Development of Antimicrobial Agent with Novel Mechanisms of Actions and 1,2,4,5-Tetrazine Click Chemistry and its Application in DNA Postsynthetic Functionalization

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DEVELOPMENT OF ANTIMICROBIAL AGENT WITH NOVEL MECHANISMS OF ACTIONS 

AND 

MECHANISTIC STUDIES OF 1,2,4,5-TETRAZINE CLICK CHEMISTRY AND ITS APPLICATION IN DNA POSTSYNTHETIC FUNCTIONALIZATION 

by 

WEIXUAN CHEN 

Under the Direction of Binghe Wang 

ABSTRACT 

SecA ATPase is a critical member of the Sec system, which is important in the translocation of membrane and secreted polypeptides/proteins in bacteria. Small molecule inhibitors can be very useful research tools as well as leads for future antimicrobial agent development. Based on previous virtual screening work, we optimized the structures of two hit compounds and obtained SecA ATPase inhibitors with IC50 in the single digit micromolar range. These represent the first low micromolar inhibitors of bacterial SecA and will be very useful for mechanistic studies. 

Post synthetic modification is an important and efficient way of DNA functionalization especially in DNA aptamer selection. In this research, the feasibility of norbornene (Neo) modified thymidine triphosphate incorporation was described. Besides, substituted tetrazines have been found to undergo facile inversed electron demand Diels-Alder reactions with “tunable” reaction rates. This finding paves the way to utilize tetrazine conjugation reactions for not only DNA but also other labeling work. 

INDEX WORDS: SecA inhibitors, Antimicrobials, Nature sources, Small molecule inhibitors, tetrazine, click chemistry, DNA post-synthetic functionalization
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WEIXUAN CHEN

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

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Georgia State University

2012
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December 2012
DEDICATION

I want to dedicate this dissertation to my parents (Guoquan Chen and Xiaoyan Ye). Without you, I would not be where I am today. You gave me spiritual as well as material support in my pursuit of knowledge. Your love and care cannot be compared by anything in the world.

“You raise me up, so I can stand on mountains;
You raise me up, to walk on stormy seas;
I am strong, when I am on your shoulders;
You raise me up to more than I can be.” -- Brendan Graham

I also dedicate my work to my wife (Yanni Lin), your love, kindness, support, and encouragements have made everything possible.

Finally, my grandfather (Huarong Chen), wish you could see my accomplishments and may you rest in peace. I love you forever.
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1 DEVELOPMENT OF ANTIMICROBIAL AGENT WITH NOVEL MECHANISMS OF ACTIONS

Abstract: This chapter is mainly based on my publications: Bioorg. Med. Chem. in 2010, from page 1617-1625. SecA ATPase is a critical member of the Sec system, which is important in the translocation of membrane and secreted polypeptides/proteins in bacteria. Small molecule inhibitors can be very useful research tools as well as leads for future antimicrobial agent development. Based on previous virtual screening work, we optimized the structures of two hit compounds and obtained SecA ATPase inhibitors with IC\textsubscript{50} in the single digit micromolar range. These represent the first low micromolar inhibitors of bacterial SecA and will be very useful for mechanistic studies.

1.1 Introduction

1.1.1 Urgent needs of antibiotics with unique mechanism of action

In the history of drug discovery, one can say with a high degree of confidence that no other field has experienced the wide swing of interest and perceived human needs than the field of antimicrobial development. Before antibiotics became widely available in the 1940’s, simple infections routinely led to death. Therefore, interests in new antibiotics were very high at that time and needs were real. However, with the advent of a large number of modern antibiotics in different classes with various mechanisms of action, it was perceived by many that no major effort would be needed to develop new antibiotics because cure could be achieved in essentially all cases of bacterial infection with some rare exceptions, which often were complicated by other factors. However, this thinking has changed in the last decade because of the wide-spread emergence of drug resistant bacterial strains. For example, methicillin-resistant \textit{Staphylococcus aureus} (MRSA) has become a major issue in hospital-related infections. It has been reported that each year there are more deaths caused by MRSA than by HIV and deaths caused by hospital-acquired infection alone stand at about 90,000 per year in the US.\textsuperscript{1} It is expected that bacterial infection will once again become a major disease killing a large number of people if no new
antimicrobials are developed. Despite the realization of the urgent human need for new antimicrobials, research in this direction has been lagging for one simple financial reason. Any new antimicrobials effective against drug resistant strains will not be used as the first line of treatment options (for good reasons). This means that there is not much money to be made. Therefore, pharmaceutical industry is essentially staying away or at least not focusing on new antimicrobials. This makes it even more important that academic labs and other non-profit institutes recognize this long-term need and devote adequate effort to the development of new antimicrobials. Along this line, it is especially important that efforts be focused on developing new therapeutic options that have novel mechanisms of actions and can overcome current drug resistant problems. To help put this review in a historical perspective, Table 1.1 lists major families of antibiotics with their mechanism of actions.

