Design, Synthesis and Biological Evaluation of Novel Trisubstituted-Benzimidazoles as a New Class of Antitubercular Agents, Targeting FtsZ

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The Graduate School

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Design, Synthesis and Biological Evaluation of Novel Trisubstituted-Benzimidazoles as a New Class of Antitubercular Agents, Targeting FtsZ

Kunal Kumar

Doctor of Philosophy in
Chemistry

Stony Brook University

2011

Despite extensive research in last 40 years, the treatment of TB is still limited to a cocktail of classical drugs that mostly target cell wall biosynthesis, DNA coiling, transcription, translation. Knowing the bacterial ability to develop resistance, there is a dire need for the discovery of new cellular targets to develop novel antitubercular agents which can surpass the resistance. In this context, FtsZ, a highly conserved and ubiquitous cytokinesis protein, offers an extremely promising therapeutic target. It has been observed that inhibition of FtsZ assembly leads to absence of septum formation, eventually causing cell lethality. Hence, compounds targeting FtsZ can be developed as novel anti-tubercular agents.

Libraries of novel trisubstituted benzimidazoles were created through rational drug design. A good number of these benzimidazoles exhibited promising MIC values in the range of 0.5-6 μg/mL (2-15 μM) against Mtb H37Rv strain with no appreciable cytotoxicity (IC50 >200 μM) against Vero cells. Moreover, five of the lead compounds also exhibited excellent activity against clinical Mtb strains with different drug-resistance profiles. In the light scattering experiments, the lead compounds clearly showed inhibition of FtsZ assembly in a dose dependent manner while enhancing the GTPase activity of Mtb FtsZ by 3-4 folds. Transmission electron microscopy (TEM) images of Mtb FtsZ treated with lead compound revealed an impressive dose-dependent inhibition of FtsZ assembly and a remarkable reduction in FtsZ protofilament formation. A unique Mtb cell morphology was observed under Scanning electron microscopy (SEM) upon treatment with lead benzimidazoles. Mtb cells were shorter in length with increased circumferences and altered polar caps. The TEM and SEM analyses strongly suggest that lead benzimidazoles have a novel mechanism of action on the inhibition of Mtb FtsZ assembly and Z-ring formation. Furthermore, as FtsZ is highly conserved in different pathogens, we also identified several hit compounds against F. tularensis, B. thailandensis and Y. pestis. Design, synthesis, optimization and biological evaluation of novel trisubstituted-benzimidazoles are presented.
Dedicated to my parents,

My brother Kushal

and

my friends
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>10-DAB</td>
<td>10-Deacetylbaccatin III</td>
</tr>
<tr>
<td>3-MBA</td>
<td>3-methoxybenzamide</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ADMET</td>
<td>Absorption, distribution, metabolism, excretion and</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>ANS</td>
<td>1-anilinonaphthalene-8-sulfonic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>Bacillus subtilis</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CA-MRSA</td>
<td>Community acquired MRSA</td>
</tr>
<tr>
<td>CDC</td>
<td>Center for disease control and prevention</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CMPF</td>
<td>3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid</td>
</tr>
<tr>
<td>CNMR</td>
<td>Carbon nuclear magnetic resonance</td>
</tr>
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<td>CYP3A4</td>
<td>CytochromeP3A4</td>
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<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
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<tr>
<td>EPR</td>
<td>Enhanced permeability and retention</td>
</tr>
<tr>
<td>EtOAc</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>F. tularensis</td>
<td>Fransicella tularensis</td>
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<tr>
<td>FAB</td>
<td>Fast atom bombardment</td>
</tr>
<tr>
<td>FDA</td>
<td>Food &amp; drug administration</td>
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<td>FRAP</td>
<td>Fluorescence recovery after photobleaching</td>
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<td>Filamentous temperature-sensitive protein Z</td>
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<td>GDP</td>
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<td>GFP</td>
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<td>GTP</td>
<td>Guanosine triphosphatase</td>
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<td>GTPase</td>
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<td>HCl</td>
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<td>HIV</td>
<td>Human immune virus</td>
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<td>HNMR</td>
<td>Proton nuclear magnetic resonance</td>
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<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>HRMS</td>
<td>High resolution mass spectrometry</td>
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<tr>
<td>HSA</td>
<td>Human serum albumin</td>
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<tr>
<td>HTP</td>
<td>High throughput</td>
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<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Half maximal inhibitory concentration</td>
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<tr>
<td>ID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Median infectious dose</td>
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<tr>
<td>IP</td>
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<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
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<td>LB</td>
<td>Lysogeny broth</td>
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<tr>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Median lethal dose</td>
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<tr>
<td>LTBI</td>
<td>Latent TB infection</td>
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<td>mp</td>
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<td>MABA</td>
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<td>MALDI</td>
<td>Matrix-assisted laser desorption/ionization</td>
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<tr>
<td>MDR</td>
<td>Multidrug resistant</td>
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<td><em>Mycobacterium tuberculosis</em></td>
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<td>NHS</td>
<td>N-hydroxysuccimide</td>
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<td>NIAID</td>
<td>National institute of allergy and infectious diseases</td>
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<td>Photoaffinity analogue</td>
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<td>Yersinia pestis</td>
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Acknowledgments

This thesis is the result of five and half years of research work where I have been accompanied and supported by many people. It is my great pleasure to express my deepest gratitude to all of them who made it possible for me.

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Chapter I

FtsZ: A Novel Target for the Discovery of New Class of Antitubercular Agents

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§ 1.1 Introduction

§ 1.1.1 Tuberculosis

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*) is a leading cause of death worldwide.\(^1\) The primary site of infection is the lungs, followed by dissemination via the circulatory system and lymphatic system to secondary sites including the central nervous system, bones, joints, liver and spleen. Symptoms of TB infection usually include severe coughing-up of blood or sputum, chest pain, fatigue, loss of weight/appetite, and fever. TB is contagious when airborne and inhalation of air containing the bacteria is the most common route of infection. In the 19\(^{th}\) century, TB was one of the most dreaded diseases and the eighth leading cause of death in children 1 to 4 years of age during the 1920s. There are two kinds of TB-related conditions: latent-TB infections (LTBI) and active-TB disease.\(^2\) It has been estimated that 90\% of those who are exposed to or infected with TB have latent-TB infections and the body is able to fight the growth of bacteria.\(^2\) People with LTBI are healthy, do not show any symptoms and can’t spread infection. However, during sickness or in cases of weak immune systems, there is a 10 \% chance that these bacteria can progress to an active-TB disease.\(^2\) Hence, LTBI, even though it is not contagious, should be eradicated to eliminate any possibilities of it growing to an active disease.

![Figure I-1. Estimated new TB cases worldwide\(^3\)](image)

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Recent statistics from World Health Organization (WHO) in the year 2009 estimate that there are an estimated 9.4 million new TB incident cases globally every year with a global mortality rate of 23% (Figure I-1). Most of these cases occurred in Asia (55%) and Africa (30%). The Global Burden of Disease study by the WHO indicates that women account for an estimated 3.3 million cases equal to 35% of all cases.

However, with the emergence of HIV/AIDS in the last couple of decades, TB has become the most common opportunistic infection for HIV/AIDS patients. Among the 9.4 million estimated TB incident cases, 1-1.2 million (11-13%) were HIV-positive, which is slightly, but not significantly, lower than the previous years (Figure I-2). An estimated 1.3 million and 0.38 million deaths occurred among the HIV-negative and HIV-positive TB cases, respectively. Furthermore, there were 0.38 million deaths among women infected with TB infection.

![Figure I-2. Estimated HIV prevalence in new TB cases worldwide](image)

Poor patient compliance and inadequate control programs have led to the emergence of multi-drug resistant (MDR) strains of *Mtb*. In 2008, the WHO estimated that up to 440,000 cases of MDR-TB emerge every year leading to more than 110,000 deaths worldwide. Treatment of MDR-TB requires treatment time of two years with estimated cost of $250,000 per patient in the US. Bacterial resistance to three or more ‘second-line’ antibiotics is classified as extremely drug resistant-TB (XDR-TB). Findings by WHO from 2000-2004 suggested that 4%
of MDR-TB cases meet the criteria for XDR-TB.\(^3\) By 2010, 58 countries and territories had reported at least one case of XDR-TB.\(^3\)

§ 1.1.2 \textit{Current TB treatments}

Primarily, TB infection can be treated by administering a combination of first-line drugs like, isoniazid, ethambutol, pyrazinamide, rifampicin, streptomycin which target cell-wall biosynthesis, RNA synthesis, protein synthesis etc (\textbf{Figure I-3} and \textbf{I-4}).\(^5,6\)

\textbf{Figure I-3. Current drugs and their targets; Adapted and reorganized from the National Institute of Allergy and Infectious Diseases’ website}

In the initial treatment phase, rifampicin and isoniazid are taken in combination for the first two months, followed by ethambutol and pyrazinamide for the continuation phase, which lasts 4-7 months.\(^6\) In cases of MDR-TB, where the bacteria become resistant to first-line drugs, such as rifampicin and isoniazid, a combination of second-line drugs comprising of thioamides, cycloserine, fluoroquinolines, cyclic peptides, and aminoglycosides are used.\(^6\) However, these second-line drugs come with several drawbacks. They may show additional side effects and the
treatment itself is 100 times more costly. Furthermore, there’s a high possibility of MDR-TB strain developing resistance to even second-line drugs, complicating the treatment.

![Chemical structures of FDA-approved current anti-TB drugs](image)

**Figure I-4. Structures of FDA-approved current anti-TB drugs**

Treatment for XDR-TB involves the administration of a cocktail of several first-line as well as second-line drugs for a long period of time with very limited chance of recovery. Widespread bacterial resistance to existing therapeutics due to poor drug compliance and bacterial flexibility to overcome drug effect has been a key hurdle for the complete treatment of the disease. Therefore, in order to counter attack the adaptive genetic machinery of bacteria, there is a dire need for the identification of novel therapeutic targets. In this context, FtsZ, an essential bacterial cytokinesis protein, is a highly promising therapeutic target since the disruption of cell division would lead to the inhibition/arrest of bacterial infection.\(^{7,8}\)

### § 1.2 FtsZ – Filamentous Temperature Sensitive Protein Z

Unlike drugs designed for eukaryotic cells, cytokinesis remains largely unexploited for the development of novel bacterial therapeutics. Filamentous temperature-sensitive protein Z (FtsZ), a tubulin homolog, is the most abundant bacterial cell division protein. In the presence of guanosine triphosphate (GTP), FtsZ polymerizes bi-directionally at the center of the cell on the inner membrane to form a highly dynamic helical structure known as the “Z-ring”.\(^{9,10,11,12,13}\) The recruitment of several other cell division proteins leads to Z-ring contraction, resulting in septum
formation and eventually cell division (Figure I-5). It was hypothesized that the inhibition of proper FtsZ assembly would cause an absence of septum formation, leading to bacterial cell division arrest. The bacterial cell continues to elongate, resulting in filamentation, which ultimately leads to cell death. Because of the central role that FtsZ plays in cell division, it is a very promising target for the development of new anti-TB drugs active against drug-resistant as well as drug sensitive Mtb strains.

Figure I-5. Graphic presentation of the Z-ring formation and cell division.

(A) Bacterial cell before the beginning of cell division with FtsZ protofilaments scattered in the cell and undergoing continuous nucleotide exchange between GTP-bound FtsZ and GDP-bound FtsZ with rapid equilibrium, favoring GTP-bound FtsZ. (B) Cell elongation and chromosome segregation, localization of FtsZ protofilaments at the mid cell. (C) Formation of the Z-ring: the ‘steady-state turnover’—GTP hydrolysis competes continuously with protofilament growth during polymerization. (D &E) Formation of septum and subsequent constriction of the Z-ring followed by membrane fluctuation to bring about cell division.

§ 1.2.1 Biological Role of Filamentous Temperature Sensitive Protein Z (FtsZ)

FtsZ is the most essential and highly conserved cytoplasmic protein in prokaryotes. In the presence of GTP, at a critical concentration of 0.5-1 µM of FtsZ, FtsZ subunits are believed to display cooperative assembly to form single stranded protofilaments. They stack in a head to tail fashion, with GTP sandwiched between two FtsZ subunits. Extensive lateral
interaction between these overlapping protofilaments leads to the formation of a highly dynamic ring structure known as the “Z-ring”. However, the existence of lateral interactions among overlapping protofilaments is still debatable as accounted by several mathematical and \textit{in vitro} models, and other alternative modes of interaction have been suggested such as annealing of protofilaments, and initial attraction of the ring. It has been proposed that association of the protofilaments does not necessarily involve physical contact; rather, they interact through electrostatic forces involving the ions between the protofilaments. Although the structure of the Z-ring formed \textit{in vivo} is not well understood, it has been suggested that the Z-ring architecture might consist of a large number of short, overlapping protofilaments rather than a continuous ring.

While many of the proteins involved in chromosomal segregation and cell division are conserved across the taxa, others appear limited to Gram-negative or Gram-positive bacteria. Analysis of the \textit{Mtb} genome sequence revealed that many of the genes that encode proteins involved in regulation of septum formation and cell division are not annotated. Much of what is known is derived from the studies in model organisms like \textit{E. coli}, \textit{B. subtilis}. It has been shown that an essential requirement for the formation of the Z-ring is the anchoring of FtsZ to the cell membrane through either FtsA or ZipA (accessory proteins), both of which are known to bind to the conserved C-terminal domain of FtsZ. However, it has been revealed that only one of them is required for Z-ring formation but both ZipA and FtsA are necessary for proper cytokinesis. Since the requirement of ZipA can be bypassed by a mutation in FtsA and given that FtsA is widely conserved compared to ZipA, FtsA is believed to be the crucial factor responsible for maintaining the Z-ring integrity.

Fluorescence recovery after photobleaching (FRAP) along with other studies have revealed that the Z ring is very dynamic and continuously exchanging the subunits from a pool of cytoplasmic FtsZ before and during constriction. The rate of the steady-state turnover depends on the GTPase activity of the FtsZ polymer. Rapid turnover with no effect on the integrity of the Z-ring further suggests that it consists of short filaments of FtsZ interacting laterally with each other, presumably held together by FtsA/ZipA. A number of accessory proteins, especially MinCDE system and Noc (\textit{B. subtilis})/SIma (\textit{E. coli}) are involved in the spatial regulation of septum formation, ensuring that the Z-ring is formed between the segregated chromosomes in the middle of the cell and not at the poles (\textbf{Figure I-6}).
In new daughter cells, Min system and NO prevents Z ring assembly at the cell poles and close to the nucleoid, respectively. During initial phase of chromosome segregation, NO continue to inhibit Z-ring formation in the cylindrical part of the cell. Finally, the progression of chromosome segregation reveals an inhibitor-free region at mid-cell, allowing the Z ring assembly.

The constant balance between assembly and disassembly of the “Z ring” is essential for bacterial cytokinesis, since small changes in FtsZ polymerization dynamics would be translated into large and rapid aberration in Z-ring structure. Thus, a proper understanding of FtsZ polymerization dynamics will aid in the development of drugs that can prevent cell division by disrupting Z ring formation or function (Figure I-7).
Figure I-7. Polymerization-depolymerization dynamics of FtsZ.

(A) Nucleotide exchange between GTP-bound FtsZ and GDP-bound FtsZ with rapid equilibrium, favoring GTP-bound FtsZ (B) Polymerization begins and long-strait protofilaments starts to form after a critical concentration of GTP-bound FtsZ is achieved. (C) The ‘steady-state turnover’-- GTP hydrolysis competes continuously with protofilament growth during polymerization. (D) After bacterial cell division occurs, distorted GDP-bound FtsZ protofilaments depolymerize to yields FtsZ monomers.

The relation between FtsZ polymerization and GTP hydrolysis to guanosine diphosphate (GDP) is crucial for integrity of the Z-ring. Romberg and Mitchison showed that GTP-bound FtsZ polymerizes into straight polymers, which triggers the GTPase activity of FtsZ, because the catalytic site is formed at the interface of two adjacent FtsZ monomers.\textsuperscript{34,35} The GTP binding domain is located in one monomer and the highly conserved tubulin-like loop 7 (T7-loop) of the other domain is located in an adjacent FtsZ monomer. During steady-state GTP turnover, the properties of the polymer are determined by the nucleotide species that predominates.\textsuperscript{34} It has been shown that GTP hydrolysis is the rate-limiting step in this process, thus suggesting GTP as the major nucleotide species in the FtsZ polymer.\textsuperscript{34} Upon GTP hydrolysis, polymers bound to GDP were thought to favor curved conformation as opposed to straight polymers.\textsuperscript{36} It has been proposed that the GTP hydrolysis generates a force during septation where nucleotide-dependent transitions from straight to a curved polymer can transmit mechanical work to the membrane leading to the constriction of the Z-ring.\textsuperscript{26,36} However, the physiological role of this force
remains controversial as accounted by several experiments where FtsZ mutants with undetectable GTPase activity could still divide suggesting that FtsZ GTPase is not the source of power during constriction of the septum but is important for symmetric invagination.\textsuperscript{37,38}

Recent experiments with liposomes (tubular membrane) and observation of curved and straight protofilaments \textit{in vivo} demonstrated that the protofilament bending is more likely to be a plausible mechanism for producing the constriction force.\textsuperscript{26,39} However it is believed that the curved conformation of the protofilaments itself can not pull the membrane to complete scission.\textsuperscript{14} It has been suggested that the final step of scission can be completed by membrane fluctuations.\textsuperscript{14} The remodeling of the cell wall, which starts from the membrane invagination, may become a positive force pushing the septum toward closure, leading to final scission.\textsuperscript{14,40,41}

\section*{§ 1.2.2 FtsZ and Tubulin: Structural and functional homology}

The fact that FtsZ is a homologue of tubulin was first realized via the identification of a short amino acid sequence, GGGTGTG, from the crystal structure of \textit{Methanococcus jannaschii} FtsZ, that is virtually identical to the tubulin signature motif, (G/A)GGTGSG, found in all $\alpha$, $\beta$ and $\gamma$-tubulins.\textsuperscript{10} Despite limited (10-18\%) sequence similarity,\textsuperscript{42} FtsZ and tubulin share a common fold, comprised of two domains linked by an $\alpha$-helix.\textsuperscript{43} The residues involved in the nucleotide binding domain and a region involved in protofilament formation in tubulin are found to be conserved.\textsuperscript{43} Like tubulin, FtsZ polymerizes in the presence of GTP into 5 nm wide protofilaments while depolymerizing upon GTP hydrolysis.\textsuperscript{11} Amidst similarity, the proteins also differ in several ways. Unlike FtsZ, where identical subunits form protofilaments, microtubules are formed from non-identical subunits, $\alpha$ and $\beta$ tubulin.\textsuperscript{11} Furthermore, the two \textit{Mtb} FtsZ subunits are associated laterally as compared to longitudinally in case of tubulin. Because of the structural and functional homology, compounds that are known to affect the assembly of tubulin into microtubules, can act as a lead for targeting FtsZ assembly. The limited sequence homology at protein level, affords the opportunity to discover FtsZ-specific compounds with limited cytotoxicity to eukaryotic cells.
§ 1.3 FtsZ-A novel target for anti-TB drug discovery

As described above, FtsZ, an essential bacterial cytokinesis protein, has emerged as a highly promising new target for the discovery of novel antibacterial agents. It was hypothesized that inhibition of proper FtsZ assembly would prevent septum formation, giving way to continued cell filamentation, ultimately leading to cell death.\textsuperscript{15,16} FtsZ offers a significant potential advantage over traditional targets. As FtsZ is highly conserved in prokaryotes, in addition to being pathogen specific, FtsZ inhibitors may be developed as broad-spectrum antibacterial agents for the treatment of a variety of bacterial infections.\textsuperscript{44} Since FtsZ is an essential and conserved protein for bacterial cell division, it is believed that acquiring resistance to the FtsZ-targeted compounds by mutations in the protein may be demanding for bacteria.\textsuperscript{45,18} Recently Haydon et al have isolated staphylococcal strains resistant to anti-FtsZ compounds with relatively low frequency, reiterating the difficulty in developing resistance.\textsuperscript{46} This suggests that resistance mechanisms against compounds developed for FtsZ inhibition may not be widespread in nature. Accordingly, FtsZ is a very promising target to develop new generation antibacterial drugs. A good number of compounds have already been identified to target FtsZ.\textsuperscript{44,47,48,49} As FtsZ and tubulin have close functional homology,\textsuperscript{50,51,52} compounds that are known to inhibit or stabilize tubulin/microtubules can serve as a good starting point for the development of novel antibacterial agents.\textsuperscript{8,49,53,47,54} Upon modification, these tubulin inhibitors can be made specific to target FtsZ with no appreciable cytotoxicity to eukaryotic cells.

Although drug development in this field is still in an early stage, several classes of compounds have been found effective against \textit{Mtb} and a number of compounds have been identified as promising leads.
§ 1.4 *Mtb* FtsZ inhibitors

Inhibitors of *Mtb* FtsZ reported so far are summarized in Table I-1 and their structures are shown in Figure I-8. Characteristics of these inhibitors are described below.

**Totarol**

Totarol is a naturally occurring diterpenoid phenol extracted from *Podocarpus totara*. Since its discovery, totarol has been shown to inhibit the proliferation of several pathogenic Gram-positive bacteria such as *S. aureus* and *Mtb*.\(^{55,56}\) Jaiswal *et al.* examined its effect on the assembly dynamics of *Mtb* FtsZ *in vitro*.\(^{57}\) In the preliminary study they found that totarol increased the length of *B. subtilis* cells by several fold, indicating its inhibitory activity against cell division. Totarol inhibited the polymerized mass of *Mtb* FtsZ by 27% at 50 µM concentration. GTPase activity was also reduced in a concentration dependent manner without affecting the binding of GTP to *Mtb* FtsZ. Transmission electron microscopy (TEM) images indicated that totarol significantly reduced the average width of FtsZ protofilaments and also induced aggregation in the absence of GTP. Totarol increased the fluorescence intensity of the FtsZ-ANS complex (ANS = 1-anilinonaphthalene-8-sulfonic acid), whereas decreased that of PM-FtsZ (PM = \(N\)-(1-pyrene)maliemide), indicating that totarol induced conformational changes in FtsZ.

**Albendazole and Thiabendazole**

Albendazole and thiabendazole are known fungicide and parasiticide, which cause degenerative alterations in the intestinal cells of worms by binding to the colchicine-sensitive site of tubulin, and inhibiting its polymerization or assembly into microtubules. Sarcina *et al.* reported that the treatment of cyanobacterial and bacterial cultures with thiabendazole resulted in cell elongation.\(^{58}\) This suggested that the cell division cycle was arrested and therefore tubulin inhibitors may act, in a similar manner, on the *fisZ* gene product. In addition, increased amount of DNA in DAPI-DNA-stained TBZ-treated *Synechococcus* 7942 cells was observed, which indicated that DNA replication still occurred in the presence of thiabendazole. Slayden *et al.* examined the effect of albendazole and thiabendazole on *Mtb* cell growth.\(^{16}\) MIC\(_{99}\) values of albendazole and thiabendazole were determined to be 61 µM and 80 µM, respectively. The cell
morphology and transcriptional response indicated cell filamentation and gene regulation causing the inhibition of septum formation in \textit{Mtb} cells. These results suggested that thiabendazole and albendazole interfered and delayed \textit{Mtb} cytokinesis.

\textbf{2-Alkoxycarbonylaminopyridines and 2-carbamoylpteridine}

Based on the premise that known tubulin inhibitors can inhibit FtsZ assembly,\textsuperscript{42} White, Reynolds and their coworkers screened a library of 200 2-alkoxycarbonylaminopyridines for antimicrobial activity against \textit{Mtb}.\textsuperscript{59} Two compounds, SRI-3072 and SRI-7614, were found to inhibit the growth of \textit{Mtb} cells at 0.15 \(\mu\text{g/mL}\) and 6.25 \(\mu\text{g/mL}\), respectively. SRI-3072 and SRI-7614 inhibited \textit{Mtb} FtsZ polymerization in a dose dependent manner with ID\textsubscript{50} values of 52 \(\mu\text{M}\) and 60 \(\mu\text{M}\), respectively. These compounds also inhibited the GTPase activity by 20-25 \% at 100 \(\mu\text{M}\) concentration. In addition, these compounds were evaluated for their inhibitory activity against tubulin polymerization. SRI-3072 was specific to FtsZ and did not exhibit tubulin polymerization inhibition at 100 \(\mu\text{M}\) concentration, whereas SRI-7614 inhibited tubulin polymerization (ID\textsubscript{50} 4 \(\mu\text{M}\)). Furthermore, SRI-3072 reduced the growth of \textit{Mtb} in mouse-derived macrophages. However, SRI-3072 had some issues in the synthesis and purification. Thus, an optimization of this lead compounds was carried out to yield a more easily accessible and more potent (8-fold better MIC than SRI-3072) 2-carbamoylpteridine analogue of SRI-3072.\textsuperscript{60}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure.png}
\caption{\textit{Mtb} FtsZ inhibitors}
\end{figure}
Table I-1. *Mtb* FtsZ inhibitors

<table>
<thead>
<tr>
<th></th>
<th>Compound</th>
<th>Mode of action</th>
<th>Assays used</th>
<th>MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Albendazole, Thiaendazole</td>
<td>filamentous phenotype response; up-regulation of gene encoding Z-ring formation</td>
<td>cell-based assay; cell morphology; transcriptional profiling.</td>
<td>Albendazole 61 µM, Thiaendazole 80 µM</td>
</tr>
<tr>
<td>2</td>
<td>Totarol</td>
<td>Dose dependent inhibition of GTPase and FtsZ polymerization; reduced width and aggregation of FtsZ protofilaments</td>
<td>GTPase and FtsZ polymerization assay; TEM</td>
<td>---</td>
</tr>
<tr>
<td>3.</td>
<td>SRI-3072, SRI-7614, 2-carbamoyl-pteridine</td>
<td>Inhibition of FtsZ polymerization and GTPase activity</td>
<td>FtsZ GTPase and polymerization assay</td>
<td>SRI-3072 0.15 µg/mL SRI-7614 6.25µg/mL 2-carbamoylpteridine 0.25 µg/mL</td>
</tr>
<tr>
<td>4</td>
<td>Taxanes</td>
<td>FtsZ polymer stabilization</td>
<td>Real time PCR-based assay, cell filamentation</td>
<td>1.25 -2.5 µM</td>
</tr>
</tbody>
</table>

**Taxanes**

Paclitaxel (Taxol®), a microtubule-stabilizing anticancer agent, exhibits modest antibacterial activity against drug-sensitive and drug-resistant *Mtb* strains (MIC 40 µM), although its cytotoxicity against human cancer cell lines (a benchmark for activity against human host cells) is three orders of magnitude more potent (IC₅₀ 0.019-0.028 µM). Following the hypothesis that compounds that affect tubulin/microtubule would serve as leads for developing FtsZ inhibitors, a library of taxanes was designed and screened for antibacterial activity against *Mtb* cells. Real time PCR-based assay was employed to screen taxanes, such as cytotoxic taxanes that stabilize microtubules and non-cytotoxic taxane-multidrug resistance (MDR) reversal agents (TRAs) that inhibit the efflux pumps of ATP-binding cassette (ABC) transporters such as P-glycoprotein (P-gp).
Table I-2. Antimicrobial activities of taxanes against drug-sensitive and multidrug-resistant 
Mtb strains

<table>
<thead>
<tr>
<th></th>
<th>Taxanes</th>
<th>MIC Mtb. (µM)</th>
<th>Cytotoxicity (IC₅₀, µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>H37Rv</td>
<td>IMCJ946.K2</td>
</tr>
<tr>
<td>1</td>
<td>Paclitaxel</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>SB-T-0032</td>
<td>5</td>
<td>1.25</td>
</tr>
<tr>
<td>3</td>
<td>SB-RA-2001</td>
<td>5</td>
<td>2.5</td>
</tr>
<tr>
<td>4</td>
<td>SB-RA-5001</td>
<td>2.5</td>
<td>1.25</td>
</tr>
<tr>
<td>5</td>
<td>SB-RA-5001MeO6</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>6</td>
<td>SB-RA-5011</td>
<td>2.5</td>
<td>1.25</td>
</tr>
<tr>
<td>7</td>
<td>SB-RA-5012</td>
<td>2.5</td>
<td>1.25</td>
</tr>
</tbody>
</table>

M. tuberculosis H37Rv is sensitive to all antibiotics tested. M. tuberculosis IMCJ946.K2 is resistant to drugs including INH, REF, EB, streptomycin, kanamycin, p-aminosalicilic acid, cycloserine and enniomycin. MCF7 and A549 cells: human breast and non-small cell lung cancer cell lines, respectively.

Of the 120 taxanes screened, several compounds exhibited significant anti-TB activity. From the MIC₉⁹ values and cytotoxicity assay, SB-T-0032 and SB-RA-2001 (Figure I-9) were selected as lead compounds for further studies. SB-RA series of taxanes bearing a (E)-3-(naphtha-2-yl)acryloyl (2-NpCH=CHCO) group at the C-13 position exhibited MIC₉⁹ between 2.5-5 µM against drug-sensitive and drug-resistant Mtb strains. A new library of taxanes based on this SB-RA series was prepared through modification of 10-deacetylbaccatin III (DAB). These SB-RA-2001 analogs were found to have higher specificity to FtsZ than microtubules and exhibited the same level of anti-TB activity as that of SB-T-0032. It has been shown that novel and effective anti-angiogenic taxoid, IDN5390,⁷¹,⁷² bearing a C-seco-baccatin moiety possesses substantially less cytotoxicity than paclitaxel. Therefore, C-seco analogs of SB-RA-2001 (Figure I-9) were designed, synthesized and evaluated. These novel C-seco-taxanes SB-RA-5001 and its congeners (Figure I-9), were found to exhibit promising anti-TB activity (MIC₉⁹ 1.25-2.5 µM) against both drug-sensitive and drug-resistant Mtb strains without appreciable cytotoxicity (IC₅₀ > 80 µM). Thus, the specificity of these novel taxanes to microtubules as compared to FtsZ was completely reversed. The scanning electron microscopy (SEM) images of Mtb cells treated with SB-RA-20018 and SB-RA-5001 clearly show substantial elongation and filamentation, a
phenotypic response to FtsZ inactivation (Figure I-10). In addition, a preliminary study on the
effect of SB-RA-5001 on polymerization-depolymerization, using the standard light-scattering
assay exhibited a dose-dependent stabilization of FtsZ against depolymerization.

Figure I-9. *Mtb* FtsZ inhibitors: Taxanes

![Taxanes](image)

SB-T-0032

SB-RA-2001: R = H
SB-RA-20018: R = N

SB-RA-5001: X = H
SB-RA-5001MeO6: X = MeO

SB-RA-5011: Y = O
SB-RA-5012: Y = S

Figure I-10. Electron micrograph of *Mtb* cells: (A) Control, (B) SB-RA-20018 and (C) SB-
RA-5001.
§ 1.5 References


# Chapter II

## Discovery of Novel Benzimidazoles as a New Class of Antitubercular Agents: Targeting Essential Cell Division Protein FtsZ

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§ 2.1 Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mt* ) is a highly infectious disease affecting one-third of the world’s population.\textsuperscript{1} In the past several decades, the increase in TB co-infection in HIV/AIDS afflicted patients along with the emergence of MDR-TB and XDR-TB has made TB the leading cause of death worldwide. Indeed, statistics from World Health Organization (WHO) estimate that there are about 9.4 million new TB cases with 1 to 1.2 million cases being HIV positive.\textsuperscript{2}

Despite considerable efforts in the past 40 years, the development of new TB treatments has been entirely limited to classical drugs which target cell wall biosynthesis, ATP synthesis, RNA synthesis, protein synthesis, and other important biological mechanisms. Knowing that bacteria have the ability to develop resistance, these scientific labors have been largely inadequate. Hence, the discovery of new cellular targets represents a brilliant opportunity for the development of more effective antitubercular agents which may surpass resistance and could very well be active against drug sensitive as well as drug resistant *Mt* strains.

*Figure II-1.FtsZ dynamics: GTP hydrolysis and Polymerization*\textsuperscript{3}

As discussed previously, FtsZ, a highly essential and indispensable cytokinesis protein, offers an excellent therapeutic target for the development of novel anti-TB agents (*Figure II-1*).\textsuperscript{3,4,5,6} The polymerization and de-polymerization of FtsZ is a dynamic and continuous process. It was hypothesized that the inhibition of either polymerization or depolymerization of FtsZ into the proper FtsZ assembly would prevent septum formation without affecting DNA replication.
and nucleoid segregation. Such conditions would give way to continued cell filamentation, ultimately leading to cell death.\textsuperscript{6,7,8} Another interesting factor is that the concentration of FtsZ remains invariant during the cell cycle. Therefore it is less probable that it will escape the drug interaction. Because of structural and functional homology,\textsuperscript{9,10} compounds that are known to affect the assembly of tubulin into microtubules can act as a lead for targeting FtsZ assembly, which on modification can be made specific to target FtsZ with no appreciable cytotoxicity to eukaryotic cells. Because of the overwhelming importance of FtsZ in the bacterial cycle, it is a very promising target for the development of new anti-TB drugs active against drug-resistant and drug sensitive \textit{Mtb} strains.

