiv.)	A (large excess)	K_2CO_3 (excess) THF, 16h	70°C	Desired Substituted Product

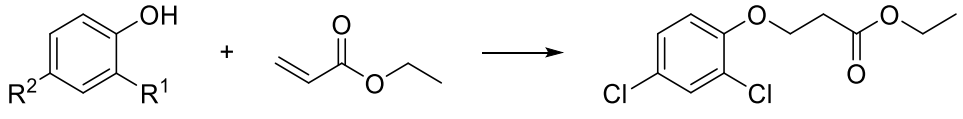
The optimization of oxygen analogs, as depicted in **Table 2-13**, was a meticulous process aimed at extending the ether chain by one carbon unit. Initial attempts involved reacting substituted phenols with corresponding bromoesters, which predominantly led to an undesired elimination

by-product rather than the targeted substituted product. This suggested a competitive scenario where elimination reactions were favored over substitution in this context. The nuances of this phenomenon are grounded in the delicate balance of thermodynamics, kinetics and the relative stability of the resulting products and the reaction conditions that favor the formation of alkenes in an elimination reaction.

To address this challenge, various strategies were employed. The reaction conditions were fine-tuned by altering the equivalents of reactants and reagents, experimenting with different bases, and varying the reaction temperature and solvents. Despite the use of a strong base like n-BuLi and chelating ligands such as TMEDA, the desired product formation was not achieved. The introduction of a phase transfer catalyst, tetrabutylammonium bromide, and the execution of the reaction in a biphasic water-chloroform system also did not steer the reaction towards the desired substitution pathway; the elimination product was still obtained.

In further trials, a large excess of the reactant ester and an excess of the base were used, which did lead to the detection of product formation via GCMS. However, this approach was not pursued to the stage of product isolation due to considerations of green chemistry principles. The excessive use of reagents and the generation of large volumes of waste conflicted with the ideals of sustainability and efficiency.

Table 2-14. Systematic Optimization Attempts for the Synthesis of Extended Ether Chain Oxygen Analogs of MAC13772 (Part B).



Reactant 1 | Reactant 2 Ethyl acrylate (**B**) | Desired product

Experiment No.	Reactant 1	Reactant 2	Reagents & Solvent	Temperature (°C)	Outcome
1	R^1= Cl R^2= Cl (1.0 equiv.)	B (1.2 equiv.)	NaH (1.5 equiv.), DMF, 16h	80°C	No Product formed
2	R^1= NO_2 R^2= H (1.0 equiv.)	B (1.2 equiv.)	NaH (1.5 equiv.), DMF, 16h	80°C	No Product formed
3	R^1= Cl R^2= Cl (1.0 equiv.)	B (1.2 equiv.)	Et_3N (1.5 equiv.), acetonitrile, 2 days	50°C	No Product formed
4	R^1= NO_2 R^2= H (1.0 equiv.)	B (1.2 equiv.)	Et_3N (1.5 equiv.), acetonitrile, 2 days	50°C	No Product formed

In the concluding phase of our optimization efforts for the oxygen analog, as detailed in **Table 2-14**, we changed our strategy in response to the unsuccessful outcomes of previous attempts. We switched our starting reactant to the undesired alkene that was previously a by-product, aiming to harness it through a conjugated addition reaction mechanism. This adjustment represented a significant shift in our synthetic approach, hoping to utilize the reactive nature of the alkene in a more favorable reaction pathway.

Despite this strategic change, we explored different solvents such as DMF and acetonitrile, and varied the bases between sodium hydride (NaH) and triethylamine (Et_3N), while carefully

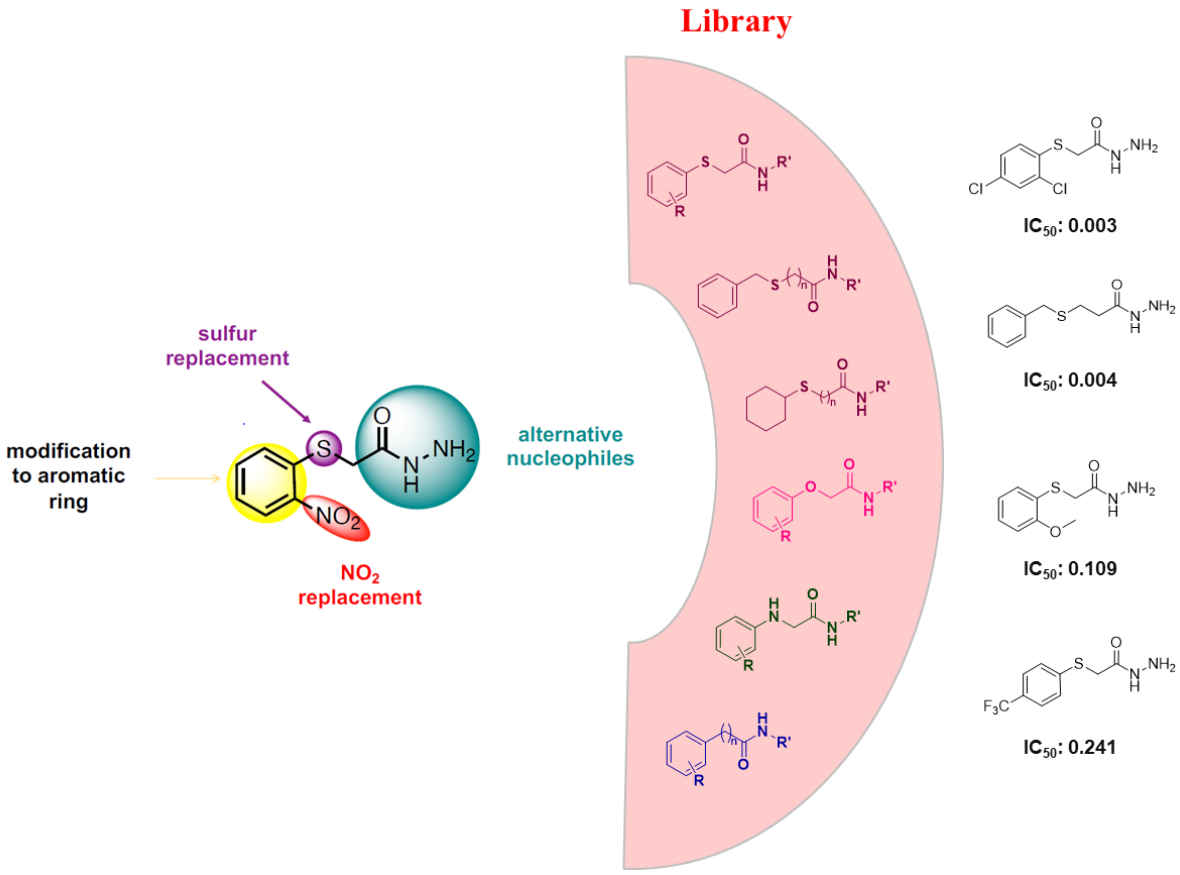
maintaining the usual stoichiometric ratios of the reactants without using excess. The temperature parameters were also modified in an attempt to optimize the reaction conditions. However, these efforts did not yield the anticipated product; instead, we consistently recovered the starting materials, indicating no progress toward the conjugated addition product.

The series of experiments underscored the complexity of the synthetic process and the challenges in manipulating reaction pathways to achieve desired outcomes. This experience, although it ended without the successful synthesis of the intended product, provided valuable insights into the reaction mechanisms and the limitations of the current methodology, which can inform future research endeavors in the field.

2.4. Conclusion of Chapter 2

MAC-13772 is an inhibitor of biotin biosynthesis that showed promising activity against Gram-negative pathogens *in vivo*. Although, the activity of MAC-13772 is limited by its low bioavailability *in vivo*. In this study, we synthesized a series of analogs in an attempt to enhance MAC-13772 potency and metabolic stability. Among the all new analogs, three demonstrated improvements in each characteristic: higher potency, increased resistance to liver metabolism, and stronger inhibition of the enzyme. However, we were unable to identify an analog that possessed all the improved properties simultaneously. Nonetheless, we hope that our findings will provide valuable guidance for future modifications to the compound's structure.

CHAPTER 3: CONCLUSION & FUTURE DIRECTIONS



Given the detadiscussed;ration of SAR for MAC13772 analogs and the comprehensive synthetic methodologies discussed, we can conclude that significant advancements have been made in understanding the structure-activity relationships of these compounds. Our work has successfully identified modifications that enhance potency and metabolic stability, offering promising directions for future antibacterial agent development. Among my synthesized molecules, four molecules exhibited better IC_{50} values compared to the lead molecule, MAC13772.

Looking ahead, there's a need for further SAR exploration, building on the foundation laid by the current SAR studies, future research should continue to explore the chemical space around MAC13772 analogs. This includes investigating additional heteroatom substitutions and further

modifications to the thioether chain, as well as exploring other potential sites for modification that have not yet been considered.

Given the improvements seen with certain analogs, a focused effort on enhancing metabolic stability through structural modifications is required. This includes systematic testing of analogs for in vivo efficacy and pharmacokinetics to identify candidates with optimized profiles for therapeutic development.

Moreover, assessing the potential for resistance development against these analogs in bacterial populations is necessary. Long-term exposure studies could help identify any emerging resistance mechanisms and guide the design of analogs to circumvent such issues.

Additionally, expanding the evaluation of these analogs against a wider range of bacterial pathogens, including Gram-positive bacteria, could uncover broad-spectrum antibacterial agents. This would significantly increase the therapeutic potential of these compounds.

Through these future directions, the work presented in this thesis lays a solid foundation for the continued development of MAC13772 analogs as potent, stable, and effective antibacterial agents.

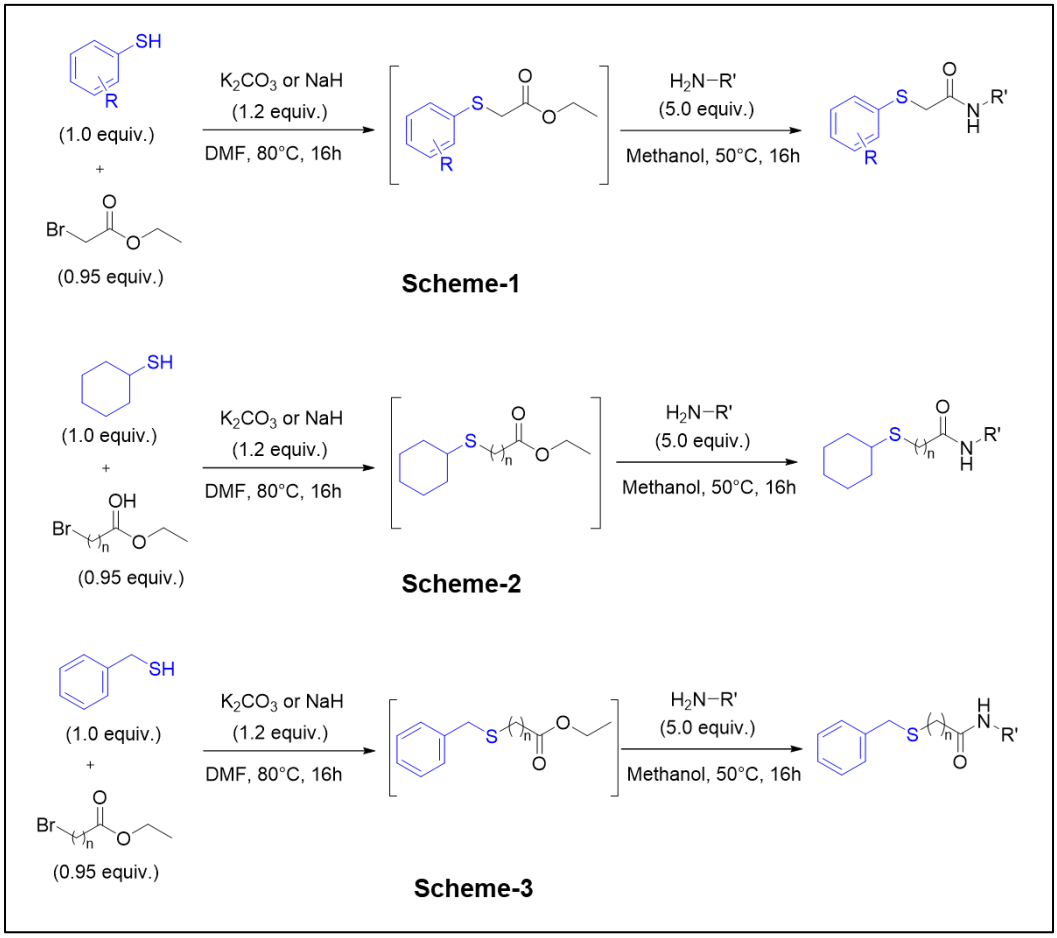
CHAPTER 4: SUPPLEMENTAL INFORMATION

4.1. General experimental

All experiments were carried out under a nitrogen atmosphere in oven-dried or flame-dried glassware using standard Schlenk techniques unless noted otherwise. Glovebox manipulations were performed in an MBraun Unilab glove-box under nitrogen atmosphere. All reagents were purchased from Sigma-Aldrich or Alfa Aesar and were used without further purification unless noted otherwise. All the *Pd-PEPPSI* precatalysts were provided by Total Synthesis Ltd (Toronto, Ontario, Canada). THF was distilled under nitrogen using SPS prior to use. Toluene was distilled under nitrogen using SPS prior to use. Analytical thin layer chromatography (TLC) was performed on EMD 60 F254 pre-coated glass plates and spots were visualized with UV light (254 nm) or a staining solution ($KMnO_4$ or CAM). Column chromatography purifications were carried out using either the flash technique on EMD silica gel 60 (230 – 400 mesh) or the Biotage Isolera Four with 10 g SNAP cartridges. NMR spectra were recorded on Bruker 300 AVANCE or Bruker 400 AVANCE or Bruker 500 AVANCE or Bruker 600 AVANCE spectrometer. The chemical shifts for ^{1}H NMR spectra are given in parts per million (ppm) referenced to the residual proton signal of the deuterated solvent (d = 7.28 ppm for $CDCl_3$); coupling constants are expressed in Hertz (Hz). ^{13}C NMR spectra were referenced to the carbon signals of the deuterated solvent (d = 76.9 ppm for $CDCl_3$). The following abbreviations are used to describe peak multiplicities: s = singlet, br s = broad singlet, d = doublet, dd = doublet of doublet, t = triplet, q = quartet, and m = multiplet. High Resolution Mass Spectrometry (HRMS) analysis was performed by the John L. Holmes Spectrometry Department at University of Ottawa, Canada.