Table 1.1. Major antibiotic classes by mechanism of action

<table>
<thead>
<tr>
<th>Mechanism of action</th>
<th>Antibiotic families</th>
<th>Representative Structures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition of cell wall synthesis</td>
<td>Beta-lactams (penicillins, cephalosporins, carbapenems, monobactams); glycopeptides (vancomycin, teicoplanin); cyclic lipopeptides (daptomycin)</td>
<td>penicillin</td>
</tr>
<tr>
<td>Inhibition of protein synthesis</td>
<td>Tetracyclines; aminoglycosides; oxazolidonones (linezolid); streptogramins (quinupristin-dalfopristin); ketolides; macrolides; lincosamides,</td>
<td>glycopeptide</td>
</tr>
<tr>
<td>Inhibition of DNA synthesis</td>
<td>Fluoroquinolones</td>
<td>Fluoroquinolones</td>
</tr>
<tr>
<td>Inhibition of RNA synthesis</td>
<td>Rifampin</td>
<td>Rifampin</td>
</tr>
</tbody>
</table>
Inhibition of folic acid synthesis | Sulfonamides; trimethoprim

Membrane disorganizing agents | Polymyxins (polymyxin-B, colistin)

Other mechanisms | Metronidazole

1.1.2  *SecA as new target for antimicrobial agent design*

In selecting a new target, the following characteristics are strongly desirable. **First,** the target plays an essential function in bacterial survival and there is no alternative pathway for mitigation and compensation. This also offers the possibility of developing broad-spectrum antimicrobials. **Second,** there is no closely related human homolog to minimize the issue of potential toxicity. **Third,** the target also contributes to bacterial virulence and lethality. With these factors in mind, we are interested in developing inhibitors of bacterial protein secretion with the specific target being **SecA** (Fig. 1), which is an indispensable ATPase of the protein translocation machinery present in all bacteria, is critical for bacterial survival, and is responsible for the secretion of many essential proteins as well as some toxins and additional virulence factors.4-7 All these characteristics make SecA an ideal target for antimicrobial development. Below we discuss in detail this target, the current state of research, and future directions.

1.1.2.1  **SecA is an ATPase**

It is said that in any given organism, membrane and secreted polypeptides/proteins comprise more than 30% of the proteome; and no less than 10% of proteins cross a membrane before arriving at their final locations of function.8,9 In bacteria, more than 30% of the proteins are located in or outside the
cellular cytoplasmic membrane. Several protein transport mechanisms exist in bacteria to allow for the needed transport and translocation.\textsuperscript{10} The Sec-dependent protein translocase consists of oligomer complex of SecYEG and SecDF as membrane proteins\textsuperscript{11,12} and SecA functions as an ATPase that provides the energy for the Sec-dependent protein translocation. When SecA is bound to the SecYEG complex, acidic phospholipids and a precursor protein such as proOmpA (the precursor of outer membrane protein A), it becomes fully active as an ATPase and a protein translocase.\textsuperscript{13,14} Details of the SecA-mediated secretion process have been discussed elsewhere.\textsuperscript{15-17} Briefly, the transport process has the following events (Fig. 1.1): (1) SecB binds the preprotein and then SecA;\textsuperscript{18,19} (2) the pre-protein bound SecA sees and binds to the lateral gate SecY\textsuperscript{20} of the SecYEG hetero-trimeric complex; (3) electrostatic gate opening\textsuperscript{21} occurs due to allosteric regulation; (4) ADP is released from the SecA motor domains\textsuperscript{19,21,22} and SecA C-terminal domain mediated suppression relieves; (5) SecA acquires the translocation ATPase activity,\textsuperscript{14,23} and (6) SecYEG channel loosens\textsuperscript{24} and gets ready for pre-protein to be pushed in by the intramolecular regulator of ATPase activity 1 domain (IRA1) helix-loop-helix element in SecA. As the final step of the secretion, the signal peptide is cleaved by signal peptidase leading to the release of the mature protein, which gets folded later. As a major component of the bacterial Sec-system, domain organization of SecA was revealed by crystallographic structures from different species\textsuperscript{25-30} and by biochemical analyses.\textsuperscript{31-35}
SecA structures have been studied using X-ray crystallography (Table 1.2) and NMR spectroscopy, cryo-EM, atomic force microscopy, small angle X-ray scattering and small angle neutron scattering (SANS). Most of these studies revealed a dimeric structure for Sec A with one exception. Interestingly, in each of the SecA structures, the protomer structures are very similar. However, the orientation of each protomer relative to one another is quite different in every dimeric structure. In most dimers, their C-domains face opposite directions creating anti-parallel structures (Fig. 1.2 A), while one has a parallel orientation (Fig. 1.2 B).