Following the hypothesis that compounds which affect tubulin/microtubule would serve as leads for developing FtsZ inhibitors,\textsuperscript{11,12,13} a library of taxanes was designed and screened for antibacterial activity against \textit{Mtb} cells. Of the 120 taxanes screened, several compounds exhibited significant anti-TB activity.\textsuperscript{14} \textit{C}-seco analogs of SB-RA-2001 (\textbf{Figure II-2}) were designed, synthesized and evaluated. Of these novel \textit{C}-seco-taxanes, SB-RA-5001 and its congeners were found to exhibit promising anti-TB activity (MIC\textsubscript{99} 1.25-2.5 \textmu M) against both drug-sensitive and drug-resistant \textit{Mtb} strains without appreciable cytotoxicity (IC\textsubscript{50} > 80 \textmu M) to normal cells. Thus, the specificity of these novel taxanes to microtubules as compared to FtsZ was completely reversed through systematic and rational drug design. The scanning electron microscopy (SEM) images of \textit{Mtb} cells treated with SB-RA-20018 and SB-RA-5001 clearly showed substantial elongation and filamentation, a phenotypic response to FtsZ inactivation.

\textbf{Figure II-2.\textit{Mtb} FtsZ inhibitors: Taxanes}\textsuperscript{14}
Albendazole and thiabendazole are known inhibitors of tubulin polymerization. Sarcina and Mullineaux demonstrated that thiabendazole caused cell elongation in *E. coli* and cyanobacteria, which suggests that these tubulin inhibitors may act in a similar manner on the *ftsZ* gene product as they do on tubulin.\(^\text{15}\)

![Figure II-3. Proposed trisubstituted benzimidazoles library](image)

Later, Slayden, *et al.*, determined that thiabendazole and albendazole interfered and delayed *Mtb* cell division processes at 16 µg/mL (*Figure II-3*)\(^\text{8}\) Based on the similarity of the benzimidazole moiety to the pyridopyrazine and pteridine pharmacophores in the FtsZ inhibitors identified by White, Reynolds and others,\(^\text{10,16,17,18}\) it was hypothesized that the benzimidazole framework might be a promising starting point for the development of novel FtsZ inhibitors.

The benzimidazole skeleton is a privileged nitrogen-heterocycle and an important pharmacophore present in a number of widely used drugs. The pharmacological, metabolic and toxicological profiles of the benzimidazole pharmacophore have been extensively studied. Several solid- or solution-phase synthetic methods for mono or di-substituted benzimidazoles have been reported, but few deal with the synthesis of trisubstituted benzimidazoles. Furthermore, based on thorough literature search, there are very few trisubstituted benzimidazoles known in literature and they have not been investigated for their antibacterial activity. Consequently, trisubstituted benzimidazoles were selected (*Figure II-3*), especially 2,5,6- and 2,5,7- trisubstituted benzimidazoles, as the basic scaffold for the synthesis of novel benzimidazole libraries using systematic and rational design (*Scheme II-1*).
Synthesis of 2,5,6-trisubstituted benzimidazole library

![Synthesis reaction](image)

Synthesis of 2,5,7-trisubstituted benzimidazole library

![Synthesis reaction](image)

Scheme II-1. Proposed synthesis of 2,5,6- and 2,5,7-trisubstituted benzimidazole library
§ 2.2 Results and Discussion

§ 2.2.1 Synthesis of 2,5,6-trisubstituted benzimidazole library

A library of 2,5,6-trisubstituted benzimidazoles was synthesized as outlined in Scheme II-2. The aromatic nucleophilic substitution of commercially available 2,4-dinitro-5-fluoroaniline with diethyl amine afforded II-1 in 95-100% yield. The acylation of II-1 with different acyl chlorides afforded II-2a~II-2h in 64-85% yield. The reduction and subsequent acid-catalyzed cyclization gave 5-aminobenzimidazoles II-3a~II-3h in 48-56% yield. The intermediates II-3a~II-3h were dissolved in methylene chloride and transferred to a 96-well plate. Subsequently, a series of 33 different acyl chlorides and/or alkyl chloroformates were added to afford library II-A of 272 compounds with good to high purity (see the experimental section for detailed structures of library).

Scheme II-2. Synthesis of 2,5,6-trisubstituted benzimidazole library II-A
§ 2.2.2 Preliminary screening, re-synthesis and accurate MIC determination

The library II-A (272 2,5,6-trisubstituted benzimidazoles) was screened in a 96-well format (single point assay). Of these, 26 compounds were identified to inhibit the growth of *Mtb* H37Rv with MIC\(_{99}\) values ≤ 5 μg/mL in triplicates. Furthermore, among the 26 hits, 8 compounds showed MIC\(_{99}\) values of 0.5-6.1 μg/mL. In order to confirm accurate MIC values, hit compounds were re-synthesized in an analytically pure form (Scheme II-3, Table II-1). Unfortunately, the re-synthesis of the hit compounds showed product selectivity problems. For reactions where acyl chlorides were used as reagents at room temperature, relatively better product selectivity in favor of the desired mono-substituted products was observed (Entry 1). Following the same reaction conditions with butychloroformate (Entry 2), showed very poor product selectivity and mostly disubstituted product was observed. The poor selectivity could be attributed to high reactivity of the chlorformates. Hence, reaction was repeated with a slow addition of 0.9 equivalents of the reagent at lower temperature and higher dilution (Entry 3-5). There was some improvement in the selectivity, but not significant.

Scheme II-3. Re-synthesis of hit compounds

Table II-1. Optimization of the reaction condition

<table>
<thead>
<tr>
<th>Entry</th>
<th>RCOCl/ROCOCl</th>
<th>Solvent [C]</th>
<th>Temp</th>
<th>Time</th>
<th>A (yield)</th>
<th>B (Yield)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>PhCOCl (1 eq.)</td>
<td>CH(_2)Cl(_2)[0.02]</td>
<td>RT</td>
<td>15 h</td>
<td>74 %</td>
<td>12 %</td>
</tr>
<tr>
<td>2.</td>
<td>BuOCOCl (1 eq.)</td>
<td>CH(_2)Cl(_2)[0.02]</td>
<td>RT</td>
<td>15 h</td>
<td>13 %</td>
<td>46 %</td>
</tr>
<tr>
<td>3.</td>
<td>BuOCOCl (0.9 eq.)</td>
<td>CH(_2)Cl(_2)[0.01]</td>
<td>0°C-RT</td>
<td>15 h</td>
<td>20 %</td>
<td>35 %</td>
</tr>
<tr>
<td>4.</td>
<td>BuOCOCl (0.9 eq.)</td>
<td>CH(_2)Cl(_2)[0.02]</td>
<td>0°C-RT</td>
<td>15 h</td>
<td>20 %</td>
<td>35 %</td>
</tr>
<tr>
<td>5.</td>
<td>BuOCOCl (0.9 eq.)</td>
<td>CH(_2)Cl(_2)[0.02]</td>
<td>-50°C-RT</td>
<td>15 h</td>
<td>20 %</td>
<td>40 %(^a)</td>
</tr>
<tr>
<td>6.</td>
<td>Bu-NHS ester (0.9 eq.)</td>
<td>CH(_2)Cl(_2)[0.02]</td>
<td>0°C-RT</td>
<td>15 h</td>
<td>61 %</td>
<td>14 %(^a)</td>
</tr>
</tbody>
</table>

\(^a\)Reaction did not go to completion. Some starting material was recovered.
Following a suggestion given by Dr. Henry Li, \(N\)-hydroxysuccinimide (NHS) esters of the corresponding chloroformates were used under the presumption that the NHS-ester will have lower reactivity compared to chloroformates. As expected, it was observed that using 0.9 equivalent of NHS ester at 0 °C - RT improved the product selectivity to a greater extent with 50-60 % yield of the desired mono-substituted product (Entry 6). The reaction may warrant additional optimization to further improve the product selectivity. Nevertheless, this procedure was used for the re-synthesis of all the hit compounds with carbamate functionality at the 5-position (Scheme II-4). (Please see the Experimental Section for the structures and synthetic procedure of the hit compounds.)

Scheme II-4. Re-synthesis of Hit compounds

The eight analytically pure hit compounds showed MIC values of 2-15 µM against drug sensitive H37Rv Mtb strain based on accurate Alamar blue assay, consistent with the preliminary screening results (Table II-2).
Table II-2. Antimicrobial Activities of Selected Trisubstituted Benzimidazoles against Drug-Sensitive and Drug-Resistant Strains of *Mtb*\(^{a}\)

<table>
<thead>
<tr>
<th>Entry</th>
<th>Benzimidazole</th>
<th>R(^3)</th>
<th>MIC(_{99}) ((\mu)M)</th>
<th>Mtb strains</th>
<th>Cytotoxicity (IC(_{50}) (\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>H37Rv</td>
<td>W210</td>
<td>NHN20</td>
</tr>
<tr>
<td>1</td>
<td>II-Aa</td>
<td></td>
<td>7.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>II-Ab</td>
<td></td>
<td>4.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>II-Ac</td>
<td>MeO</td>
<td>7.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>II-Ad</td>
<td></td>
<td>4.2</td>
<td>4.2</td>
<td>4.2</td>
</tr>
<tr>
<td>5</td>
<td>II-Ae</td>
<td>Cl</td>
<td>14.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>II-Af</td>
<td></td>
<td>15.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>II-Ag</td>
<td></td>
<td>2.0</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>8</td>
<td>II-Ah</td>
<td></td>
<td>3.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>II-Ba</td>
<td></td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>10</td>
<td>II-Bb</td>
<td></td>
<td>3.7</td>
<td>3.7</td>
<td>3.7</td>
</tr>
<tr>
<td>11</td>
<td>II-257</td>
<td>Me</td>
<td>1.6</td>
<td>4.6</td>
<td>4.6</td>
</tr>
</tbody>
</table>

*Mtb* H37Rv is a standard drug sensitive strain. *Mtb* W210, NHN20, NHN335, NHN382 and TN587 are clinical strains with differing drug resistant profiles.
None of the pure lead compounds showed any appreciable cytotoxicity (IC$_{50}>200$ μM) against Vero cells (Table II-2). Two lead compounds, II-Ad and II-Ag, were also evaluated for their activities against clinical isolates of \textit{Mtb} strains W210 (drug-sensitive),$^{19}$ NHN20 (drug-sensitive),$^{19}$ NHN335-2 (isoniazide-resistant; KasA G269S mutation),$^{20}$ NHN382 (isoniazide-resistant; Kat G S315t mutation)$^{19}$ and TN587 (isoniazide-resistant; KatG 3315T mutation),$^{19}$ which showed no difference between the drug-sensitive and drug-resistant strains, as anticipated, exhibiting excellent MIC$_{99}$ values in the range of 0.39-1.56 μg/mL (1-4.6 μM). These compounds have been advanced to the \textit{in vivo} rapid model assay in mice. The remaining 26 hit compounds have also been re-synthesized for extensive structure activity relationship (SAR) studies and their biological evaluation is currently in progress (Scheme II-5) (Please see the experimental section for the synthetic procedure and the structures).

Scheme II-5. Re-synthesis of hit compounds
§ 2.2.3 Preliminary SAR study and optimization of the 6-position of 2,5,6-trisubstituted benzimidazoles

Preliminary SAR studies of 2,5,6-trisubstituted lead compounds indicated that cyclohexyl and diethyl amino groups at the 2- and 6-position, respectively, were critically important for antibacterial activity (Figure II-4).

Based on these observations, another library (II-B) of 238 2,5,6-trisubstituted benzimidazoles was synthesized with various diethylamino-like substituents at the 6-position retaining the cyclohexyl substituent at the 2-position (Scheme II-6) (procedures are similar to the synthesis of library II-A, see experimental section for the structures of library II-B). As the final acid catalyzed cyclization step to form the benzimidazole intermediates with desired substitution pattern was giving low yield, it was decided to optimize the reaction conditions. During the process, it was observed that one pot reaction using tin (II) chloride dihydrate and 4 N HCl improved the yield drastically. Also the reaction was much cleaner with single spot on TLC and baseline impurities, making purification simpler.
Based on the Alamar Blue Assay, 54 compounds were identified from library II-B, which inhibited the cell growth with MIC\textsubscript{99} values \leq 5 \, \mu\text{g/mL}. Among the 54 hits, re-synthesized pure II-Ba and II-Bb showed MIC\textsubscript{99} values of 0.39 \, \mu\text{g/mL} (1.2 \, \mu\text{M}) and 1.56 \, \mu\text{g/mL} (13 \, \mu\text{M}), respectively (Table II-2). Two lead compounds, II-Ba and II-Bb, were also assayed for their activities against clinical isolates of \textit{Mtb} strains W210 (drug-sensitive)\textsuperscript{19}, NHN20 (drug-sensitive)\textsuperscript{19}, NHN335-2 (isoniazide-resistant; KasA G269S mutation)\textsuperscript{20}, NHN382 (isoniazide-resistant; Kat G S315T mutation)\textsuperscript{19} and TN587 (isoniazide-resistant; KatG 3315T mutation)\textsuperscript{19}, which showed no difference in activity against drug-sensitive and drug-resistant strains. As anticipated, II-Ba and II-Bb exhibited excellent MIC\textsubscript{99} values in the range of 0.39-1.56 \, \mu\text{g/mL} (1-4.6 \, \mu\text{M}). These compounds have been advanced to the \textit{in vivo} rapid model assay in mice.

Scheme II-6. Optimization of the 6-position of 2,5,6-trisubstituted benzimidazole library
§ 2.2.4 Optimization of the 2-position of 2,5,6-trisubstituted benzimidazoles

While we were waiting for the results from the 6-position optimization, the 2-position optimization with the piperidine group at the 6-position was carried out. The 2-position was modified with heteroaromatic substituents which are found in many drug-like molecules (Figure II-5).

In addition, diphenyl and benzyl groups were also installed at the 2-position to assess the flexibility of the 2-position (Figure II-5). With these modifications, another library (II-C) of 147 2,5,6-trisubstituted benzimidazoles was synthesized with various aromatic and heteroaromatic substituents at the 2-position (Scheme II-7) (procedures are similar to the synthesis of library II-A, see experimental section for the structures of library II-C).
Scheme II-7. Optimization of the 2-position of 2,5,6-trisubstituted benzimidazole library

Based on the Alamar Blue Assay, 12 compounds were identified, which inhibited cell growth with MIC$_{99}$ values of $\leq 10 \mu$g/mL. Among the 12 hits, 3 compounds exhibited MIC$_{99}$ values of $\leq 5 \mu$g/mL and 5 compounds exhibited MIC$_{99}$ values of $\leq 10 \mu$g/mL (Figure II-6). Some of the compounds have been re-synthesized and sent for accurate MIC$_{99}$ determination. The rest of the compounds are currently being re-synthesized.
As mentioned before, a pyrrolidine substituent at the 6-position is critical for anti-TB activity. Thus, the 2-position optimization was done using a pyrrolidine substituent at the 6-position (Scheme II-8). These intermediates are being used to synthesize active compounds which may exhibit improved biological activity.

**Scheme II-8. Synthesis of 2,5,6-trisubstituted benzimidazoles with pyrrolidine substituent at the 6-position**
§ 2.2.5 Optimization of the 5-position of 2,5,6-trisubstituted library

So far only amide or carbamate functionalities have been explored at the 5-position using a limited number of acyl chlorides and chloroformates. In order to expand the scope of the library and investigate other functionalities such as urea, thiourea, and sulfonamide, substituent optimization at the 5-position was evaluated using various isocyanates, isothiocyanates, and sulfonyl chlorides as outlined (Figure II-7).

For the optimization of the 5-position, a new library (II-D) of 180 compounds was synthesized using various isocyanate, isothiocyanate and sulfonyl chlorides as reagents (Figure II-8). In a preliminary screening against drug sensitive H37Rv cells, compound II-Da, with a thiourea functionality at the 5-position, was found to exhibit good activity (MIC value of ≤ 5 µg/mL). Moreover, for two compounds, II-Db and II-Db no visible growth with active respiration was observed. This led us to belief that these compounds may be bacteriostatic: rather than killing the bacteria the compounds either slow down or inhibit their growth. These findings need to be confirmed using analytically pure compounds.
Furthermore, acylating reagents that have been used so far are very limited in their structure versatility and functional properties. Mostly, we have focused on alkyl or aromatic acylating reagents. Hence it was important to extend the series to assorted reagents with diverse structural and functional features. In an attempt to diversify the 5-position, a series of different acylating reagents was used for the synthesis of library II-E (Figure II-9). The biological evaluation of this library of compounds is currently in progress.

**Figure II-8. Synthesis of the 5-position optimized 2,5,6-trisubstituted benzimidazole library II-D**
§ 2.2.6 Optimization of 2,5,7-trisubstituted benzimidazoles

Previously in the Ojima lab, a library of 77 2,5,7-trisubstituted benzimidazoles was synthesized for the discovery of novel compounds against \textit{Mtb}. In a preliminary screening against \textit{Mtb} strains, one compound \textbf{II-257} was identified, which inhibited cell growth with an MIC\textsubscript{99} value of \( \leq 0.5 \mu\text{g/mL} \). Upon accurate MIC\textsubscript{99} determination, \textbf{II-257} exhibited MIC\textsubscript{99} value of 0.5 \( \mu\text{g/mL} \) and 1.56 \( \mu\text{g/mL} \) against drug-sensitive and drug-resistant cell lines, respectively (Table II-2). In order to optimize the lead compounds, a more diverse library of 2,5,7-trisubstituted benzimidazoles with different substitution patterns was synthesized (Figure II-10). In the case of 2,5,6-trisubstituted benzimidazole library, a cyclohexyl group at the 2–position proved to be very crucial for anti-TB activity. Thus, a cyclohexyl moiety was used at the 2-position for the 2,5,7-trisubstituted library as well. Moreover, in addition to ethoxycarbamate substituent at the 5-position, we also decided to expand the scope by using various other carbamates as well.
A library of 2,5,7-trisubstituted benzimidazole was synthesized as outlined in Scheme II-9. Earlier, the synthesis of a 2,5,7-trisubstituted library was carried out starting with 4-amino-3,5-dinitrobenzamide (II-12). Unfortunately, this compound is no longer commercially available or very expensive. Hence, we decided to start the synthesis from relatively cheaper, commercially available 4-chlorobenzoic acid (II-11). Nitration of II-11 in the presence of potassium nitrate and sulfuric acid followed by the substitution of the chloride by an amino moiety using ammonia solution in methanol generated the intermediate 4-amino-3,5-dinitrobenzoic acid (II-12) as yellow solid in 85 % yield (2 steps). The intermediate II-12 was converted to acyl chloride II-13. The reaction of II-13 with sodium azide afforded the corresponding acyl azide, which was subjected to the Curtius rearrangement to give the corresponding isocyanate. The isocyanate was subsequently reacted with $t$-butanol or ethanol to give the corresponding carbamates II-14a (R$^1$ = Et) or II-14b (R$^1$ = $t$-Bu) in 78-84 % yield (for 4 steps) as a bright red solid. The reduction of II-14a/II-14b followed by cyclocondensation with the bisulfite salts of different aldehydes afforded 7-aminobenzimidazoles II-15a~II-15f in 40-64 % yield. The final acylation of II-15a~II-15f in the same manner as that for the library of II-A gave the library of 126 2,5,7-trisubstituted benzimidazoles II-F with good to high purity (see the experimental section for the detailed experimental procedures, structures of intermediates and library).
Unexpectedly, most of the compounds were found to be cytotoxic at 5 µg/ml based on a more accurate Alamar Blue Assay. We hypothesized that the extreme cytotoxicity of these compounds could arise from the residual DMF in the 96 well plates. Hence in order to confirm this premise, we are currently resynthesizing the compounds using volatile solvents such as ethanol, tetrahydrofuran, and acetonitrile.
§ 2.2.7 Early ADMET results and synthesis of metabolically stable benzimidazoles analogues

Considering the encouraging *in vitro* results of our lead compounds, evaluation of early *in vitro* adsorption, distribution, metabolism, excretion and toxicology (ADMET) properties of SB-P3G2 was carried out by our collaborators at Sanofi-Aventis laboratory. The compound exhibited relatively reasonable drug-like ADMET properties with good membrane permeability, promising cardiac safety (no effect on hERG calcium-ion channel), negligible inhibition of CYP 3A4 and clean kinase profile. Additionally, the plasma stability of the compound in human plasma was excellent. However, the plasma stability in mouse plasma was poor which improved significantly on addition of PMSF (phenylmethylsulfonyl fluoride), a hydrolase inhibitor. Also, it was observed that the metabolic stability in mouse and rat microsome was suggestively low.

**Figure II-11. Amide congeners of SB-P3G2 and SB-P8B2**

As all preclinical studies will be done with mice or rats, it is necessary to develop a metabolically more stable compound that can be used for mice or rats. Knowing that the compound showed improved stability on addition of PMFS; we suspected that carbamate functionality is one possible site which is getting cleaved during the process. Therefore, it was decided to synthesize the amide congeners of two lead compounds, SB-P3G2 and SB-P8B2 (*Figure II-11*). However, these compounds need to retain their anti-tubercular activities after modification. Currently, the biological evaluation of these compounds is in progress, and depending on the results, the metabolic stability of the compounds will be evaluated. Furthermore, among the 11 hit compounds, several benzimidazoles with amide functionality at the 5 position were identified, however with lower activities compared to SB-P3G2. The
ADMET properties of these compounds will also be evaluated in collaboration with Sanofi-Aventis.

§ 2.3 Conclusion

A library of 349 novel trisubstituted-benzimidazoles was synthesized using multi-step polymer-assisted solution phase synthesis through a rational and systematic design. In preliminary screening, 27 compounds exhibited MIC99 values of ≤ 0.5 μg/mL against Mtb H37RV strain. Upon resynthesis and accurate MIC determination, 9 lead compounds were identified which exhibited MIC99 in the range of 0.56-6.1 μg/mL. Following the initial discovery, an optimized library of 238 trisubstituted-benzimidazoles was synthesized and screened. 54 additional compounds with MIC99 values ≤ 5 μg/mL were identified as solid hits. Among the 54 hits, 2 compounds showed MIC99 values of 0.39 and 1.56 μg/mL, respectively, based on the Alamar blue assay. The lead compounds showed excellent activities against drug-resistant clinical isolates of Mtb with MIC99 values in the range of 0.39-1.56 μg/mL without any appreciable cytotoxicity. Based on preliminary SAR studies, optimization of substituents at the 2-, 5-, and 6-position was carried out by synthesizing another series of libraries of ~1000 compounds. Unfortunately, some of the compounds were found to be cytotoxic, whereas other compounds didn’t show any improvement in activities over the previous compounds. Currently, other series of libraries have been synthesized which are being screened against Mtb. Moreover, the lead compound SB-P3G2 has exhibited reasonable drug-like ADMET properties.
§ 2.4 Experimental Section

**General Methods:** $^1$H and $^{13}$C NMR spectra were measured on a Varian 300, 400 or 500 MHz NMR spectrometer. Melting points were measured on a Thomas Hoover Capillary melting point apparatus and are uncorrected. TLC was performed on Merck D Calufolien with Kieselgel 60F-254 and column chromatography was carried out on silica gel 60 (Merck; 230-400 mesh ASTM). High-resolution mass spectra were obtained from Mass Spectrometry Laboratory, University of Illinois at Urbana-Champaign, Urbana, IL. Purity was determined by Agilent 1100 series HPLC assembly. Two analytical conditions were used and noted as a part of the characterization data for re-synthesized compounds. **HPLC1:** Luna PFP, 3 µm, 100 Å (pore size), 3.0 x 150 mm column, Solvent A – H$_2$O:ACN, 95:5 (20 mM Ammonium acetate, pH 6.5), Solvent B = H$_2$O:ACN, 5:95 (20 mM Ammonium acetate, pH 6.5), Temperature 30 °C, flow rate 0.6 mL/min, t = 0-13.5 min, gradient 15-90 % solvent B. **HPLC2:** Kinetex PFP, 2.6 µm, 100 Å (pore size), 4.6 x 100 mm column, Solvent A – H$_2$O:ACN, 95:5 (25 mM Ammonium acetate, pH=6.5), Solvent B = H$_2$O:ACN, 5:95 (25 mM Ammonium acetate, pH=6.5), Temperature 25 °C, flow rate 1.0 mL/min, t = 0-15 min, gradient 20-95 % solvent B.

**Materials:** All chemicals were purchased from Aldrich Co., Synquest Inc., Fisher Scientific and VWR and purified before use by standard methods. Tetrahydrofuran was freshly distilled from sodium metal and benzophenone. Dichloromethane was also distilled immediately prior to use under nitrogen from calcium hydride. Aminomethylated polystyrene resin EHL (200-400 mesh) 2 % DVB was purchased from Novagen Biochem.

§ 2.4.1 Synthesis of 2,5,6-Trisubstituted Benzimidazole Library II-A

5-N,N-Diethylamino-2,4-dinitroaniline (II-1): 

![Chemical Structure](image)

To a solution of 2,4-dinitro-5-fluoroaniline (500 mg, 2.48 mmol) and diisopropylethylamine (DIPEA) (476.3 µL, 2.73 mmol) in tetrahydrofuran (25 mL) was added a
solution of diethylamine (360 μL, 3.48 mmol) in tetrahydrofuran (22 mL) dropwise. The reaction mixture was stirred at room temperature for 4 h. The solvent was evaporated and water was added to give a yellow precipitate. The solution was extracted with dichloromethane (30 mL x 3). The organic layer was collected, dried over anhydrous magnesium sulfate and evaporated to give II-1 as yellow solid (626 mg, 99 % yield); mp 108-109 °C; ¹H NMR (500 MHz, CDCl₃) δ 1.18 (t, 6 H, J = 7 Hz), 3.23 (q, 4 H, J = 6.5 Hz), 6.14 (s, 1 H), 6.49 (broad s, NH), 8.71 (s, 1 H); ¹³C NMR (125 MHz, CDCl₃) δ 12.07, 45.72, 102.7, 123.2, 128.1, 131.5, 147.7, 149.3. MS (ESI) m/z 253.1 (M+1)⁺.

1-Benzamido-5-N,N-diethylamino-2,4-dinitrobenzene (II-2a):

\[
\text{A solution of II-1a (500 mg, 1.96 mmol) and benzoyl chloride (329 mg, 2.35 mmol) in pyridine (8 mL) was refluxed overnight. After completion of the reaction, pyridine was removed invacuo to give a brown solid. This crude mixture was washed with methanol and collected by filtration to afford pure II-2a as a yellow solid (570 mg, 81 % yield); mp 168-170 °C; ¹H NMR (400 MHz, CDCl₃) δ 1.30 (t, 6 H, J = 7.2 Hz), 3.41 (q, 4 H, J = 7.2 Hz), 7.53 (dd, 2 H), 7.59 (dd, 1 H), 7.98 (d, 2 H, J = 7.2 Hz), 8.78 (s, 1 H), 8.81 (s, 1 H), 11.84 (s, 1 H); ¹³C NMR (100 MHz, CDCl₃) δ 12.23, 46.53, 107.5, 125.1, 127.3, 129.1, 132.9, 133.5, 138.8, 149.0, 166.1. MS (ESI) m/z 359.1 (M+1)⁺.}

Compounds II-2a–II-2h were prepared in the same manner.

1-(4-Methylbenzamido)-5-N,N-diethylamino-2,4-dinitrobenzene (II-2b):

\[
\text{Yellow solid; 80 % yield; mp 200-202 °C; ¹H NMR (500 MHz, CDCl₃) δ 1.30 (t, 6 H, J = 7 Hz), 2.45 (s, 3 H), 3.41 (q, 4 H, J = 7 Hz), 7.34 (d, 2 H, J = 8 Hz), 7.89 (d, 2 H, J = 8.5 Hz), 8.80 (s, 1}

H), 8.83 (s, 1 H), 11.82 (s, NH); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 12.29, 21.57, 46.56, 107.5, 125.1, 127.3, 129.8, 130.8, 132.9, 139.0, 143.8, 149.0, 166.2. MS (ESI) m/z 373.1 (M+1)$^+$. 

1-(4-Methoxybenzamido)-5-$N,N$-diethylamino-2,4-dinitrobenzene (II-2c):

Yellow solid; 85 % yield; mp 166-167 °C; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 1.29 (t, 6 H, $J$ = 7.2 Hz), 3.41 (q, 4 H, $J$ = 6.8 Hz), 3.39 (s, 3 H), 7.01 (d, 2 H, $J$ = 8.4 Hz), 7.96 (d, 2 H, $J$ = 8.4 Hz), 8.82 (d, 2 H, $J$ = 8 Hz), 11.77 (s, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 12.26, 46.53, 55.53, 107.4, 114.3, 125.0, 125.7, 127.2, 129.4, 132.8, 139.1, 149.0, 163.4, 165.6. MS (ESI) m/z 389.1 (M+1)$^+$. 

1-(2-Methoxybenzamido)-5-$N,N$-diethylamino-2,4-dinitrobenzene (II-2d):

Yellow solid; 64 % yield; mp 155-157 °C; $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 1.28 (t, 6 H, $J$ = 7.5 Hz), 3.39 (q, 4 H, $J$ = 7 Hz), 4.10 (s, 3 H), 7.05 (d, 1 H, $J$ = 8.5 Hz), 7.11 (dd, 1 H, $J$ = 7 Hz), 7.53 (m, 1 H), 8.21 (d, 1 H, $J$ = 9 Hz), 8.79 (s, 1 H), 8.95 (s, 1 H), 12.5 (s, 1 H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 12.30, 46.42, 55.67, 109.4, 111.4, 121.2, 126.1, 127.0, 132.6, 134.3, 138.8, 148.8, 157.6, 164.9. MS (ESI) m/z 389.1 (M+1)$^+$. 

1-(4-Fluorobenzamido)-5-$N,N$-diethylamino-2,4-dinitrobenzene (II-2e):

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Yellow solid; 78 % yield; mp 173-174 °C; $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 1.30 (t, 6 H, $J = 7$ Hz), 3.41 (q, 4 H, $J = 7$ Hz), 7.21 (d, 2 H, $J = 8$ Hz), 8.02 (d, 2 H, $J = 8$ Hz), 8.79 (s, 1 H), 8.81 (s, 1 H), 11.82 (s, 1 H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 12.26, 46.59, 107.5, 116.4, 125.1, 127.2, 129.8, 133.0, 138.7, 149.0, 164.5, 165.0, 166.6. MS (ESI) m/z 377.1 (M+1)$^+$.  

1-(4-tert-Butylbenzamido)-5,N,N-diethylamino-2,4-dinitrobenzene (II-2f):

$\begin{align*}
\text{N} & \text{O} \\
\text{NH} & \text{O}_2\text{N} \\
\text{N} & \text{O} \\
\text{NH} & \text{O}_2\text{N} \\
\end{align*}$

Yellow solid; 78 % yield; mp 171-173 °C; $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 1.31 (t, 6 H, $J = 7$ Hz), 1.36 (s, 9 H), 3.41 (q, 4 H, $J = 7$ Hz), 7.57 (d, 2 H, $J = 8.5$ Hz), 7.93 (d, 2 H, $J = 8.5$ Hz), 8.81 (s, 1 H), 8.84 (s, 1 H), 11.82 (s, 1 H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 12.29, 31.07, 35.14, 46.55, 107.5, 125.2, 126.13, 127.29, 130.76, 132.9, 139.0, 149.0, 156.8, 166.2. MS (ESI) m/z 415.2 (M+1)$^+$.  

1-(Cyclohexanecarboxamido)-5,N,N-diethylamino-2,4-dinitrobenzene (II-2g):

$\begin{align*}
\text{N} & \text{O} \\
\text{NH} & \text{O}_2\text{N} \\
\text{N} & \text{O} \\
\text{NH} & \text{O}_2\text{N} \\
\end{align*}$

Yellow solid; 81 % yield; mp 129-131 °C; $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 1.25 (t, 6 H, $J = 7$ Hz), 1.33-1.5 (m, 5 H), 1.69 (m, 1 H), 1.83 (m, 2 H), 2.02 (m, 2 H), 2.36 (m, 1 H), 3.33 (q, 4 H, $J = 7$ Hz), 8.62 (s, 1 H), 8.71 (s, 1 H), 10.91 (s, 1 H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 12.19, 25.24, 25.51, 28.71, 29.35, 46.40, 47.29, 107.6, 124.9, 127.0, 132.6, 138.6, 148.9, 175.9. MS (ESI) m/z 365.1 (M+1)$^+$.  