4.2. Synthetic Procedures

4.2.1. General Procedure A: Sulfur analogs

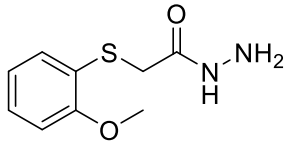


Schemes 4-1, 4-2 & 4-3. General Synthetic Procedures for Sulfur analogs

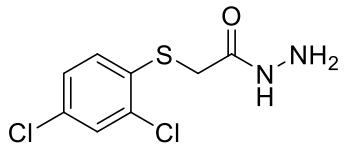
Procedure adapted from literature.[142–148] In a dry 5 mL glass vial, relevant arylthiol (1 mmol, 1 equiv.) or cyclohexyl thiol (1 mmol, 1 equiv.) and K_2CO_3 (1.5 mmol, 1.5 equiv.) or NaH (60% dispersion in mineral oil, 3.3 mmol, 1.1 equiv.) were added. The vial was fitted with a screw cap and Teflon septa, purged, and refilled with N_2. Next, 3 mL of DMF was added. The reaction mixture was stirred at 80 °C for 30 min, followed by the addition of ethyl bromoacetate (0.95 mmol, 0.95 equiv.) or ethyl 3-bromopropionate (0.95 mmol, 0.95 equiv.). After stirring for 16 h,

the reaction mixture was diluted with ethyl acetate, the organic layer was extracted with water (5 x 10 mL) and the mixture washed with brine and dried over magnesium sulfate ($MgSO_4$). The crude mixture was concentrated under reduced pressure and purified by flash column chromatography (0 to 20% gradient of ethyl acetate in hexanes). The purified product was dissolved in methanol (3 mL), and hydrazine hydrate (5 equiv.) added. The reaction mixture was stirred at 50 °C for 16h. The solvent was evaporated under reduced pressure to afford the crude product that was then diluted with water and washed with ethyl acetate (3 x 10 mL). The combined organic layers were dried over anhydrous magnesium sulfate and concentrated under reduced pressure to yield the final product.

Experimental data

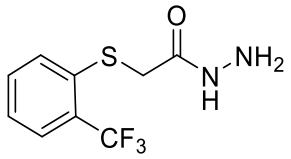


Following General Procedure A, 185.5 mg of **24** (77% yield) was isolated as a fluffy white solid. Mp: 185-187 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.06 (br s, 1H), 7.30-7.27 (m, 1H), 7.27-7.24 (m, 1H), 6.94 (td, *J* = 7.6, 1.2 Hz, 1H), 6.89 (dd, *J* = 8.2, 1.2 Hz, 1H), 3.91 (s, 3H), 3.82 (br s, 2H), 3.62 (s, 2H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 169.0, 157.8, 131.2, 129.1, 122.1, 121.7, 111.1, 56.1, 35.9. The spectral data are consistent with those reported in the literature.[149]

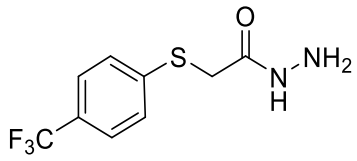


Following General Procedure A, 125.4 mg of **12** (42% yield) was isolated as a yellow powder. Mp: 147-149 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.71 (br s, 1H), 7.42 (d, *J* = 2.2 Hz, 1H), 7.23 (dd, *J* = 8.4, 2.2 Hz, 1H), 7.18 (d, *J* = 8.4 Hz, 1H), 3.85 (br s, 2H), 3.67 (s, 2H); ^{13}C NMR (125

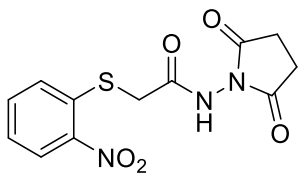
MHz, $CDCl_3$) δ 167.9, 134.3, 133.3, 132.4, 130.0, 129.4, 128.1, 35.2; HRMS (EI) [M+] calculated for $C_8H_8Cl_2N_2OS$, 249.9734; found 249.9746.



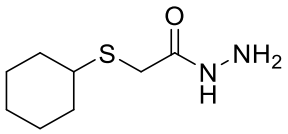
Following General Procedure A, 207.6 mg of **33** (83% yield) was isolated as a white powder. Mp: 212-214 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (br s, 1H), 7.65 (d, J = 7.8 Hz, 1H), 7.48 (t, J = 7.8 Hz, 1H), 7.40 (d, J = 7.8 Hz, 1H), 7.31 (t, J = 7.8 Hz, 1H), 3.69 (s, 2H), 3.47 (br s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.1, 133.9, 129.9 (quart, $^2J_{CF}$ = 31 Hz), 129.7 (quart, $^3J_{CF}$ = 24 Hz), 127.2 (quart, $^3J_{CF}$ = 4 Hz), 127.0, 125.1 (quart, $^1J_{CF}$ = 217 Hz), 122.4, 36.2; HRMS (EI) [M+] calculated for $C_9H_9F_3N_2OS$, 250.0388; found 250.0395.



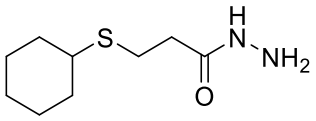
Following General Procedure A, 172.5 mg of **32** (69% yield) was isolated as a white powder. Mp: 175-177 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.30 (br s, 1H), 7.59 (d, J = 8.3 Hz, 2H), 7.49 (d, J = 8.3 Hz, 2H), 4.30 (br s, 2H), 3.68 (s, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.3, 143.0, 127.4, 127.1 (quart, $^2J_{CF}$ = 43 Hz), 126.0 (quart, $^3J_{CF}$ = 4 Hz), 123.4 (quart, $^1J_{CF}$ = 270 Hz), 33.9; HRMS (EI) [M+] calculated for $C_9H_9F_3N_2OS$, 250.0388; found 250.0330.



Using the General Procedure A the starting material was prepared and then the hydrazide product was stirred in glacial acetic acid (6 mL) with succinic anhydride or methyl succinic anhydride at 110 °C for 16h. The reaction mixture was diluted with H_2O, then washed with EtOAc (3x10mL). The combined organic phase was washed with brine, dried over anhydrous magnesium sulfate ($MgSO_4$) and concentrated under reduced pressure. A yellow powder of **69** was isolated with a yield of 196.07 mg (53% yield). Mp: 185-188 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.88 (br s, 1H), 8.25-8.19 (m, 1H), 7.75-7.67 (m, 2H), 7.44 (ddd, J = 8.4, 6.5, 2.0 Hz, 1H), 4.06 (s, 2H), 2.76 (s, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.9, 166.3, 145.4, 135.6, 134.3, 127.7, 125.9, 125.8, 33.6, 26.3; HRMS (EI) [M+] calculated for $C_{12}H_{11}N_3O_5S$ 309.0419; found 309.0429.

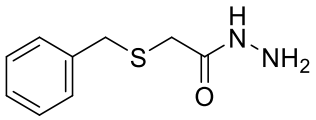


Following General Procedure A, 140.5 mg of **53** (71% yield) was isolated as a yellow powder. Mp: 154-156 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.44 (br s, 1H), 4.20 (br s, 2H), 3.26 (s, 2H), 2.67 (s, 1H), 1.93 (s, 2H), 1.73 (s, 2H), 1.61 (d, J = 10.0 Hz, 1H), 1.36-1.17 (m, 5H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.1, 49.8, 44.4, 33.1, 32.7, 32.4, 25.8, 25.6. The spectral data are consistent with those reported in the literature.[150] HRMS (EI) [M+] calculated for $C_8H_{16}N_2OS$ 188.0983; found 188.0969.

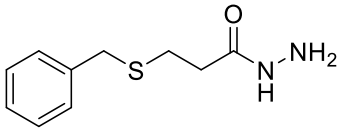


Following General Procedure A, 155.2 mg of **54** (73% yield) was isolated as a brown powder. Mp: 157-159 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.12 (br s, 1H), 3.46 (br s, 2H), 2.81 (td, J = 7.2,

1.2 Hz, 2H), 2.64 (tt, *J* = 10.6, 4.0 Hz, 1H), 2.42 (td, *J* = 7.2, 1.4 Hz, 2H), 1.97-1.88 (m, 2H), 1.80-1.66 (m, 2H), 1.63-1.54 (m, 1H), 1.36-1.14 (m, 5H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.2, 43.7, 34.9, 33.5, 25.9, 25.6, 25.5. HRMS (EI) [M-$NHNH_2$]+ calculated for $C_9H_{16}OS$ 172.0922; found 171.0903.

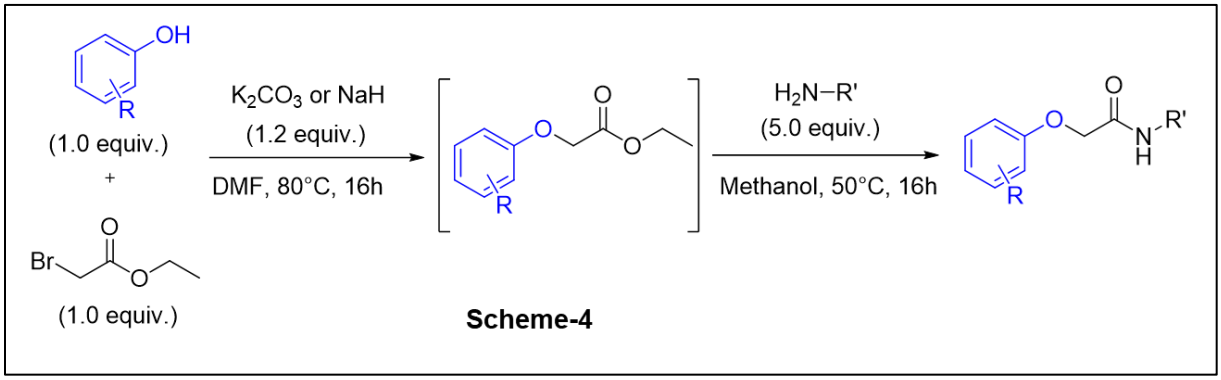


Following General Procedure A, 162.3 mg of **55** (76% yield) was isolated as a white powder. Mp: 168-170 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.50 (br s, 1H), 7.35-7.30 (m, 2H), 7.29-7.25 (m, 3H), 4.89 (br s, 2H), 3.74 (s, 2H), 3.14 (s, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.5, 137.0, 128.9, 128.5, 127.3, 36.9, 33.1. The spectral data are consistent with those reported in the literature.[151,152]



Following General Procedure A, 80.78 mg of **56** (76% yield) was isolated as a pale brown powder. Mp: 155-157 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35-7.29 (m, 4H), 7.27-7.23 (m, 1H), 6.79 (br s, 1H), 3.73 (s, 2H), 2.74 (t, *J* = 7.1 Hz, 2H), 2.32 (t, *J* = 7.1 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.3, 138.5, 129.1, 128.9, 127.5, 36.9, 34.7, 27.4. HRMS (EI) [M-$NHNH_2$]+ calculated for $C_{10}H_{12}OS$ 180.0609; found 180.0619.

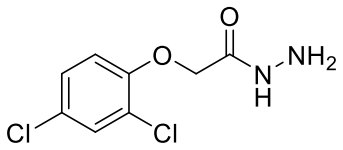
4.2.2. General Procedure B: Oxygen analogs



Schemes 4-4. General Synthetic Procedures for Oxygen analogs

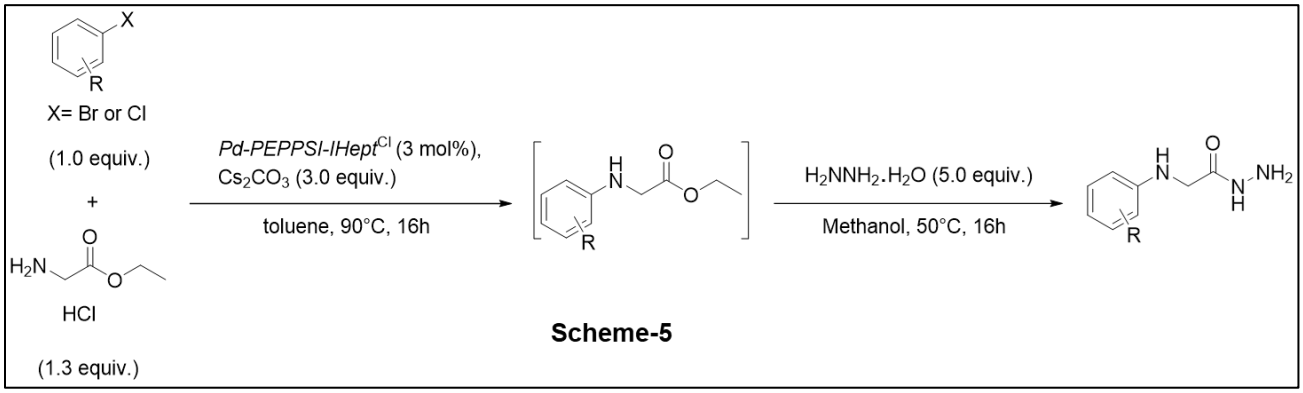
Procedure adapted from literature.[153–155] In a dry 5 mL glass vial, the appropriate aryl alcohol (1 mmol, 1 equiv.) and K_2CO_3 (1.2 mmol, 1.2 equiv.) were added. The vial was fitted with a screw cap and Teflon septum, then purged and refilled with N_2 after which 3 mL of DMF was added. The reaction mixture was stirred at 80 °C for 30 min, after which ethyl bromoacetate (1 mmol, 1 equiv.) was added. Following a 16h stirring period, the reaction mixture was diluted with ethyl acetate, and the organic layer extracted with water (5 x 10 mL). The mixture was then washed with brine, dried over anhydrous magnesium sulfate ($MgSO_4$), concentrated under reduced pressure and the crude product purified by flash column chromatography (0 to 20% gradient of ethyl acetate in hexanes). The purified product was dissolved in methanol (3 mL), hydrazine hydrate (5 equiv.) was added, and the reaction was stirred at 50 °C for 16h. The solvent was evaporated under reduced pressure to afford the crude product; then diluted with water and washed with ethyl acetate (3 x 10 mL). The combined organic layers were dried over magnesium sulfate and concentrated under reduced pressure to yield the final product.