Table 1.2. List of X-ray crystallography structures of SecA

<table>
<thead>
<tr>
<th>Organism</th>
<th>PDB entry</th>
<th>Ligand</th>
<th>Additional structures</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. tuberculosis</td>
<td>1NL3</td>
<td></td>
<td>1NKT (ADP, Mg2+)</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>2IBM</td>
<td>ADP</td>
<td></td>
</tr>
<tr>
<td>T. thermophiles</td>
<td>2IPC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>2FSF</td>
<td></td>
<td>2FSG (ATP), 2FSH (AMP-PNP), 2FSI (ADP)</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>1M6N</td>
<td></td>
<td>1M74 (ADP, Mg2+, SO4^2-)</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>3JV2</td>
<td>ADP, Mg2+, peptide</td>
<td></td>
</tr>
<tr>
<td>B. subtilis</td>
<td>3DL8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. subtilis</td>
<td>1TF5</td>
<td></td>
<td>1TF2 (ADP, Mg2+)</td>
</tr>
<tr>
<td>T. maritima</td>
<td>3JUX</td>
<td>ADP, Mg2+</td>
<td></td>
</tr>
<tr>
<td>T. maritima</td>
<td>3DIN</td>
<td>ADP, Mg2+, BEF</td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>3BXZ</td>
<td>ADP, Mg2+, spermidine</td>
<td></td>
</tr>
</tbody>
</table>
Dimeric SecA proteins were structurally aligned on one of their protomers (the nucleotide binding domain NBD, red; the intramolecular regulator of ATPase 2 IRA2, dark blue; the protein binding domain PBD, yellow and the C-domain, purple) so as to demonstrate the variable position that the second (grey) protomer occupies; arrows indicate C terminus. The figure was created using the UCSF Chimera package. The structures used are: *Escherichia coli* (ecSecA 1;2FSF), *Thermus thermophilus* (ttSecA; 2IPC). Bottom: Residue boundaries of SecA domains of different organisms.

Each SecA protomer contains several sub-structural domains (Fig. 1.2). The nucleotide binding domain (NBD) and the intramolecular regulator of ATPase activity 2 domain (IRA2) forms the “DEAD” motor, the main catalytic moiety of SecA. The ATP binding site of SecA is located at the interface of the NBD and the IRA2 domains. SecA is a member of the superfamily II (SF2) DExH/D proteins, the majority of which are helicases. Other than helicases, to achieve the unique functions of SecA, the preprotein binding domain (PBD) and the C-terminal domain are two key domains that contribute to SecA substrate specificity. The PBD is composed of two sub-domains: an antiparallel β strand (stem) connects PBD and NBD, and a bilobate globular domain (bulb). The C-terminal domain is composed of four substructures: the wing domain (WD), the α-helical scaffold domain (SD), the intramolecular regulator of ATP activity 1 domain (IRA1), and a C-terminal linker (CTL).
1.1.2.2 The ATPase activity of SecA is regulated by the NBD and the IRA2 domains

The opening and closing of the nucleotide-binding cleft, which is located in the gap between the NBD and IRA2 domains, play the key role in SecATPase activity regulation. The complex conformational changes upon binding the DEAD motor and C-terminal domain of SecA to SecYEG complex control the interactions between NBD and IRA2. Before binding to SecYEG, cytoplasmic SecA has high affinity for ADP. Therefore this ADP-bound SecA shows low intrinsic ATPase activity and low affinity for the SecYEG complex. Binding with SecYEG in the membrane weakens the interactions between the NBD and IRA2 domains and stimulates the ATPase activity. The SecA with elevated ATPase activity is named “membrane ATPase.” Besides, binding of the DEAD motor and C-terminal domain of SecA to the SecYEG complex triggers conformational changes at the interface among the IRA1, PBD, and CTL domains. These allosteric changes increase the SecA affinity for signal peptides as well. Consequently, binding of the signal peptide near the stem region of the PBD-NBD interface causes a large rotation of the bulb domain, which drives trapping of the first amino-terminal segment of mature...
pre-protein domains. This binding further increases the ATPase activity of SecA and converts it into a translocation ATPase. At this stage, the C-terminal domain inhibition effect is totally relieved and the Gate 1 salt bridge is open at the base of the DEAD motor. Next, the DEAD motor ADP affinity is lost while the IRA2 detaches from the NBD and becomes disordered. ADP released from the DEAD motor induces the PBD (with the trapped preprotein) conformational changes and the dissociation of SecB. Later ATP binds to the empty nucleotide-binding cleft, brings subsequent conformational changes that causes insertion of SecA along with bound pre-protein into the SecYEG channel. Finally, ATP hydrolysis drives a small segment of the pre-protein release from SecA into the SecYEG channel. The SecA returns to the NBD-IRA2 tight interaction state that forces SecA to exit the SecYEG channel while the PBD moves further along the pre-protein chain and re-attaches onto the following pre-protein segment, which causes ADP release and allows a new round of ATP binding and hydrolysis. In order for the whole pre-protein to cross the channel, the co-insertion of pre-protein through the channel in segments is approximately 30 residues at a time.