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1-(Naphthalene-1-carboxamido)-5-N,N-diethylamino-2,4-dinitrobenzene (II-2h):

Orange solid; 80 % yield; mp 135-137 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 1.30 (t, 6 H, $J = 7.2$ Hz), 3.41 (q, 4 H, $J = 7$ Hz), 7.57 (m, 3 H), 7.86 (t, 2 H, $J = 8$ Hz), 7.98 (d, 1 H, $J = 8.4$ Hz), 8.50 (d, 1 H, $J = 8.4$ Hz), 8.72 (s, 1 H), 8.82 (s, 1 H), 11.47 (s, NH); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 12.16, 46.43, 107.4, 124.6, 124.8, 125.8, 126.6, 127.0, 127.6, 128.4, 130.0, 132.3, 132.7, 133.7, 138.5, 148.8, 167.9. MS (ESI) m/z 409.1 (M+1)$^+$. 

5-Amino-6-N,N-diethylamino-2-phenyl-1H-benzo[d]imidazole (II-3a):

To a solution of II-2a (700 mg, 1.95 mmol) in dioxane (50 mL) and ethanol (50 mL) was added ammonium formate (3.22 g) and 10 wt. % Pd-C (300 mg) with stirring. The reaction mixture turned from yellow to red and then to colorless in 45-60 min at room temperature. The catalyst and excess ammonium formate were filtered off to give the product solution. Concentrated hydrochloric acid was added to this solution and adjusted to 4 N HCl concentration. Then the mixture was refluxed for 4-6 h. The reaction mixture was basified by addition of ammonium hydroxide, extracted with ethyl acetate (40 mL x 3) and dried over anhydrous magnesium sulfate. The crude product was purified by flash chromatography on silica gel using ethyl acetate/hexanes (gradient 20-40 % ethyl acetate/hexanes) as eluent to give pure II-3a as a brown solid (250 mg, 50 % yield); mp 105-106 °C; $^1$H NMR (300MHz, CDCl$_3$) δ 0.96 (t, 6 H, $J = 6.9$ Hz), 2.92 (q, 4 H, $J = 6.9$ Hz), 6.89 (s, 1 H), 7.32 (m, 4 H), 8.05 (d, 2 H, $J = 9.5$ Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 15.27, 51.23, 100.2, 113.9, 128.9, 131.5, 132.0, 132.9, 137.9, 144.1, 153.0; HRMS (FAB) m/z calcd for C$_{17}$H$_{20}$N$_4$H$: 281.1758$, Found: 281.1766 (Δ = -2.8 ppm). HPLC(2): 9.02 min, purity > 99 %.

Compounds II-3a ~ II-3h were prepared in the same manner.
5-Amino-6-N,N-diethylamino-2-(4-methylphenyl)-1H-benzo[d]imidazole (II-3b):

Brown solid; 52 % yield; mp 114-116 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 0.90 (t, 6 H, $J$ = 6.8 Hz), 2.30 (s, 3 H), 2.85 (q, 4 H, $J$ = 6.8 Hz), 6.77 (s, 1 H), 7.12 (d, 2 H, $J$ = 8 Hz), 7.21 (s, 1 H), 7.97 (d, 2 H, $J$ = 8.4 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 12.55,21.27, 48.53, 97.78, 110.88, 126.2, 127.5, 129.5, 134.4, 135.0, 139.3, 141.2, 150.8; HRMS (FAB) m/z calcd for C$_{18}$H$_{22}$N$_4$H$^+$: 295.1919, Found: 295.1923 ($\Delta$ = -1.4 ppm). HPLC(2): 7.20 min, purity > 99 %.

5-Amino-6-N,N-diethylamino-2-(4-methoxyphenyl)-1H-benzo[d]imidazole (II-3c):

Brown solid; 56 % yield; mp 100-103 °C; $^1$H NMR (400MHz, CDCl$_3$) δ 0.88 (t, 6 H, $J$ = 6.8 Hz), 2.83 (q, 4 H, $J$ = 7.2 Hz), 3.71 (s, 3 H), 6.78 (m, 3 H), 7.18 (s, 1 H) 8.05 (d, 2 H, $J$ = 8.8 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 12.55, 48.49, 55.17, 97.85, 110.4, 114.2, 122.7, 128.0, 133.8, 135.0, 135.1, 141.1, 150.7, 160.5; HRMS (FAB) m/z calcd for C$_{18}$H$_{22}$N$_4$OH$^+$: 311.1864, Found: 311.1872 ($\Delta$ = -2.6 ppm). HPLC(1): 9.22 min, purity > 89 %.

5-Amino-6-N,N-diethylamino-2-(2-methoxyphenyl)-1H-benzo[d]imidazole (II-3d):

Brown solid; 45 % yield; mp 171-175 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 0.97 (t, 6 H, $J$ = 6.8 Hz), 2.97 (q, 4 H, $J$ = 7.2 Hz), 3.96 (s, 3 H), 6.88 (s, 1 H), 6.97 (d , 1 H, $J$ = 8.4 Hz), 7.06 (t, 1 H, $J$ = 8 Hz), 7.31 (t, 1 H, $J$ = 8.5 Hz), 7.37 (s, 1 H), 8.49 (d, 1 H, $J$ = 9.6 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 12.49, 48.52, 55.73, 97.31, 111.3, 118.2, 121.4, 129.4, 130.2, 133.1, 134.9, 141.2,
148.1, 156.2; HRMS (FAB) m/z calcd for C_{18}H_{22}N_{4}OH^+: 311.1867, Found: 311.1872 (Δ = -1.6 ppm). HPLC(2): 7.99 min, purity > 99 %.

5-Amino-6-N,N-diethylamo-2-(4-fluorophenyl)-1H-benzo[d]imidazole (II-3e):

Brown solid; 55 % yield; mp 105-107 °C; \(^1\)H NMR (300MHz, CDCl\(_3\)) δ 0.96 (t, 6 H, J = 7 Hz), 2.92 (q, 4 H, J = 7.2 Hz), 6.85 (s, 1 H), 7.04 (t, 2 H, J = 8.0 Hz), 7.31 (s, 1 H), 7.98 (ddd, 2 H, J = 1.8, 5.4, 8.7 Hz); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) δ 12.53, 48.44, 97.37, 110.8, 115.9, 116.0, 125.9, 128.2, 133.9, 134.4, 135.6, 141.7, 149.0, 162.4, 164.4; HRMS (FAB) m/z calcd for C_{17}H_{19}FN_{4}H^+: 299.1668, Found: 299.1672 (Δ = -1.3 ppm). HPLC(2): 9.54 min, purity > 96 %.

5-Amino-6-N,N-diethylamo-2-(4-tert-butylphenyl)-1H-benzo[d]imidazole (II-3f):

Brown solid; 50 % yield; mp 95-96 °C; \(^1\)H NMR (400 MHz, CDCl\(_3\)) δ 0.90 (t, 6 H, J = 6.8 Hz), 1.25 (s, 9 H), 2.82 (q, 2 H, J = 6.8 Hz), 6.81 (s, 1 H), 7.26 (s, 1 H), 7.32 (d, 2 H, J = 8.4 Hz), 8.15 (d, 2 H, J = 8.4 Hz); \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) δ 12.57, 31.02, 34.55, 48.39, 97.15, 110.4, 125.6, 126.3, 127.4, 134.1, 135.0, 135.5, 140.9, 151.0, 152.3; HRMS (FAB) m/z calcd for C\(_{21}\)H\(_{28}\)N\(_4\)H^+: 337.2393, Found: 337.2392 (Δ = -2.7 ppm). HPLC(2): 9.16 min, purity > 99 %.

5-Amino-2-cyclohexyl-6-N,N-diethylamo-1H-benzo[d]imidazole (II-3g):

Dark brown solid; 50 % yield; mp 102-104 °C; \(^1\)H NMR (300 MHz, CDCl\(_3\)) δ 1.27 (t, 6 H, J = 6.9 Hz), 1.52 (m, 5 H), 1.75 (m, 1 H), 1.89 (m, 2 H), 2.04 (m, 2 H), 2.39 (m, 1 H), 3.37 (t, 4 H, J = 7.2 Hz), 8.65 (s, 1 H), 8.76 (s, 1 H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) δ 12.55, 25.62, 25.90, 31.84,
HRMS (FAB) $m/z$ calcd for $C_{17}H_{26}N_4H^+$: 287.2231, Found: 287.2236 ($\Delta = -1.7$ ppm). HPLC(2): 8.36 min, purity > 98 %.

5-Amino-6-$N,N$-diethylamino-2-(naphth-1-yl)-1$H$-benzo[d]imidazole (II-3h):

![Chemical Structure]

Off white solid; 48 % yield; mp 101-104 °C; $^1$H NMR (500MHz, CDCl$_3$) $\delta$ 0.94 (t, 6 H, $J = 7.2$ Hz), 2.95 (broad s, 4 H, $J = 7.2$ Hz), 7.54 (m, 3 H), 7.68 (s, 1 H), 7.91 (m, 3 H), 8.81 (d, 2 H, $J = 8$ Hz), 8.99 (s, 1 H), 9.4 (s, 1 H), 12.48 (s, 1 H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 12.94, 24.49, 50.65, 100.9, 113.7, 124.8, 126.1, 126.3, 127.0, 127.9, 128.1, 128.5, 129.9, 131.2, 132.3, 132.5, 133.8, 134.9, 140.4, 152.2, 168.5; HRMS (FAB) $m/z$ calcd for $C_{21}H_{22}N_4H^+$: 331.1929, Found: 331.1923 ($\Delta = -1.8$ ppm). HPLC(2): 8 min, purity > 99 %.

Synthesis of the library of 2,5,6-trisubstituted benzimidazoles II-A:

1. $R^3$COCl or $R^3$COOCl, CH$_2$Cl$_2$, overnight, RT
2. Aminomethylated resin, CH$_2$Cl$_2$, overnight, RT
3. Filter

X = $R^3$, $R^3O$
List of Acylating reagents

The intermediates II-3a~II-3h (0.005 mmol), thus synthesized, were dissolved in dichloromethane and transferred into 96 well plates, and to these wells were added 33 different acyl chlorides (1.1 eq.) or alkyl chloroformates (1 eq) and reacted overnight on a shaker. Acyl chlorides and alkyl chloroformates used for the library synthesis are shown in Figure S1. Aminomethylated polystyrene resin EHL (200-400 mesh), 2 % DVB (10 eq) was added to scavenge excess or unreacted acyl chlorides or alkyl chloroformates. After reacting on a shaker overnight, the resin was filtered to provide the library of 2,5,6-trisubstituted benzimidazoles II-A. The products were analyzed by LC-MS/UV for their purity and confirmation of the structure. The purity was in the range of 80-90 %.

§ 2.4.2 Synthesis of 2,5,6-Trisubstituted Benzimidazole Library II-B

2,4-dinitro-5-(4-Methylpiperazin-1-yl)aniline (II-4a):
To a solution of 2,4-dinitro-5-fluoroaniline (2 g, 9.95 mmol) and DIPEA (2.01 mL, 11.94 mmol) in tetrahydrofuran (50 mL) was added a solution of N-methylpiperazine (1.19 g, 11.94 mmol) in tetrahydrofuran (50 mL), dropwise. The mixture was stirred at room temperature for 4 h. The reaction mixture was diluted with dichloromethane, washed with water (30 mL x 3) and dried over anhydrous magnesium sulfate. The solvent was evaporated to afford II-4a as a yellow solid (1.40 g, 98% yield): mp 148-150 °C; \(^1\)H NMR (400 MHz, acetone-d\(_6\)) δ 2.26 (s, 3 H), 2.50 (t, 4 H, \(J = 4.8\) Hz), 3.11 (t, 4 H, \(J = 4.8\) Hz), 6.56 (s, 1 H), 7.50 (broad s, 2 H), 8.79 (s, 1 H); \(^{13}\)C NMR (100 MHz, acetone-d\(_6\)) δ 46.28, 51.55, 55.25, 105.4, 124.9, 128.6, 132.0, 150.2, 151.8; MS (ESI) \(m/z\) 282.1 (M+1)

In the same manner, compounds II-4b~ II-4g were prepared.

**2,4-Dinitro-5-(pyrrolidin-1-yl)aniline (II-4b):**

![Structure of II-4b]

Yellow solid; quantitative yield; mp 169-171 °C; \(^1\)H NMR (500 MHz, CDCl\(_3\)) δ 2.0 (m, 4 H), 3.27 (m, 4 H), 5.91 (s, 1 H), 6.30 (broad s, NH), 8.71 (s, 1 H); \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) δ 25.63, 50.96, 98.26, 122.8, 127.9, 130.1, 146.7, 147.2; MS (ESI) \(m/z\) 255.1 (M+1). 

**2,4-dinitro-5-Morpholinoaniline (II-4c):**

![Structure of II-4c]

Yellow solid; 93% yield; mp 194-195 °C; \(^1\)H NMR (400 MHz, acetone-d\(_6\)) δ 3.11 (t, 4 H, \(J = 4.8\) Hz), 3.77 (t, 4 H, \(J = 4.8\) Hz), 6.60 (s, 1 H), 7.54 (broad s, 2 H), 8.78 (s, 1 H); \(^{13}\)C NMR (100 MHz, acetone-d\(_6\)) δ 52.04, 66.93, 105.5, 125.2, 128.7, 131.9, 150.3, 151.9; MS (ESI) \(m/z\) 260 (M+1).
3-(N',N',N-Triethylethylenediamino)-4,6-dinitroaniline (II-4d):

Brown oil; 99% yield; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 0.99 (t, 6 H, $J = 7.2$ Hz), 1.20 (t, 3 H, $J = 7.2$ Hz), 2.50 (q, 4 H, $J = 7.2$ Hz), 2.60 (t, 2 H, $J = 7.5$ Hz), 3.25 (m, 4 H), 6.17 (s, 1 H), 6.37 (broad s, 2 H), 8.75 (s, 1 H). $^{13}$C NMR (100 MHz, acetone-d$_6$) $\delta$ 12.54, 12.74, 47.51, 48.05, 51.20, 51.40, 104.6, 124.1, 128.2, 132.5, 149.7, 150.3; Mass (ESI) m/z 326.2 (M+1)$^+$. 

3-(N',N',N-Trimethylethylenediamino)-4,6-dinitroaniline (II-4e):

Yellow solid; 93% yield; mp 134–135 °C; $^1$H NMR (400 MHz, acetone-d$_6$) $\delta$ 2.16 (s, 6 H), 2.55 (t, 2 H, $J = 6.4$ Hz), 2.93 (s, 3 H), 3.28 (t, 2 H, $J = 6.9$ Hz), 6.48 (s, 1 H), 7.37 (broad s, 2 H), 8.62 (s, 1 H); $^{13}$C NMR (100 MHz, acetone-d$_6$) $\delta$ 40.17, 45.89, 53.17, 57.13, 102.7, 123.9, 128.5, 131.5, 149.7, 151.1; MS (ESI) m/z 284.1 (M+1)$^+$. 

3-(N',N'-Dimethyl-N-ethylethylenediamino)-4,6-dinitroaniline (II-4f):

Yellow solid; 88% yield; mp 83-85 °C; $^1$H NMR (400 MHz, acetone-d$_6$) $\delta$ 1.18 (t, 3 H, $J = 7.0$ Hz), 2.16 (s, 6 H), 2.48 (t, 2 H, $J = 7.2$ Hz), 3.29 (m, 4 H), 6.56 (s, 1 H), 7.41 (broad s, 2 H), 8.60 (s, 1 H); $^{13}$C NMR (100 MHz, acetone-d$_6$) $\delta$ 12.67, 45.93, 47.45, 50.54, 57.33, 104.5, 124.0, 128.2, 132.4, 149.8, 150.3; MS (ESI) m/z 298.1 (M+1)$^+$. 

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2,4-Dinitro-5-(piperidin-1-yl)aniline (II-4g):

Yellow solid; 98% yield; mp 115-116 °C; ¹H NMR (400 MHz, acetone-δ₆) δ 1.64 (m, 6 H), 3.08 (t, 4 H, J = 4.8 Hz), 6.51 (s, 1 H), 7.43 (broad s, 2 H), 8.69 (s, 1 H); ¹³C NMR (100 MHz, acetone-δ₆) δ 24.64, 26.27, 52.72, 104.81, 124.6, 128.5, 131.9, 150.2, 152.2; MS (ESI) m/z 267.1 (M+1)+.

1-Cyclohexanecarboxamido-2,4-dinitro-5-(4-methylpiperazin-1-yl)benzene (II-5a):

A solution of II-4a (2.79 g, 9.94 mmol) and cyclohexanecarbonyl chloride (2.03 mL, 14.91 mmol) in pyridine (30 mL) was refluxed overnight. The reaction mixture was diluted with dichloromethane, washed with water (30 mL x 3) and dried over anhydrous magnesium sulfate. The crude product was purified by flash chromatography on silica gel using ethyl acetate as eluent to give II-5a as yellow solid (3.28 g, 74 % yield): mp 90-92 °C; ¹H NMR (400 MHz, CDCl₃) δ 1.23-1.39 (m, 3 H), 1.50 (m, 2 H, J = 12.4 Hz), 1.70 (d, 1 H, J = 12 Hz), 1.83 (m, 2 H, J = 12 Hz), 2.04 (m, 2 H, J = 12 Hz), 2.37 (m, 4 H), 2.58 (t, 4 H, J = 4.5 Hz), 3.33 (t, 4 H, J = 4.5 Hz), 8.62 (s, 1 H), 8.85 (s, 1 H), 10.91 (s, 1 H); ¹³C NMR (100 MHz, CDCl₃) δ 25.44, 29.36, 45.76, 47.28, 50.52, 54.15, 108.7, 126.2, 127.2, 132.8, 139.4, 150.2, 175.9. MS (ESI) m/z 392.1 (M+1)+.

In the same manner, compounds II-5b~II-5g were prepared.

1-Cyclohexanecarboxamido-2,4-dinitro-5-(pyrrolidin-1-yl)benzene (II-5b):
Yellow solid; 83 % yield; mp 181-182 °C; $^1$H NMR (500 MHz, CDCl$_3$) δ 1.6-1.3 (m, 7 H), 1.73 (m, 1 H), 1.87 (m, 2 H), 2.02 (m, 6 H), 2.37 (m, 1 H), 3.37 (m, 4 H), 8.46 (s, 1 H), 8.71 (s, 1 H), 10.95 (s, 1 H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 25.43, 25.46, 29.33, 47.21, 51.26, 104.8, 124.2, 126.8, 130.8, 138.2, 146.5, 175.8. Ms (ESI) m/z 363.1 (M+1)$^+$. 

1-Cyclohexanecarboxamido-2,4-dinitro-5-N-morpholinobenzene (II-5c): 

Yellow solid; 93% yield; mp 160-161 °C; $^1$H NMR (300 MHz, CDCl$_3$) δ 1.23-1.54 (m, 4 H), 1.74 (m, 2 H), 1.84-2.04 (m, 4 H), 2.37 (m, 1 H), 3.28 (t, 4 H, $J = 4.5$ Hz), 3.85 (t, 4 H, $J = 4.5$ Hz), 8.64 (s, 1 H), 8.87 (s, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 25.43, 28.76, 29.36, 47.28, 50.97, 66.09, 108.7, 126.6, 127.1, 132.9, 139.5, 150.4, 176.0; MS (ESI) m/z 379.2 (M+1)$^+$. 

1-Cyclohexanecarboxamido-2,4-dinitro-5-N,N',N'-triethylethylenediaminobenzene (II-5d): 

Yellow oil; 94 % yield; $^1$H NMR (400 MHz, CDCl$_3$) δ 1.01 (t, 6 H, $J = 7.2$ Hz), 1.24 (t, 3 H, $J = 7.2$ Hz), 1.37-1.72 (m, 5 H), 1.99 (m, 2 H), 2.35 (m, 1 H), 2.61 (q, 4 H, $J = 6.8$ Hz), 2.74 (t, 2 H, $J = 7.6$ Hz), 3.38 (q, 2 H, $J = 6.8$ Hz), 3.45 (t, 2 H, $J = 6.8$ Hz), 8.62 (s, 1 H), 8.71 (s, 1 H), 10.93 (s, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 10.72, 12.25, 25.52, 25.86, 29.3, 43.86, 46.6, 47.28,
47.56, 49.35, 49.58, 107.9, 125.3, 126.9, 132.9, 138.7, 149.1, 175.7, 180.6; MS (ESI) m/z 436.2 (M+1)^+.

1-Cyclohexanecarboxamido-2,4-dinitro-5-\(N',N',N\)-trimethylethylenediaminobenzene (II-5e):

![Structure of II-5e]

Yellow solid; 83% yield; mp 160-161 °C; \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 1.19-1.33 (m, 3 H), 1.47 (m, 2 H, \(J = 12.4\) Hz), 1.67 (m, 1 H), 1.82 (m, 2 H), 1.97 (m, 2 H), 2.22 (s, 6 H), 2.37 (m, 1 H), 2.67 (t, 2 H, \(J = 6.8\) Hz), 2.96 (s, 3 H), 3.5 (t, 2 H, \(J = 6.8\) Hz), 8.58 (s, 1 H), 8.71 (s, 1 H), 10.90 (s, 1 H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 25.39, 25.47, 29.29, 40.52, 45.5, 47.22, 52.48, 55.79, 106.6, 124.9, 127.1, 131.7, 138.6, 149.7, 175.6; MS (ESI) m/z 394.2 (M+1)^+.

1-Cyclohexanecarboxamido-2,4-dinitro-5-\(N',N\)-dimethyl-\(N\)-ethylethylenediaminobenzene (II-5f):

![Structure of II-5f]

Yellow solid; 50% yield; mp 212-213 °C; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 1.23 (t, 3 H, \(J = 7.2\) Hz), 1.35 (m, 2 H), 1.51 (m, 2 H), 1.68 (m, 1 H), 1.82 (m, 2 H), 1.98 (m, 2 H), 2.22 (s, 6 H), 2.36 (m, 1 H), 2.54 (t, 2 H, \(J = 7.2\) Hz), 3.40 (m, 4 H), 8.64 (s, 1 H), 8.73 (s, 1 H), 10.93 (s, 1 H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 12.35, 25.47, 25.53, 29.38, 45.52, 47.32, 50.05, 56.09, 108.0, 125.2, 126.9, 132.9, 138.7, 149.2, 175.8; MS (ESI) m/z 408.3 (M+1)^+.

1-Cyclohexanecarboxamido-2,4-dinitro-5-piperidinylbenzene (II-5g):
Yellow solid; 96% yield; mp 154-155 °C; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 1.30 (m, 3 H), 1.47 (m, 2 H), 1.69 (m, 7 H), 1.82 (m, 2 H), 2.00 (m, 2 H), 2.38 (m, 1 H), 3.22 (t, 4 H, $J$ = 4.2 Hz), 8.57 (s, 1 H), 8.77 (s, 1 H), 10.96 (s, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 25.52, 25.24, 25.36, 29.26, 47.15, 51.88, 108.3, 125.5, 127.2, 132.4, 139.1, 150.5, 175.80; MS (ESI) m/z 377.1 (M+1)$^+$.  

5-Amino-2-cyclohexyl-6-(4-methylpiperazinyl)-1H-benzo[d]imidazole (II-6a): 

To a solution of II-5a (2.06 g, 5.28 mmol) in ethanol (30 mL) and dioxane (36 mL) was added solid stannous chloride dihydrate (6.99 g, 37 mmol). Concentrated hydrochloric acid (33 mL) was added to the reaction mixture such that the final concentration of HCl is 4 N in the reaction flask. The reaction mixture was refluxed for 4 h. Upon completion, the reaction mixture was basified with 30% sodium hydroxide solution. Excess stannous chloride formed a soluble salt in excess base. The desired product was extracted with dichloromethane and purified by flash chromatography on silica gel using ethyl acetate/ethanol (98/2) as eluent to afford II-6a as a pale brown solid (1.32 g, 82 % yield): mp 143-145 °C; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 1.25 (m, 3 H), 1.61 (m, 3 H), 1.75 (m, 2 H), 2.02 (m, 2 H), 2.31 (s, 3 H), 2.50 (broad m, 4 H), 2.78 (m, 1 H), 2.86 (broad s, 4 H), 6.74 (s, 1 H), 7.16 (s, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 25.70, 25.95, 30.50, 31.87, 38.47, 46.01, 51.78, 55.85, 98.89, 106.9, 132.5, 134.7, 136.4, 137.7, 158.0; HRMS (FAB) m/z calcd for C$_{18}$H$_{27}$N$_3$H$: 314.2345$, Found: 314.2348 ($\Delta = 1.0$ ppm). HPLC(1): 6.37 min, purity > 99 %.

In the same manner, compounds II-6b~II-6g were prepared.

5-Amino-2-cyclohexyl-6-(pyrrolidin-1-yl)-1H-benzo[d]imidazole (II-6b):
Off white solid; 50 % yield; mp 138-140 °C; $^1$H NMR (300 MHz, CDCl$_3$) δ 1.29-1.12 (m, 3 H), 1.5-1.79 (m, 5 H), 1.85 (broad s, 4 H), 2.07 (m, 2 H), 2.81 (m, 1 H), 2.97 (q, 4 H, $J = 5.4$ Hz), 6.80 (s, 1 H), 7.23 (s, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 23.97, 25.78, 26.01, 31.94, 38.48, 51.92, 98.81, 106.1, 132.9, 133.8, 134.78, 138.28, 157.53, HRMS (FAB) $m/z$ calcd for C$_{17}$H$_{24}$N$_4$H$^+$: 285.2072, Found: 285.2079 ($\Delta = -2.5$ ppm). HPLC(2): 7.84 min, purity > 99 %.

5-Amino-2-cyclohexyl-6-(morpholino)-1H-benzo[d]imidazole (II-6c):

Brown solid; 79% yield; mp 124-125 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 1.23-1.39 (m, 4 H), 1.59-1.84 (m, 5 H), 2.10 (m, 2 H), 2.82 (m, 1 H), 2.89 (t, 4 H, $J = 4.5$ Hz), 3.85 (t, 4 H, $J = 4.5$ Hz), 6.83 (s, 1 H), 7.26 (s, 1 H), 10.94 (s, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 25.80, 29.58, 31.78, 38.30, 45.63, 52.33, 67.66, 98.19, 107.4, 136.3, 138.1, 157.3; HRMS (FAB) $m/z$ calcd for C$_{17}$H$_{24}$N$_4$OH$^+$: 301.2028, Found: 301.2022 ($\Delta = -2.0$ ppm). HPLC(1): 7.09 min, purity > 99 %.

5-Amino-2-cyclohexyl-6-(N',N',N-triethylethylenediamino)-1H-benzo[d]imidazole (II-6d):

Pale brown solid; 81 % yield; mp: turned black at 120 °C; $^1$H NMR (300 MHz, CDCl$_3$) δ 0.95 (t, 9 H, $J = 7.2$ Hz), 1.20-1.35 (m, 5 H), 1.59-1.81 (m, 6 H), 2.05 (m, 2 H), 2.42 (m, 6 H), 2.79 (m, 1 H), 2.90 (m, 2 H, $J = 6.9$ Hz), 2.98 (t, 2 H, $J = 6.9$ Hz), 6.78 (s, 1 H), 7.27 (s, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 11.28, 12.33, 25.43, 25.51, 25.81, 31.75, 38.38, 46.94, 49.08, 51.01, 52.98, 98.05, 109.8, 133.0, 133.9, 140.1, 158.0; HRMS (FAB) $m/z$ calcd for C$_{21}$H$_{35}$N$_5$H$^+$: 358.2971, Found: 358.2975 ($\Delta = 1.1$ ppm). HPLC(1): 8.74 min, purity > 96 %.
5-Amino-2-cyclohexyl-6-(N',N',N-trimethylethylenediamino)-1H-benzo[d]imidazole (II-6e):

Pale brown solid; 59% yield; mp: turned black at 120 °C; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 1.25 (m, 3 H), 1.60-1.76 (m, 5 H), 2.04 (m, 2 H), 2.24 (s, 6 H), 2.42 (t, 2 H, $J = 6.4$ Hz ), 2.59 (s, 3 H), 2.80 (m, 1 H), 2.92 (t, 2 H, $J = 6.4$ Hz), 6.75 (s, 1 H), 7.22 (s, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 25.71, 25.97, 31.89, 38.42, 42.96, 45.45, 54.74, 57.36, 98.20, 108.3, 132.6, 134.2, 136.7, 139.1, 157.8; HRMS (FAB) $m/z$ calcd for C$_{18}$H$_{29}$N$_5$H$: 316.2501$, Found: 316.2498 ($\Delta = -0.9$ ppm). HPLC(1): 6.57 min, purity $> 98$ %.

5-Amino-2-cyclohexyl-6-(N',N'-dimethyl-N-ethylethylenediamino)-1H-benzo[d]imidazole (II-6f):

Pale brown solid; 74% yield; mp: turned black at 120 °C; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 0.89 (t, 3 H, $J = 7.2$ Hz), 1.12-1.29 (m, 3 H), 1.56-1.74 (m, 5 H), 2.01 (m, 2 H), 2.12 (s, 6 H), 2.28 (t, 2 H, $J = 6.8$ Hz), 2.73 (m, 1 H), 2.84 (q, 2 H, $J = 6.8$ Hz), 2.97 (t, 2 H, $J = 6.8$ Hz), 6.72 (s, 1 H), 7.23 (s, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 12.48, 25.68, 25.95, 31.85, 38.50, 45.54, 49.91, 52.99, 57.43, 63.38, 98.01, 110.3, 133.5, 133.9, 134.6, 140.4, 158.1; HRMS (FAB) $m/z$ calcd for C$_{19}$H$_{31}$N$_5$H$: 330.2658$, Found: 330.2649 ($\Delta = -2.7$ ppm). HPLC(1): 7.61 min, purity $> 99$ %.

5-Amino-2-cyclohexyl-6-piperidinyl-1H-benzo[d]imidazole (II-6g):

Pale brown solid; 82% yield; mp > 200 °C; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 1.24 (m, 4 H), 1.65 (m, 8 H), 1.77 (m, 2 H), 2.07 (m, 2 H), 2.84 (broad m, 5 H), 6.89 (s, 1 H), 7.22 (s,1 H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 24.15, 25.72, 26.01, 26.89, 32.00, 38.58, 53.58, 98.57, 107.0, 132.8, 134.3,
137.8, 138.0, 157.97; HRMS (FAB) m/z calcd for C_{18}H_{26}N_{4}H^+: 299.2236, Found: 299.2229 (Δ = -2.3 ppm). HPLC(1): 10.59 min, purity > 99%.

**Synthesis of 2,5,6-Trisubstituted Benzimidazole Library II-B**

![Synthesis Scheme](image)

**List of reagents**

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§ 2.4.3 Synthesis of analytically pure trisubstituted benzimidazoles

5-Butoxycarbonylamino-2-cyclohexyl-6-N,N-diethylamino-1H-benzo[d]imidazole (II-Ag):

To a solution of 2-cyclohexyl-5-N,N-diethylamino-6-amino-1H-benzo[d]imidazole (II-3g) (486 mg, 1.71 mmol) in dichloromethane (20 mL) was added a solution of N-butoxycarbonyloxsuccinimide (340 mg, 1.71 mmol) in dichloromethane (20 mL) dropwise at room temperature. After the addition, the reaction mixture was stirred at room temperature overnight. After the completion of the reaction, the reaction mixture was concentrated. The crude was purified via flash chromatography on silica gel (gradient 20-40% EtOAc/hexanes) to give II-Agas white solid (336 mg, 51% yield): mp 171-172 ºC; \(^1\)HNMR (500 MHz, CDCl\(_3\)) \(\delta\) 0.91 (t, 6 H, \(J = 7.2\) Hz), 0.96 (t, 3 H, \(J = 7.5\) Hz), 1.23-1.46 (m, 5 H), 1.57-1.72 (m, 5 H), 1.81 (m, 2 H, \(J = 10\) Hz), 2.07 (m, 2 H, \(J = 10\) Hz), 2.85 (m, 1 H), 2.92 (q, 4 H, \(J = 7.2\) Hz), 4.19 (t, 2 H, \(J = 6.5\) Hz), 7.46 (s, 1 H), 8.24 (s, 1 H), 8.51 (s, 1 H); \(^13\)C NMR (125 MHz, CDCl\(_3\)) \(\delta\) 12.72, 13.75, 19.11, 25.81, 26.02, 31.07, 31.78, 38.46, 50.43, 64.87, 98.88, 107.3, 113.3, 131.2, 132.2, 134.3, 138.5, 154.1, 158.8; HRMS (FAB) \(m/z\) calcd for C\(_{22}\)H\(_{34}\)N\(_4\)O\(_2\)H\(^+\): 387.2763, Found: 387.2760 (\(\Delta = 0.8\) ppm). HPLC(2): 10.6 min, purity >99%.