Experimental data



Following General Procedure C, 206.8 mg of **7** (88% yield) was isolated as a white powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.22 (br s, 1H), 7.53 (d, *J* = 2.4 Hz, 1H), 7.33 (dd, *J* = 8.8, 2.4 Hz, 1H), 7.03 (d, *J* = 8.8 Hz, 1H), 4.56 (s, 2H), 4.32 (br s, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ166.4, 153.0, 129.8, 128.4, 125.5, 122.9, 115.7, 67.5. HRMS (EI) [M+] calculated for $C_8H_8Cl_2N_2O_2$ 233.9963; found 233.9960. The spectral data are consistent with those reported in the literature.[156,157]

4.2.2. General Procedure C: Nitrogen analogs



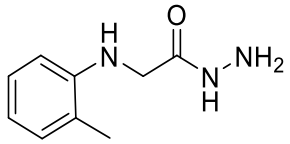
Scheme-5

Schemes 4-5. General Synthetic Procedures for Nitrogen analogs

Procedure adapted from literature.[158] A dry Schlenk flask, equipped with a stir bar, was charged with *Pd-PEPPSI-IHept^{Cl}* (3 mol%), followed by the addition of Cs_2CO_3 (3 mmol, 3.0 equiv.) and glycine ethyl ester hydrochloride (1.3 mmol, 1.3 equiv.). If the aryl halide was in solid form, it was added at this point (1 mmol, 1 equiv.). The flask was then evacuated and backfilled with nitrogen three times. If the aryl halide was in liquid form, it was added via a microliter syringe

after the evacuation and backfilling process. Toluene (6 mL) was added to the flask, and the resulting mixture was stirred for five min at room temperature. The reaction mixture was then stirred with heating (90 °C) for 16h after which it was allowed to cool to room temperature and filtered through a small pad of Celite, washing with diethyl ether. The filtrate was evaporated, and the residue was purified by silica gel column chromatography. The purified product was dissolved in methanol (3 mL), hydrazine hydrate (5 equiv.) was added, and the reaction was stirred at 50 °C for 16h. The solvent was evaporated under reduced pressure to afford the crude product after which it was diluted with water and washed with ethyl acetate (3 x 10 mL). The combined organic layers were dried over anhydrous magnesium sulfate ($MgSO_4$) and concentrated under reduced pressure to yield the final product.

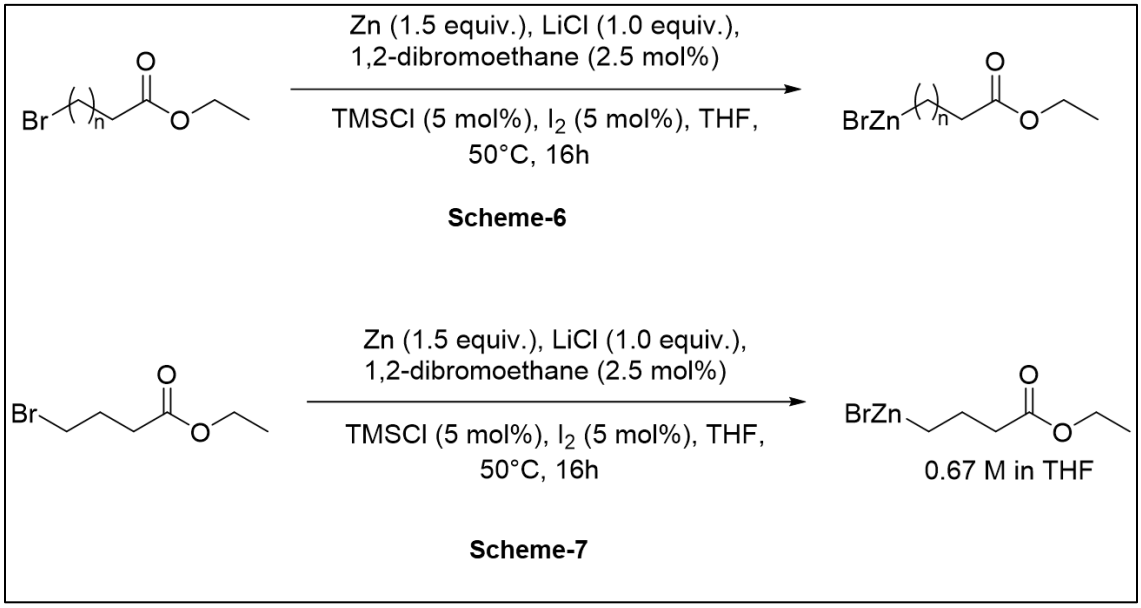
Experimental data



Following General Procedure B, 152.4 mg of **21** (88% yield) was isolated as a white powder. Mp: 163-165 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.30 (br s, 1H), 6.98-6.96 (m, 2H), 6.52-6.47 (m, 1H), 6.35 (t, *J* = 7.2 Hz, 1H), 4.91 (br s, 2H), 4.06 (d, *J* = 5.6 Hz, 1H), 3.78 (d, *J* = 5.6 Hz, 1H), 2.08 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.8, 146.3, 130.2, 127.3, 121.9, 116.5, 109.8, 46.2, 17.8. HRMS (EI) [M+] calculated for $C_9H_{13}N_3O$ 179.1059; found 179.1048. The spectral data are consistent with those reported in the literature.[159]

4.2.4. General Procedure D: Carbon analogs

Preparation of organozinc reagent

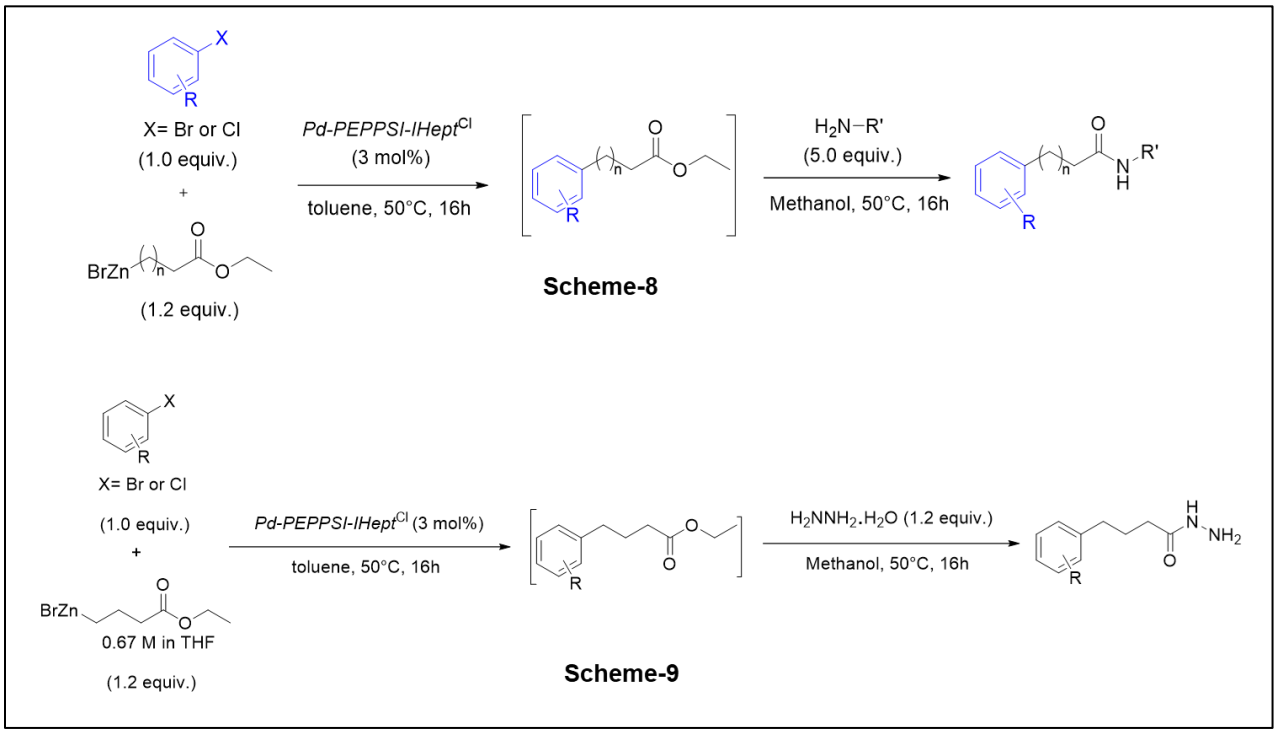


Schemes 4-6 & 4-7. General Synthetic Procedures for Preparation of organozinc reagent

Procedure adapted from literature.[160 161 162] For **Scheme-7**, a 10 mL round-bottom flask was oven dried, sealed with a rubber septum and subjected to three cycles of purging with nitrogen gas to create an inert atmosphere. Under high vacuum, the flask was heated with a heat gun for 15 min. Lithium chloride (LiCl, 217.35 mg, 5.13 mmol, 1 equiv.) and zinc powder (Zn, 516.50 mg, 7.9 mmol, 1.5 equiv.) were added to the round-bottom flask under an inert atmosphere. The flask was then sealed and again purged with nitrogen gas 3X after which THF (4.5 mL) was added via syringe. The mixture was heated at 60 °C with stirring for 10 min, after which 1,2-dibromoethane (11 μL, 0.13 mmol, 0.025 equiv.) was added dropwise. Heating was continued for an additional 10 min or until the appearance of bubbling.

The reaction mixture was then allowed to cool to room temperature. Trimethylsilyl chloride (TMSCl, 32.5 µL 0.25 mmol, 0.05 equiv.) was added to the flask, followed by the addition of iodine solution (65 mg, 0.25 mmol, 0.05 equiv.) in THF (0.63 mL) via syringe. The reaction mixture was stirred at room temperature for 20 min and ethyl 4-bromopropionate (1 g, 5.127 mmol, 1 equiv.) was added drop-wise to the reaction mixture over 5 min. The mixture was then heated at 50 °C with stirring for 16h where upon it was cooled to room temperature and allowed to stand without stirring for 24h. The concentration of the organozinc solution was determined by iodometric titration of the resulting supernatant using Knochel's procedure.

General Procedure for Negishi Cross-Coupling of Aryl Halides with organozinc reagents

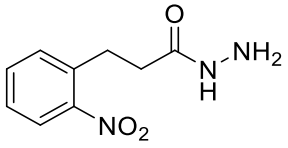


Schemes 4-8 & 4-9. General Synthetic Procedures for Carbon analogs

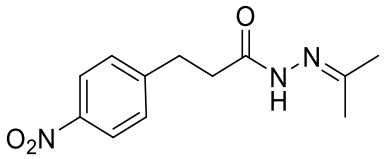
An oven-dried Schlenk flask, equipped with a stir bar, was charged with *Pd-PEPPSI-IHept*^{Cl} (0.03 mmol, 0.03 equiv.), If the aryl halide was in solid form, it was added at this point (1 mmol, 1 equiv.). The flask was then evacuated and backfilled with nitrogen three times. If the aryl

halide was in liquid form, it was added via a microliter syringe after the evacuation and backfilling process. Toluene (2 mL) was added to the flask, and the resulting mixture was stirred for five minutes at room temperature. A THF solution of the relevant organozinc reagent (1.2 mmol, 1.2 equiv.) was added and the reaction mixture was then heated and stirred at 50 °C for 16h after which it was allowed to cool to room temperature and was then filtered through a small pad of Celite, washing with diethyl ether. The filtrate was evaporated, and the residue was diluted with water and washed with ethyl acetate (3 x 10 mL). The combined organic layers were dried over magnesium sulfate and concentrated under reduced pressure. The purified product was dissolved in methanol (3 mL), and hydrazine hydrate (5 equiv.) was added. This reaction was stirred at 50 °C for 16h. The solvent was evaporated under reduced pressure to afford the crude product; then diluted with water and washed with ethyl acetate (3 x 10 mL). The combined organic layers were dried over magnesium sulfate ($MgSO_4$) and concentrated under reduced pressure to yield the final product.

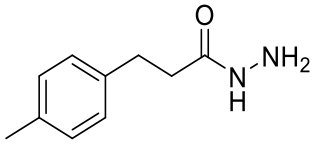
Experimental data



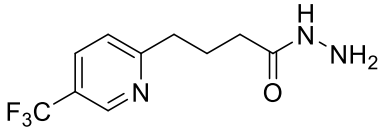
Following General Procedure D, 162.0 mg of **4** (68% yield) was isolated as an orange powder. Mp: 145-147 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.98 (br s, 1H), 7.92 (dd, *J* = 8.1, 1.3 Hz, 1H), 7.64 (td, *J* = 7.6, 1.4 Hz, 1H), 7.51-7.44 (m, 2H), 4.16 (s, 2H), 3.04 (dd, *J* = 8.4, 6.9 Hz, 2H), 2.38 (dd, *J* = 8.3, 7.0 Hz, 2H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 170.7, 149.6, 135.9, 133.8, 132.3, 128.2, 124.8, 34.4, 28.2. The spectral data are consistent with those reported in the literature.[163] HRMS (EI) [M+] calculated for $C_9H_{11}N_3O_3$ 209.0800; found 209.0890.



Following General Procedure D, 181.9 mg of **72** (73% yield) was isolated as a white powder. Mp: 161-164 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.0 (br s, 1H), 8.12-8.08 (m, 2H), 7.49-7.45 (m, 2H), 2.94 (t, J = 7.7 Hz, 2H), 2.54 (t, J = 7.7 Hz, 2H), 1.84 (d, J = 7.4 Hz, 3H), 1.76 (d, J = 7.4 Hz, 3H); ^{13}C NMR (500 MHz, DMSO-d_6) δ 173.4, 167.6, 150.6, 146.3, 130.1, 123.8, 34.9, 31.1, 25.6, 17.4. HRMS (EI) [M+] calculated for $C_{12}H_{15}N_3O_3$, 249.1113; found 249.1108.



Following General Procedure D, 112 mg of **20** (63% yield) was isolated as a white powder. Mp: 137-140 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.91 (br s, 1H), 7.03 (s, 4H), 4.12 (br s, 2H), 2.72 (t, J = 7.4 Hz, 2H), 2.24 (t, J = 7.4 Hz, 2H), 2.21 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.3, 138.6, 135.2, 129.3, 128.5, 35.7, 31.1, 21.1; HRMS (EI) [M+] calculated for $C_{10}H_{14}N_2O$, 178.1106; found 178.1178.



Following General Procedure D, 182.0 mg of **46** (73% yield) was isolated as a white powder. Mp: 162-164 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.76 (d, J = 2.4 Hz, 1H), 8.48 (br s, H), 7.83 (dd, J = 8.2, 2.4 Hz, 1H), 7.29 (d, J = 8.2 Hz, 1H), 3.64 (br s, 2H), 2.89 (t, J = 7.4 Hz, 2H), 2.22 (t, J = 7.2 Hz, 2H), 2.08 (p, J = 7.4 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.4, 165.3, 146.2 (quart, $^{3}J_{CF}$ = 4 Hz), 133.8 (quart, $^{3}J_{CF}$ = 4 Hz), 124.6 (quart, $^{2}J_{CF}$ = 33 Hz), 123.7 (quart, $^{1}J_{CF}$ =

270 Hz), 122.9, 37.0, 33.4, 25.3. HRMS (EI) [M+] calculated for $C_{10}H_{12}F_3N_3O$ 247.0932; found 247.0915.

4.2. Supporting Info for Chapter 2

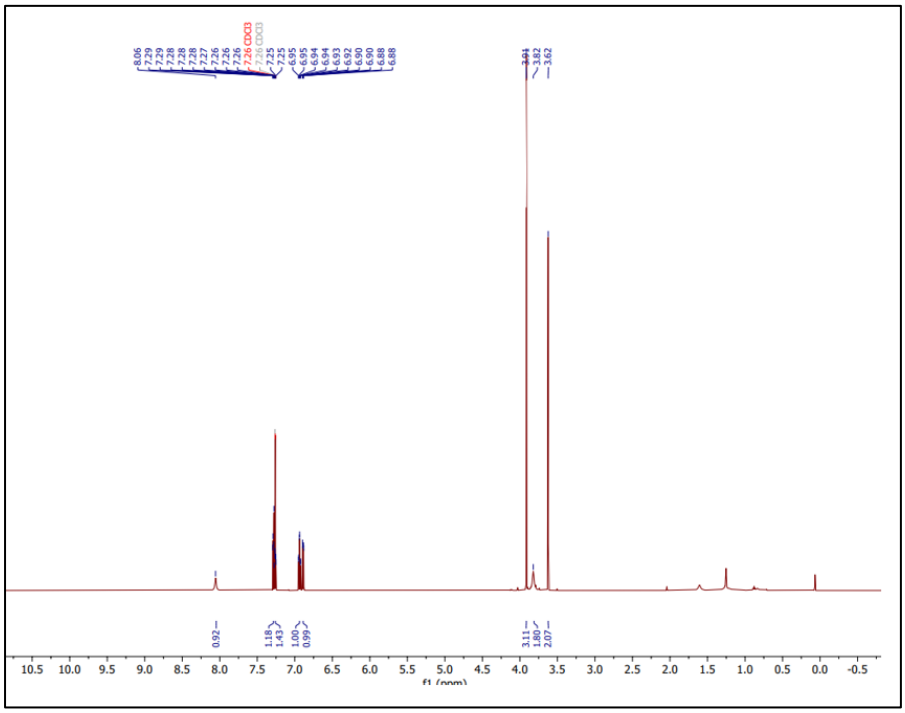


Figure 1. ^{1}H NMR spectrum of **24**.

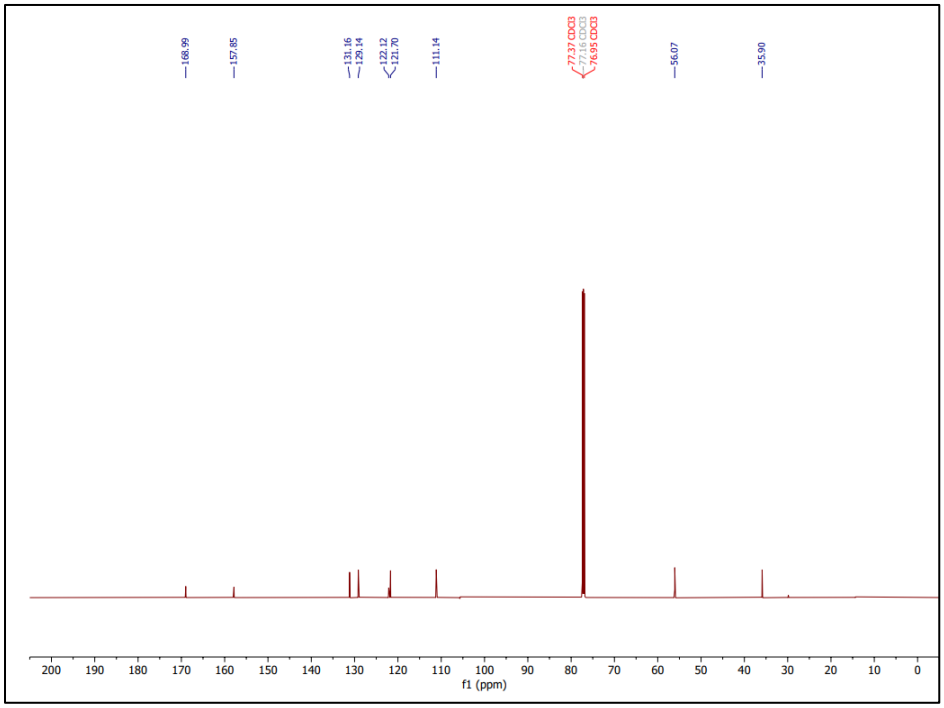


Figure 2. ^{13}C NMR spectrum of **24**.

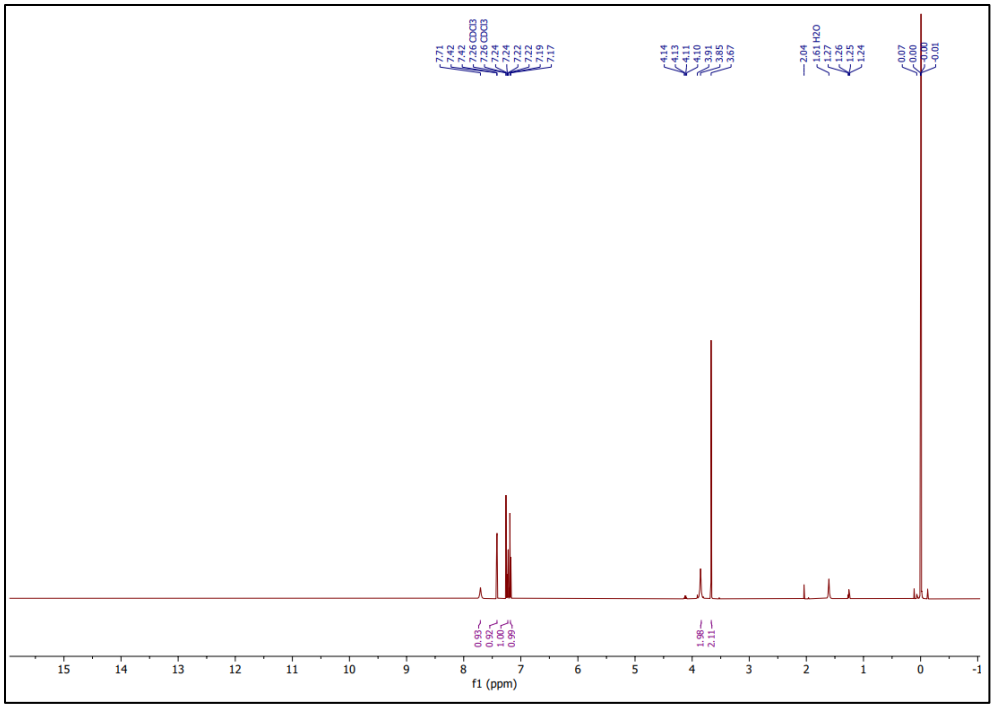


Figure 3. ^{1}H NMR spectrum of **12**.

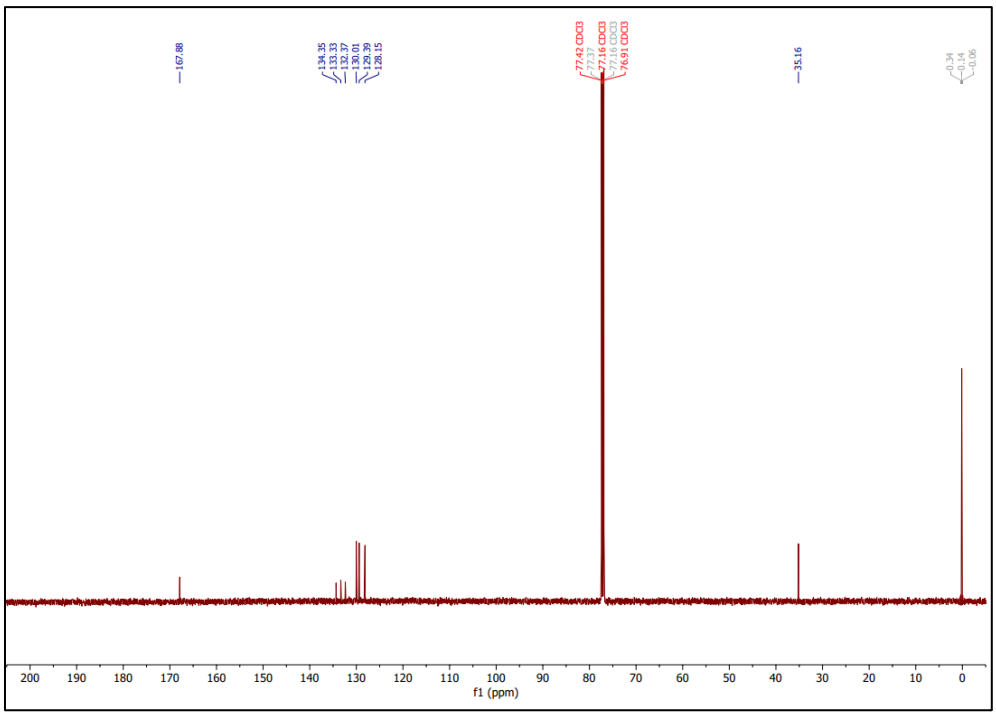


Figure 4. ^{13}C NMR spectrum of **12**.

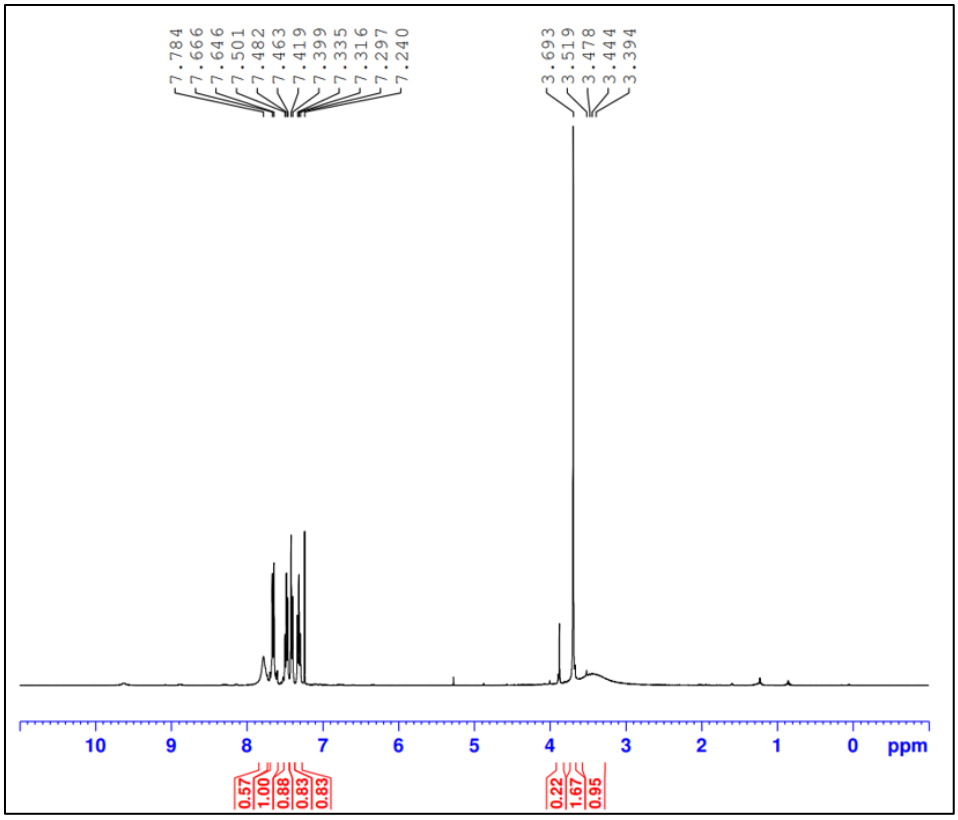


Figure 5. ^{1}H NMR spectrum of **33**.

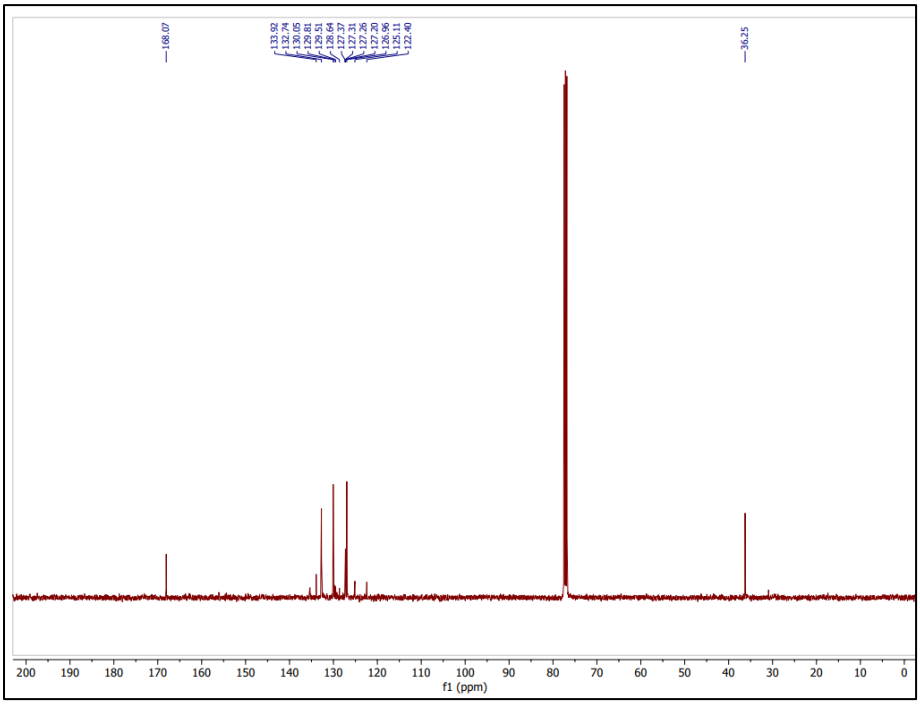


Figure 6. ^{13}C NMR spectrum of **33**.