In a similar manner, II-Ab, II-Ad, II-Ah, II-Ba and II-Bb were synthesized and characterized.

2-Cyclohexyl-6-N,N-diethylamino-5-propoxycarbonylamino-1H-benzo[d]imidazole (II-Ab):
White solid; 63 % yield; mp 80-181 °C; ^1^HNMR (300 MHz, CDCl\textsubscript{3}) δ 0.92 (t, 6 H, J = 7.2 Hz), 0.99 (t, 3 H, J = 7.5 Hz), 1.25-1.41 (m, 4 H), 1.60-1.76 (m, 5 H), 1.86 (m, 2 H), 2.10 (m, 2 H), 2.87 (m, 1 H), 2.92 (m, 4 H, J = 6.9 Hz), 4.14 (t, 2 H, J = 6.6 Hz), 7.47 (s, 1 H), 8.23 (s, 1 H), 8.51 (s, 1 H); ^1^C NMR (125 MHz, CDCl\textsubscript{3}) δ 10.28, 12.67, 22.28, 25.71, 25.96, 31.75, 38.44, 50.38, 66.56, 100.0, 112.0, 132.1, 132.4, 134.3, 136.92, 154.1, 159.0; HRMS (FAB) m/z calcd for C\textsubscript{21}H\textsubscript{32}N\textsubscript{4}O\textsubscript{2}H\textsuperscript{+}: 373.2601, Found: 373.2604 (Δ = -0.8 ppm). HPLC(2): 9.98 min, purity >99 %.

5-But-3-enoxycarbonylamino-2-cyclohexyl-6,N,N-diethylamino-1H-benzo[d]imidazole (II-Ad):

![Structure](image)

White solid; 51 % yield; mp 143-145 °C; ^1^HNMR (500 MHz, CDCl\textsubscript{3}) δ 0.91 (t, J = 7 Hz, 6 H), 1.16-1.31 (m, 3 H), 1.56-1.66 (m, 3 H), 1.77 (d, 2 H, J = 13.5 Hz), 2.04 (d, 2 H, J = 12.5 Hz), 2.47 (t, 2 H, J = 6.5 Hz), 2.83 (m, 1 H), 2.88 (q, 4 H, J = 7.5 Hz), 4.23 (t, 2 H, J = 7 Hz), 5.11 (m, 2 H), 5.84 (m, 1 H), 7.41 (s, 1 H), 8.24 (s, 1 H), 8.52 (s, 1 H); ^1^C NMR (125 MHz, CDCl\textsubscript{3}) δ 12.66, 25.72, 25.96, 31.76, 33.47, 50.39, 63.98, 99.97, 112.1, 117.1, 131.9, 132.0, 131.9, 133.9, 134.3, 137.3, 153.9, 159.2; HRMS (FAB) m/z calcd for C\textsubscript{22}H\textsubscript{32}N\textsubscript{4}O\textsubscript{2}H\textsuperscript{+}: 385.2604, Found: 385.2604 (Δ = 0.0 ppm). HPLC(2): 10.1 min, purity >99 %.

5-Benzylxycarbonylamino-2-cyclohexyl-6,N,N-diethylamino-1H-benzo[d]imidazole (II-Ah):

![Structure](image)

White solid; 61 % yield; mp 208-209 °C; ^1^HNMR (300 MHz, CDCl\textsubscript{3}) δ 0.90 (t, 6 H, J = 7.2 Hz), 1.38 (m, 4 H), 1.84-1.62 (m, 5 H), 2.11 (m, 2 H), 2.84 (m, 1 H), 2.91 (m, 4 H, J =7.2 Hz), 5.22
(s, 2 H), 7.45-7.35 (m, 5 H), 8.25 (s, 1 H), 8.61 (s, 1 H); \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) \(\delta\) 12.62, 25.77, 25.97, 31.77, 38.46, 50.41, 66.68, 99.02, 113.2, 128.1, 128.5, 131.3, 132.0, 134.2, 136.3, 138.2, 153.7, 159.0; HRMS (FAB) \(m/z\) calcd for C\(_{25}\)H\(_{32}\)N\(_4\)O\(_2\)H\(^+\): 421.2601, Found: 421.2604 (\(\Delta = -0.5\) ppm). HPLC(2): 10.7 min, purity >99 %.

5-Butoxycarbonylamino-2-cyclohexyl-6-(pyrrolidin-1-yl)-1H-benzo[d]imidazole (II-Ba):

![Chemical structure](https://example.com/structure_Ba)

White solid; 51 % yield; mp 128-129 °C; \(^1\)HNMR (500 MHz, CDCl\(_3\)) \(\delta\) 0.94 (t, 3 H, \(J = 6.5\) Hz), 1.26-1.19 (m, 5 H), 1.73-1.55 (m, 7 H), 1.92 (bs, 4 H), 2.00 (m, 2 H), 2.76 (m, 1 H), 2.93 (bs, 4 H), 4.18 (t, \(J = 6.5\) Hz, 2 H), 7.40 (s, 1 H), 7.95 (s, 1 H), 8.17 (s, 1 H); \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) \(\delta\) 13.68, 19.03, 24.24, 25.71, 25.94, 30.99, 31.75, 38.45, 53.41, 64.90, 101.3, 109.80, 129.3, 131.8, 135.6, 137.5, 154.3, 159.1. HRMS (FAB) \(m/z\) calcd for C\(_{22}\)H\(_{32}\)N\(_4\)O\(_2\)H\(^+\): 385.2594, Found: 385.2604 (\(\Delta = -1.1\) ppm). HPLC(2): 10 min, purity >99 %.

5-Benzylxycarbonylamino-2-cyclohexyl-6-(pyrrolidin-1-yl)-1H-benzo[d]imidazole (II-Bb):

![Chemical structure](https://example.com/structure_Bb)

White solid; 68 % yield; mp 86-87 °C \(^1\)HNMR (500 MHz, CDCl\(_3\)) \(\delta\) 1.21-1.28 (m, 4 H), 1.59-1.65 (m, 4 H), 1.76 (m, 2 H), 1.91 (s, 4 H), 2.04 (d, 2H, \(J = 12.5\) Hz), 2.82 (m, 1 H), 2.93 (s, 4 H), 5.22 (s, 2 H), 7.34-7.48 (m, 5 H aromatic), 8.08 (s, 1 H), 8.22 (s, 1 H); \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) \(\delta\) 24.29, 25.69, 25.91, 31.72, 38.31, 53.54, 66.75, 101.5, 108.7, 128.1, 128.5, 129.6, 131.9, 136.0, 136.2, 153.8, 158.9. HRMS (FAB) \(m/z\) calcd for C\(_{25}\)H\(_{30}\)N\(_4\)O\(_2\)H\(^+\): 419.2448, Found: 419.2447 (\(\Delta = 0.2\) ppm). HPLC(2): 10.1 min, purity >99 %.
2-Cyclohexyl-6-N,N-diethylamino-5-(4-methoxybenzamido)-1H-benzo[d]imidazole (II-Ac):

To a solution of II-3g (120 mg, 0.41 mmol) in dichloromethane (10 mL) was added a solution of 4-methoxybenzoyl chloride (69 mg, 0.41 mmol) in dichloromethane (15 mL) dropwise at room temperature. After the addition, the reaction mixture was stirred at room temperature overnight. After the completion of the reaction, the reaction mixture was washed with saturated solution of sodium bicarbonate. The organic layer was dried over anhydrous magnesium sulfate and concentrated. The crude was purified by flash chromatography on silica gel (gradient: 20-40% ethyl acetate/hexanes) to give II-Ac as a white solid (139 mg, 79%): mp 198-199 °C; ¹H NMR (400 MHz, CDCl₃) δ 0.97 (t, 6 H, J = 7.2 Hz), 1.16 (m, 3 H), 1.53-1.68 (m, 5 H), 1.98 (m, 2 H), 2.71 (m, 1 H), 3.02 (q, 4 H, J = 7.2 Hz), 3.9 (s, 3 H), 7.05 (d, 2 H, J = 8.8 Hz), 7.55 (s, 1 H), 7.95 (d, 2 H, J = 8.8 Hz), 8.95 (s, 1 H), 10.32 (s, 1 H); ¹³C NMR (100 MHz, CDCl₃) δ 13.36, 25.85, 26.18, 31.96, 38.67, 51.02, 55.75, 101.2, 113.2, 114.2, 127.9, 128.8, 129.0, 132.1, 139.3, 135.2, 160.0, 162.6, 164.9; HRMS (FAB) m/z calcd for C₂₅H₃₂N₄O₂H⁺: 421.2601, Found: 421.2604 (Δ = -0.7 ppm). HPLC(2): 9.5 min, purity >99 %.

The same procedure was used for the synthesis of II-Aa, II-Ae, II-Af, II-Ai~II-Ao, II-16a and II-16b.

5-Benzamido-2-cyclohexyl-6-N,N-diethylamino-1H-benzo[d]imidazole (II-Aa):

White solid; 74% yield; mp 180-181 °C; ¹H NMR (400 MHz, CDCl₃) δ 0.97 (t, 6 H, J = 7.2 Hz), 1.16 (m, 3 H), 1.65 (m, 5 H), 1.98 (m, 2 H), 2.71 (m, 1 H), 3.03 (m, 4 H, J = 6.8 Hz), 7.57 (m, 4
H), 7.98 (d, 2 H, J = 6.2 Hz), 8.97 (s, 1 H), 10.4 (s, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 13.09, 21.48, 25.64, 25.91, 31.73, 38.40, 50.77, 100.9, 113.1, 126.9, 129.5, 131.6, 132.7, 135.15, 139.5, 159.8, 165.1; HRMS (FAB) m/z calcd for C$_{24}$H$_{30}$N$_4$OH$^+$: 391.2486, Found: 391.2498 (Δ = -3.1 ppm). HPLC(2): 9.50 min, purity >99 %.

5-(4-Chlorobenzamido)-2-cyclohexyl-6,N,N-diethylamino-1H-benzo[d]imidazole (II-Ae):

White solid; 64 % yield; mp 216-217 °C (turned brown); $^1$HNMR (400 MHz, CDCl$_3$) δ 0.96 (t, 6 H, J = 7.2 Hz), 1.21-1.32 (m, 2 H), 1.59-1.81 (m, 5 H), 2.08 (m, 2 H), 2.79 (m, 1 H), 3.02 (m, 4 H, J = 7 Hz), 7.50 (d, 2 H, J = 6.8 Hz), 7.58 (s, 1 H), 7.88 (d, 2 H, J = 8.8 Hz), 8.82 (s, 1 H), 10.31 (s, 1 H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 13.12, 25.75, 25.97, 29.69, 31.76, 38.47, 50.78, 100.5, 113.6, 128.3, 129.1, 131.3, 131.8, 133.9, 135.2, 137.9, 139.6, 159.5, 163.7; HRMS (FAB) m/z calcd for C$_{24}$H$_{29}$N$_4$OClH$^+$: 425.2108, Found: 425.2108 (Δ = -0.0 ppm). HPLC(2): 10.5 min, purity >99 %.

2-Cyclohexyl-6,N,N-diethylamino-5-(4-methylbenzamido)-1H-benzo[d]imidazole (II-Af):

White solid; 65 % yield; mp 183-184 °C; $^1$HNMR (300 MHz, CDCl$_3$) δ 0.97 (t, 6 H, J = 6 Hz), 1.2 (m, 3 H), 1.58-1.78 (m, 5 H), 2.06 (m, 2 H), 2.45 (s, 3 H), 2.76 (m, 1 H), 3.03 (q, 4 H, J = 7.2 Hz), 7.34 (d, 2 H, J = 8.4 Hz) 7.59 (s, 1 H), 7.87 (d, 2 H, J = 8.1 Hz), 8.91 (s, 1 H), 10.31 (s, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 13.09, 21.48, 25.63, 25.92, 31.72, 38.40, 50.75, 100.9, 113.1, 126.8, 129.6, 131.8, 132.7, 135.0, 139.4, 142.1, 159.8, 165.1; HRMS (FAB) m/z calcd for C$_{25}$H$_{32}$N$_4$OH$^+$: 405.2654, Found: 405.2654 (Δ = -0.0 ppm). HPLC(2): 10.1 min, purity >99 %.
2-Cyclohexyl-6-N,N-diethylamino-5-(4-tert-butylbenzamido)-1H-benzo[d]imidazole (II-Ai):

White solid; 48 % yield; mp 206-208 °C; $^1$HNMR (400 MHz, CDCl$_3$) $\delta$ 0.99 (t, 6 H, $J$ = 7.2 Hz), 1.2 (m, 3 H), 1.38 (s, 9 H), 1.58-1.67 (m, 5 H), 1.97 (m, 2 H) 2.68 (m, 1 H), 3.03 (q, 4 H, $J$ = 6.8 Hz), 7.58 (s, 1 H), 7.57 (d, 2 H, $J$ = 8.4 Hz), 7.95 (d, 2 H, $J$ = 8.4 Hz), 9.02 (s, 1 H), 10.41 (s, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 13.09, 25.58, 25.87, 29.67, 31.16, 31.73, 31.90, 35.02, 38.40, 50.82, 100.8, 113.2, 125.9, 126.8, 131.9, 132.6, 134.9, 155.2, 159.8, 165.1; HRMS (ESI) m/z calcd for C$_{28}$H$_{38}$N$_4$OH$^+$: 447.3124, Found: 447.3120 ($\Delta$ = -0.9 ppm).

2-Cyclohexyl-5-cyclopentylamido-6-N,N-diethylamino -1H-benzo[d]imidazole (II-Aj):

White solid; 74 % yield; mp 202-204 °C; $^1$HNMR (400 MHz, CDCl$_3$) $\delta$ 0.92 (t, 6 H, $J$ = 7.2 Hz), 1.24 (m, 3 H), 1.67-2.05 (m, 15 H), 2.91 (m, 2 H), 3.03 (q, 4 H, $J$ = 7.2 Hz), 7.50 (s, 1 H), 8.65 (s, 1 H), 9.41 (s, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 12.92, 25.77, 25.79, 30.53, 31.75, 38.45, 47.70, 50.57, 101.1, 112.6, 131.9, 134.6, 138.5, 159.4, 174.1; HRMS (ESI) m/z calcd for C$_{23}$H$_{34}$N$_4$OH$^+$: 383.2911, Found: 383.2913 ($\Delta$ = 0.5 ppm).
2-Cyclohexyl-6-N,N-diethylamino-5-(3-phenylpropanamido)-1H-benzo[d]imidazole (II-Ak):

White solid; 55 % yield; \(^1\)HNMR (400 MHz, CDCl\(_3\)) \(\delta\) 0.83 (t, 6 H, \(J = 6.8\) Hz), 1.23-1.29 (m, 5 H), 1.67-1.83 (m, 4 H), 2.08 (m, 2 H), 2.78 (t, 2 H, \(J = 7.2\) Hz), 2.85 (q, 4 H, \(J = 7.2\) Hz), 3.11 (t, 2 H, \(J = 7.2\) Hz), 7.25 (m, 5 H), 7.49 (s, 1 H), 8.69 (s, 1 H), 9.33 (s, 1 H); \(^1^3\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 12.81, 25.80, 26.04, 29.66, 31.89, 31.98, 38.53, 40.18, 50.48, 101.1, 112.5, 126.3, 128.2, 128.4, 131.7, 134.5, 138.5, 140.3, 159.4, 170.1; MS (FIA) \(m/z\) 419.2 (M+1)

2-Cyclohexyl-6-N,N-diethylamino-5-(pentanamido)-1H-benzo[d]imidazole (II-Al):

White solid; 70 % yield; mp 124-126 °C; \(^1\)HNMR (400 MHz, CDCl\(_3\)) \(\delta\) 0.92 (t, 6 H, \(J = 6.8\) Hz), 0.95 (t, 3 H, \(J = 7.2\) Hz), 1.27-1.48 (m, 5 H), 1.65-1.84 (m, 7 H), 2.08 (m, 2 H), 2.45 (t, 2 H, \(J = 7.2\) Hz), 2.84 (m, 1 H), 2.93 (q, 4 H, \(J = 7.2\) Hz), 7.51 (s, 1 H), 8.72 (s, 1 H), 9.45 (s, 1 H); \(^1^3\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 12.93, 13.78, 22.42, 25.82, 26.11, 28.09, 31.59, 38.48, 50.63, 101.1, 112.6, 131.6, 132.0, 134.4, 138.8, 159.6, 171.2; HRMS (FAB) \(m/z\) calcd for C\(_{22}\)H\(_{34}\)N\(_4\)OH\(^+\): 371.2911, Found: 371.2909 (\(\Delta = -0.9\) ppm).

5-(Butanamido)-2-cyclohexyl-6-N,N-diethylamino-1H-benzo[d]imidazole (II-Am):

White solid; 65 % yield; mp 134-136 °C; \(^1\)HNMR (400 MHz, CDCl\(_3\)) \(\delta\) 0.92 (t, 6 H, \(J = 7.2\) Hz), 1.06 (t, 6 H, \(J = 7.2\) Hz), 1.31 (m, 3 H), 1.66-1.84 (m, 7 H), 2.12 (m, 2 H), 2.45 (t, 2 H, \(J = 7.2\) Hz).
Hz), 2.84 (m, 1 H), 2.93 (q, 4 H, \( J = 7.2 \) Hz), 7.75 (s, 1 H), 8.73 (s, 1 H), 9.45 (s, 1 H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \( \delta \) 12.93, 13.82, 19.60, 25.84, 26.13, 31.62, 38.62, 40.68, 50.68; HRMS (ESI) \( m/z \) calcd for C\(_{21}\)H\(_{32}\)N\(_4\)OH\(^+\): 357.2654, Found: 357.2653 (\( \Delta = -0.3 \) ppm).

6-\( N,N\)-diethylamino-5-(4-methylbenzamido)-2-toluoyl-\( 1\)H-benzo[d]imidazole (II-An):

![Chemical Structure]

White solid; 53 % yield; mp > 210 °C; \(^{1}\)HNMR (400 MHz, CDCl\(_3\)) \( \delta \) 0.97 (t, 6 H, \( J = 7.2 \) Hz), 2.93 (s, 3 H), 2.45 (s, 3 H), 3.03 (q, 4 H, \( J = 7.2 \) Hz), 6.95 (d, 2 H, \( J = 7.6 \) Hz), 7.29 (d, 2 H, \( J = 8 \) Hz), 7.64 (s, 1 H), 7.84 (m, 4 H), 9.10 (s, 1 H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \( \delta \) 12.99, 21.40, 50.54, 101.5, 113.3, 126.5, 127.0, 127.1, 129.3, 129.6, 132.7, 135.6, 139.5, 142.0, 152.6, 165.5; HRMS (ESI) \( m/z \) calcd for C\(_{26}\)H\(_{28}\)N\(_4\)OH\(^+\): 413.2341, Found: 413.2341 (\( \Delta = -0.0 \) ppm).

\( \S \) 2.4.4 Synthesis of 2,5,6-Trisubstituted Benzimidazole Library II-C:

2,4-Dinitro-5-piperidinyl-1-tetrahydropyrancarboxamido-benzene (II-7a):

![Chemical Structure]

To a solution of 1-tetrahydropyrancarboxylic (300 mg, 2.32 mmol) in CH\(_2\)Cl\(_2\) (5 mL) was added oxalyl chloride (0.3 mL, 3.48 mol) and a few drops of dimethylformamide. The reaction mixture was stirred for 90 minutes and the solvent was evaporated in vacuo to give the corresponding acyl chloride. The crude product was taken to the next step. A solution of II-4g (329 mg, 1.24 mmol) and 1-tetrahydropyrancarbonyl chloride (274 mg, 1.86 mmol) in pyridine (5 mL) was refluxed overnight. The reaction mixture was diluted with dichloromethane, washed with water (30 mL x 3) and dried over anhydrous magnesium sulfate. The crude product was purified by flash chromatography on silica gel using ethyl acetate as eluent to give II-7a as a
yellow solid (398mg, 85 % yield); mp 134-136 °C; \[^1\]H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 1.69-1.73 (m, 6 H), 1.81-1.93 (m, 4 H), 2.65 (m, 1 H), 3.27 (m, 4 H), 3.41-3.52 (m, 2 H), 4.09 (m, 2 H), 8.61 (s, 1 H), 8.84 (s, 1 H), 11.0 (s, 1 H); \[^{13}\]C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 23.59, 25.35, 28.39, 28.84, 39.60, 44.00, 52.01, 66.98, 108.4, 125.6, 127.4, 132.7, 138.9, 150.7, 174.0. MS (ESI) \(m/z\) 379.3 (M+1)^+.

In the same manner, compounds II-7b–II-7e were prepared.

**2,4-dinitro-1-(2-Furyl)carboxamido-5-piperidinylbenzene (II-7b):**

![2,4-dinitro-1-(2-Furyl)carboxamido-5-piperidinylbenzene (II-7b)](image)

Yellow solid; 81 % yield; mp 196-198° C; \[^1\]H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 1.72 (m, 6 H), 3.30 (t, 4 H, \(J = 4.8\) Hz), 6.61 (dd, 2 H, \(J = 1.2\) Hz, 2.4 Hz), 7.30 (d, 1 H, \(J = 3.6\) Hz), 7.63 (m, 1 H), 8.68 (s, 1 H), 8.85 (s, 1 H); \[^{13}\]C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 23.60, 25.34, 52.01, 108.4, 112.9, 116.9, 125.8, 127.4, 132.7, 138.7, 145.7, 146.9, 150.5, 156.7; MS (ESI) \(m/z\) 361.3 (M+1)^+.

**2,4-dinitro-1-(2-Thiophenyl)carboxamido-5-piperidinylbenzene (II-7c):**

![2,4-dinitro-1-(2-Thiophenyl)carboxamido-5-piperidinylbenzene (II-7c)](image)

Orange solid; 85 % yield; mp 178-180° C; \[^1\]H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 1.72 (m, 6 H), 3.30 (t, 4 H, \(J = 4.8\) Hz), 7.19 (dd, 1 H, \(J = 0.8\) Hz, 4 Hz), 7.67 (d, 1 H, \(J = 4.8\) Hz), 7.75 (d, 1 H, \(J = 4\) Hz), 8.66 (s, 1 H), 8.86 (s, 1 H), 11.7 (s, NH); \[^{13}\]C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 23.60, 25.36, 52.03, 108.1, 125.5, 127.5, 127.6, 128.3, 129.5, 132.7, 133.0, 138.6, 139.0, 150.6, 160.6; MS (ESI) \(m/z\) 377.3 (M+1)^+.  

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1-Benzylcarboxamido-2,4-dinitro-5-piperidinylbenzene (II-7d):

Yellow solid; 82 % yield; mp 128-130°C; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 1.71 (m, 6 H), 3.25 (t, 4 H, \(J = 4.8\) Hz), 3.83 (s, 2 H), 7.35-7.44 (m, 5 H), 8.58 (s, 1 H), 8.78 (s, 1 H), 10.8 (s, NH); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 23.59, 25.32, 45.96, 51.98, 108.3, 125.6, 127.3, 128.0, 129.3, 129.5, 132.6, 138.7, 150.5, 170.8; MS (ESI) \(m/z\) 385.3 (M+1)^+.

1-Diphenylmethylcarboxamido-2,4-dinitro-5-piperidinylbenzene (II-7e):

Yellow solid; 65 % yield; mp 116-119 °C; \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 1.69 (s, 6 H), 3.24 (d, 4 H, \(J = 5.7\) Hz), 5.16 (s, 1 H), 7.28-7.41 (m, 10 H), 8.66 (s, 1 H), 8.79 (s, 1 H), 10.9 (s, 1 H); MS (ESI) \(m/z\) 462.1 (M+1)^+.

5-Amino-2-tetrahydropyran-6-piperidinyl-1H-benzo[d]imidazole (II-8a):

To a solution of II-7a (308 mg, 0.81 mmol) in dioxane (20 mL) and ethanol (20 mL) was added ammonium formate (2.0 g) and 10 wt. % Pd-C (200 mg) with stirring. The reaction mixture turned from yellow to red and then to colorless in 45-60 min at room temperature. The catalyst and excess ammonium formate were filtered off to give the product solution.
Concentrated hydrochloric acid was added to this solution and adjusted to 4 N HCl concentration. Then the mixture was refluxed for 4-6 h. The reaction mixture was basified by addition of ammonium hydroxide, extracted with ethyl acetate (40 mL x 3) and dried over anhydrous magnesium sulfate. The crude product was purified by flash chromatography on silica gel using ethyl acetate/hexanes (gradient 20 % ethyl acetate/hexanes) as eluent to give pure **II-8a** as a brown solid (160 mg, 65 % yield); $^1$H NMR (400MHz, CDCl$_3$) δ 1.70 (m, 6 H), 1.98 (m, 4 H), 3.06 (m, 1 H), 3.45 (m, 2 H), 3.99 (m, 2 H), 6.80 (s, 1 H), 7.22 (s, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 24.19, 26.92, 31.43, 35.67, 53.61, 67.49, 98.18, 107.4, 133.0, 134.1, 138.5, 155.3; MS (ESI) m/z 301.4 (M+1)$^+$. 

Compounds **II-8b**~ **II-8e** were prepared in the same manner.

**5-Amino-2-furyl-6-piperidinyl-1H-benzo[d]imidazole (II-8b):**

![5-Amino-2-furyl-6-piperidinyl-1H-benzo[d]imidazole](image)

Dark brown solid; 55 % yield; mp 124-126 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 1.68 (m, 6 H), 2.78 (broad s, 4 H), 6.45 (m, 1 H), 6.85 (s, 1 H), 7.09 (m, 1 H), 7.24 (s, 1 H), 7.40 (s, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 24.19, 26.88, 53.48, 98.04, 107.5, 109.4, 112.1, 133.3, 133.9, 139.2, 139.3, 142.1, 143.0, 145.6; HRMS (ESI) m/z calcd for C$_{16}$H$_{18}$N$_4$OH$^+$: 283.1559, Found: 283.1561 (Δ = 0.7 ppm).

**5-Amino-6-piperidinyl -2-thiophenyl-1H-benzo[d]imidazole (II-8c):**

![5-Amino-6-piperidinyl -2-thiophenyl-1H-benzo[d]imidazole](image)

Pale yellow solid; 50 % yield; mp 145-148°C; $^1$H NMR (400 MHz, CDCl$_3$) δ 1.64 (m, 6 H), 2.73 (broad s, 4 H), 6.80 (s, 1 H), 6.94 (t, 1 H, J = 4.8 Hz), 7.19 (s, 1 H), 7.24 (d, 1 H, J = 5.2 Hz), 7.64 (d, 1 H, J = 3.6 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 24.17, 26.88, 53.44, 98.05, 107.4, 125.9, 127.0, 127.9, 133.4, 134.5, 139.0, 139.2, 145.6; HRMS (ESI) m/z calcd for C$_{16}$H$_{18}$N$_4$SH$^+$: 299.1330, Found: 299.1330 (Δ = 0.0 ppm).
5-Amino-2-benzyl-6-piperidinyl-1H-benzo[d]imidazole (II-8d):

Brown solid; 53% yield; mp 82-84 °C; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 1.68 (m, 6 H), 2.76 (broad s, 4 H), 4.12 (s, 2 H), 6.72 (s, 1 H), 7.15 (s, 1 H), 7.21 (m, 5 H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 24.22, 26.92, 35.50, 53.44, 98.33, 107.1, 126.9, 128.7, 128.8, 132.7, 134.2, 136.8, 138.4, 138.5, 151.4; HRMS (ESI) $m/z$ calcd for C$_{19}$H$_{22}$N$_4$H$: 307.1923$, Found: 307.1921 ($\Delta$ = - 0.7 ppm).

5-Amino-2-diphenylmethyl-6-piperidinyl-1H-benzo[d]imidazole (II-8e):

Off-white solid; 43% yield; mp 136-138°C; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 1.69 (m, 6 H), 2.76 (broad s, 4 H), 5.69 (s, 1 H), 6.89 (s, 1 H), 7.20-7.30 (m, 10 H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 25.52, 28.16, 52.95, 55.04, 100.4, 108.0, 128.1, 129.7, 130.1, 139.9, 140.5, 142.4, 155.4; HRMS (ESI) $m/z$ calcd for C$_{25}$H$_{26}$N$_4$H$: 383.2236$, Found: 383.2234 ($\Delta$ = - 0.5 ppm).

**Synthesis of 2,5,6-Trisubstituted Benzimidazole Library II-C:**

1. R$^3$COCl or R$^3$COOCl, CH$_2$Cl$_2$, overnight, RT
2. Aminomethylated resin, CH$_2$Cl$_2$, overnight, RT
3. Filter

147 trisubstituted benzimidazoles

$R^2 =$ *cyclohexyl, furan Michael acceptor, thienyl, benzyl, benzene*
List of reagents

§ 2.4.5 Synthesis of 2,5,6-Trisubstituted Benzimidazole Library II-D:

\[
\begin{align*}
\text{R}^{\prime\prime}N & \quad \text{R}^\prime \quad \text{R}^2 \\
\text{H}_2\text{N} & \quad 1. \text{R}^3\text{COCl} \text{ or } \text{R}^3\text{COOC}\text{Cl} , \text{CH}_2\text{Cl}_2, \text{ overnight, RT} \\
\text{N} & \quad 2. \text{Aminomethylated resin, CH}_2\text{Cl}_2, \text{ overnight, RT} \\
\text{N} & \quad 3. \text{Filter} \\
\end{align*}
\]

180 trisubstituted benzimidazoles

\[
\begin{align*}
\text{R}^2 &= & \text{R}^{\prime}\text{R}^\prime\prime\text{N} \\
\text{R}^2 &= & \text{R}^3 \text{Me} & \text{R}^3 \text{F} & \text{R}^3 \text{OMe} & \text{R}^3 \text{Th} \\
\text{R}^{\prime}\text{R}^\prime\prime\text{N} &= & \text{N}^* & \text{N}^* & \text{N}^* & \text{N}^* \\
\end{align*}
\]
List of reagents

§ 2.4.6 Synthesis of 2,5,6-Trisubstituted Benzimidazole Library II-E:

1. R³COCl or R³COOC₂H₅, CH₂Cl₂, overnight, RT
2. Aminomethylated resin, CH₂Cl₂, overnight, RT
3. Filter

180 trisubstituted benzimidazoles
List of reagents

[Chemical structures of reagents]

1-Benzylcarboxamido-2,4-dinitro-5-pyrrolidinylbenzene (II-9a):

Yellow solid; 74 % yield; mp 134-138°C; 1H NMR (400 MHz, CDCl$_3$) δ 2.01 (m, 4 H), 3.34 (t, 4 H, $J = 4.8$ Hz), 3.82 (s, 2 H), 7.33-7.41 (m, 5 H), 8.44 (s, 1 H), 8.71 (s, 1 H); 13C NMR (100 MHz, CDCl$_3$) δ 25.50, 45.97, 51.30, 104.8, 124.2, 126.9, 128.0, 129.3, 129.5, 129.7, 131.1, 132.8, 137.8, 146.4, 170.8; MS (ESI) $m/z$ 371.3 (M+1)$^+$.  

1-Diphenylmethylcarboxamido-2,4-dinitro-5-pyrrolidinylbenzene(II-9b):

Yellow solid; 65 % yield; mp 115-117°C; 1H NMR (400 MHz, CDCl$_3$) δ 1.92 (m, 4 H), 3.28 (m, 4 H, $J = 5.7$ Hz), 5.11 (s, 1 H), 7.18-7.32 (m, 10 H), 8.48 (s, 1 H), 8.67 (s, 1 H); 13C NMR (100 MHz, CDCl$_3$) δ 25.49, 51.35, 56.95, 61.25, 104.9, 124.3, 126.9, 127.4, 127.8, 128.6, 128.9, 129.0, 131.9, 137.7, 137.8, 172.2, 178.3; MS (ESI) $m/z$ 447.4 (M+1)$^+$.  

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5-Amino-2-benzyl-6-pyrrolidinyl-1H-benzo[d]imidazole (II-10a):

Brown solid; 82 % yield; mp 82 °C; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 1.87 (m, 4 H), 2.95 (broad s, 4 H), 4.1 (s, 2 H), 6.71 (s, 1 H), 7.16 (s, 1 H), 7.21 (m, 5 H); \(^1\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 24.22, 35.92, 52.14, 98.99, 106.3, 127.2, 129.1, 133.7, 134.1, 135.3, 137.0, 138.8, 151.6; HRMS (ESI) \(m/z\) calcd for C\(_{18}\)H\(_{20}\)N\(_4\)H\(^+\): 293.1766, Found: 293.1766 (\(\Delta = 0.0\) ppm).