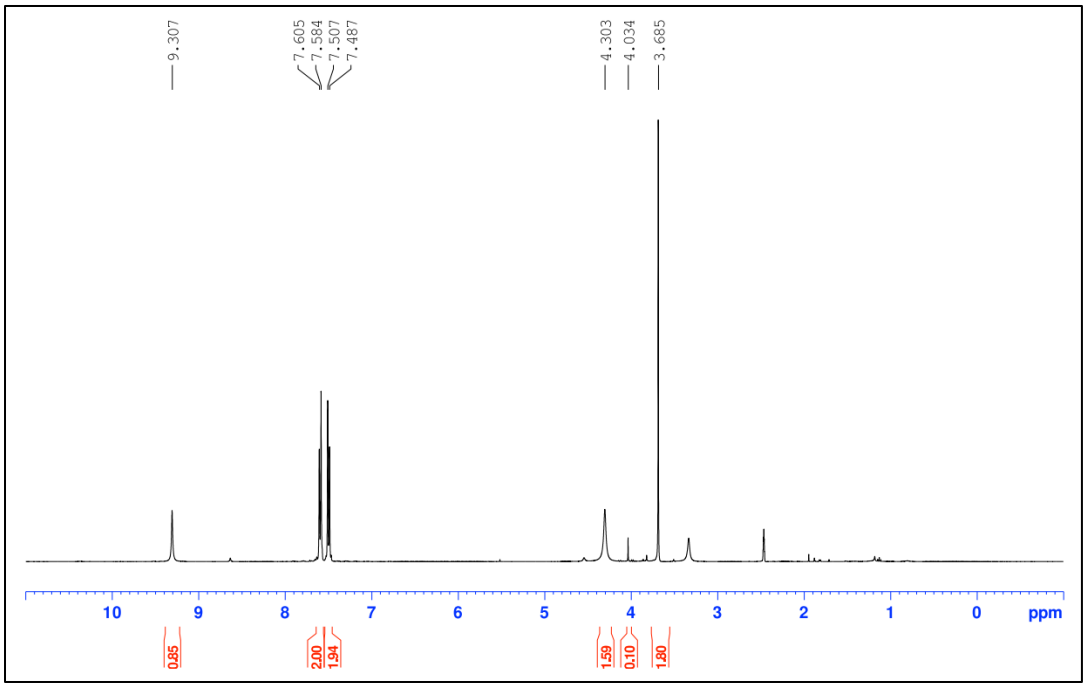


Figure 7. ^{1}H NMR spectrum of **32**.

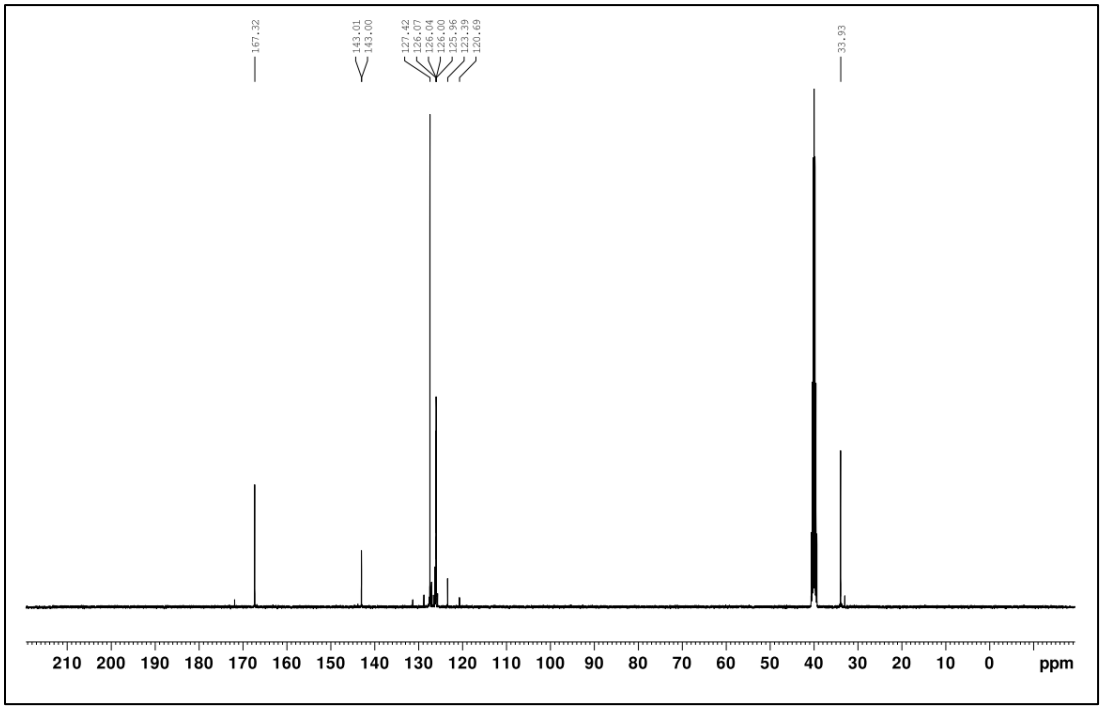


Figure 8. ^{13}C NMR spectrum of **32**.

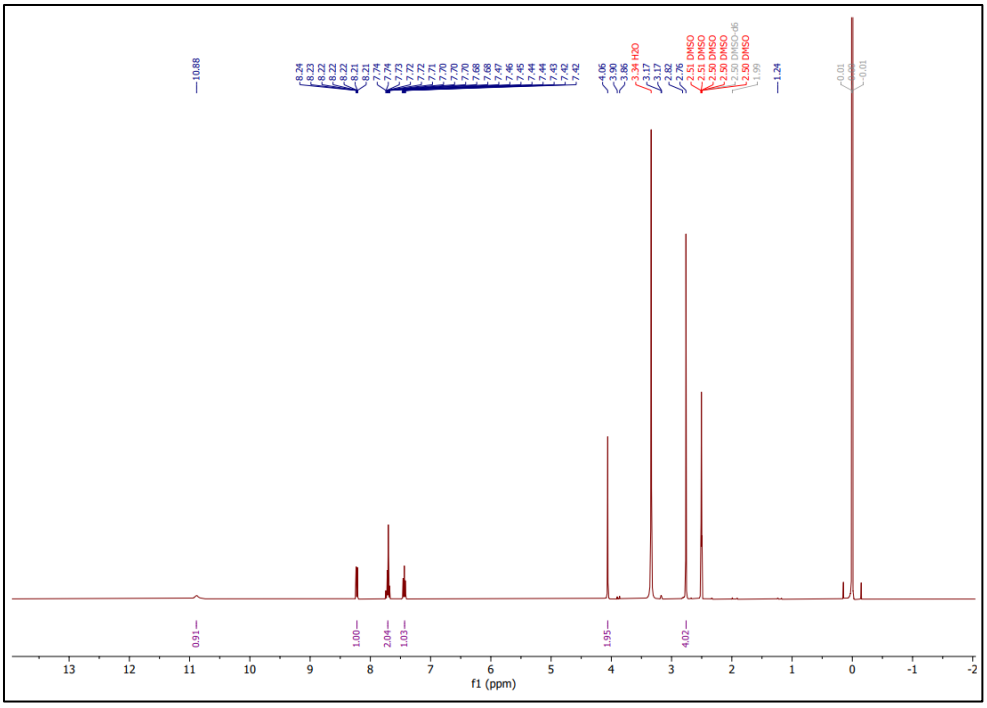


Figure 9. ^{1}H NMR spectrum of **69**.

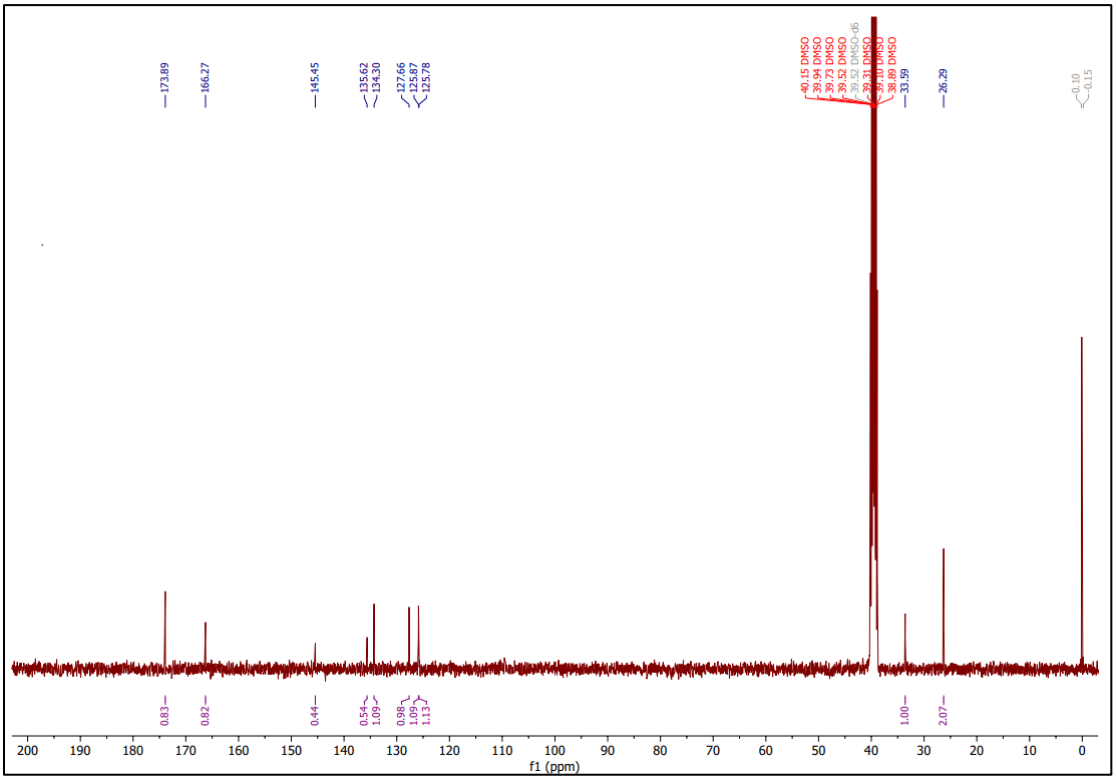


Figure 10. ^{13}C NMR spectrum of **69**.

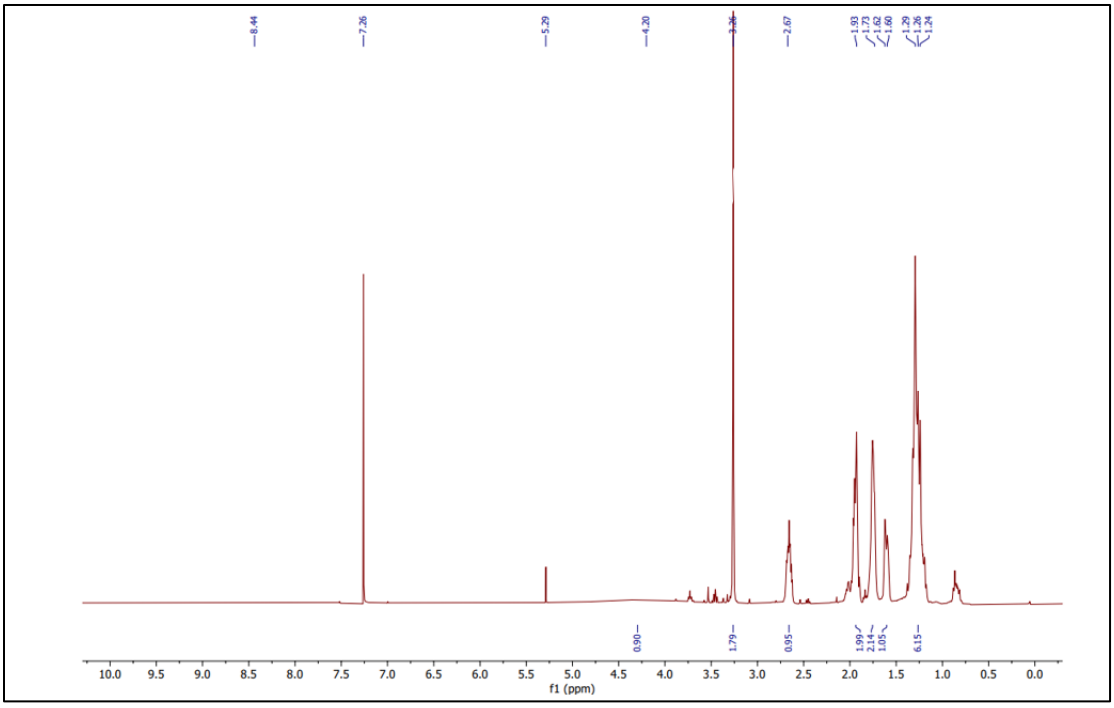


Figure 11. ^{1}H NMR spectrum of **53**.

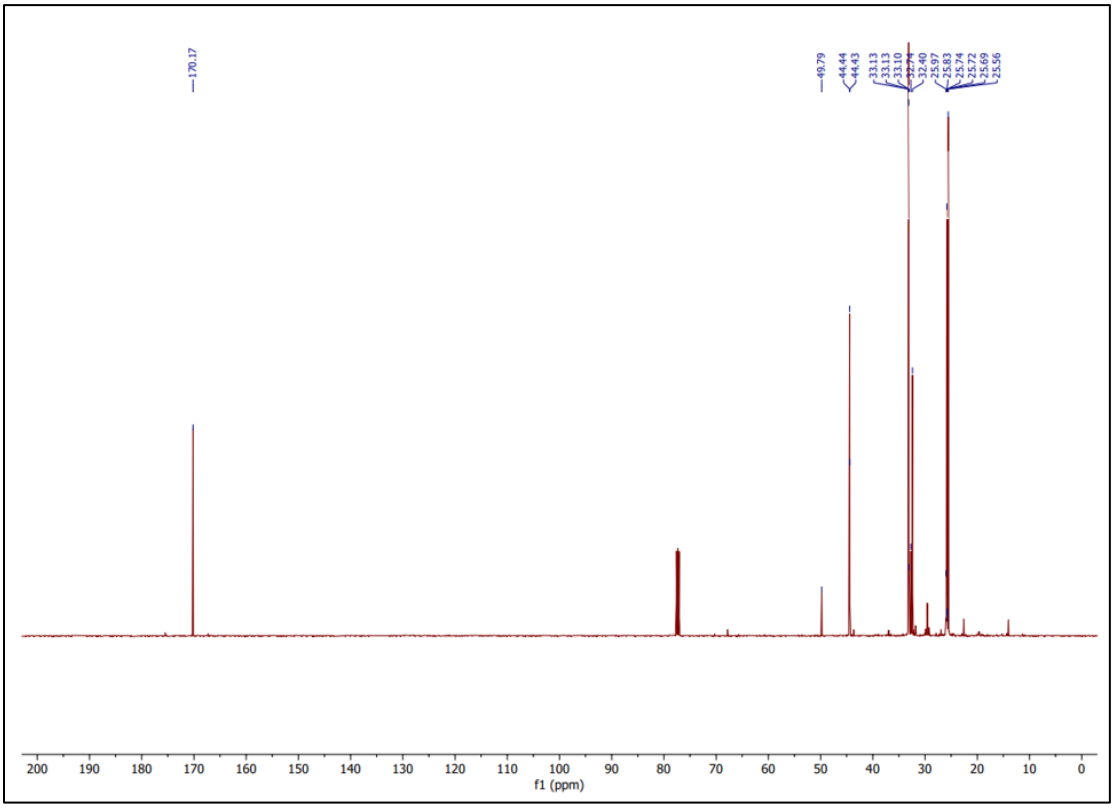


Figure 12. ^{13}C NMR spectrum of **53**.