5-Amino-2-diphenylmethyl-6-pyrrolidinyl-1H-benzo[d]imidazole (II-10b):

Off-white solid; 84 % yield; mp 123-126°C; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 1.87 (m, 4 H), 2.95 (broad s, 4 H), 5.68 (s, 1 H), 6.68 (s, 1 H), 7.14-7.27 (m, 11 H); \(^1\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 23.99, 29.16, 51.84, 100.4, 127.1, 128.7, 128.9, 135.2, 138.7, 140.9, 153.1; HRMS (ESI) \(m/z\) calcd for C\(_{24}\)H\(_{24}\)N\(_4\)H\(^+\): 369.2079, Found: 369.2080 (\(\Delta = 0.3\) ppm).

§ 2.4.7 Synthesis of 2,5,7-Trisubstituted Benzimidazole Library II-F:

4-Amino-3,5-dinitro-1-(ethoxycarbonylamino)benzene(II-14a):

4-chlorobenzoic acid (1.52 g, 9.74 mmol) was dissolved in sulfuric acid (25 mL) at 70-100 °C and KNO\(_3\) (5.02g) was added in portion. The reaction mixture was heated to 120-130 °C for 15 hours, cooled down to ~20 °C and poured onto ice. The precipitate was filtered to give light yellow solid, m.p. 153-154 °C (Lit m.p. 153-156 °C).\(^{21}\)Product from the previous reaction was dissolved in methanol (50 mL) and aqueous NH\(_3\) solution was added gradually. The reaction
mixture was refluxed. After the completion of reaction, 1:1 solution of HCl was added to the reaction mixture. On stirring the precipitate was formed which was filtered and washed with water to obtain 4-amino-3,5-dinitrobenzoic acid (II-12) in 85 % yield (2 steps); \(^1\)HNMR (300 MHz, DMSO-d\(_6\)) \(\delta\) 8.80(s, 2H). \(^{14}\)Amino-3,5-dinitrobenzoic acid II-12(614 mg, 2.70 mmol) was dissolved in thionyl chloride (4 mL) and refluxed overnight. The reaction mixture was cooled down to room temperature and excess thionyl chloride was distilled off to give compound II-13. The crude product was immediately dissolved in acetone (2.4 mL) in an ice-bath. To this solution was added dropwise NaN\(_3\) (448 mg, 5.94 mmol) in ice-water (0.88 mL). The mixture was stirred for 20 min at 0 °C until a solid precipitated out. After dilution with ice-water (12 mL), the reaction mixture was extracted with dichloromethane (6 mL x 2), dried over anhydrous magnesium sulfate at 0 °C for 1 h, and filtered. The filtrate was concentrated on a rotary evaporator (below room temperature), and the residue dissolved in toluene (15 mL). After refluxing for 2 h, the reaction mixture was cooled down to room temperature, and ethanol (10 mL) was added. After stirring overnight at room temperature, the reaction mixture was concentrated in vacuo and purified by flash chromatography on silica gel (hexane/ethylacetate = 1/1) to afford 4-amino-3,5-dinitro-1-(ethoxycarbonylamino)benzene (II-14a) as a bright red solid (570 mg, 78 % yield); \(^1\)HNMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.64 (s, 2 H), 6.59 (bs, 1 H), 4.26 (q, 2 H, J = 6.9 Hz), 1.33 (t, 3 H, J = 6.9 Hz ); \(^{13}\)C NMR (100 MHz, CD\(_3\)OD) \(\delta\) 15.00, 62.53, 125.2, 127.9, 136.3, 139.0, 156.1; HRMS (FAB) \(m/z\) calcd for C\(_9\)H\(_{10}\)N\(_4\)O\(_6\)H\(^+\): 271.0677, Found: 271.0679 (\(\Delta = -0.7\) ppm).

4-Amino-3,5-dinitro-1-(tert-butoxycarbonylamino)benzene (II-14b):

\[
\begin{align*}
\text{O} & \quad \text{O} \\
\text{N} & \quad \text{N} \\
\text{NO}_2 & \quad \text{NO}_2 \\
\text{NH}_2 & \\
\end{align*}
\]

Compound II-14b was prepared in the same manner: bright red solid; 84 % yield; mp 178-180°C; \(^1\)H NMR (400 MHz, DMSO-d\(_6\)) \(\delta\) 1.48 (s, 9 H), 8.19(s, 2 H), 8.65 (s, 2 H), 9.69 (s, 1 H); \(^{13}\)C NMR (100 MHz, DMSO-d\(_6\)) \(\delta\)28.02, 79.97, 123.3, 126.6, 134.3, 136.9, 152.8; MS (ESI) \(m/z\) 299.2 (M+1).
7-Amino-5-ethoxycarbonylamino-2-phenyl-1H-benzo[d]imidazole (II-15a):

To a suspension of II-14a (324 mg, 1.2 mmol) in ethanol (24 mL), was added ammonium formate (1.8 g) and 10 wt. % Pd-C (120 mg) under nitrogen. The mixture was stirred at room temperature overnight. The catalyst and excess ammonium formate were filtered off. The filtrate was treated with the sodium bisulfite adduct of benzaldehyde (252 mg, 1.2 mmol) at 0 °C. After the solution was stirred for 12-16 h at room temperature under nitrogen, a trace of insoluble material was removed by filtration and the filtrate was concentrated on a rotary evaporator until approximately 60-70% of the solvent was removed. To the residue an equal volume of ethyl acetate was added, and the mixture was transferred to a separatory funnel. The organic layer was separated, and the water layer was extracted with ethyl acetate (40 mL). The combined organic layers were washed with brine, dried over anhydrous sodium sulfate. The crude product was purified by flash chromatography on silica gel (hexane/ethyl acetate = 1/1) to afford II-15a (610 mg, 48% yield) as brown solid; mp 114-115 °C; $^1$H NMR (300MHz, CD$_3$OD) $\delta$ 1.30 (t, 3H, J = 6.9 Hz), 4.16 (q, 2H, J = 7.2 Hz), 6.51 (s, 1H), 7.18 (s, 1H), 7.44 (m, 3H), 8.01 (m, 2H); $^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$ 15.11, 62.90, 93.72, 100.95, 127.30, 127.40, 129.11, 130.17, 130.90, 131.32, 137.19, 138.28, 139.11, 151.32, 156.47; HRMS (FAB) m/z calcd for C$_{16}$H$_{16}$N$_4$O$_2$H$: 297.1244, Found: 297.1352 (Δ = -2.7 ppm).HPLC (2): 4.50 min, purity > 99 %.

The compounds II-15b~II-15f were prepared in the same manner.

7-Amino-2-cyclohexyl-5-ethoxycarbonylamino-1H-benzo[d]imidazole (II-15b):

Brown solid; 42 % yield; mp > 200 °C; $^1$H NMR (400MHz, CD$_3$OD) $\delta$ 1.27 (t, 3 H, J = 7.2 Hz), 1.32-1.43 (m, 2 H), 1.51-1.61 (m, 2 H), 1.69-1.83 (m, 4 H), 1.98-2.04 (m, 2 H), 2.83 (m, 1 H), 4.15 (q, 2 H, J = 7.2 Hz), 6.57 (s, 1 H), 8.56 (s, 1 H); $^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$ 15.09,
7-Amino-2-benzyl-5-ethoxycarbonylamino-1H-benzo[d]imidazole (II-15c):

Brown solid; 64 % yield; mp > 200 °C; $^1$H NMR (400MHz, CD$_3$OD) δ 1.28 (t, 3H, $J=7.2$ Hz), 4.15 (q, 2H, $J=7.2$ Hz), 4.21 (s, 2H), 6.55 (s, 1H), 7.10 (s, 1H), 7.29 (m, 5 H), 8.35 (s, 1H); $^{13}$C NMR (100 MHz, CD$_3$OD) δ 15.07, 35.46, 61.93, 93.51, 101.3, 125.3, 128.4, 129.9, 136.8, 137.5, 138.0, 152.7, 156.4, 167.2; HRMS (ESI) m/z calcd for C$_{16}$H$_{22}$N$_4$O$_2$H$: 303.1821, Found: 303.1821 (Δ = 0.0 ppm).


Brown solid; 51 % yield; mp > 200 °C; $^1$H NMR (400MHz, CD$_3$OD) δ 1.36 (s, 9 H), 1.54-1.63 (m, 4 H), 1.75-1.88 (m, 4 H), 2.07 (m, 2 H), 2.88 (m, 1 H), 6.51 (s, 1 H), 7.10 (s, 1H), 8.44 (s, 1 NH); $^{13}$C NMR (100 MHz, CD$_3$OD) δ 25.81, 27.50, 27.60, 31.33, 37.89, 79.46, 92.18, 99.70, 123.0, 135.0, 136.4, 154.2, 156.6; HRMS (ESI) m/z calcd for C$_{18}$H$_{26}$N$_4$O$_2$H$: 331.2137, Found: 331.2137 (Δ = 0.9 ppm).

7-Amino-2-benzyl-5-(tert-butoxycarbonylamino)-1H-benzo[d]imidazole (II-15e):

Brown solid; 63 % yield; mp > 200 °C; $^1$H NMR (400MHz, CD$_3$OD) δ 1.49 (s, 9 H), 4.17 (s, 2 H), 6.5 (s, 1 H), 7.07 (s, 1 H), 7.28 (m, 5 H); $^{13}$C NMR (100 MHz, CD$_3$OD) δ 27.59, 34.37,
79.43, 92.35, 99.94, 124.9, 126.9, 128.5, 135.8, 136.1, 136.5, 136.7, 151.3, 154.3; HRMS (ESI) m/z calcd for C_{19}H_{22}N_{4}O_{2}H^{+}: 339.1821, Found: 339.1862 (Δ = 1.5 ppm).

7-Amino-2-phenyl-5-(tert-butoxycarbonylamino)-1H-benzo[d]imidazole (II-15f):

Brown solid; 59 % yield; mp > 200 °C; $^1$H NMR (400MHz, CD$_3$OD) $\delta$ 1.51 (s, 9 H), 6.49 (s, 1 H), 7.15 (s, 1 H), 7.48 (m, 3 H), 8.00 (d, 2 H, $J = 8.4$ Hz); $^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$ 28.94, 80.71, 93.64, 101.0, 127.4, 130.1, 130.8, 131.4, 137.4, 139.1, 151.2, 155.7; HRMS (ESI) m/z calcd for C_{18}H_{20}N_{4}O_{2}H^{+}: 325.1665, Found: 325.1669 (Δ = 1.2 ppm).

**Synthesis of 2,5,7-Trisubstituted Benzimidazole Library II-F:**

![Synthesis diagram](image)

1. R$_2$COCl or R$_3$COOCl, CH$_2$Cl$_2$, overnight, RT
2. Aminomethylated resin, CH$_2$Cl$_2$, overnight, RT
3. Filter

126 trisubstituted benzimidazoles

R$_2$ = ![Structural diagram](image)

R$_1$ = ![Structural diagram](image)
List of reagents

2-Cyclohexyl-6-N,N-diethylamino-5-(hexanamido)-1H-benzo[d]imidazole (II-16a):

White solid; 56 % yield; mp 183-184 °C;\textsuperscript{1}HNMR (400 MHz, CDCl\textsubscript{3}) $\delta$ 0.91 (m (overlapping triplets), 9 H), 1.23-1.39 (m, 7 H), 1.63-1.83 (m, 7 H), 2.11 (m, 2 H), 2.47 (t, 2 H, $J$ = 7.2 Hz), 2.87 (m, 1 H), 2.94 (q, 4 H, $J$ = 7.2 Hz), 7.50 (s, 1 H), 8.74 (s, 1 H), 9.46 (s, 1 H);\textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}) $\delta$ 12.89, 13.81, 22.39, 25.88, 26.11, 31.43, 31.55, 38.59, 38.72, 50.60, 101.0, 112.6, 131.6, 132.0, 134.4, 139.0, 159.7, 171.1; HRMS (ESI) $m/z$ calcd for C\textsubscript{23}H\textsubscript{36}N\textsubscript{4}OH\textsuperscript{+}: 385.2963, Found:385.2967 ($\Delta$ = -1.0 ppm).

2-Cyclohexyl-5-hexanamido-6-pyrrolidinyl-1H-benzo[d]imidazole (II-16b):
White solid; 61 % yield; mp 127-129 °C; $^1$HNMR (400 MHz, CDCl$_3$) δ 0.91 (t, 3 H, $J = 7.2$ Hz), 1.2-1.43 (m, 7 H), 1.63-1.85 (m, 7 H), 1.97 (broad s, 4 H), 2.07 (m, 2 H), 2.47 (t, 2 H, $J = 7.2$ Hz), 2.84 (m, 1 H), 2.99 (broad s, 4 H), 7.52 (s, 1 H), 8.59 (s, 1 H), 8.91 (s, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 13.86, 22.43, 24.33, 25.84, 26.09, 31.43, 31.78, 38.57, 53.72, 102.3, 109.7, 129.4, 131.4, 135.5, 138.5, 159.4, 171.2; HRMS (ESI) m/z calcd for C$_{23}$H$_{34}$N$_4$OH$^+$: 383.2906, Found: 383.2911 ($\Delta = -1.3$ ppm).

5-Allythioamido-6-piperidinyl-2-thiophene-$1H$-benzo[d]imidazole (II-Da):

![Chemical structure](image)

White solid; 64 % yield; mp 128-130 °C; $^1$HNMR (400 MHz, CDCl$_3$) δ 1.64 (broad s, 6 H), 2.72 (broad s, 4 H), 4.22 (broad s, 2 H), 5.12 (dd, 2 H), 5.83 (m, 1 H), 6.45 (m, 1 H), 7.11 (m, 1 H), 7.27 (s, 1 H), 7.41 (m, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 23.85, 24.46, 47.62, 53.75, 106.9, 109.1, 110.6, 112.6, 117.1, 128.5, 133.1, 136.7, 143.3, 143.7, 144.6, 145.0, 180.1; HRMS (ESI) m/z calcd for C$_{20}$H$_{23}$N$_5$OSH$^+$: 382.1703, Found: 382.17022911 ($\Delta = 0.3$ ppm).

§ 2.4.8 Antibacterial and cytotoxicity activity determination:

**Bacterial Strains and Growth.** H37Rv, the drug sensitive laboratory strain of *Mtb* as well as clinical *Mtb* strains W210, NHN20, HN355, NHN382 and TN587 exhibit different resistant profiles to isoniazid and rifampicin.$^{10,31}$ For evaluation of drug sensitivity all strains were grown in 7H9 media containing 10% OADC enrichment and 0.05% Tween-80 and assessed at mid log phase growth.

**Antibacterial Activity.** The minimum inhibitory concentration (MIC) was determined by the MicroplateAlamar Blue assay (MABA) as described previously.$^{32}$ Briefly, stock solutions of the compounds were prepared in DMSO and were serially diluted 2 fold in 96 well microtiter plates and each *Mtb* strains was added to each well to an OD$_{600}$ of 0.005. Plates were incubated for 6 days at 37°C. AlamarBlue® (Invitrogen) was added to the plates and the plates were
incubated for an additional 24 h at 37 °C. Plates were monitored for color change, and MIC was determined in triplicate.

**Cytotoxicity Assay.** The cytotoxicity of the compounds was tested against VERO cells. Epithelial cells from the kidneys of the African Green Monkey were used to start the VERO cell line. Vero cells were grown in L15 media without CO₂. Serial two fold dilutions of the drugs were prepared in the 96 well microtiter plates. The cells were added to the plates in media containing the appropriate amount of AlamarBlue®. The cells were added to a final concentration of 2.5 x 10⁴ / well. The plates were incubated for 3 days at 37°C. The LD₅₀ was calculated according to manufacturer directions.
§ 2.5 References


2. Tuberculosis: Data and Country Profiles. World Health Organization (WHO)


Chapter III
Target Confirmation and Biological Evaluation of Novel Benzimidazole-Based Anti-tubercular Agents

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§ 3.1 Introduction

Bacterial cell division has been proven to be very crucial for the sustenance and growth of the bacteria. However, the basic mechanism behind the cytokinesis is poorly understood and continuous efforts have been dedicated towards identifying gene products involved in cell division and their associated function. Among this pool of cytokinesis proteins, FtsZ is the most heavily studied, and has been very deservingly found to be the most essential and ubiquitous protein. The polymerization and de-polymerization of FtsZ is a dynamic and continuous process. During cell division, the recruitment of several other cell division proteins leads to Z-ring contraction, resulting in septum formation and eventually cell division (Figure III-1).\textsuperscript{1}

![Diagram of bacterial cell division](image)

**Figure III-1. The Z-ring formation: Bacterial Cell division**\textsuperscript{1}

It was hypothesized that the inhibition of proper FtsZ assembly by either inhibiting polymerization or depolmerization of FtsZ would prevent septum formation without affecting DNA replication and nucleoid segregation.\textsuperscript{1} Such conditions would give way to continued cell filamentation, ultimately leading to cell death.\textsuperscript{2,3} Because of the overwhelming importance of FtsZ in bacterial cycle; it is a very promising target for the development of new anti-Tuberculosis (TB) drugs active against drug-resistant \textit{Mtb} strains.\textsuperscript{4} Based on this premise, several compounds have been identified to inhibit FtsZ assembly.\textsuperscript{5,6}
Following the hypothesis, a library of benzimidazole-based compounds were designed and synthesized as described in the previous chapter (Figure III-2). As expected, several compounds which exhibited Minimum Inhibitory Concentration (MIC) value of ≤ 6 µg/mL.
against drug sensitive as well as drug resistant *Mtb* strains were indentified. However, such cell-based assays do not provide enough insightful information about the mode of action. In order to confirm that these compounds inhibit bacterial growth by interfering with the FtsZ assembly, the effect of our lead compounds on the FtsZ assembly was investigated by using several basic kinetic assays pertinent to *Mtb* FtsZ including light scattering assays, GTPase assay, TEM and SEM imaging etc.

§ 3.2 Results and discussion

§ 3.2.1 Polymerization Assay

A cell-based assay was carried out wherein these re-synthesized lead compounds were screened against drug-sensitive H37Rv strain to obtain the accurate MIC. However, such cell-based assays are not informative about the mode of action. In order to confirm that these compounds inhibit *Mtb* growth by interfering with the FtsZ assembly, a light scattering experiment was carried out to investigate the effect of the lead compounds on FtsZ (de)polymerization. In this experiment, an examination of the extent of light scattering due to FtsZ (de)polymerization as a function of lead compound’s concentration was studied. Initially, the critical concentration of FtsZ required for the assay was determined. FtsZ (10 and 15 μM) was incubated at room temperature in polymerization buffer (50 mM MES, 5mM MgCl2, 50 mM KCl, pH 6.5). Upon addition of GTP to a final concentration of 100 μM GTP, an immediate increase in light scattering was observed, reaching a plateau in about 3 min. The value for the critical concentration required for maximum polymerization was determined to be 15 μM. To study the effect of the compounds on FtsZ assembly, 15 μM FtsZ in polymerization buffer was incubated with different concentration of the compound for 3 min, after which GTP was added to a final concentration of 100 μM to initiate FtsZ polymerization. As anticipated, a reduction in the extent of light scattering was observed with increasing concentration of compounds, indicating the inhibition of FtsZ polymerization.

The assay confirmed that the compounds inhibited polymerization of FtsZ in a dose dependent manner (Figure III-3 and III-4). In case of SB-P3G5, 50 % inhibition was observed at 10-20 μM concentration and the polymerization was completely inhibited at 40 μM concentration. In the case of SB-P1G2, 50 % inhibition was observed at 20 μM concentration.
Strangely, there was no significant inhibition at 10 μM concentration. For SB-P1G8 and SB-P3G2, similar patterns in inhibition were observed; 50% inhibition at 10 μM and polymerization was completely shut down at 20 μM concentration. The polymerization assay confirmed that benzimidazoles inhibited the bacterial (Mtb) growth by inhibiting FtsZ polymerization.

Figure III-3. Inhibition of FtsZ polymerization by lead benzimidazoles
3.2.2 GTPase Assay

The assembly and disassembly of FtsZ protein has been shown to be GTP dependent. Since FtsZ has GTPase activity, the Malachite green assay was performed to monitor the amount of inorganic phosphate (Pi) released upon treatment of *Mtb* FtsZ with some of the lead compounds.

FtsZ protein at a concentration of 20 µM in polymerization buffer was incubated with SB-P3G2 and polymerization was initiated by the addition of GTP. Two controls were maintained for this set of experiments. The first control was untreated protein with GTP, while the second was lead compound suspended in polymerization buffer. Upon addition of Malachite green reagent, formation of solid precipitate (believed to be the protein) in the wells containing protein was observed, therefore, accurate absorbance measurements could not be made. To prevent the precipitation of the protein, its concentration in the reaction mixture was reduced to 10 µM. Also an additional control i.e. protein suspended in polymerization buffer without any

![Graphs showing inhibition of FtsZ polymerization by lead benzimidazoles](image-url)
GTP was included to eliminate any background reading. Unfortunately, this control showed absorbance upon the addition of the reagent. This indicated that the protein was already bound to inorganic phosphate, the source of which was probably the phosphate buffer used to purify the protein. The protein was washed with MES buffer (pH 6.5) to remove this inorganic phosphate. The GTPase assay was performed again with SB-P3G2 and SB-P3G5. Upon treatment of Mtb FtsZ with the two compounds, an enhancement in GTPase activity was observed (Figure III-5).

Figure III-5. Enhancement of GTPase activity of FtsZ by lead benzimidazoles

This unique behavior is analogous to the effect curcumin has on recombinant E.coli FtsZ. Enhancement in the GTPase activity in combination with the previous data illustrating polymerization inhibition of FtsZ upon treatment with these compounds led to the conclusion that the increased GTPase activity causes the instability of FtsZ polymer. As a result of the instability, FtsZ is unable to polymerize and hence polymerization inhibition is observed.

§ 3.2.3 Transmission Electron Microscopy of FtsZ assembly

To visualize the effect of the hit compounds on polymerization of FtsZ protein, TEM imaging of the treated and untreated FtsZ protein was carried out. FtsZ (12 μM) polymerized in the presence of GTP (50 μM) was transferred onto a carbon coated copper-mesh grid for TEM imaging. Unfortunately the filaments were too dense; therefore FtsZ concentration was reduced to 6 μM however, similar results were obtained. At 3 μM concentration of FtsZ, the filaments were too scattered. The method which proved successful was using 5 μM FtsZ, 25 μM GTP and diluting the sample 5 times before loading onto the grid. With the above described optimized
protocol, FtsZ protein treated with 40 µM or 80 µM SB-P3G2 was visualized. Colchicine treated protein was used as a reference in this set of experiments (Figure III-6).

![Figure III-6. Transmission electron microscope (TEM) images of FtsZ](image)

FtsZ (5µM) was polymerized by GTP in the absence (A, B) and presence of SB-P3G2 at 40 µM (C, D) and 80 µM (E, F). TEM images of the inhibition of FtsZ polymerization in the presence of colchicine at 200 µM (G, H) are also shown for comparison. Images (A, C, E, G) are at 30,000x magnification (scale bar 500 nm) and (B, D, F, H) are at 68,000x magnification (scale bar 200 nm).

As expected, there was considerable reduction in the extent of FtsZ protofilament formation upon treatment with SB-P3G2 in comparison to the control experiment. At 40 µM concentration of SB-P3G2, the density of FtsZ protofilaments was considerably reduced. Furthermore the filaments were shorter and very thin which became more apparent at higher concentration of 80 µM. On the other hand, treatment of Mtb FtsZ protein with 200 µM colchicine clearly had a less profound effect on the inhibition of FtsZ polymerization. Similar formation of tiny aggregates was not observed as compared to SB-P3G2 treated protein, reiterating the efficacy of the compound. This result definitely confirms that these novel benzimidazoles exhibit a remarkable FtsZ assembly inhibitory activity.

§ 3.2.4 Scan Electron Microscopy of Mtb cells

Visualization of bacteria treated with 2xMIC and 4xMIC SB-P3G2 for 2-days using scanning electron microscopy (SEM) revealed aberrant cell division indicative of FtsZ assembly
inhibition (Figure III-7). Specifically, cell division and elongation was inhibited in a dose dependent manner resulting in shorter bacterial cells with increased circumferences and altered polar caps. The observed altered bacterial morphology upon exposure to SB-P3G2 is thought to result from inhibition of FtsZ leading to disruption in septum formation and recruitment of septum associated proteins involved in later steps of division and septum resolution.

Figure III-7. SEM of Mtb cells treated with SB-P3G2 (20,000X).\textsuperscript{7}

\text{(A) Control; (B) 2x MIC; (C) 4 x MIC; (D) distribution of bacterial cell length: Control (black), 2x MIC (grey), 4x MIC (light grey)}

§ 3.2.5 Photoaffinity labeling (PAL) of FtsZ for binding site determination

Though FtsZ kinetic assays confirmed that the novel trisubstituted benzimidazoles restrict the Mtb growth by inhibiting FtsZ polymerization, the mechanism of its action and binding site are still unknown. Furthermore, identifying the binding site could very well open a new dimension for structure-based design of more efficient inhibitors. In order to investigate the binding site of benzimidazoles, there are several experiments which can be carried out, including photoaffinity-labeling of FtsZ, co-crystallization of FtsZ and molecular docking (Figure III-8). Co-crystallization of Mtb FtsZ with our lead benzimidazole as well as taxane based-anti TB
agents could potentially be the most promising experiments to determine the binding site. For this experiment, lead taxanes as well as benzimidazoles which will be discussed in later chapters were synthesized.

Photoaffinity labeling (PAL) of FtsZ is another experiment whereby photolyzing FtsZ with the lead compounds containing photo labile group such as, azide, benzophenone will enable the identification of amino acid residues in the binding site. Thus, photoaffinity analogues of taxane-based and trisubstituted benzimidazole-based anti-TB agents were designed and synthesized to perform photoaffinity labeling experiments (Figure III-8). It should be noted that attachment of photoreactive groups, in this case azide and benzophenone, should not affect binding to the target proteins and hence should maintain good anti-TB activities. The taxoid based photoaffinity analogue (PAA) SB-RA-50012 was previously investigated for its anti-TB activity and was found to show appreciable activity.8

The synthesis of photoaffinity analogue (PAA), 4-azido-SB-P1G8 II-3 was carried out starting with diazotization of p-aminobenzoic acid and subsequent nucleophilic substitution by azido group to form p-azidobenzoic acid II-1 in 96 % yield. Treatment with phosphorous
pentachloride gave $p$-azidobenzoyl chloride II-2 in 80 percent yield. Finally coupling with the respective benzimidazole (SB-P1G8) gave the desired product II-3 in 50 % yield (Scheme III-1).

![Scheme III-1. Synthesis of 4-azido SB-P1G8](image)

In order to check the activity of 4-azido-SB-P1G8 (III-3) against *Mtb*. FtsZ, polymerization assay was carried out (Figure III-9). In comparing the inhibition of polymerization by III-3 with that of SB-P1G8, it was concluded that no significant change was observed, confirming its activity.

![Figure III-8. Effect of 4-azido-SB-P1G8 on FtsZ polymerization](image)
In the PAL experiment, FtsZ (polymerized and unpolymerized) was incubated with 4-azido-SB-P1G8 for 3 minutes following the polymerization conditions. The samples were then photolyzed at 360 nm for 30 minutes. After photolyzing the samples, FtsZ was precipitated using acetone method. In solution tryptic digestion of the precipitated protein was carried out, followed by MALDI analysis of the digested sample. The two control samples, polymerized and non-polymerized FtsZ with and without inhibitor were photolyzed. In the MALDI spectrum, it was expected to observe disappearance of a peptide peak and appearance of a new peptide peak with additional molecular weight (of the PAA). Unfortunately, when the MALDI spectrums were compared, no such phenomenon was observed (Figure III-9 and Figure III-10).

Figure III-9. MALDI of digested samples of non-polymerized FtsZ with and without PAA
At this end, PAL experiments have been tried with 4-azido-SB-P1G8. However, no relevant information about the binding site amino acid sequence has been gathered. Next, taxoid–based PAA will be used along with the optimization of photolysis time.

§ 3.2.6 Preliminary in vivo assays and formulation studies

The evidence presented by above mentioned experiments strongly suggests that the novel benzimidazoles directly inhibit FtsZ assembly by destabilizing the FtsZ polymer. The lead benzimidazole, SB-P3G2 was advanced to in vivo testing. SB-P3G2 was tested for efficacy against M. tuberculosis H37Rv in the M tuberculosis rapid infection model. SB-P3G2 was delivered intraperitoneally (IP) at a concentration of 150 mg/kg for 1 dose and 75 mg/kg for the last 8 doses. In the preliminary studies, it was observed the delivery of the lead compound was partial due to its poor solubility. Nevertheless, the compound demonstrated promising efficacy
based on greater than 0.3 reduction in $\log_{10}$ CFU cutoff (Figure III-11). As a result, an increased effort was required to improve the solubility of our lead compounds via formulation (using surfactant) or by synthesizing water soluble prodrug.

![Figure III-11. Mean CFU in lung and spleen after 22 days of administration of compound](image)

Eight groups of 5 mice each were treated for 9 days and then were assessed for bacterial burden in the lungs and spleen. SB-P3G2 was diluted in a solution of 12.5% ethanol, 10% solutol at 150mg/kg for 1 dose and 75mg/kg for the last 8 doses. There were 2 control groups (negative control group 1 received 20ul of 75% Ethanol and Positive control group 2 received isoniazid administered at 25mg/kg in water). All treatments and controls were delivered IP. Dosing was started at 12 days of infection and continued till day 21 of infection. On day 12 a pretreatment group was sacrificed and the bacterial loads for each mouse was determined prior to treatments. On day 22 the mice were sacrificed and the lungs and spleens were homogenized and plated on 7H11agar plates for CFU enumeration.

In order to develop a suitable formulation method, Solutol HS 15 was used as a solubilizing agent. The solubilizer Solutol HS 15 (polyethylenglycol 660 –12-hydroxystearate) is a non-ionic surfactant used for pharmaceutical purposes without showing any apparent toxicity. Solutol HS 15 is very efficient in solubilizing substances like fat-soluble vitamins, and active ingredients of hydrophobic nature. It has been observed that the solubilizing capacity of compounds increases linearly due to the formation of spherical micelles. To develop a suitable formulation, it is important to make sure that after injection the compound solution in solutol HS 15 should stay homogeneous without any immediate precipitation. In order to judge that, the homogeneity of the solution of our lead compounds in 1:1 (w/w) solution of solutol HS15 and EtOH when diluted by phosphate buffer saline (PBS pH 7.4) was examined. A solution of SB-P3G2, SB-
**P8B2** and **SB-P8B4** in solutol HS 15/EtOH (1:1) at a concentration 20 mg/mL was diluted with PBS (Table III-1).

### Table III-1. Formulation of lead benzimidazoles

<table>
<thead>
<tr>
<th>A.</th>
<th>Solubility test of SB-P3G2 at room temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>100 µL of 20 mg/mL in solutol HS 15 (1:1)</td>
</tr>
<tr>
<td></td>
<td>Added 300 µL of PBS - <strong>No precipitate</strong></td>
</tr>
<tr>
<td></td>
<td>Added 100 µL of PBS - <strong>Compound precipitated</strong></td>
</tr>
<tr>
<td>2.</td>
<td>100 µL of 10 mg/mL in solutol HS 15 (1:1)</td>
</tr>
<tr>
<td></td>
<td>Added 500 µL of PBS - Few floating particles</td>
</tr>
<tr>
<td></td>
<td>Added 200 µL of PBS - More floating particles after 30 minutes</td>
</tr>
<tr>
<td></td>
<td>Added 200 µL of PBS - <strong>Compound precipitated</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B.</th>
<th>Solubility test of SB-P8B2 and SB-P8B4 at room temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>100 µL of 20 mg/mL in solutol HS 15 (1:1)</td>
</tr>
<tr>
<td></td>
<td>Added 800 µL of PBS – <strong>No precipitate</strong></td>
</tr>
<tr>
<td></td>
<td>Left for 2 days - <strong>Homogeneous solution</strong></td>
</tr>
<tr>
<td>2.</td>
<td>100 µL of 10 mg/mL in solutol HS 15 (1:1)</td>
</tr>
<tr>
<td></td>
<td>Added 1000 µL of PBS - <strong>No precipitate</strong></td>
</tr>
<tr>
<td></td>
<td>Left for 2 days - <strong>Homogeneous solution</strong></td>
</tr>
</tbody>
</table>

**SB-P8B2** and **SB-P8B4** showed better solubility results as compared to **SB-P3G2** at concentrations of 20 mg/mL and 10 mg/mL. **SB-P3G2** solution started to precipitate after 4-6 times dilution by PBS at room temperature. However, in case of **SB-P8B2** and **SB-P8B4**, the solution remained homogeneous even after 8-10 times dilution and 2 days at room temperature. Thus, it was concluded that administering 1 dose of 4 mg or 2 doses of 2 mg of the lead compound would be ideal for overcoming the solubility issues. In other words, just before injection, stock solution of the lead compounds (40 mg/mL or 20 mg/mL in solutol HS 15/EtOH) can be diluted with appropriate volume of PBS (to keep the ethanol volume to a permissible level) so that each dose has either 4 mg or 2×2 mg. Presently, the *in vivo* assays are being repeated by our collaborator Prof. Slayden laboratory following our formulation.
§ 3.3 Conclusion

Several novel trisubstituted-benzimidazoles were designed and synthesized through rational and systematic design. In preliminary cell-based screening against Mtb H37Rv strain, several of these compounds exhibited MIC$_{99}$ values in the range of 1.2-15 µM along with limited cytotoxicity. In a light scattering experiment, several of these novel lead compounds exhibited inhibition of FtsZ assembly in a dose dependent manner. Additionally, GTPase activity of Mtb FtsZ was enhanced by 3-4 folds as observed in malachite green assay.

SEM images of Mtb cells treated with SB-P3G2 showed dose dependent shortening of cell length. A visible reduction in the extent of FtsZ polymerization was observed under transmission electron microscope. The FtsZ protofilaments were shorter in length in comparison to the untreated FtsZ which became more apparent at higher concentrations of SB-P3G2. At this end, photoaffinity labeling of FtsZ with 4-azido-SB-P1G8 haven’t been able to provide relevant information about the binding site. In the preliminary in vivo studies, although the delivery of our lead compounds was partially limited due to poor solubility, SB-P3G2 demonstrated promising efficacy based on greater than 0.3 reduction in log$_{10}$CFU cutoff.

The evidence presented in this study strongly suggests that these novel benzimidazoles can directly inhibit proper FtsZ assembly by destabilizing the FtsZ polymer which is unequivocally confirmed by SEM and TEM images. Further optimization and in vivo evaluation of these newly discovered lead compounds are actively underway in our laboratories.
§ 3.4 Experimental Section

General Methods: $^1$H and $^{13}$C NMR spectra were measured on a Varian 300, 400 or 500 MHz NMR spectrometer. Melting points were measured on a Thomas Hoover Capillary melting point apparatus and are uncorrected. TLC was performed on Merck DCalufolien with Kieselgel 60F-254 and column chromatography was carried out on silica gel 60 (Merck; 230-400 mesh ASTM). SensoLyte® MG phosphate assay kit from Anaspec was used for Malachite Green assay. Light scattering assays were performed using a Fluorolog fluorimeter from ISA instruments. Scanning electron micrographs were obtained with a JOEL JSM-6500F scanning electron microscope. FEI Tecnai12 BioTwinG transmission electron microscope with an AMT XR-60 CCD digital camera system was used to acquire transmission electron microscopy images.

Materials: BL21(DE3)pLysS cells, Ni-NT and the buffers were purchased from Novagen. BCA kit for protein concentration determination was purchased from Sigma Aldrich. Malachite Green Carbinol Base was bought from Sigma Aldrich. Ammonium molybdate (VI) tetrahydrate was provided by Acros. Buffer salts (reagent grade or better), solvents (HPLC grade or better), and all the other chemicals were purchased from Fisher Scientific Co. (Pittsburgh, PA). The chemicals were purchased from Sigma Aldrich and Synquest Inc. and purified before use. Tetrahydrofuran was freshly distilled from sodium metal and benzophenone. Dichloromethane was distilled immediately prior to use under nitrogen from calcium hydride.

§ 3.4.1 Mtb FtsZ Protein Preparation

The appropriate (pET 15 b) plasmid (1.5 μL and 3 μL) was transformed into 100 μL of BL21(DE3) pLys cells. The transformed cells were plated onto LB plates, containing 100 μg/mL ampicillin. The antibiotic concentration was kept the same for the following steps. The plates were incubated overnight at 37 °C. The colonies were picked and grown in 10 mL of LB media at 37 °C at 250 rpm shake rate. The inoculum was transferred to 1 L of LB media in a 4 L flask and grown to an OD of 0.4 at A600. Then, 1 mM IPTG was added to induce protein expression overnight at 32 °C at 250 rpm shake rate. Next day the cells were harvested at 5K rpm for 30 min and re-suspended in approximately 20-30 mL binding buffer (300 mM NaCl, 50 mM NaH$_2$PO$_4$, 10 mM imidazole, pH = 8). The re-suspended cells were lysed using ultra-sonicator. The lysate
was centrifuged in an ultracentrifuge at 33K rpm for 90 min. The supernatant was filtered and loaded onto Ni\textsuperscript{2+}-NTA column washed with 50 mL of binding buffer and eluted using a gradient of binding buffer with 50-250 mM imidazole. The eluted protein was immediately loaded onto a G-25 size exclusion chromatography column, pre-equilibrated with the polymerization buffer (50 mM MES, 5mM MgCl\textsubscript{2}, 50 mM KCl, pH 6.5). The protein fractions were concentrated and stored at -80 °C for further use. Since the number of aromatic residues in \textit{Mtb} FtsZ protein are low (Tyr: 1, Trp: 0), it is not reliable to follow concentration of protein by scanning at A280. The concentration of protein was therefore ascertained using the BCA kit from Sigma.

§ 3.4.2 \textit{FtsZ} Polymerization Inhibitory Assay\textsuperscript{9}

The inhibitory activity of lead benzimidazoles for \textit{Mtb} FtsZ polymerization was determined by light scattering on a PTI Fluorescence Master Systems. The 90° light scattering was measured at 30 °C, using excitation and emission wavelength of 400 nm with slit width of 2 nm. The gain was set at 950 V. \textit{Mtb} FtsZ (15 µM) was incubated in the polymerization buffer (50 mM MES, 5mM MgCl\textsubscript{2}, 50 mM KCl, pH 6.5) to establish a baseline. Then, 5 mM GTP (20 µL) was added to make the final volume 1,000 µL in a 1.5 mL cuvette. The light scattering was measured for a period of 30-60 min. When DMSO was used as a solvent for the inhibitor, the control also contained the same amount of DMSO. For inhibition studies, a lead benzimidazole at various concentrations was incubated with the polymerization buffer to establish a base line. This was followed by addition of FtsZ (15 µM). Once the baseline was stabilized, 5 mM GTP (20 µL) was added to make the final volume 1,000 µL in a 1.5 mL cuvette and the light scattering was measured.

§ 3.4.3 GTPase Assay

The amount of inorganic phosphate (Pi) released during the assembly of FtsZ was measured using a standard Malachite Green/ammonium molybdate assay. Briefly, FtsZ protein (10 µM) was incubated with or without a benzimidazole at different concentrations (0, 20, 40 and 80 µM) in polymerization buffer (50 mM MES, 5 mM MgCl\textsubscript{2}, 50 mM KCl, pH 6.5) at room temperature for 15 min. Then, 50 µM GTP was added to the reaction mixture and incubated at 37 °C to start the hydrolysis reaction. After 30 min of incubation, Malachite green reagent (20% v/v) was
added to the reaction mixtures to quench the reaction. The reaction mixtures were centrifuged at 13,000 rpm for 90 sec to remove the protein debris. The samples (100 µL) were transferred to a 96 well plate and the absorbance of each well was measured at 620 nm. The background absorbance was subtracted from all the readings. A phosphate standard curve was prepared using phosphate standard provided with a Malachite Green assay kit (SensoLyte®). All the solutions were prepared in polymerization buffer.

§ 3.4.4 Transmission Electron Microscopy (TEM) Analysis

*Mtb* FtsZ (5 µM) was incubated with 40 µM or 80 µM of **SB-P3G2** in the polymerization buffer (50 mM MES, 5mM MgCl₂, 50 mM KCl, pH 6.5) for 15-20 min. To each solution was added GTP to a final concentration of 25 µM. The resulting solution was incubated at 37 °C for 30 min. The incubated solution was diluted 5 times with the polymerization buffer and immediately transferred to carbon coated 300 mesh formvor copper grid and negatively stained with 1 % uranyl acetate. The samples were viewed with a FEI Tecnai12 BioTwinG transmission electron microscope at 80 kV. Digital images were acquired with an AMT XR-60 CCD Digital Camera System.

§ 3.4.5 Ultrastructural analysis via scanning electron microscopy (SEM)

*Mtb* H37Rv bacteria were exposed to 2 x MIC and 4 x MIC **SB-P3G2** for 2 days. The bacteria were prepared for scanning electron microscopy as described by Slayden et. al.³ Briefly, bacteria were prepared for SEM treatment with 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.2), 5mM CaCl₂ and 5 mM MgCl₂ and incubated at room temperature for 1-2 h. The fixed bacteria were harvested by centrifugation and washed in PBS, and subjected to 2.5% glutaraldehyde overnight at 4 °C. Final preparation for SEM was achieved by treatment with 1% OsO₄ in sodium cacodylate buffer and dehydration in a graded alcohol series. The bacteria were then examined using a JOEL JSM-6500F scanning electron microscope. Bacteria from all treatment groups were measured for their lengths. Size frequency graphs were prepared for each treatment group.
3.4.6 Photoaffinity Labeling of FtsZ using 4-azido-SB-P1G8

500 µL of 15 µM FtsZ (polymerized and unpolymerized) was incubated with 2µL of 10 mM photoaffinity analogues 4-azido-SB-P1G8 in polymerization buffer for 3 minutes following the polymerization conditions. Two control samples, polymerized and non-polymerized FtsZ with and without inhibitor, were photolyzed at 360 nm for 30 minutes using southern RMR3500 2 lamps. After photolyzing the samples, FtsZ was precipitated using acetone method. Four volumes of cold acetone was mixed with one volume of photolyzed protein sample and stored at -20 °C for 20-30 minutes. The solution was centrifuged at 15000g for 15 minutes. The supernatant was discharged carefully and the protein pellets were air dried. In solution tryptic digestion of the precipitated protein was carried out. The pellets were mixed with 10 M urea and 1 M Tris-HCl (pH 8.5) to bring up the solution to 8 M urea and 100 mM Tris-HCl. 100 mM TCEP (reducing agent) was added to a final concentration of 5 mM TCEP and incubated at room temperature for 20 minutes. Later, 500 mM iodoacetamide (freshly prepared) was added to a final concentration of 10 mM and incubated at room temperature in dark for 15 minutes. The above samples were diluted by a factor of four with 100 mM Tris-HCl (pH 8.5) followed by addition of 100 mM CaCl₂ to a final concentration of 1 mM. Finally trypsin (0.4 µg/µL) was added to the above solution, incubated overnight and quenched with 5 % formic acid solution. These samples were then subjected to MALDI analysis.

3.4.7 Synthesis of photoaffinity analogue 4-Azido-SB-P1G8

4-azido-N-(2-cyclohexyl-6-(diethylamino)-1H-benzimidazol-5-yl)benzamide (III-3):

4-Aminobenzoic acid (1.0 g, 7.3 mmol) dissolved in TFA (25 mL) was diazotized in the dark at 0 °C with NaNO₂ (1.0 g, 14.6 mmol). After stirring for 30 minutes, sodium azide (4.75 g, 73 mmol) was slowly added to the reaction mixture over a period of 5 minutes. After an additional 15 minutes, diethyl ether (20 mL) was added and the mixture was stirred for 1 hour. The reaction mixture was washed with water, extracted with diethyl ether, and dried over magnesium sulfate.
Removal of excess TFA as an azeotrope with benzene gave III-1 as a white powder (1.12 g, 96 % yield) with m.p. 182-184 °C (lit. m.p. 185 °C)\textsuperscript{11}.

4-Azidobenzoic acid (259 mg, 1.58 mmol) was dissolved in diethyl ether (10 mL) followed by the addition of phosphorous pentachloride (352 mg, 1.74 mmol) The reaction mixture was stirred for 30 minutes. The diethyl ether was removed under vacuum to obtain yellow oil which crystallized under vacuum. The solid was triturated with hexane at 0 °C to give III-2 as white solid (230 mg, 80 % yield) with m.p. 55-56 °C (Lit. m.p. 57-58 °C)\textsuperscript{12}.

To a solution of 5-Amino-6-N,N-diethylamino-2-cyclohexyl-1\textsubscript{H}benzo[d]imidazole (38.8 mg, 0.135 mmol) in methylene chloride (mL) was added III-2 (23 mg, 0.121 mmol) and stirred overnight. After the completion of reaction, the solvent was removed and the crude was purified by flash chromatography (gradient 20-40 % EtOAc/hexanes) to give III-3 (15 mg, 50 % yield).\textsuperscript{1}$^1$H NMR (300 MHz, CDCl\textsubscript{3}) $\delta$ 0.97 (m, 6 H, $J = 7.0$ Hz), 1.25-1.28 (m, 5 H), 1.57-1.58 (m, 3H), 2.04 (m, 2 H), 2.77 (m, 1 H), 3.02 (q, 4 H, $J = 6.9$ Hz), 7.18 (d, 2 H, $J = 4.2$ Hz), 7.26 (s, 1 H), 7.58 (s, 1 H), 7.97 (d, 2 H, $J = 4.2$ Hz), 8.86 (s, 1 H), 10.3 (s, NH); $^{13}$C NMR (100 MHz, CDCl\textsubscript{3}) $\delta$ 13.13, 25.71, 25.97, 31.73, 38.46, 50.79, 100.5, 113.4, 119.3, 128.6, 131.8, 135.1, 139.5, 143.5, 159.6, 163.8. MS (ESI) $m/z$ 432.1 (M+1).
§ 3.5 References


Chapter IV

Discovery of Novel Trisubstituted Benzimidazole-Based Antibacterial Agents Against Other Pathogens

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§ 4.1 Introduction

Bacterial infections due to pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *enterococci* (VRE), *F. tularensis*, *Y. pestis*, *B. thailandensis*, and others have re-emerged as major health concerns throughout the world. Infections due to VRE and MRSA are a particular nosocomial problem in hospitalized or immuno-compromised individuals as well as healthcare professionals. These organisms are often resistant to multiple antibiotics. In the past two decades, there has been a rapid increase in the incidence of VRE and MRSA infections. Community-acquired (CA) MRSA infections among young persons have emerged in several areas worldwide during the last decade which represents a new challenge for healthcare professionals. According to the Center for Disease Control and Prevention (CDC), the estimated number of people developing invasive MRSA infection in 2005 was about 94,360, while approximately 18,650 people died during a hospital stay related to these MRSA infections in the United States. In spite of the serious situation, there are only a small number of FDA-approved therapeutic drugs including, linezolid, daptomycin, tigecycline, quinupristin, platensimycin, clindamycin available to treat these infections. However, these drugs have been found to show limited efficacy and furthermore, drug resistance has already been observed.

*Francisella tularensis* is a highly virulent gram-negative bacterium and the causative agent of tularemia. Due to its high virulence and difficult diagnosis, *F. tularensis* is classified as a Class A agent pathogen by the U.S. government. Tularemia can be treated to a certain extent by a cocktail of antibiotics such as streptomycin, gentimycin, doxycycline, and ciprofloxacin.

*Yersinia pestis* is another gram-negative rod-shaped bacterium, belonging to the *enterobacteriaceae* family. *Y. pestis* infection takes three main forms, i.e., pneumatic, septicemic, and the notorious bubonic plagues, which have been responsible for a number of high-mortality epidemics in the past. More recently, *Y. pestis* has gained attention as a possible biological warfare agent and CDC has classified it as a category A pathogen requiring preparation for a possible terrorist attack. About 14% (1 in 7) of all plague cases in the United States are fatal. Globally, the WHO reports 1,000 to 3,000 cases of plague every year. The traditional first line treatment for *Y. pestis* has been streptomycin, chloramphenicol, tetracycline, and fluoroquinolones. In addition, there are various other neglected diseases caused by bacterial infections such as buruli ulcer, trachoma, leprosy, cholera, lyme disease, etc., which
need special attention due to their highly epidemic nature in areas such as Africa, Asia, and Russia.\textsuperscript{15}

The treatment regimen for above mentioned infections are still limited to rather classical antibacterial drugs that target cell wall biosynthesis, nucleic acid synthesis, protein synthesis, etc.\textsuperscript{16} Furthermore, the widespread bacterial resistance to existing therapeutics due to poor drug compliance has been a crucial obstacle in finding suitable and more efficient treatments. Therefore, in order to circumvent the adaptive genetic machinery of bacteria, there is a pressing requirement for the identification of novel therapeutic targets. As mentioned in previous chapters, FtsZ offers an excellent therapeutic target since the disruption of cell division would lead to the inhibition/arrest of bacterial growth (Figure IV-1).\textsuperscript{17,18}

\textbf{Figure IV-1. Graphic representation of Z-ring formation and cell division.}\textsuperscript{19}

\begin{enumerate}
\item Bacterial cell before the beginning of cell division with FtsZ (O) scattered in the cell and undergoing continuous nucleotide exchange between GTP-bound FtsZ and GDP-bound FtsZ with rapid equilibrium, favoring GTP-bound FtsZ. A circular chromosome with a single origin of replication (\blacktriangleleft) is located at midcell.
\item-4) After chromosome replication initiates, the origins of replication separate and move to opposite poles of the cell. After completion of replication, the chromosomes separate, leaving a nucleoid-freespace.
\item Z-ring formation coincides with chromosome segregation.
\item During cytokinesis the Z-ring constricts leading to septum formation. Complete disassembly of the Z-ring forms the daughter cells.
\end{enumerate}

FtsZ offers a significant potential advantage over traditional targets as it is a highly conserved protein in prokaryotes. Thus, in addition to being pathogen specific, FtsZ inhibitors
could potentially be developed as broad-spectrum antibacterial agents for the treatment of a variety of bacterial infections.\textsuperscript{20} Since FtsZ is an essential and conserved protein for bacterial cell division, it is believed that escaping the FtsZ-targeted compounds by mutating the protein may be demanding for bacteria, suggesting that resistance mechanisms against compounds developed for FtsZ inhibition may not be widespread in nature.\textsuperscript{21,22} Furthermore, extensive structural and functional homology\textsuperscript{23,24,25} between FtsZ and tubulin provides an opportunity to use known tubulin/microtubules (de)stabilizer as an excellent starting point for the development of novel antibacterial agents. Considering the limited sequence homology between tubulin and FtsZ at protein level, compounds specific to FtsZ should have no appreciable cytotoxicity to eukaryotic cells.\textsuperscript{26} As described in previous chapters, a number of active trisubstituted benzimidazoles against \textit{Mtb} FtsZ have already discovered. Therefore, our library of trisubstituted benzimidazoles was screened against various pathogens to identify broad-spectrum inhibitors of FtsZ assembly.

\section*{\textsection 4.2 Compounds targeting FtsZ from other pathogens}

Representative FtsZ inhibitors for bacterial strains other than \textit{Mtb} are summarized in Tables IV-1 \& IV-2. The structures of these FtsZ inhibitors are shown in Figure IV-2 and Figure IV-3. These inhibitors have relevance to the discovery and development of broad-spectrum FtsZ inhibitors since FtsZ structure is well conserved in all bacterial species.

\textit{Zantrins}

A high throughput (HTP) protein-based screening of 18,320 molecules lead to the identifications of five structurally diverse compounds named “zantrins”, which inhibited \textit{E. coli} FtsZ GTPase activity by 50 \% at 4-25 \textmu{}M concentration.\textsuperscript{17,27} Zantrins were also examined for inhibitory activity against FtsZ orthologue from \textit{Mtb}. Majority of zantrins inhibited the GTPase activity of \textit{Mtb} FtsZ with IC\textsubscript{50} values in the range of 50-70 \textmu{}M.
It was proposed that these FtsZ-destabilizing zantrins (Z1 and Z4) may bind to a pocket between two FtsZ subunits to block the loop T7 of one monomer to come in optimal contact with GTP bound to the T1-T6 loops of the neighboring monomer, which is essential for polymerization. In contrast, some zantrins (Z2, Z3 and Z5) appeared to stabilize the FtsZ assembly by contracting the movement of T3 loop that causes a bend in the filament upon GTP hydrolysis.

**Viriditoxin**

Viriditoxin, a potent *E. coli* FtsZ inhibitor, was identified from a pool of more than 100,000 extracts of microbial fermentation broths and plants using FtsZT65C-fluorescein polymerization assay. It exhibited *E. coli* FtsZ polymerization inhibiton with an IC$_{50}$ of 8.2 µg/mL and GTPase inhibition with an IC$_{50}$ of 7µg/mL. Furthermore, Viriditoxin was also evaluated for its broad-spectrum antibacterial activity. It inhibited the growth of *S. aureus* and MRSA with MIC values of 4-8 µg/mL and *enterococcus faecalis* and VRE with MIC values of 2-16 µg/mL.

**GTP analogues**

Lappchen *et al.* designed a novel inhibitor of *E. coli* FtsZ, 8-bromogunosine-5’-triphosphate (BrGTP), based on the natural substrate GTP. GTPase assay and polymerization
assay indicated that BrGTP is a reversible competitive inhibitor of *E. coli* FtsZ. Paradis-Bleau *et al.* synthesized a library of GTP analogues with core structures composed of a guanine-like moiety linked to an alanine side chain (GAL analogues). All the GAL analogues moderately inhibited the GTPase activity of *Psuedomonas aeruginosa* FtsZ (IC$_{50}$ 450 µM ~ 2.6 mM).

**Sanguinarine**

Sanguinarine, a benzophenanthridine alkaloid derived from the rhizomes of *Sanguinaria canadensis*, is known to have a broad range of antimicrobial activity (Figure IV-3). Beuria *et al.* reported that sanguinarine inhibited cytokinesis in both gram-positive and gram-negative bacteria by arresting *E. coli* FtsZ assembly. Sanguinarine inhibited the extent of light scattering developing from FtsZ polymerization. Electron microscopic images of FtsZ polymer treated with sanguinarine indicated reduced thickness and significantly shorter lengths of FtsZ bundles.

![Mtb](image)

**Figure IV-3. FtsZ inhibitors of bacteria other than *Mtb***
Table IV-1. Compounds targeting FtsZ of bacteria other than *Mtb*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Target Organism</th>
<th>Mode of action</th>
<th>MIC/IC&lt;sub&gt;50&lt;/sub&gt; (cell growth inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Viriditoxin</td>
<td><em>E. Coli</em>, <em>S. aureus, MRSA, VRE, Enterococcus faecalis</em></td>
<td>Inhibits GTPase and FtsZ polymerization</td>
<td>4-8 µg/mL (<em>S. aureus and MRSA</em>) 2-16 µg/mL (<em>enterococcus faecalis</em> and VRE)</td>
</tr>
<tr>
<td>2 Zantrins</td>
<td><em>Mtb, E. coli</em>, MRSA, VRE</td>
<td>Inhibits GTPase, Z1, Z2, Z4 destabilizes FtsZ polymer, Z5 stabilizes FtsZ assembly</td>
<td>-</td>
</tr>
<tr>
<td>3 GTP analogues 1. BrGTP</td>
<td><em>E. coli</em></td>
<td>Inhibits GTPase and FtsZ polymerization Assay in reversible competitive mode</td>
<td>-</td>
</tr>
<tr>
<td>2.GAL Analogues</td>
<td><em>E. Coli</em>, <em>S. aureus</em></td>
<td>Inhibits GTPase activity (<em>E. coli FtsZ</em>),</td>
<td>0.4-20 mg/mL (<em>S. aureus</em>)</td>
</tr>
<tr>
<td>4 Sanguinarine</td>
<td><em>B. subtilis, E. coli</em></td>
<td>Inhibits FtsZ assembly and protofilament bundling, thinner and shorter FtsZ protofilaments (<em>E. coli</em>)</td>
<td>-</td>
</tr>
<tr>
<td>5 N-Benzyl-3-sulfonamidopyrrolidines</td>
<td><em>E. Coli</em></td>
<td>Lethal cell filamentation, moderate inhibition of GTPase activity, No effect on FtsZ polymerization</td>
<td>Compound 14 -10 μM Compound 15-20 μM</td>
</tr>
<tr>
<td>6 (±)-Dichamanetin</td>
<td><em>S. aureus</em>, <em>B. subtilis</em>, <em>M. smegmatis</em>, <em>E. coli</em></td>
<td>Inhibits GTPase activity of <em>E. coli FtsZ</em></td>
<td>1.7-3.4 μM (<em>S. aureus, B. subtilis, M. smegmatis, E. coli</em>)</td>
</tr>
<tr>
<td>7 (±)-2''-Hydroxy-5''-benzylisouvarinol-B</td>
<td><em>S. aureus</em>, <em>B. subtilis</em>, <em>M. smegmatis</em>, <em>E. coli</em></td>
<td>Inhibits GTPase activity of <em>E. coli FtsZ</em></td>
<td>2.3-10.7 μM (<em>S. aureus, B. subtilis, M. smegmatis, E. coli</em>)</td>
</tr>
</tbody>
</table>

**N-Benzyl-3-sulfonamidopyrrolidines**

A high define HTP (HTP) screening of a library of compounds at the Broad Institute of Harvard University and Mass Institute of Tech (MIT) identified several compounds that caused lethal cell filamentation in *E. coli* without significantly inhibiting the GTPase activity of FtsZ. For example, the lead compound, 534F6 (Figure IV-3), displayed only weak inhibition (20 %) of *E. coli* FtsZ GTPase activity at 100 μM concentration and no effect on FtsZ polymerization. Based on this lead compound, an optimized library of 45 compounds was synthesized and
evaluated against *E. coli* and compound 14 was found to be the most potent with MIC value of 10 µM. *E. coli* culture treated with 5 µM of compound 14 and 10 µM of compound 15 showed extensive filamentation.

*(±)-Dichamanetin and (±)-2‴-hydroxy-5‴-benzylisouvarinol-B*

Dichamanetin and 2‴-hydroxy-5‴-benzylisouvarinol-B, polyphenolic natural products, were isolated from *U. chamae* and *X. aificana*, respectively (Figure IV-3). These compounds show a high level of activity against gram-positive bacteria, such as, *S. aureus*, *B. subtilis*, *M. smegmatis* and *E. coli*, with MIC values in the range of 1.7-3.4 µM for dichamanetin and 2.3-10.7 µM for hydroxybenzylisouvarinol-B. Dichamanetin and hydroxybenzylisouvarinol-B were found to be potent GTPase inhibitors of *E. coli* FtsZ (IC$_{50}$ 8-12 µM).

*Cinnamaldehyde*

Cinnamaldehyde, a natural product, was shown to exhibit broad-spectrum anti-bacterial activity (MIC: 1 µg/mL for *E. coli*; 0.5 µg/mL for *B. subtilis*; 0.25 µg/mL for MRSA). *E. coli* FtsZ polymerization and GTPase activity were strongly inhibited with an IC$_{50}$ value of 6.86±2.2 µM and 5.81±2.2 µM, respectively. Electron microscopy images and confocal imaging of live *E. coli* cells with GFP-tagged FtsZ further confirmed the reduction in Z-ring formation. STD-NMR in conjunction with AutoDock computer modeling suggested that cinnamaldehyde binds around the T7 loop in the FtsZ monomer, inducing conformational change that block optimal contact with the GTP binding T1-T6 loop in a neighboring monomer.

3-{(5-[4-Oxo-2-thioxo-3-(3-trifluoromethylphenyl)-thiazolidin-5-ylidene methyl]-furan-2-yl)-benzoic acid (OTBA)}

Beuria *et al.* screened their library of 81 compounds with 29 diverse scaffolds using a sedimentation assay of *E. coli* FtsZ polymer, and found that OTBA promoted *E. coli* FtsZ assembly *in vitro* and inhibited the *B. subtilis* cell proliferation, leading to filamentation (MIC 2 µM). The light scattering assay showed that OTBA enhanced the extent of *E. coli* FtsZ and *B. subtilis* FtsZ polymerization by 3-fold. Electron microscopic analysis showed that the thickness of FtsZ bundles was increased in the presence of cinnamaldehyde. Inhibition in GTPase activity
was also observed. It was proposed that OTBA inhibited the Z-ring dynamics by promoting the assembly and stability of FtsZ protofilaments.

**Curcumin**

Curcumin is a naturally occurring polyphenolic compound that has been used as an important dietary ingredient for centuries. Curcumin has also shown antibacterial activity against a number of gram-positive and gram-negative bacteria. Rai et al. reported that curcumin induced the filamentation of *B. subtilis* 168 cells, and inhibited the cell proliferation of *B. subtilis* and *E. coli* (MIC: 17±3 µM for *B. subtilis*; 100 µM for *E. coli*).\(^\text{41}\) Immunofluorescence microscopy indicated the inhibition of Z-ring formation without any effect on segregation and organization of the nucleoids. Curcumin was found to inhibit FtsZ polymerization, while enhancing GTPase activity. The result indicated that curcumin decreased the stability of FtsZ protofilaments by increasing its GTPase activity.

**Berberine**

Berberine is a natural product isolated from various species of *Berberis*. Berberine inhibits the over growth of pathogenic gram-positive as well as gram-negative bacteria such as *B. subtilis*, *Staphylococcus aureus*, *E. coli* and MRSA.\(^\text{42,43,44}\) Domadia et al. showed that berberine inhibited FtsZ assembly (IC\(_{50}\) 10±2.5 µM) and GTPase activity (IC\(_{50}\) 16.01±5 µM).\(^\text{45}\) Electron microscopy images further supported the destabilization of FtsZ protofilaments by berberine, wherein FtsZ filaments were found to be thin, short and curved. Fluorescence study of FtsZ-bound TNP-GTP (TNP = 2,4,6-trinitro-2,5-cyclohexadienyldiene) implied that the binding site of berberine was in the vicinity of GTP binding pocket, overlapping with hydrophobic residues of the active site of FtsZ, which was supported by AutoDock and saturation transfer difference (STD)-NMR.

**Stathmin-derived I19L peptide**

Clement et al. showed I19L, a peptide derived from the N-terminal region of stathmin, hampered tubulin polymerization.\(^\text{46}\) Since tubulin and FtsZ exhibit similar folding patterns, the effect of I19L on FtsZ polymerization was also studied.\(^\text{47}\) Sedimentation assay on *E.coli* FtsZ
showed that I19L co-sedimented mainly with FtsZ bundles and thereby affected the bundling of FtsZ. A combination of docking studies and Saturation Transfer Difference–NMR (STD-NMR) experiments indicated a possible binding site of I19L in *E. coli* FtsZ near the GTP binding pocket.

**4,6-Diamidino-2-phenylindole (DAPI)**

Extending the study on tubulin-DAPI binding,48,49,50 Nova *et al.* investigated the characterization of DAPI interaction with *E. coli* FtsZ.51 Polymerization kinetics indicated that DAPI binding increased FtsZ polymer stability in a concentration dependent manner. The GTPase assay illustrated that DAPI behaved as a non-competitive inhibitor of *E.Coli* FtsZ with a $K_i$ of 29.4±0.3 µM. Anisotropy fluorescence measurements suggested that the binding of one mole of DAPI per mole of *E.Coli* FtsZ was responsible for the bundling of protofilaments and the inhibition of GTPase activity.

**PC58538 and PC170942**

Stokes *et al.* screened a library of 105,000 synthetic compounds against *B. subtilis* by adopting newly designed microtiter plate assay in combination with phase-contrast microscopy, and identified **PC58538**.52 The FtsZ GTPase assay showed that **PC58538** inhibited FtsZ assembly in a dose-dependent manner (IC$_{50}$ 362 µM; K$_i$ 82 µM). **PC170942**, an analog of **PC58538**, was found to be more potent than its parent molecule (IC$_{50}$ 44 µM; K$_i$ 10µM). In addition, fluorescent imaging of GFP-FtsZ expressing *B. subtilis*, treated with **PC58538** (128 µg/mL) lacked the band of FtsZ-GFP formed at the midpoint of the untreated cell.

**PC190723**

Ohashi *et al.* reported the effect of 3-methoxybenzamide (3-MBA) on septation via inhibition of FtsZ cytokinesis.53 Haydon *et al.* designed and screened more than 500 analogs of 3-MBA against various pathogenic bacterial strains and found that **PC190723** exhibited potent antibacterial activity against *B. subtilis, MRSA* and *MDRSA* with MIC values in the range of 0.5-1 µg/mL.54 **PC190723** inhibited the GTPase activity of *S. aureus* FtsZ in a dose dependent
manner (IC$_{50}$ 55 ng/mL). The docking study of **PC190723** suggested that the interaction of **PC190723** with H7 could contribute to the inhibition of GTPase activity.