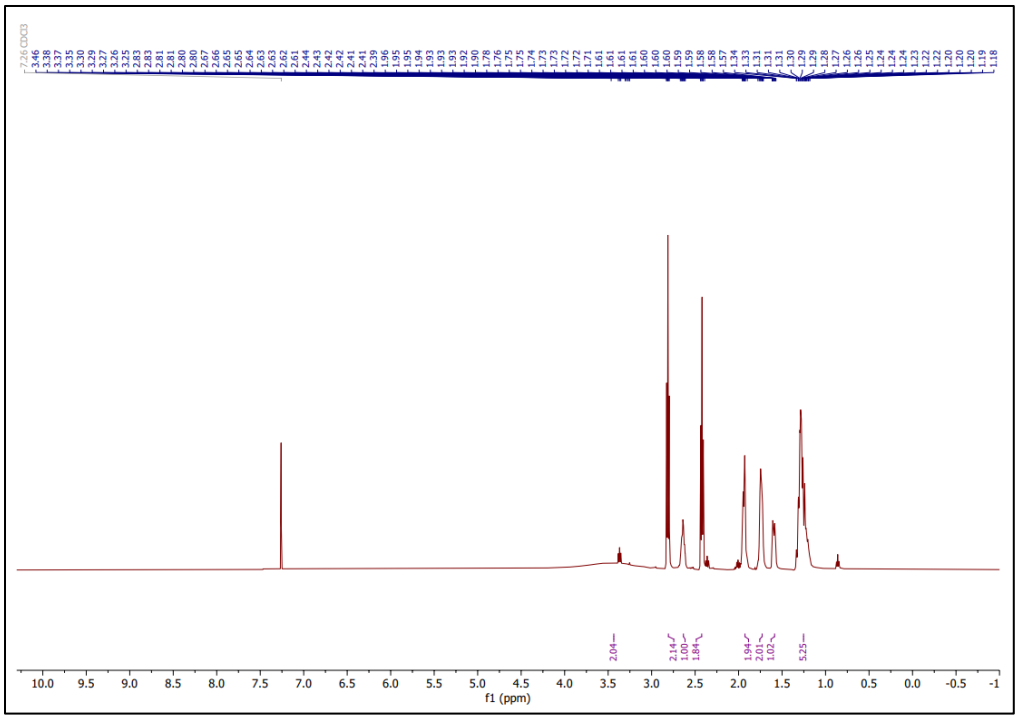


Figure 13. ^{1}H NMR spectrum of **54**.

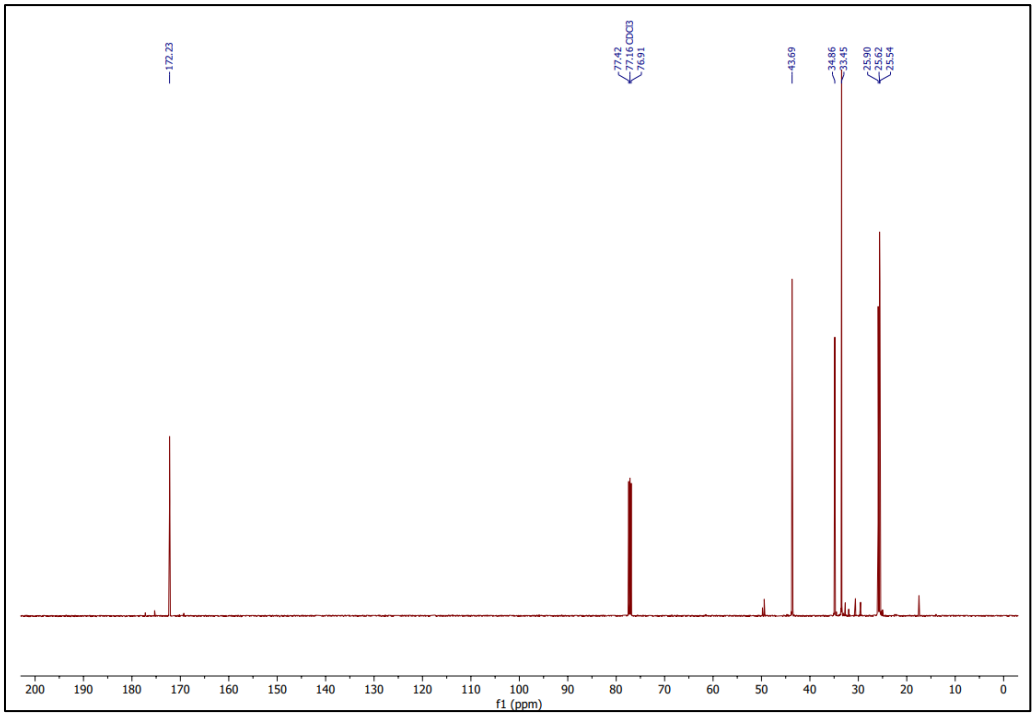


Figure 14. ^{13}C NMR spectrum of **54**.

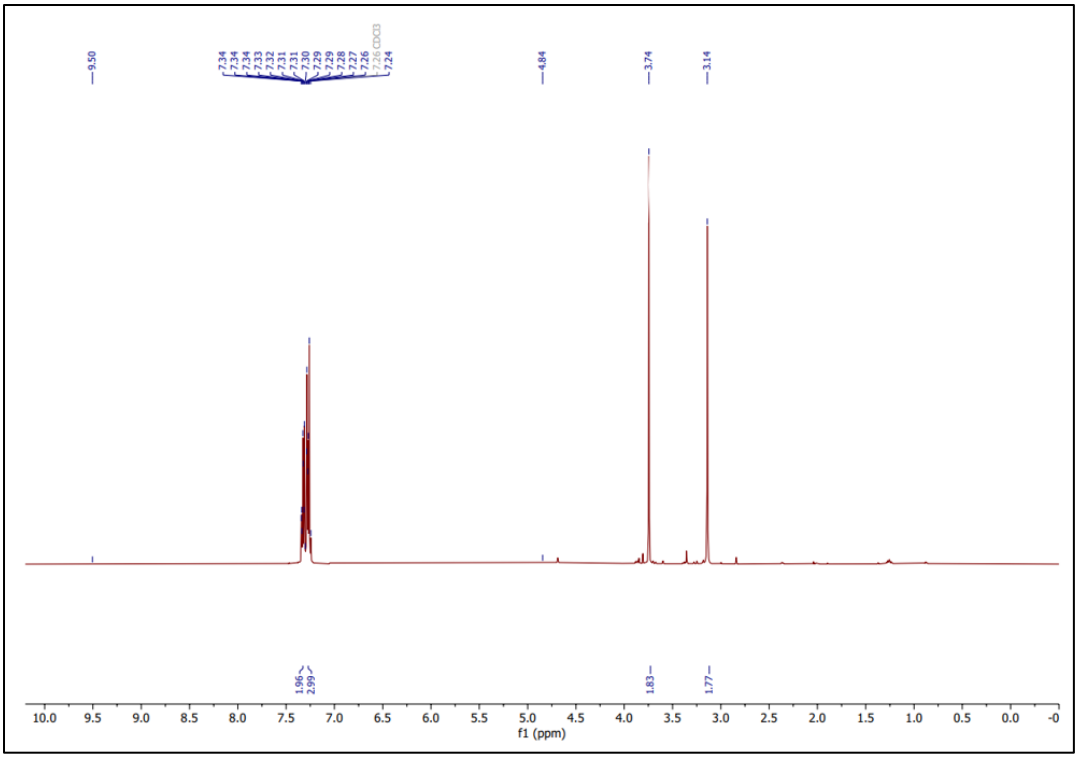


Figure 15. ^{1}H NMR spectrum of **55**.

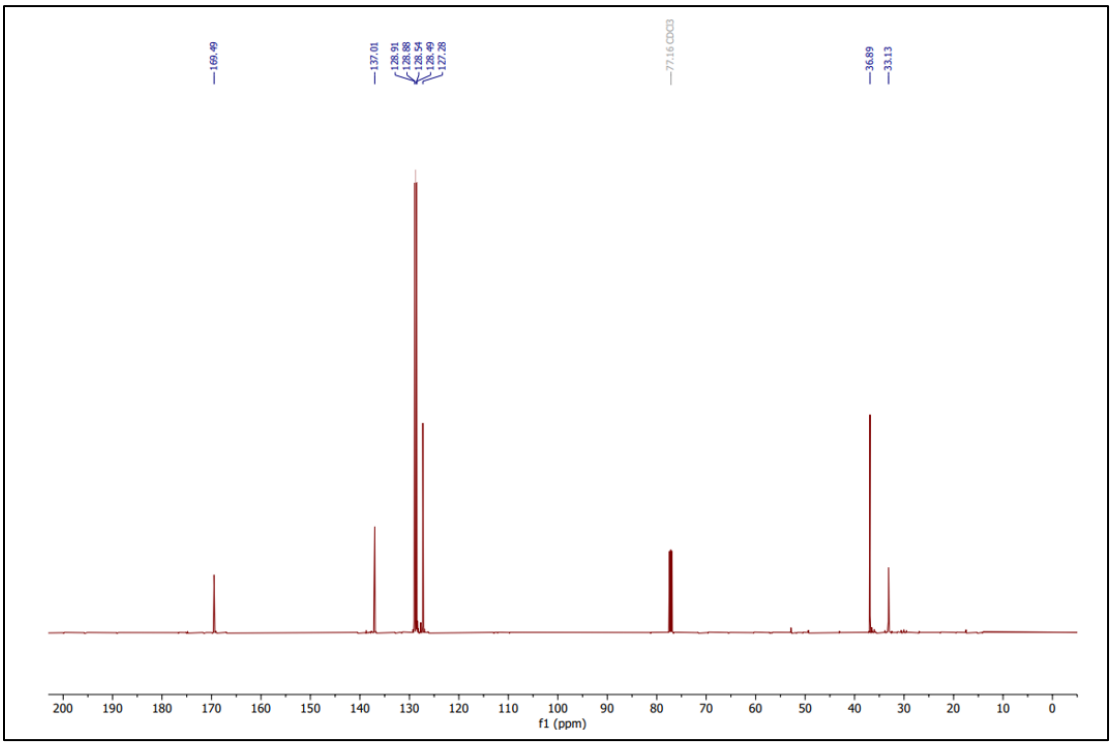


Figure 16. ^{13}C NMR spectrum of **55**.

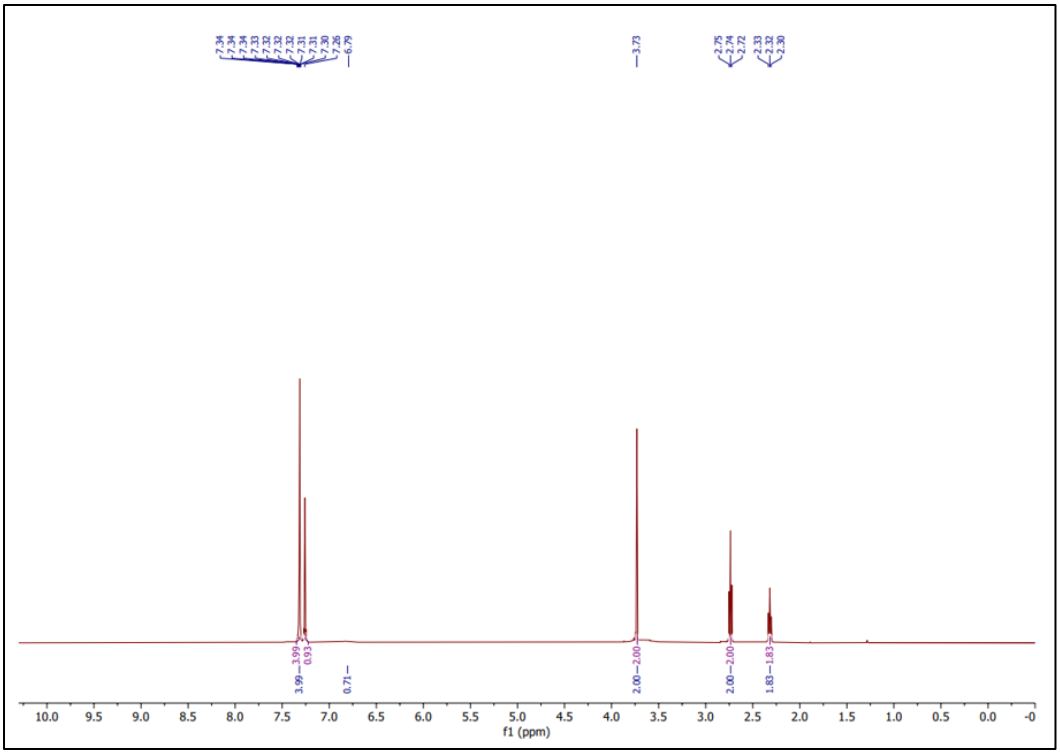


Figure 17. ^{1}H NMR spectrum of **56**.