**Table IV-2. Compounds targeting FtsZ for bacteria other than Mtb**

<table>
<thead>
<tr>
<th></th>
<th>Compound</th>
<th>Target species</th>
<th>Mode of action</th>
<th>MIC/IC$_{50}$ (cell growth inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cinnamaldehyde</td>
<td><em>E. coli, B. subtilis, MRSA</em></td>
<td>Inhibits GTPase activity, FtsZ polymerization and FtsZ protofilament bundling (<em>E. coli</em>); reduction in Z-ring/unit cell; conformational change blocking optimal contact with the GTP binding T1-T6 loop in a neighboring monomer</td>
<td>1 µg/mL (<em>E. coli</em>) 0.5 µg/mL (<em>B. subtilis</em>) 0.25 µg/mL (MRSA)</td>
</tr>
<tr>
<td>2</td>
<td>OTBA</td>
<td><em>E. coli, B. subtilis</em></td>
<td>Inhibition of GTPase activity and enhanced FtsZ polymerization (<em>E. coli and B. subtilis</em>); promoting assembly and stabilization of FtsZ polymer.</td>
<td>2 µM (<em>B. subtilis</em>)</td>
</tr>
<tr>
<td>3</td>
<td>Curcumin</td>
<td><em>E. coli, B. subtilis</em></td>
<td>Filamentation of <em>B. subtilis</em> 168 cells, inhibition of Z-ring formation without affecting organization of nucleoids; inhibition of FtsZ polymerization and enhancement of GTPase assay (<em>E. coli</em>)</td>
<td>17 µM against (<em>B. Subtilis</em>) 100 µM (<em>E. coli</em>)</td>
</tr>
<tr>
<td>4</td>
<td>Berberine</td>
<td><em>E. coli, B. subtilis, S. aureus</em></td>
<td>Inhibits GTPase and FtsZ polymerization (<em>E. coli</em>); destabilization of FtsZ assembly-thin, short and curved FtsZ protofilaments; binding site in the vicinity of GTP binding site.</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>PC190723</td>
<td><em>B. subtilis, S. aureus</em></td>
<td>Inhibits GTPase activity of <em>S. aureus</em> FtsZ by possible interaction with H7 (docking study); discrete foci throughout the elongated <em>B. subtilis</em> 168 cell, indicating mislocalization of Z-ring.</td>
<td>0.5-1 µg/mL against *B. subtilis, S. aureus, MRSA, MDRSA</td>
</tr>
<tr>
<td>6</td>
<td>PC58538 and PC170942</td>
<td><em>B. subtilis, S. aureus, MRSA</em></td>
<td>Long aseptate filaments; inhibition of GTPase activity in a dose dependent manner.</td>
<td><em>B. subtilis</em> 168 PC58538 128 µg/mL PC170942 16 µg/mL S. Aureus PC170942 64 µg/mL</td>
</tr>
<tr>
<td>7</td>
<td>DAPI</td>
<td><em>E. coli</em></td>
<td>Enhancing FtsZ protofilament stability while inhibiting GTPase activity; bundling of FtsZ protofilament</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Statmin-derived 119L peptide</td>
<td><em>E. coli</em></td>
<td>Inhibition of FtsZ polymerization, bundling of FtsZ protofilaments; proposed binding site near GTP binding pocket.</td>
<td>-</td>
</tr>
</tbody>
</table>
§ 4.3 Results and discussion

§ 4.3.1 Preliminary screening against different bacterial strains

As discussed previously, FtsZ is a highly conserved and ubiquitous protein in various bacterial strains. Therefore, in collaboration with Professor Slayden’s laboratory at Colorado State University, a library of trisubstituted benzimidazoles (~1000 compounds) was screened against several pathogens other than *Mtb*. Remarkably, a large number of compounds showed significant activity exhibiting greater than 90 % growth inhibition at 1 µg/mL against *F. tularensis* (Figure IV-4).

![Structures of 2,5,6-trisubstituted benzimidazoles against *F. tularensis* at 1 µg/mL with > 90 % growth inhibition](image)

- R = Benzylxoy, X=S, SB-P10C5
- R = 2-methoxyphenyl, X=S, SB-P9C8
- R = 2-methoxyphenyl, X=O, SB-P9B8
- R = 1-naphthoyl, X=O, SB-P9B9

**Figure IV-4. Structures of 2,5,6-trisubstituted benzimidazoles against *F. tularensis* at 1 µg/mL with > 90 % growth inhibition**

Unexpectedly, in comparison to *Mtb* FtsZ inhibitors where we identified only one 2,5,7-trisubstituted benzimidazole SB-P5C1, there were a large number of 2,5,7-trisubstituted benzimidazole hits against *F. tularensis* (Figure IV-5).
Figure IV-5. Structures of 2,5,7-trisubstituted benzimidazoles against *F. tularensis* at 1 µg/mL with > 90 % growth inhibition

In addition, several hits against *F. tularensis* exhibiting 50-60 % growth inhibition at 0.2 µg/mL were also identified (Figure IV-6).

Figure IV-6. Structures of hit benzimidazoles against *F. tularensis* with 40-50 % growth inhibition 0.2 µg/mL

Furthermore, several of these benzimidazoles showed moderate growth inhibitory activities (50-80 %) at 1 µg/mL against *Y. pestis* and *B. thailandensis* as well (Figure IV-7 and Figure IV-8).
§ 4.3.2 Ex-vivo efficacy of hit benzimidazoles against B. thailandensis and F. tularensis

Since a good number of lead compounds active against F. tularensis and B. thailandensis was identified, ex-vivo efficacy of these compounds were evaluated. The effect of three active benzimidazoles, SB-P8G10, SB-P4B10 and SB-P5D11 on B. thailandensis Bt38 efflux mutant infection of RAW macrophages was studied. As shown in the Figure IV-5, SB-P8G10 and SB-P4B10 significantly reduced the B. thailandensis growth ex vivo at 25 µg/mL and 10 µg/mL, respectively (Figure IV-9). SB-P4B10 and SB-P5D11 would probably have been more effective at higher concentrations, but RAW cell toxicity of these compounds, which was found to be 25 µg/mL, prevented testing at higher concentrations. Since these assays were done by using compounds from the plate which are not analytically pure, the assays will be repeated with pure
compounds to obtain more accurate results. Nevertheless, **SB-P8G10** at 25 µg/mL resulted in a 2 log reduction in *B. thailandensis* compared to untreated control cells.

![Graph](image)

**Figure IV-9. Effect of benzimidazoles on *B. thailandensis* Bt38 efflux mutant infection of RAW macrophages**

RAW macrophages infected with *B. thailandensis* Bt38 efflux mutants were treated with various benzimidazoles and different concentrations. After the treatment duration the cells were homogenized and transferred to 7H11 agar plates for CFU enumeration. (ns: non-significant reduction in log_{10} CFU)

Similarly, lead compounds active against *F. tularensis*, **SB-P9C8**, **SB-P9B8**, **SB-P8B8**, **SB-P9B9**, and **SB-P10C5**, were subjected to ex-vivo efficacy assay against *F. tularensis* LVS (live vaccine strain) ex vivo infection of RAW macrophages. With the exception of **SB-P8B8**, the compounds were highly effective in reducing infection at the concentration range of 10-50 µg/mL (**Figure IV-10**). There was a 2-3 Log reduction in the colony forming units (CFU) per mL which is considered to be significant. The assays strongly suggest that these compounds are very effective in controlling infections in macrophages. As mentioned before, these assays will be repeated using analytically pure compounds to confirm the results.
Figure IV-10. Effect of benzimidazoles on *F. tularensis* LVS infection of raw macrophages

RAW macrophages infected with *F. tularensis* LVS were treated with various benzimidazoles and different concentrations. After the treatment duration the cells were homogenized and transferred to 7H11 agar plates for CFU enumeration.

§ 4.3.3 Resynthesis of 2,5,6-trisubstituted hit benzimidazoles for accurate MIC\textsubscript{99} determination:

For accurate MIC determination, the 2,5,6-trisubstituted benzimidazole intermediates required for the final acylation to obtain the library of analytically pure active compounds were resynthesized. **Scheme IV-1** outlines the synthesis described in the **Chapter II** (refer to chapter-II for synthesis and characterization details of the intermediates).
Scheme IV-1. Synthesis of 2,5,6-trisubstituted benzimidazole intermediates

These intermediates were then used for the final acylation to generate the hit benzimidazoles in relatively moderate yield, as outlined in Scheme IV-2 (Table IV-3).

Scheme IV-2. Synthesis of hit compounds
### Table IV-3. Re-synthesis of the hit compounds

<table>
<thead>
<tr>
<th>Entry</th>
<th>Compound</th>
<th>( R'R'' )</th>
<th>( R_2 )</th>
<th>( X )</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV-1</td>
<td>SB-P4B1</td>
<td>( \text{N} )</td>
<td>( \text{Ph} )</td>
<td>( \text{CO} )</td>
<td>58%</td>
</tr>
<tr>
<td>IV-2</td>
<td>SB-P4D1</td>
<td>( \text{N} )</td>
<td>( \text{Ph} )</td>
<td>( \text{MeO} )</td>
<td>42%</td>
</tr>
<tr>
<td>IV-3</td>
<td>SB-P4E1</td>
<td>( \text{N} )</td>
<td>( \text{Ph} )</td>
<td>( \text{F} )</td>
<td>48%</td>
</tr>
<tr>
<td>IV-4</td>
<td>SB-P2A5</td>
<td>( \text{N} )</td>
<td>( \text{Ph} )</td>
<td>( \text{Ph} )</td>
<td>44%</td>
</tr>
<tr>
<td>IV-5</td>
<td>SB-P8A11</td>
<td>( \text{N} )</td>
<td>( \text{Ph} )</td>
<td>( \text{Ph} )</td>
<td>51%</td>
</tr>
<tr>
<td>IV-6</td>
<td>SB-P10C5</td>
<td>( \text{N} )</td>
<td>( \text{S} )</td>
<td>( \text{Ph} )</td>
<td>48%</td>
</tr>
<tr>
<td>IV-7</td>
<td>SB-P9C8</td>
<td>( \text{N} )</td>
<td>( \text{S} )</td>
<td>( \text{Ph} )</td>
<td>52%</td>
</tr>
<tr>
<td>IV-8</td>
<td>SB-P9B8</td>
<td>( \text{N} )</td>
<td>( \text{F} )</td>
<td>( \text{Ph} )</td>
<td>56%</td>
</tr>
<tr>
<td>IV-9</td>
<td>SB-P8G11</td>
<td>( \text{N} )</td>
<td>( \text{Ph} )</td>
<td>( \text{Ph} )</td>
<td>57%</td>
</tr>
<tr>
<td>IV-10</td>
<td>SB-P1D11</td>
<td>( \text{N} )</td>
<td>( \text{Ph} )</td>
<td>( \text{Ph} )</td>
<td>54%</td>
</tr>
<tr>
<td>IV-11</td>
<td>SB-P4B10</td>
<td>( \text{N} )</td>
<td>( \text{Ph} )</td>
<td>( \text{NH} )</td>
<td>45%</td>
</tr>
</tbody>
</table>
Figure IV-11. Percent growth inhibition of *F. tularensis* LVS 110408 by lead benzimidazoles

The analytically pure re-synthesized active compounds were sent to our collaborator Prof. Slayden’s laboratory for accurate MIC determination using Alamar Blue Assay. The results for the first few compounds indicated that most of the lead benzimidazoles inhibited *F. tularensis* LVS 110408 growth with MIC\(_{90}\) values in the range of 1.8-12.9 µg/mL (*Figure IV-11 & Figure IV-12*).

![Chemical structures](image)

*Figure IV-12. MIC\(_{90}\) of lead benzimidazoles against *F. tularensis* LVS 110408*
§ 4.4 Conclusion

As FtsZ is a highly conserved protein in different bacterial strains, in-house libraries of trisubstituted benzimidazoles were screened against several pathogens. Remarkably, a large number of hit compounds against F. tularensis, Y. pestis, B. thailandensis exhibiting greater than 90% growth inhibition at 1 μg/mL was identified. Based on more accurate alamar blue assay, analytically pure compounds exhibited MIC₉₀ values in the range of 1.16-12.9 μg/mL against F. tularensis. Ex-vivo efficacy data indicated that SB-P8G10 and SB-P4B10 significantly reduced the B. thailandensis growth at 25 μg/mL and 10 μg/mL, respectively. Furthermore, with the exception of SB-P8B8, several compounds were highly effective in reducing F.tularensis LVS infection at the concentration range of 10-50 μg/mL. There was a 2-3 Log reduction in the colony forming units (CFU) per mL which is considered to be exceedingly significant.
§ 4.5 Experimental Section

Methods: $^1$H and $^{13}$C NMR spectra were measured on a Varian 300, 400 or 500 MHz NMR spectrometer. Melting points were measured on a Thomas Hoover Capillary melting point apparatus and are uncorrected. TLC was performed on Merck DCalufolien with Kieselgel 60F-254 and column chromatography was carried out on silica gel 60 (Merck; 230-400 mesh ASTM). High-resolution mass spectra were obtained from Mass Spectrometry Laboratory, University of Illinois at Urbana-Champaign, Urbana, IL. Purity was determined by Agilent 1100 series HPLC assembly.

Materials: The chemicals were purchased from Sigma Aldrich Co., Synquest Inc. and purified before use by standard methods. Tetrahydrofuran was freshly distilled from sodium metal and benzophenone. Dichloromethane was also distilled immediately prior to use under nitrogen from calcium hydride.

Synthesis of analytically pure trisubstituted benzimidazoles

5-Benzoyloxycarbonylamino-6-piperidinyl-2-thiophen-$^1$H-benzo[d]imidazole (SB-P10C5, IV6).

To a solution of 5-amino-2-thiophenyl-6-(N-piperidinyl-$^1$H-benzo[d]imidazole (200 mg, 0.67 mmol) in dichloromethane (10 mL) was added a solution of N-benzyloxycarbonyloxsuccinimide (167 mg, 0.67 mmol) in dichloromethane (5 mL) dropwise at room temperature. After the addition, the reaction mixture was stirred at room temperature overnight. After the completion of the reaction, the reaction mixture was concentrated under reduced pressure. The crude was purified via flash chromatography on silica gel (gradient 20-40% ethyl acetate/hexanes) to give SB-P10C5 (IV-6) as white solid (206 mg, 48 % yield): mp 105-107 ℃; $^1$H NMR (400 MHz, CDCl$_3$) δ 1.69 (m, 6 H), 2.72 (broad s, 4 H), 5.22 (s, 2 H), 6.93
(t, 1 H, \( J = 3.9 \) Hz), 7.27 (d, 1 H, \( J = 4.8 \) Hz), 7.37 (m, 6 H), 8.21 (s, 1 H), 8.27 (s, 1 H); \( ^{13} \)C NMR (100 MHz, CDCl\(_3\)) \( \delta 24.12, 25.80, 27.03, 54.60, 66.97, 101.2, 109.9, 126.5, 127.8, 128.1, 128.2, 128.4, 128.7, 130.1, 133.0, 136.5, 139.8, 147.0, 153.9, 171.3, 173.6; HRMS (ESI) m/z calcd for C\(_{24}\)H\(_{24}\)N\(_4\)O\(_2\)SH\(^+\): 433.1698, Found: 433.1698 (\( \Delta = -0.0 \) ppm).

6-N,N-Diethylamino-5-(cyclobutanamido)-2-tolyl-1\( \text{H} \)-benzo[d]imidazole (SB-P4B1, IV-1):

To a solution of 5-amino-6-N,N-diethylamino-2-(4-methylphenyl)-1\( \text{H} \)-benzo[d]imidazole (115 mg, 0.39 mmol) in dichloromethane (5 mL) was added a solution of cyclobutanecarbonyl chloride (0.45 ml, 0.39 mmol) in dichloromethane (4 mL) dropwise at room temperature. After the addition, the reaction mixture was stirred at room temperature overnight. After the completion of the reaction, the reaction mixture was washed with saturated solution of sodium bicarbonate. The organic layer was dried over anhydrous magnesium sulfate, filtered and concentrated under reduced pressure. The crude was purified by flash chromatography on silica gel (gradient: 20-40% ethyl acetate/hexanes) to give SB-P4B1 (IV-1) as a white solid (85 mg, 58 %): mp >200 °C; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta 0.92 \) (t, 6 H, \( J = 7.2 \) Hz), 1.83 (m, 1 H), 1.95 (m, 1 H), 2.22-2.37 (m, 7 H), 2.92 (q, 4 H, \( J = 7.2 \) Hz), 3.29 (m, 1 H), 7.17 (d, 2 H, \( J = 8 \) Hz), 7.52 (s, 1 H), 7.99 (d, 2 H, \( J = 8 \) Hz), 8.79 (s, 1 H), 9.40 (s, 1 H); \( ^{13} \)C NMR (100 MHz, CDCl\(_3\)) \( \delta 12.90, 17.93, 21.27, 25.69, 41.44, 50.30, 101.6, 112.7, 126.6, 127.4, 129.3, 132.0, 133.1, 135.5, 139.6, 152.6, 173.1; HRMS (ESI) m/z calcd for C\(_{23}\)H\(_{28}\)N\(_4\)OH\(^+\): 377.2341, Found: 377.2341 (\( \Delta = 0.5 \) ppm).

Similar procedure was used for the synthesis of following compounds.
5-Cyclobutanamido-2-(2-methoxyphenyl)-6-N,N-diethylamino-1H-benzo[d]imidazole (SB-P4D1, IV-2)

White solid; 42 % yield; mp 110-112 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 0.95 (t, 6 H, $J = 7.2$ Hz), 1.93 (m, 1 H), 2.01 (m, 1 H), 2.28 (m, 2 H), 2.35 (m, 2 H), 2.96 (m, 4 H, $J = 7$ Hz), 3.23 (m, 1 H), 4.07 (s, 3 H), 7.05 (d, 1 H, $J = 8$ Hz), 7.10 (t, 1 H, $J = 8$ Hz), 7.39 (m, 1 H), 7.59 (s, 1 H), 8.51 (d, 1 H, $J = 7.6$ Hz), 8.69 (s, 1 H), 9.26 (s, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 12.90, 18.01, 25.53, 41.40, 50.35, 55.94, 100.4, 111.4, 113.1, 117.9, 121.6, 129.6, 130.8, 132.6, 135.5, 149.8, 156.6, 172.8; HRMS (ESI) m/z calcld for C$_{23}$H$_{28}$N$_4$O$_2$H$: 393.2289$, Found: 393.2291 ($\Delta = -0.5$ ppm).

5-Cyclobutanamido-2-(4-fluorophenyl)-6-N,N-diethylamino-1H-benzo[d]imidazole (SB-P4E1, IV-3)

White solid; 48 % yield; mp 170-172 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 0.93 (t, 6 H, $J = 7.2$ Hz), 1.81 (m, 1 H), 2.02 (m, 1 H), 1.35-2.19 (m, 4 H), 2.94 (q, 4 H, $J = 7$ Hz), 3.30 (m, 1 H), 7.06 (t, 2 H, $J = 8.8$ Hz), 7.55 (s, 1 H), 8.08 (m, 2 H), 8.78 (s, 1 H), 9.41 (s, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 12.91, 17.96, 25.71, 41.44, 50.32, 101.7, 112.9, 115.6, 115.9, 126.4, 128.7, 132.2, 133.2, 135.9, 139.3, 162.6, 164.8, 173.3; HRMS (ESI) m/z calcld for C$_{22}$H$_{25}$N$_4$FOH$: 381.2091$, Found: 381.2090 ($\Delta = -0.3$ ppm).
5-cyclobutanamido-2-(4-fluorophenyl)-6-\(N,N\)-diethylamino-1\(H\)-benzo[d]imidazole (SB-P2A5, IV-4)

White solid; 44 % yield; mp > 200 °C; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 0.96 (t, 6 H, \(J = 7.2\) Hz), 2.44 (s, 3 H), 3.02 (q, 4 H, \(J = 7\) Hz), 7.09 (t, 2 H, \(J = 7.6\) Hz), 7.24 (m, 3 H), 7.64 (s, 1 H), 7.84 (d, 2 H, \(J = 8.4\) Hz), 7.94 (d, 2 H, \(J = 7.6\) Hz), 9.14 (s, 1 H), 10.36 (s, 1 H); \(^13\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 12.98, 21.48, 50.53, 101.6, 113.3, 126.5, 127.0, 128.5, 129.3, 129.6, 129.9, 132.3, 132.5, 133.1, 135.9, 142.1, 152.5, 165.6; HRMS (ESI) \(m/z\) calcd for C\(_{25}\)H\(_{26}\)N\(_4\)OH\(^+\): 399.2185, Found: 399.2184 (\(\Delta = -0.3\) ppm).

5-Cyclobutanamido-2-cyclohexyl-6-\((N\text{-methylpiperazino})\) -1\(H\)-benzo[d]imidazole(SB-8A11, IV-5)

White solid; 51 % yield; mp > 200 °C; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 1.24-1.36 (m, 4 H), 1.5-1.94 (m, 6 H), 2.08 (m, 3 H), 2.29-2.40 (m, 7 H), 2.64 (broad s, 4 H), 2.81-2.91 (m, 5 H), 3.31 (m, 1 H), 7.48 (s, 1 H), 8.57 (s, 1 H), 8.91 (s, 1 H); \(^13\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 17.95, 25.74, 26.00, 31.71, 38.37, 41.35, 45.96, 52.67, 55.98, 102.4, 110.1, 128.8, 131.9, 137.2, 159.4, 173.0; HRMS (ESI) \(m/z\) calcd for C\(_{23}\)H\(_{33}\)N\(_5\)OH\(^+\): 396.2763, Found: 396.2769 (\(\Delta = 1.5\) ppm).
5-Cyclobutanamido-2-cyclohexyl-6-piperidinyl-1H-benzo[d]imidazole (SB-P8G11, IV-9)

[Chemical structure image]

White solid; 57% yield; mp > 200 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 1.29-1.36 (m, 3 H), 1.63-1.83 (m, 9 H), 2.05 (m, 1 H), 2.08 (m, 3 H), 2.32-2.45 (m, 5 H), 2.84 (m, 5 H), 3.31 (m, 1 H), 7.47 (s, 1 H), 8.59 (s, 1 H), 9.05 (s, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 17.96, 23.92, 25.76, 26.03, 27.15, 31.75, 38.40, 41.44, 54.47, 101.9, 110.1, 128.8, 131.8, 138.1, 138.9, 159.2, 173.1; HRMS (ESI) m/z calcd for C$_{23}$H$_{32}$N$_4$OH$: 381.2654, Found: 381.2654 (Δ = -0.0 ppm).

5-(2-methoxybenzamido)-2-(2-methoxyphenyl)-6-N,N-diethylamino-1H-benzo[d]imidazole (SB-P1D11, IV-10)

[Chemical structure image]

White solid; 54% yield; mp > 200 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 0.96 (t, 6 H, $J = 7.2$ Hz), 3.10 (q, 4 H, $J = 7$ Hz), 4.04 (s, 3 H), 4.06 (s, 3 H), 7.04 (t, 2 H, $J = 7.6$ Hz), 7.09 (q, 2 H, $J = 7.2$ Hz), 7.39 (t, 1 H, $J = 3.2$ Hz), 7.45 (t, 1 H, $J = 3.2$ Hz), 7.65 (s, 1 H), 8.35 (d, 1 H, $J = 8$ Hz), 8.54 (d, 1 H, $J = 8$ Hz), 9.00 (s, 1 H), 11.50 (s, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 12.32, 50.54, 55.62, 55.89, 101.8, 111.2, 11.5, 113.3, 117.9, 121.2, 121.6, 122.6, 129.6, 130.8, 132.4, 132.7, 133.9, 135.9, 149.8, 155.6, 157.2, 162.8; HRMS (ESI) m/z calcd for C$_{26}$H$_{28}$N$_4$O$_3$H$: 445.2240, Found: 445.2240 (Δ = -0.0 ppm).
5-(phenylurea)-6-N,N-diethylamino-2-tolyl-1H-benzo[d]imidazole (SB-P4B10, IV-11)

White solid; 45 % yield; mp > 200 °C; ¹H NMR (400 MHz, CDCl₃) δ 0.53 (t, 6 H, J = 7.2 Hz), 2.30 (s, 3 H), 2.56 (q, 4 H, J = 7 Hz), 7.18 (d, 2 H, J = 8 Hz), 7.23-31 (m, 3 H), 7.39 (m, 2 H), 7.95 (d, 2 H, J = 8 Hz), 8.18 (s, 1 H), 9.34 (s, 1 H), 10.04 (s, 1 H); ¹³C NMR (100 MHz, CDCl₃) δ 11.86, 21.37, 49.17, 102.7, 112.5, 126.3, 126.5, 126.9, 127.5, 129.5, 132.3, 136.3, 140.4, 152.5, 176.5; HRMS (ESI) m/z calcd for C₂₅H₂₇N₅O⁺: 413.2261, Found: 413.2261 (Δ = 0.0 ppm).

5-(2-Methoxybenzamido)-6-piperidinyl-2-thiophen-1H-benzo[d]imidazole(SB-P9C8, IV-7).

White solid; 52 % yield; mp 165-167 °C; ¹H NMR (400 MHz, CDCl₃) δ 1.65 (m, 6 H), 2.74 (broad s, 4 H), 4.01 (s, 3 H), 6.71 (t, 1 H, J = 3.6 Hz), 7.01 (m, 2 H), 7.17 (d, 1 H, J = 4.8 Hz), 7.37 (d, 1 H, J = 3.2 Hz), 7.43 (m, 2 H), 8.89 (s, 1 H), 10.70 (s, 1 H); ¹³C NMR (100 MHz, CDCl₃) δ 23.87, 26.65, 54.39, 56.16, 104.0, 109.6, 111.5, 121.3, 122.6, 126.1, 127.1, 127.5, 129.5, 132.1, 132.9, 133.6, 139.6, 140.8, 147.6, 157.3, 164.0; HRMS (ESI) m/z calcd for C₂₄H₂₄N₄O₂SH⁺: 433.1698, Found: 433.1699 (Δ = 0.2 ppm).

5-(2-methoxybenzamido)-2-furyl-6-piperidinyl-1H-benzo[d]imidazole (SB-P9B8, IV8)

137
White solid; 56 % yield; mp 155-157 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 1.66 (m, 6 H), 2.74 (broad s, 4 H), 4.06 (s, 3 H), 6.32 (d, 1 H, $J = 3.6$ Hz), 6.97 (d, 2 H, $J = 3.6$ Hz), 7.06 (m, 2 H), 7.42 (s, 1 H), 7.51 (t, 1 H, $J = 8.4$ Hz), 9.01 (s, 1 H), 10.81 (s, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 23.86, 26.62, 54.33, 56.20, 104.1, 109.6, 111.5, 111.8, 121.3, 122.6, 126.1, 129.8, 131.4, 132.5, 132.9, 139.2, 140.9, 142.9, 144.1, 145.7, 157.3, 163.9; HRMS (ESI) m/z calcd for C$_{24}$H$_{24}$N$_4$O$_3$H$: 417.1927, Found: 417.1924 (Δ = -0.7 ppm).
§ 4.6 References


Chapter V

Synthesis of CMPF for Dual-Targeting Taxoid Conjugate

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§ 5.1. Introduction

Cancer is the collection of diseases which is characterized by the uncontrolled growth of undifferentiated cells known as tumor cells. Despite successful research in discovering antitumor drugs such as discodermolide, epothilone, doxorubicin, there is still no significant reduction in the death rates caused due to cancer. Cancer is the second leading cause of death in the U.S causing 7 million deaths every year—or 12.5% of deaths worldwide.¹

One of the key problems associated with conventional chemotherapy is its inability to differentiate between the tumor cells and healthy cells because of their similar physiological and biochemical processes. This lack of selectivity not only leads to undesirable side effects such as hair loss, liver and kidney damage, and bone marrow defects but also lowers drug efficiency. In order to achieve effective tumor specific drug delivery, it is required to understand the basic difference between the physiological and biochemical processes of normal and malignant cells. Rapidly growing tumor cells require large amount of nutrients and vitamins in comparison to normal cells. Therefore, tumor cells overexpress certain receptors which are specific to folic acid²-³, biotin⁴, benzamide-riboside⁵, intestinal oligopeptides⁶, hyaluronic acid, monoclonal antibodies⁷ etc. These tumor specific receptors can be used as targets to selectively deliver cytotoxic agents into malignant cell.⁸ Consequently, the cytotoxic warheads can be attached to tumor targeting moieties through a suitable linker to form a drug conjugate (Figure V-1).

![Figure V-1. Basic structure of drug conjugate](image-url)
Before internalization, the drug conjugate should be non-toxic and the linker should be stable during circulation. Many linkers like peptides, hydrazones, esters etc. have been designed and successfully used for drug delivery. In Ojima research laboratory, disulfide linkers, which are cleaved inside the tumor cells through disulfide exchange with intracellular thiol such as glutathione, are used. Based on the results from preclinical models, disulfide linkers have been found to be more efficient in comparison to several other linkers.

Another morphological difference between the malignant and normal cells is defective tumor vasculature and under developed lymphatic drainage system which cause abnormal molecular and fluid transport in malignant cells. This phenomenon of enhanced permeability and retention of large molecules and lipids in solid tumors is termed ‘enhanced permeability and retention’ (EPR) or passive effect as they are receptor independent. Thus, macromolecular cytotoxic agents or cytotoxic macromolecular drug conjugates may be expected to show enhanced degree of selectivity to malignant cell. Various approaches have been investigated to generate macromolecular cytotoxic drug conjugates by using synthetic polymers, dendrimers, proteins, carbon nanotubes etc. as macromolecule components. One of the promising approaches is the use of endogenous proteins like albumin, transferrin as macromolecular conjugates. The advantage with endogenous proteins lies in the fact that they can easily be taken up by malignant cells providing additional selectivity.

![Figure V-2. Dual targeting taxoid conjugate](image)

Albumin is the most abundant blood plasma protein which helps the transportation of long chain fatty acids. In recent studies, it was shown that malignant cells exhibit enhanced albumin uptake. Hence, an albumin-targeting conjugate containing the
cytotoxic component can be another effective approach towards achieving drug specificity.\textsuperscript{17} On the basis of these findings, dual-targeting taxoid conjugate composed of cytotoxic warhead, tumor targeting moiety, albumin targeting moiety, linker and spacer has been proposed (Figure V-2). The dual-targeting taxoid conjugates are designed to target the tumor cells at two different levels. As discussed earlier, tumor targeting moieties direct the drug conjugate to the overexpressed receptors on the tumor cells where upon internalization the active drug is released. The other aspect of dual-targeting is the presence of albumin targeting moieties. The attachment of drug conjugate to albumin will generate a macromolecular conjugate. According to the EPR effect, the macromolecular drug conjugate will show enhanced permeability and retention to the cancer cells.\textsuperscript{12}

An endogenous ligand, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) will be used as albumin-targeting moiety because of its favored water solubility and high binding affinity ($K_A =$4.6×10$^6$ M$^{-1}$) to albumin.\textsuperscript{18} As both the carboxyl groups in CMPF are required for the efficient binding to albumin, it can be attached to the conjugate by introducing an amino group at the propyl terminal. In the previous work by Ojima lab, different CMPF derivatives were prepared (Figure V-3) and their binding affinity to bovine serum albumin (BSA) and human serum albumin (HSA) was evaluated using isothermal titration calorimetry (ITC). Except for the fluorescent CMPF derivative, other derivatives either showed very weak or negligible binding affinity. This led to the assumption that the presence of a polar amino group affects the binding of CMPF derivative to albumin.

![Figure V-3. Derivatives of CMPF with their binding affinities to albumin](image-url)
This study led to the suggestion that a tert-butylthiol derivative of CMPF can tolerate the polarity of the molecule. Unfortunately, the binding affinity of tert-butylthiol derivative of CMPF was weaker than that of CMPF and higher than that of SJ183 or SJ172 (Figure V-4). This implied that the propyl group on CMPF is important for strong binding. The results suggested that increasing the hydrophobicity of the propyl arm by longer alkyl chain may enhance the binding affinity of CMPF.

Figure V-4. Binding affinity if methyl ester and N-methyl amide derivatives of CMPF to HSA.