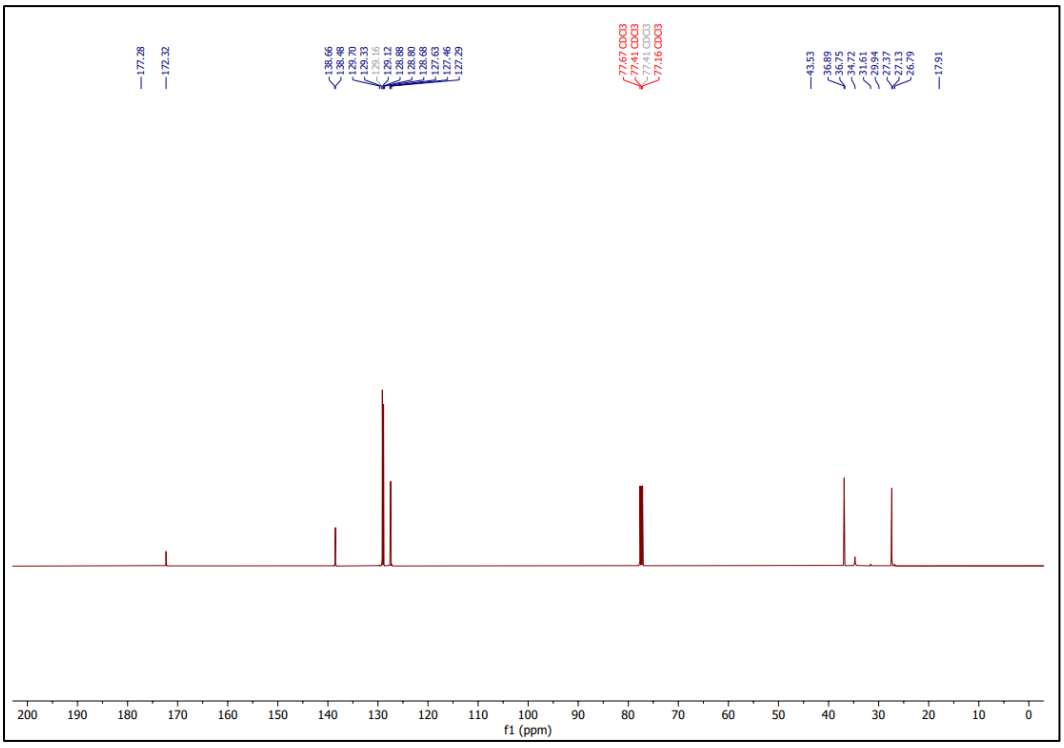


Figure 18. ^{13}C NMR spectrum of **56**.

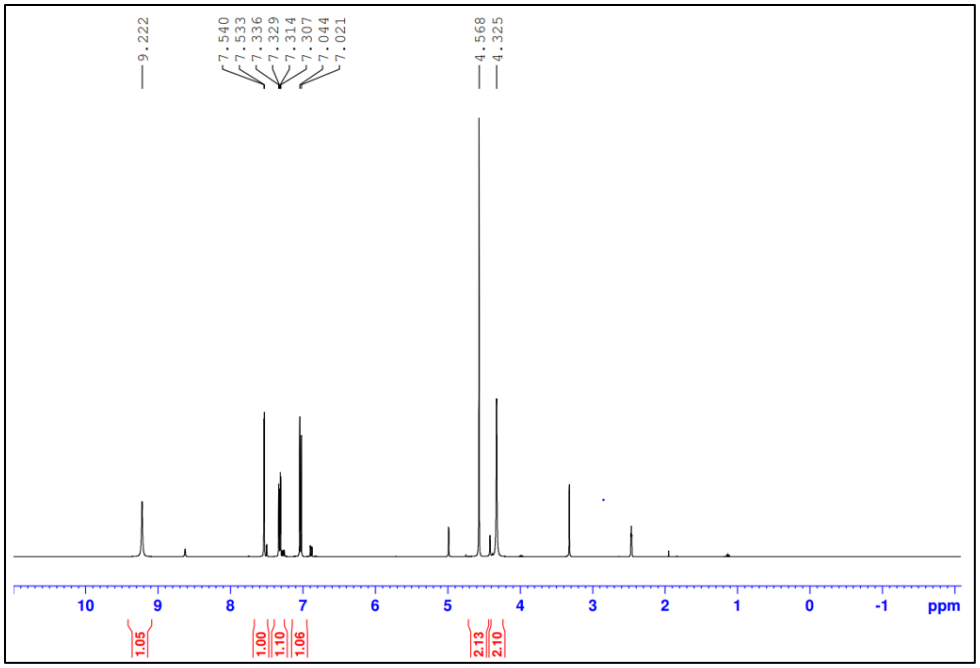


Figure 19. ^{1}H NMR spectrum of **7**.

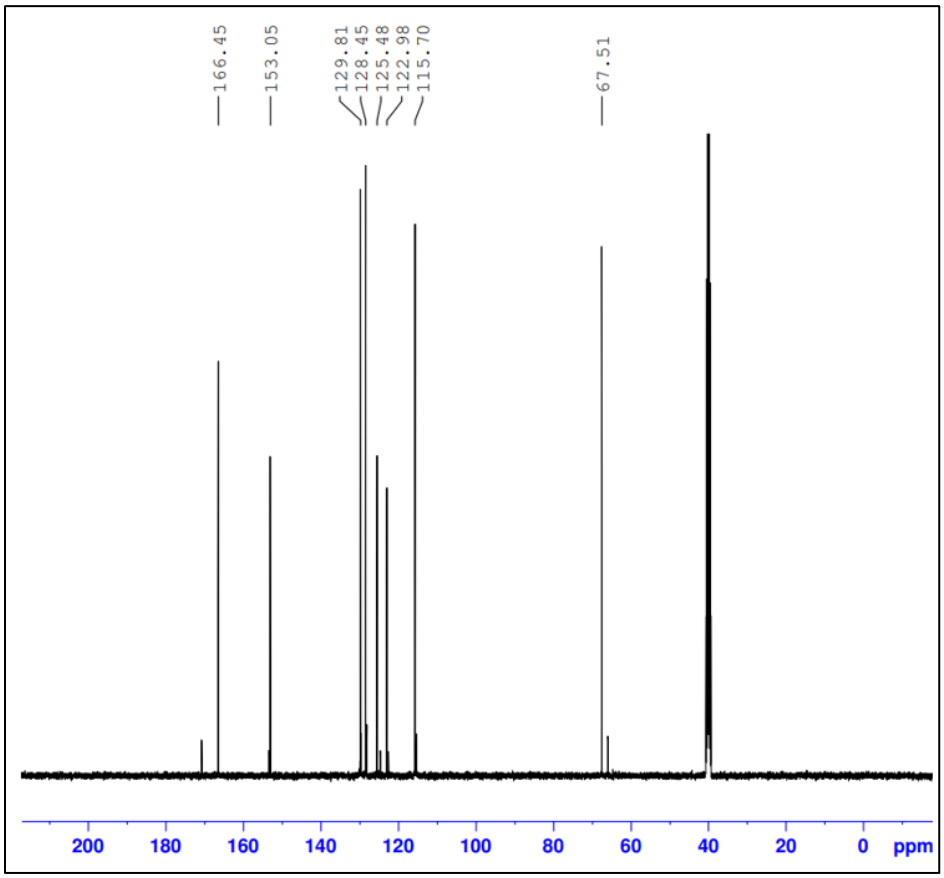


Figure 20. ^{13}C NMR spectrum of **7**.

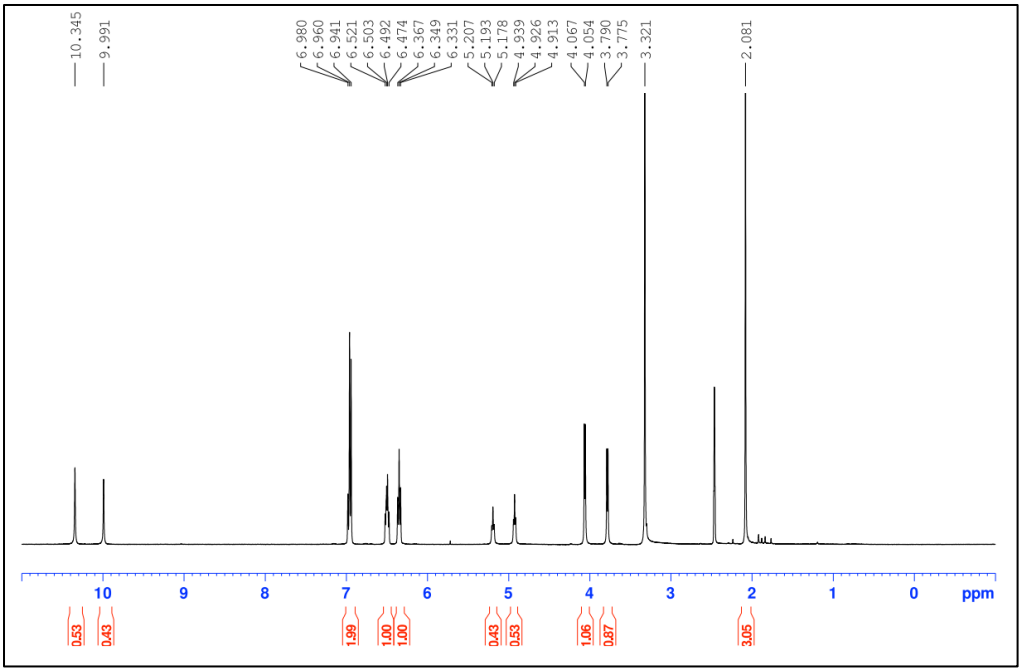


Figure 21. ^{1}H NMR spectrum of **21**.

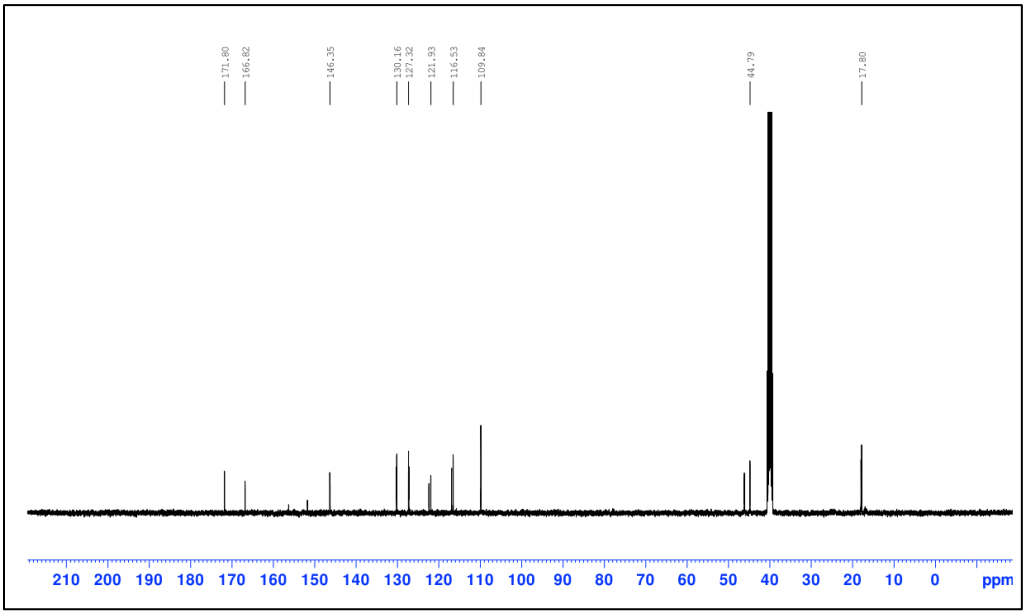


Figure 22. ^{13}C NMR spectrum of **21**.

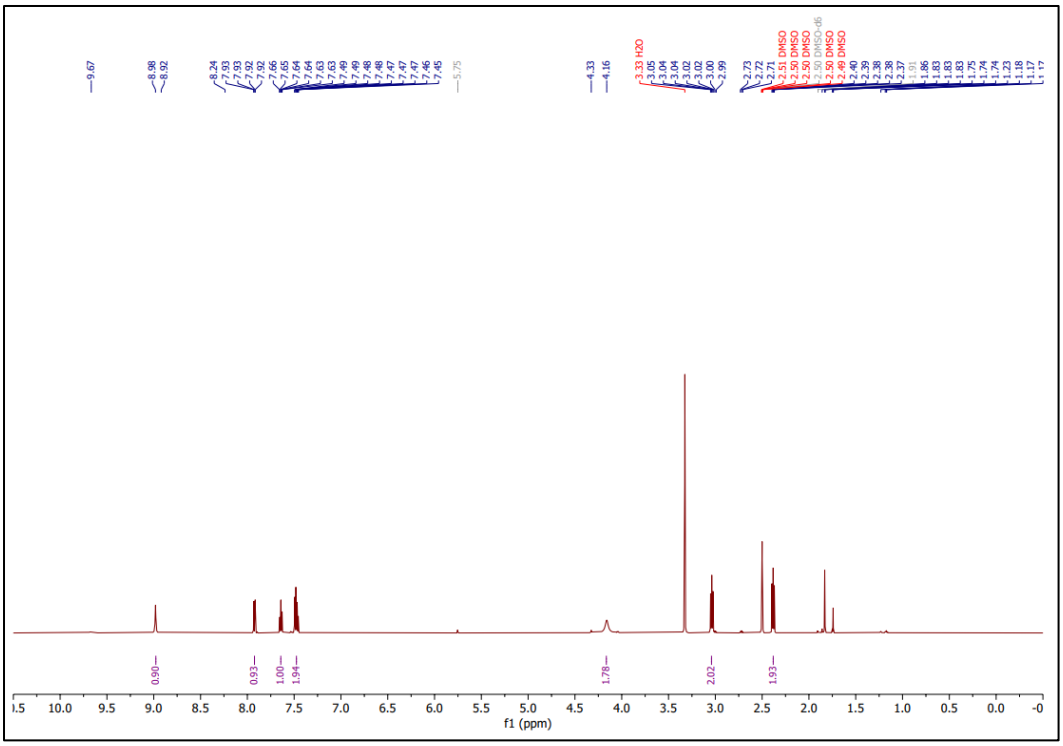


Figure 23. ^{1}H NMR spectrum of **4**.

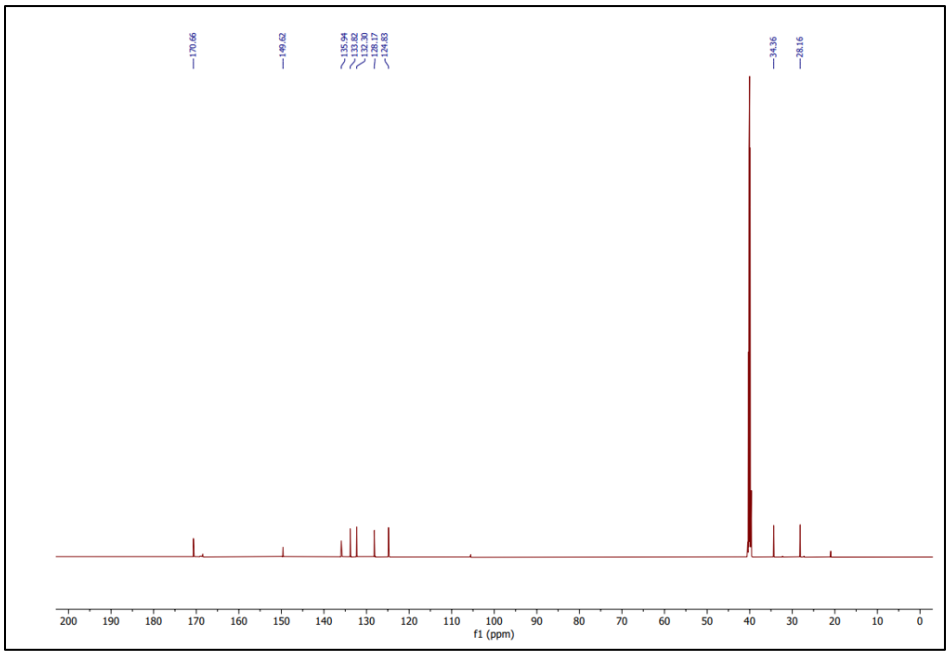


Figure 24. ^{13}C NMR spectrum of **4**.