In order to come up with alternative attachment site to the drug conjugate, the binding affinity of methyl ester and N-methylamide-CMPF derivatives were evaluated using ITC. The mono-amides showed weaker binding than the corresponding esters and the bis-amide showed no activity. The binding affinity of CMPF-2-NHMe (7.07 × 10^4 M^-1) was higher than that of CMPF-3-NHMe (1.85 × 10^3 M^-1) (Figure V-4). Though the binding affinity of CMPF-2-NHMe is 24 times weaker than that of the parent CMPF, the binding mode was still conserved. Thus CMPF may be attached to the dual-targeting conjugate through the amide linkage at the 2-position of CMPF (Figure V-5).
As previously discussed, the propyl arm in CMPF cannot be replaced with polar or bulky substituents. It is suggested that the substitution of the propyl arm by longer alkyl chains may improve the binding affinity. Accordingly, synthesis of CMPF derivatives having one and two carbon units more than parent CMPF molecule at the position-5 was proposed (Scheme V-1). Subsequently, their binding affinity to albumin will also be evaluated by ITC.

Scheme V-1. Proposed synthesis of CMPF analogues
§ 5.2 Results and Discussion

§ 5.2.1 Synthesis of CMPF derivatives

The synthesis of CMPF derivatives started with the preparation of Methyl 5-Formyl-2-(3-methoxy-3-oxopropyl)-4-methylfuran-3-carboxylate (V-3). The condensation of potassium 3-methoxy-3-oxopropanoate with 4-methoxy-4-oxobutanoic acid in the presence of N,N'-carbonyldiimidazole and magnesium chloride gave 3-oxohexanedioic acid dimethyl ester V-1 in quantitative yield. Zinc chloride-catalyzed coupling of V-1 with acetol gave V-2 in 58% yield. Using Vilsmeier-Hack formylation, previously stirred solution of phosphorous oxychloride in N,N'-dimethylformamide was added to V-2 to generate V-3 in 94% yield (Scheme V-2).

Scheme V-2. Synthesis of CMPF precursor

Alkyltriphenylphosphonium salts were synthesized by refluxing alkyl bromide and triphenylphosphine in acetonitrile. Propyltriphenylphosphonium bromide V-4a and butyltriphenylphosphonium bromide V-4b were obtained in 89% and 55% yields, respectively (Scheme V-3).

Scheme V-3. Synthesis of alkyltriphenylphosphonium salts
Alkyltriphenylphosphonium salts $\text{V-4a}$ and $\text{V-4b}$ were treated with n-BuLi to form the corresponding phosphorous ylides. To these phosphonium salts, $\text{V-3}$ was added in THF at -78 °C to form $\text{V-5a}$ and $\text{V-5b}$. These products were subjected to ambient pressure hydrogenation to form $\text{V-6a}$ and $\text{V-6b}$ with two steps yield of 70 % and 71 %, respectively. Subsequent hydrolysis of $\text{V-6a}$ and $\text{V-6b}$ with aqueous LiOH gave the final CMPF derivatives $\text{V-7a}$ and $\text{V-7b}$ (Scheme V-4).

Scheme V-4. Synthesis of CMPF analogues

§ 5.2.2 Evaluation of binding affinity of CMPF derivatives to human serum albumin (HSA)

In order to evaluate the effect of substitution at the 5-position, it is necessary to evaluate their binding affinities to HSA by ITC. ITC is a thermodynamic technique that allows the study on the interactions of two species. When these two species interact, heat is either generated or absorbed. By measuring the heat of absorption/generation, binding constants ($K$), reaction stoichiometry ($n$), and thermodynamic parameters including enthalpy ($\Delta H$) and entropy ($\Delta S$) can be accurately determined.
Phosphate buffer of pH 7.4 was used to prepare the solution of HSA and CMPF derivatives V-7a and V-7b. The concentrations of HSA and ligands (CMPF derivatives) used for the ITC measurement were 0.1 mM and 2 mM. The binding constants of V-7a and V-7b to HSA were measured to be $K_A = 7.45 \times 10^5 \text{M}^{-1}$ and $K_A = 3.729 \times 10^4 \text{M}^{-1}$ respectively (Figure V-6). On comparing the binding constants for these derivatives with that of the parent CMPF molecule, we observed that the binding constant of V-7a was one sixth of parent CMPF whereas that of V-7a was almost negligible. The increase in the hydrophobicity of the propyl arm by adding one carbon unit showed significant decrease in the binding constant. The binding constant of CMPF derivative having additional two carbon units was negligible in comparison to parent CMPF molecule.

§ 5.4 Conclusion

An endogenous ligand, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) is known to bind to albumin and can be used as a tumor targeting moiety because of its favored water solubility and high binding affinity to albumin. Several CMPF analogues were designed and synthesized for targeted drug delivery using multi-step organic synthesis in excellent yield. The binding affinity of these analogues to albumin was also studied. In comparison to CMPF, the binding constant of V-7a was one sixth of that of CMPF whereas that of V-7a was almost negligible. It was concluded that the increase in the hydrophobicity of the propyl arm by adding one carbon unit showed significant decrease in the binding constant.
§ 5.5 Experimental Section

General Methods: All chemicals were obtained from commercial sources. Tetrahydrofuran and dichloromethane were dried by passing through drying columns on the Innovative Technologies, Inc. solvent purification system. NMR spectra were recorded on a Varian 300 NMR spectrometer or a Varian 400 NMR spectrometer. The $^1$H and $^{13}$C spectra were calibrated using residual solvent peak as the internal standard (CDCl$_3$: 7.26/77.00 ppm). The $^{31}$P spectra were not calibrated. Melting points were measured on a Thomas Hoover Capillary melting point apparatus and are uncorrected. TLC was performed on Merck DC-alufolien with Kieselgel 60F-254 and column chromatography was carried out on silica gel 60 (Merck; 230-400 mesh ASTM). Isothermal titration calorimetry was performed on MicroCal Instrument at 37 °C. The concentration of human serum albumin and ligands were 0.1 mM and 2 mM respectively in phosphate buffer of pH7.4. The solutions were degassed in a mild vacuum for 5-10 min prior to each measurement. The size of the measurement cell is 1.43 mL and the solution of the ligand was added at 5 μL increments to reach up to 10 equivalents of ligand to albumin.

1,6-dimethyl-3-oxohexanediote (V-1):$^{19}$

To a solution of succinic acid monomethyl ester (3.09 g, 23.44 mmol) in dry THF (20 mL) was added N,N'-carbonyldiimidazole (4.56 g, 28.12 mmol) at 0 °C. The reaction flask was removed from the ice bath and stirred for 1 h under nitrogen atmosphere. The carbon dioxide was released. To the reaction mixture MgCl$_2$ (2.67 g, 28.12 mmol) was added followed by potassium malonate monomethyl ester (4.39 g, 28.12 mmol) and the suspension was stirred at 45 °C under nitrogen for 7.5 h. The reaction was quenched with 1M H$_2$SO$_4$ (30 mL), extracted with EtOAc, dried over MgSO$_4$ and solvent was evaporated. The crude product was purified by column chromatography on silica gel (20 – 35 % EtOAc in hexanes) to afford V-1 as colorless oil (4.41 g, 100 % yield):$^1$H NMR (300 MHz, CDCl$_3$) δ 2.60 (t, 2 H, $J = 7.5$ Hz), 2.85 (t, 2 H, $J = 7.5$ Hz), 3.65 (s, 3 H), 3.49 (s, 1 H), 3.72 (s, 3 H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 27.66, 37.38, 51.87, 52.39, 167.3, 172.7, 200.8. All data are in agreement with literature values.$^{19}$
Methyl 2-(3-methoxy-3-oxopropyl)-4-methylfuran-3-carboxylate (V-2):^21

To a solution of V-1 (4.17 g, 22.1 mmol) and hydroxyacetone (1.64 g, 1 equiv.) in MeOH (2 mL) was added ZnCl$_2$ (2.97 g, 22.1 mmol). The mixture was stirred at 65 °C under nitrogen. After 14 h the reaction mixture was cooled down, quenched with water (20 mL) followed by extraction with EtOAc (3×) and dried over MgSO$_4$. The crude product was purified by column chromatography on silica gel (5 - 7 % EtOAc in hexanes) to afford V-2 as colorless oil (2.91 g, 58 % yield): $^1$HNMR (300 MHz, CDCl$_3$) δ 2.12 (s, 3 H), 2.67 (t, 2 H, $J = 7.6$ Hz), 3.27 (t, 2 H, $J = 7.8$ Hz), 3.68 (s, 3 H), 3.82 (s, 3 H). All data are consistent with the literature values.^21

Methyl 5-Formyl-2-(3-methoxy-3-oxopropyl)-4-methylfuran-3-carboxylate (V-3):^25

Phosphorous oxychloride (2.32 mL, 24.84 mmol) was stirred in DMF (51 mL) at 0 °C for 1 h. The solution was stirred into a flask with V-2 (2.81 g, 12.42 mmol) and stirred for 24 h at room temperature. The solution was poured into ice-cold water (600 mL) followed by saturated aqueous solution of NaHCO$_3$ (120 mL). After stirring for 1 h the solution was extracted four times with EtOAc and dried over MgSO$_4$. The crude product was purified and column chromatography on silica gel (20 – 35 % EtOAc in hexanes) to give V-3 (2.93 g, 95 % yield) $^1$H NMR (300 MHz, CDCl$_3$) δ 2.52 (s, 3 H), 2.76 (t, 2 H, $J = 7.6$), 3.37 (t, 2 H, $J = 7.5$), 3.87 (s, 3 H), 9.72 (s, 3 H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 99.3, 23.9, 30.98, 51.57, 51.81, 116.2, 134.6, 147.3, 163.3, 165.5, 172.0, 177.08; MS(m/z) 255.08 [M$^+$].
Propyltriphenylphosphonium bromide (V-4a): 22

A solution of PPh$_3$ (3.00 g, 11.43 mmol) and bromopropane (1.24 mL, 13.71 mmol) in acetonitrile (5 mL) was refluxed under nitrogen for 12 h. After cooling to room temperature, ether 10 mL was added gradually. The product was precipitated and V-4a collected (3.91 g, 89 % yield): $^1$H NMR (300 MHz, CDCl$_3$) δ 1.22 (t, 3H, $J = 7.3$Hz), 1.68 (m, 2 H), 3.75 (m, 2 H), 7.86-7.26 (m, 15 H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 15.05, 16.32, 23.96, 24.61, 117.4, 118.6, 130.2, 130.4, 133.3, 133.5, 134.8, 134.9; $^{31}$P NMR (121 MHz, CDCl$_3$) δ 25.18. All data are in agreement with literature values. 23

Butyltriphenylphosphonium bromide (V-4b): 22

The same procedure were used for the synthesis of V-4b (1.99 g, 55 % yield): $^1$H NMR (300 MHz, CDCl$_3$) δ 0.91 (t, 3 H, $J = 7$Hz), 1.59 (m, 2 H), 1.69 (m, 2 H), 3.8 (m, 2 H), 7.9- 7.4 (m, 15 H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 13.63, 22.13, 23.49, 23.70, 24.42, 24.48, 117.5, 118.6, 130.3, 133.4, 133.5, 134.9; $^{31}$P NMR (300 MHz, CDCl$_3$) δ 25.39. All data are in agreement with literature values. 23

Methyl 5-(but-1-enyl)-2-(3-methoxy-3-oxopropyl)-4-methylfuran-3-methylcarboxylate (V-5a): 24
To a solution of propyltriphenylphosphonium bromide (130.96 mg, 0.34 mmol) in THF (2 mL) under inert atmosphere at -78 °C was slowly added n-BuLi in hexanes (1.6 M, 0.34 mmol). After stirring for 35 min a solution of V-3 (72 mg, 0.280 mmol) in THF (0.55 mL) was added and the mixture was stirred for 1 h at -78 °C and then 2 h at 0 °C. The reaction was quenched with phosphate buffer (5 mL, pH=3 - 4), extracted with 1:1 EtOAc/hexanes (3×) and dried with MgSO₄. The crude product was purified by flash chromatography (gradient 10 – 20 % EtOAc in hexanes) and the fractions were evaporated in the dark to give the product V-5a as a mixture of E/Z regioisomers. The wet product was immediately subjected to the next step.

**Methyl 2-(3-methoxy-3-oxopropyl)-4-methyl-5-(pent-1-enyl)furan-3-methylcarboxylate (V-5b):**

The same procedure was used for the synthesis of V-5b and the wet product was immediately used in the next step. Butyltriphenylphosphonium bromide (202.22 mg, 0.50 mmol, 1.2 equiv. to V-3) in THF (3 mL), n-BuLi in hexanes (1.6 M,1 equiv. to butyltriphenylphosphonium bromide), V-3 (107.3 mg, 0.280 mmol) in THF (0.82 mL).

**Methyl 5-butyl-2-(3-methoxy-3-oxopropyl)-4-methylfuran-3-carboxylate (V-6b):**

To a solution of V-5b from the previous step in EtOH (5 mL) was carefully added 10 % Pd/C (15 mg) and the suspension was vigorously stirred under 1 atm of H₂ for 24 h. The catalyst was filtered off through celite and the crude product was purified by column chromatography on silics gel(gradient 10 – 20 % EtOAc in hexanes) to give V-6b as colorless oil (44.6 mg, 35.6 % yield): 

**1H NMR (300 MHz, CDCl₃)** δ 0.88 (t, 3 H, J = 6.9 Hz), 1.27 (m, 4 H), 1.54 (m, 2 H), 2.05
(s, 3 H), 2.49 (t, 2 H, J = 7.5), 2.66 (t, 2 H, J = 7.6 Hz), 3.24 (t, 2 H, J = 7.8 Hz), 3.68 (s, 3 H), 3.80 (s, 3 H).

**Methyl 2-(3-methoxy-3-oxopropyl)-4-methyl-5-pentylfuran-3-carboxylate (V-6a):**

![Methyl 2-(3-methoxy-3-oxopropyl)-4-methyl-5-pentylfuran-3-carboxylate](image.png)

Same procedure was used for the synthesis of **V-6a** (24.0 mg, 30% yield). $^1$H NMR (300 MHz, CDCl$_3$): δ 0.89 (t, 3 H, J = 7.2 Hz), 1.30 (m, 2 H), 1.55 (m, 2 H), 2.05 (s, 3 H), 2.50 (t, 2 H, J = 7.2 Hz), 2.65 (t, 2 H, J = 7.9 Hz), 3.24 (t, 2 H, J = 7.8 Hz), 3.68 (s, 3 H), 3.80 (s, 3 H).

**5-butyl-2-(2-carboxyethyl)-4-methylfuran-3-carboxylic acid (V-7a):**

![5-butyl-2-(2-carboxyethyl)-4-methylfuran-3-carboxylic acid](image.png)

To **V-6a** (20.5 mg, .07 mmol) was added 1 M aqueous solution of LiOH (0.36 ml, 0.36 mmol). The emulsion was stirred at room temperature for 42 h. The solution was washed three times with 30 % EtOAc in hexanes and then acidified with a few drops of concentrated aqueous HCl. The precipitated product was extracted with EtOAc, dried over MgSO$_4$ and the solvent was evaporated to yield the product **V-7a** (15.1 mg, 81 % yield): mp. 115-120 °C; $^1$H NMR (300 MHz, CDCl$_3$) δ 0.91 (t, 3 H, J = 7.2 Hz), 1.30 (m, 2 H), 1.55 (m, 2 H), 2.07 (s, 3 H), 2.52 (t, 2 H, J = 7.3 Hz), 2.72 (t, 2 H, J = 7.8 Hz), 3.31 (t, 2 H, J = 7.6 Hz); $^{13}$CNMR (100 MHz, CDCl$_3$) δ 9.61, 13.75, 22.12, 23.63, 25.17, 30.41, 32.12, 113.3, 114.8, 151.0, 160.0, 170.2, 178.5; MS (ESI) m/z 255.1 (M+1) $^+$
2-(2-carboxyethyl)-4-methylfuran-5-pentyl-3-carboxylic acid (V-7b):

The same procedure was used for the synthesis of V-7b (25.2 mg, 80 % yield): mp. 107-109 °C; 

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 0.88 (t, 3 H, $J = 6.7$ Hz), 1.29 (m, 4 H), 1.57 (m, 2 H), 2.07 (s, 3 H), 2.51 (t, 2 H, $J = 7.6$ Hz), 2.71 (t, 2 H, $J = 7.6$ Hz), 3.31 (t, 2 H, $J = 7.8$ Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 9.63, 13.97, 22.34, 23.95, 25.42, 27.95, 31.20, 32.05, 113.2, 114.9, 151.06, 160.0, 170.5, 178.9; MS (ESI) m/z 269.2 (M+1)$^+$
§ 5.6 References


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1927, 60B, 119-22.
Chapter VI

Synthesis of Taxoid-Based Antitubercular Agents for Co-Crystallization With FtsZ and X-ray Study

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§ 6.1 Introduction

Paclitaxel (Taxol®), a microtubule-stabilizing anticancer agent, exhibits modest antibacterial activity against drug-sensitive and drug-resistant *Mtb* strains (MIC 40 µM), although its cytotoxicity against human cancer cell lines (a benchmark for activity against human host cells) is three orders of magnitude more potent (IC$_{50}$ 0.019-0.028 µM). Following the hypothesis that compounds that affect tubulin/microtubule would serve as leads for developing FtsZ inhibitors$^{1-4}$ a library of taxanes was designed and screened for antibacterial activity against *Mtb* cells (*Table VI-1*). Several taxanes exhibited significant anti-TB activity.$^5$ Moreover, it has been shown that novel anti-angiogenic taxoid, IDN539$^{6,7}$, bearing a C-seco-baccatin moiety possesses substantially less cytotoxicity than paclitaxel. Therefore, C-seco analogs of SB-RA-2001 (*Figure VI-1*) were designed, synthesized and evaluated. These novel C-seco-taxanes SB-RA-5001 and its congeners (*Figure VI-1*) were found to exhibit promising anti-TB activity (MIC$_{99}$ 1.25-2.5 µM) against both drug-sensitive and drug-resistant *Mtb* strains without appreciable cytotoxicity (IC$_{50}$ > 80 µM). Thus, the specificity of these novel taxanes to microtubules as compared to FtsZ was completely reversed through systematic rational drug design.

![Figure VI-1. Taxanes as *Mtb* FtsZ inhibitors](image-url)
### Table VI-1. Antimicrobial activities of taxanes against drug-sensitive and multidrug-resistant *Mtb* strains

<table>
<thead>
<tr>
<th></th>
<th>Taxanes</th>
<th>MIC <em>Mtb.</em> (µM)</th>
<th>Cytotoxicity (IC&lt;sub&gt;50&lt;/sub&gt;, µM)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Paclitaxel</td>
<td>40</td>
<td>0.019</td>
<td>0.028</td>
</tr>
<tr>
<td>2</td>
<td>SB-T-0032</td>
<td>5</td>
<td>0.65</td>
<td>0.65</td>
</tr>
<tr>
<td>3</td>
<td>SB-RA-2001</td>
<td>5</td>
<td>4.5</td>
<td>15.7</td>
</tr>
<tr>
<td>4</td>
<td>SB-RA-5001</td>
<td>2.5</td>
<td>&gt;80</td>
<td>&gt;80</td>
</tr>
<tr>
<td>5</td>
<td>SB-RA-5001MeO6</td>
<td>2.5</td>
<td>&gt;80</td>
<td>&gt;80</td>
</tr>
<tr>
<td>6</td>
<td>SB-RA-5011</td>
<td>2.5</td>
<td>&gt;80</td>
<td>&gt;80</td>
</tr>
<tr>
<td>7</td>
<td>SB-RA-5012</td>
<td>2.5</td>
<td>&gt;80</td>
<td>&gt;80</td>
</tr>
</tbody>
</table>

*M. tuberculosis* H37Rv is sensitive to all antibiotics tested. *M. tuberculosis* IMCJ946.K2 is resistant to nine drugs including INH, REF, EB, streptomycin, kanamycin, ethionamide, *p*-aminosalicylic acid, cycloserine and enniomycin. MCF7 and A549 cells: human breast and non-small cell lung cancer cell lines, respectively.

In collaboration with Dr. Lowe, we decided to carry out the co-crystallization of FtsZ with some of the active taxoid-based anti-TB agents to get some information about the binding site of the taxanes-based anti-TB agents. The synthesis of taxoid-based anti-TB agents are shown below (Scheme VI-1 and VI-2).

**Scheme VI-1. Synthesis of SB-RA-2001**
Scheme VI-2. Synthesis of SB-RA-200101
§ 6.2 Results and discussions

Starting with 10-DAB, C-7 hydroxy protection by TES chloride was carried out followed by C-10 hydroxy protection with TES chloride to obtain VI-2 in 94 % yield. C-13 coupling of compound VI-2 with naphthoyl acrylic acid and subsequent deprotection of C-7 and C-10 hydroxy groups lead to VI-4 (SB-RA-2001) (Scheme VI-3).

![Scheme VI-3. Synthesis of SB-RA-2001 (VI-4)](image)

C-7 hydroxy of VI-4 was protected by TES chloride followed by C-10 coupling with N,N-dimethyl glycine to afford compound VI-6. Finally, deprotection of C-7 TES group with HF/pyridine generated compound VI-7 (Scheme VI-4).
§ 6.3 Conclusion

In order to determine the binding site of taxane-based anti-TB agents (previously discovered in our lab), the co-crystallization of FtsZ with taxane is planned to be studied in collaboration with Dr. Lowe. Two taxane-based anti-TB agents were synthesized using multi-step synthetic methodologies developed in our laboratory.
§ 6.4 Experimental Section

Materials: All chemicals were obtained from commercial sources. Tetrahydrofuran and dichloromethane were dried by passing through drying columns on the Innovative Technologies, Inc. solvent purification system. NMR spectra were recorded on a Varian 300 NMR spectrometer or a Varian 400 NMR spectrometer. The $^1$H and $^{13}$C spectra were calibrated using residual solvent peak as the internal standard (CDCl$_3$: 7.26/77.00 ppm). Melting points were measured on a Thomas Hoover Capillary melting point apparatus and are uncorrected. TLC was performed on Merck DC-alufolien with Kieselgel 60F-254 and column chromatography was carried out on silica gel 60 (Merck; 230-400 mesh ASTM).

7-(triethylsilyl)-10-deacetylbaccatin III (VI-1)$^5$: 

![Chemical Structure](image)

To a solution of 10-DAB (500 mg, 0.918 mmol) and imidazole (249.8 mg, 3.67 mmol) in dry $N,N$-dimethylformamide (17.5 mL) was added chlorotriethylsilane (0.46 mL, 2.75 mmol) dropwise via syringe at room temperature, and then the reaction mixture was stirred for 1 h at room temperature and diluted with EtOAc (60 mL). The mixture was washed with water (15 mL x 3), brine (10 mL), dried over anhydrous MgSO$_4$ and concentrated in vacuo. The crude product was purified on a silica gel column using hexanes/EtOAc (2/1 followed by 1/1) as the eluant to give VI-1 as a white solid (568 mg, 96 % yield). $^1$H NMR (300 MHz, CDC$_3$) $\delta$ 0.5 (m, 6H), 0.97 (m, 9H), 1.21 (s, 3H), 1.58 (s, 3H), 1.73 (s, 3H), 1.85 (dt, 1H), 1.99 (s, 3H), 2.23 (s, 3H), 2.24 (s, 3H), 2.47 (ddd, 1H), 3.94 (d, 1H, $J$ = 7.4 Hz), 4.14 (d, 1H, $J$ = 8.4 Hz), 4.32 (d, 1H, $J$ = 8.1 Hz), 4.41 (d, 1H, $J$ = 6.3 Hz), 4.84 (t, 1H), 4.94 (d, 1H, $J$ = 8.4 Hz), 5.14 (s, 1H), 5.19 (s, 1H), 5.58 (d, 1H, $J$ = 7.2 Hz), 7.40 (t, 2H), 7.54 (t, 1H), 8.10 (d, 2H); $^{13}$C NMR (75 MHz, CDC$_3$) $\delta$ 5.1, 6.7, 9.9, 15.1, 19.5, 22.6, 26.8, 37.2, 38.6, 42.7, 47.0, 57.9, 67.9, 72.9, 74.7, 74.8, 76.5, 78.8, 80.7, 84.2, 128.6, 129.4, 130.0, 133.6, 135.1, 141.9, 167.0, 170.7, 210.3. All data are in agreement with literature values.$^5$
7, 10-Bis (triethylsilyl)-10-deacetylbaccatin III (VI-2)⁵:

To a solution of VI-1 (568 mg, 0.862 mmol) in THF was added LiHMDS (0.85 mL of 1.2 M solution) at -40 °C. After the reaction mixture was stirred for 10 min, TES chloride (.173 mL, 1.03 mmol) was added dropwise at -40 °C. The mixture was warmed to 0 °C over a period of 30-60 min and then concentrated in vacuo. Purification of the crude product by silica gel chromatography (hexanes/EtOAc = 4/1 to 1/1) afforded VI-2 as white solid (626 mg, 94 % yield). All data are in agreement with literature values.⁵

13-[3-(2-Naphthyl)prop-2-enoyl]-10-deacetylbaccatin III (VI-4)⁵:

To a solution of 7, 10-Bis (triethylsilyl)-10-deacetylbaccatin III (354 mg, 0.457 mmol), DMAP (279 mg, 2.28 mmol) and the corresponding acid (199 mg, 0.457 mmol) in toluene/dichloromethane (1/1) was added DIC (403 mg, 3.199 mmol) at room temperature with stirring. After stirring for 10-15 h, the solvent was evaporated in vacuo. Purification of the crude product by short silica gel chromatography using ethyl acetate/hexane (4:1) as eluant afforded TES protected C-13 coupling product VI-3 contaminated by DIC-acid complex, which were directly used in the next step.

To a solution of TES protected C-13 coupling product VI-3 (334 mg, 0.350 mmol) in a (1:1) mixture of pyridine and acetonitrile (4 mL/100mg of starting material) at 0 °C was added a 70 % solution of HF in pyridine (1 mL/100 mg of starting material) with stirring. After stirring overnight at room temperature, the reaction was quenched with NaHCO₃ solution and extracted.
with ethyl acetate. The combined organic layers were washed with a saturated solution of copper sulfate and with water, then dried over magnesium sulfate, filtered and concentrated. Purification of the crude product by silica gel chromatography (hexanes/EtOAc = 1/1) afforded to afford VI-\textsuperscript{4} as white solid (Yield: 75 %); \textsuperscript{1}HNMR (300 MHz, CDCl\textsubscript{3}) \( \delta \) 1.15 (s, 3H), 1.26 (s, 3H), 1.70 (m, 2H), 1.79 (s, 3H), 1.93 (m, 1H), 2.09 (s, 3H), 2.24 (m, 1H), 2.30 (s, 3H), 2.46 (dd, \( J = 9.2, 16.0 \) Hz, 1H), 2.65 (m, 1H), 4.02 (d, 1H, \( J = 7.0 \) Hz), 4.26 (m, 4H), 4.99(d, 1H, \( J = 8.3\)Hz), 5.31(s, 1H), 5.71 (d, 1H, \( J = 7.0 \) Hz), 6.19 (m, 1H), 6.61 (d, 1H, \( J = 16.0\) Hz), 7.51 (m, 4H), 7.60 (m, 3H), 7.70 (d, 1H, \( J = 9.2 \) Hz), 7.89 (m, 2H), 8.03(m, 3H). All data are in agreement with literature values.\textsuperscript{5}

\textbf{7-triethylsilyl-13-[3-(2-naphthyl)prop-2-enoyl]-10-deacetylbaccatin III (VI-5)}\textsuperscript{5}:

![Chemical structure of 7-triethylsilyl-13-[3-(2-naphthyl)prop-2-enoyl]-10-deacetylbaccatin III (VI-5)](image)

To a solution of 13-[3-(2-naphthyl)prop-2-enoyl]-10-deacetylbaccatin III (150 mg, 0.206mmol) and imidazole (42 mg, 0.517 mmol) in \( N,N \)-dimethylformamide (DMF, 4 mL) was added chlorotriethylsilane (0.087 mL, 0.898 mmol) dropwise via syringe at 0 \( ^\circ \)C. The reaction mixture was stirred for 1 h at room temperature and diluted with EtOAc (50 mL). The mixture was then washed with H2O (20 mL x 3), brine (20 mL), dried over anhydrous MgSO4 and concentrated. The crude product was purified on a silica gel column using hexanes/EtOAc (1/1) as eluant to give\textit{7-triethylsilyl-13-[3-(2-naphthyl)prop-2-enoyl]-10-deacetylbaccatin III (VI-5)} as a white solid (130 mg, 75%): \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) \( \delta \) 0.81 (m, 6H), 1.00 (m, 9H), s, 3H), 1.26 (s, 3H), 1.70 (m, 2H), 1.79 (s, 3H), 1.93 (m, 1H), 2.09 (s, 3H), 2.24 (m, 1H), 2.30 (s, 3H), 2.46 (dd, 1H, \( J = 9.2, 16.0 \) Hz), 2.65 (m, 1H), 4.02 (d, 1H, \( J = 7.0 \) Hz), 4.26 (m, 3H), 4.51(m, 1H), 4.99(d, 1H, \( J = 8.3\)Hz), 5.31(s, 1H), 5.71 (d, 1H, \( J = 7.0 \) Hz), 6.19 (m, 1H), 6.61 (d, 1H, \( J = 16.0\) Hz), 7.51 (m, 4H), 7.60(m, 3H), 7.70 (d, 1H, \( J = 9.2 \) Hz), 7.89 (m, 2H), 8.03(m, 3H).All data are in agreement with literature values.\textsuperscript{5}
10-\textit{N}, \textit{N}-Dimethylglycyl-13-[3-(2-naphthyl) prop-2-enoyl]-10-deacetylbaccatin III (VI-7)\textsuperscript{5}:

To a magnetically stirred solution of VI-5 (150 mg, 0.178 mmol) in dry DMF/CH\textsubscript{2}Cl\textsubscript{2} (1/2) were added \textit{N},\textit{N}-dimethyl glycine (55.29 mg, 0.536 mmol), DMAP (0.89 mmol), and DIC (0.89 mmol) at room temperature under N\textsubscript{2}. After stirring for overnight, the solvent was removed on a rotary evaporator and the residue was purified by short silica gel chromatography using ethyl acetate/hexane (2:1) as eluent to afford TES protected C- 10 coupling product VI-6 contaminated by DIC-acid complex, which were directly used in the next step.

To a solution of TES protected C-10 coupling product VI-6 in a (1:1) mixture of pyridine and acetonitrile (4 mL/100mg of starting material) at 0 \textdegree C was added a 70 \% solution of HF in pyridine (1 mL/100 mg of starting material) with stirring. After stirring overnight at room temperature, the reaction was quenched with NaHCO\textsubscript{3} solution and extracted with ethyl acetate. The combined organic layers were washed with a saturated solution of copper sulfate and with water, then dried over magnesium sulfate, filtered and concentrated. Purification of the crude product by silica gel chromatography (hexanes/EtOAc/TEA = 1/2/0.1) afforded to afford VI-7 as white solid. Yield: 78\%; \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) \(\delta\) 1.15 (s, 1 H), 1.26 (s, 3H), 1.70 (m, 2H), 1.79 (s, 3H), 1.93 (m, 1H), 2.09 (s, 3H), 2.24 (m, 7H), 2.30 (s, 3H), 2.46 (dd, 1H, \(J = 9.2, 16.0 \text{ Hz}\), 2.65 (m, 1H), 3.45 (s, 2H), 3.91 (d, 1H, \(J = 7.0 \text{ Hz}\)), 4.26 (m, 4H), 4.52(m, 1H), 5.02(d, 1H, \(J = 8.3\text{Hz}\)), 5.71 (d, 1H, \(J = 7.0\text{ Hz}\)), 6.24 (m,1H), 6.43(s, 1H), 6.63 (d, 1H, \(J = 16.0 \text{ Hz}\)), 7.49 (m, 4H), 7.58(m, 3H), 7.71 (d, 1H, \(J = 9.2\text{ Hz}\)), 7.90 (m, 2H), 8.09 (m, 3H); \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}) \(\delta\) 9.5, 15.9, 22.0, 22.9, 27.3, 35.7, 36.3, 36.5, 36.9, 43.3, 46.1, 58.9, 66.4, 70.4, 72.9, 75.3, 76.6, 76.7, 79.7, 81.4, 84.8, 117.1, 123.4, 127.2, 127.9, 128.1, 128.9, 129.2, 129.5, 130.3, 130.8, 131.6, 133.4, 133.5, 133.9, 134.7, 143.8, 147.0, 150.3, 156.4, 195 159.8, 166.6, 167.2, 170.0, 182.2, 206.0. All data are in agreement with literature values.\textsuperscript{5}
§ 6.5 References


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### Chapter VI


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18.87
25.57
25.77
30.82
31.54
38.22
50.18
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98.63
113.06
132.02
134.08
153.91
158.56
231

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