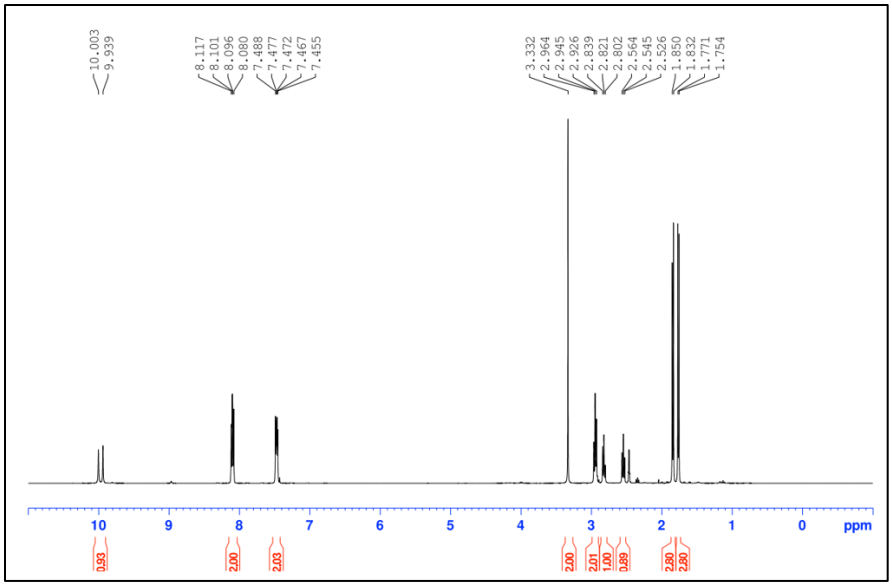


Figure 25. ^{1}H NMR spectrum of **72**.

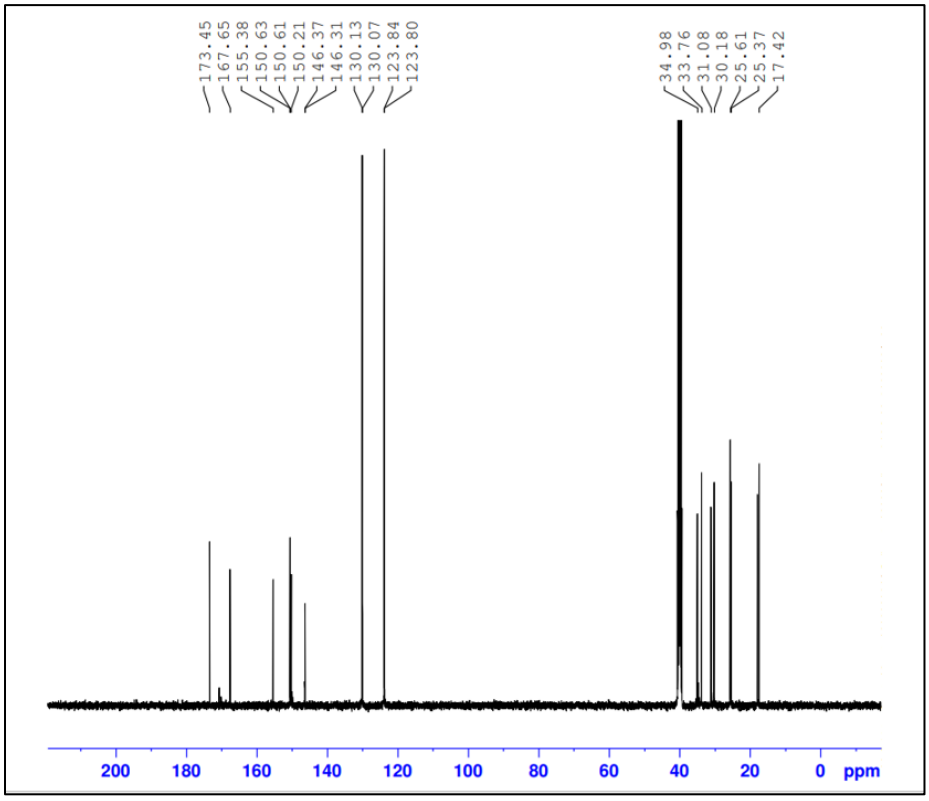


Figure 26. ^{13}C NMR spectrum of **72**.

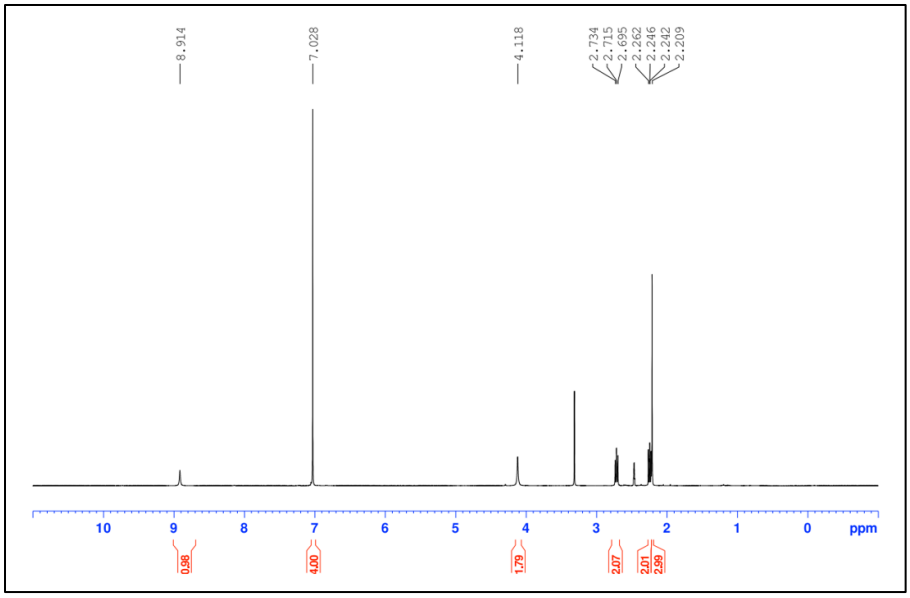


Figure 27. ^{1}H NMR spectrum of **20**.

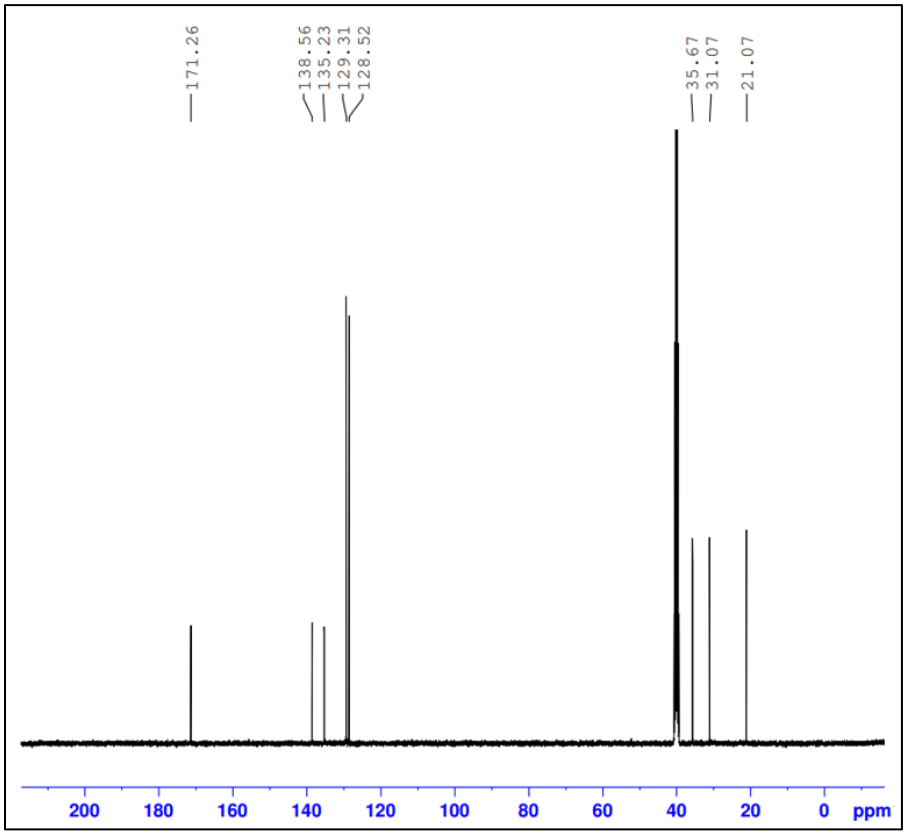


Figure 28. ^{13}C NMR spectrum of **20**.

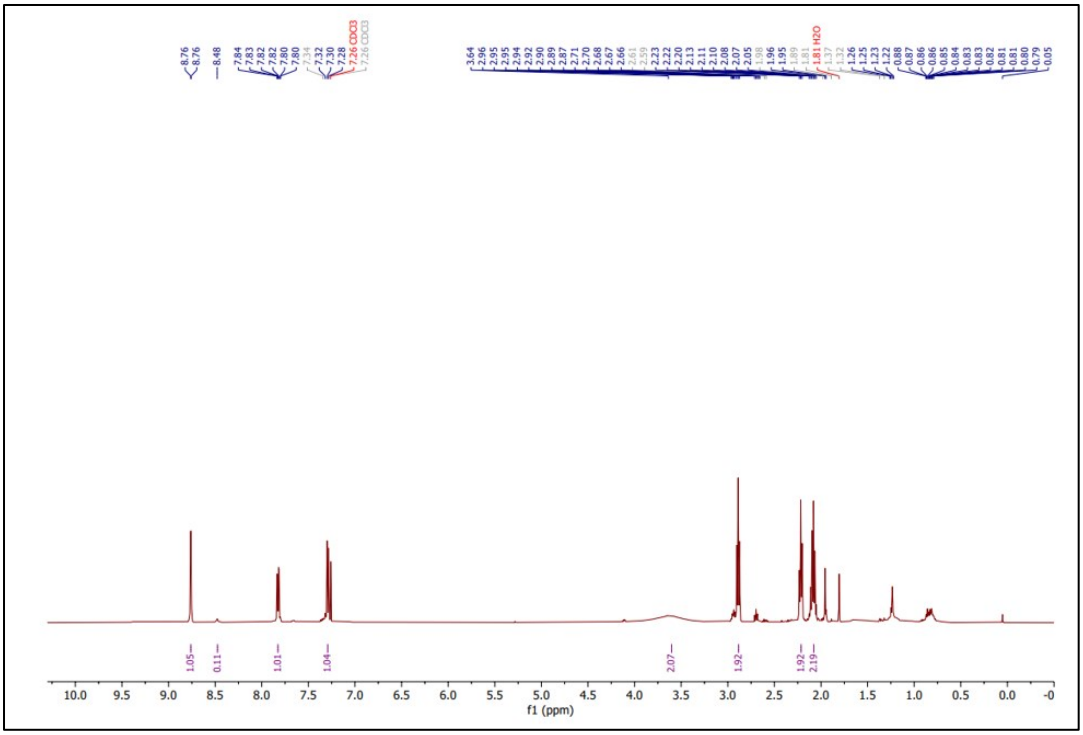


Figure 29. ^{1}H NMR spectrum of **46**.

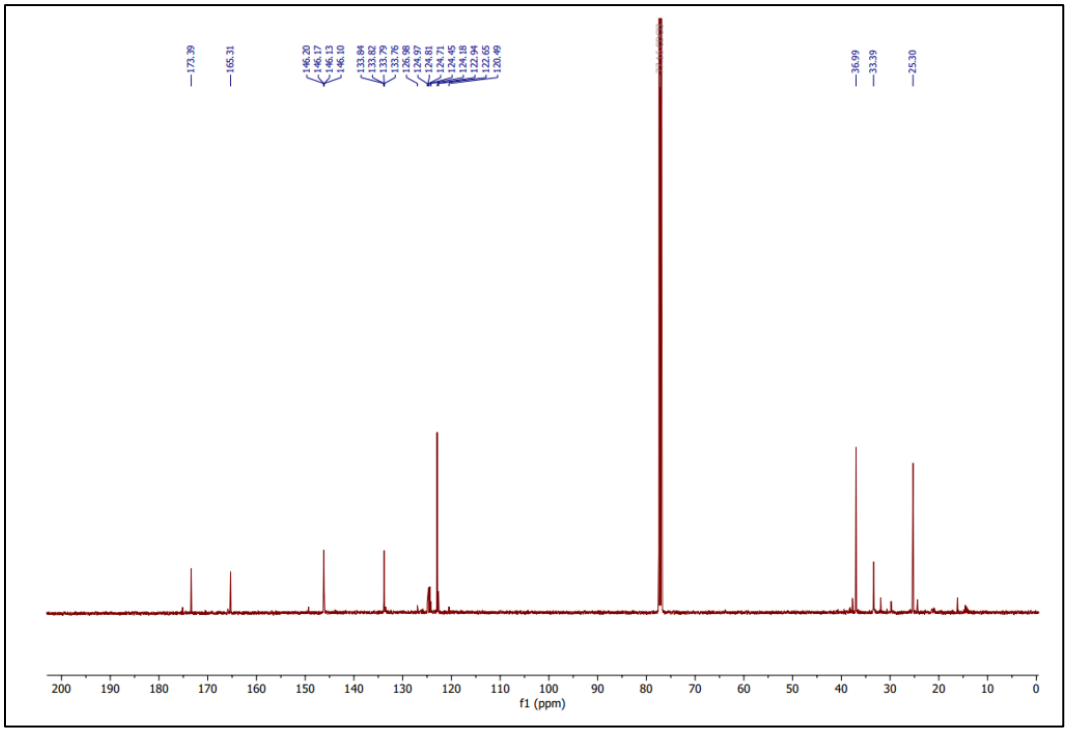


Figure 30. ^{13}C NMR spectrum of **46**.

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