1.1.2.3 Established methods to test inhibitory effects on the functions of SecA

Given the essential role that SecA plays, it is easy to understand that this can be an attractive target for the development of broad-spectrum antibacterial agents with a novel mechanism of action. However, effort in developing inhibitors for SecA has been lacking. One reason could be the complexity and difficulties in assaying compounds for inhibitory activities. An added question is how the enzyme assay results would correlate with what is relevant in whole bacteria.

A number of SecA assays are available including ATPase activity assay, protein translocation assay, an electrophysiology assay, and of course ultimately whole cell assay. All ATPase assays rely on the determination of free inorganic phosphate formed as a result of ATP hydrolysis. In the doing so, the malachite green colorimetric method is most commonly used.

In screening for potential inhibitors, the most obvious one is the use of the whole SecA in determining how an inhibitor modifies the ATPase activity. However, whole SecA is in a regulated,
closed-state largely controlled by its regulatory C-terminal (C34) domain. Therefore this is a minimally active state of SecA. Thus results generated using the whole SecA do not truly reflect the ability for the inhibitor to inhibit the fully active ATPase, which is the case when SecA is in membrane. Another assay method uses truncated SecA with only the catalytic domain (N68) in solution (e.g., EcSecA-N68, unregulated ATPase Fig. 1.4). Because of the lack of the inhibitory C-terminal domain, this assay is very sensitive. It has also been demonstrated that results from assays using the truncated form parallels that of the in vivo and membrane SecA assays.60 Another way of avoiding the intrinsic inhibition effect of the C-terminal domain is by using a mutant with elevated intrinsic activities. For example, in EcSecA residue W775 is important for the C34 regulation,64 which is located at the interface of the SD and the IRA1 domains (Fig. 1.3). Replacing the bulky tryptophan with a small hydrophobic alanine weakens the interaction of the SD and the IRA1 domains and elevates the intrinsic ATPase activity by 5-fold compared to the wild-type EcSecA.52,64 Therefore, using this mutated W775A EcSecA allows the benefit of the full-length enzyme without the intrinsic inhibition effect of the C-terminal domain.17

Because the functional form of SecA as a motor exists in the membrane alone (membrane ATPase) or in complexation with SecYEG (translocation ATPase), these two forms can also be used in inhibitor screening.14,65 The only thing is that experimentally they are more cumbersome than the solution-phase enzyme assays described above. For functional assays, in vitro translocation of proOmpA into membrane vesicles (protein translocation assay) developed by the Tai lab is commonly used.66,67 As an easy demonstration that SecA alone can form protein-conducting channel,68 assay for SecA activities in liposome was also developed. Therefore, an array of in vitro assays is available for examining the SecA ATPase activity and protein translocation ability and for cross-validating results.

As with any assay, truly mimicking the physiological conditions is a critical issue. This is especially true when it involves a membrane protein. Along this line, a semi-physiological assay for electrophysiological measurement of protein-channel activity in the oocytes was developed by the Tai lab.68,69 This assay is useful because of the ease of use, the small amount of materials (nanograms) needed, and the ability to study individual oocytes. The large size of oocytes can easily accommodate various
manipulations and electrode penetration and the recording noise is very low due to the large number of channels measured in such experiments (calculated to be 200-1,000,000 channels).\textsuperscript{69} The activity is strictly dependent on the injection of exogenous SecA and membrane vesicles. Results from this assay parallels that of antimicrobial assays very well, further supporting the near physiological nature of this assay system.

It is important to emphasize that because of the membrane environment needed for SecA function as a transporter, inhibition studies need to involve different assay techniques in order to achieve a thorough understanding of the ability for these inhibitors to inhibit SecA functions. It is also possible that different inhibitors may exhibit varying affinities for the different forms of SecA. However, the results from the truncated SecA assay and the electrophysiology oocyte assays seem to parallel that of antimicrobial results. One of our recent publications has a detailed discussion of this aspect.\textsuperscript{60}

**Figure 1.4.** Two separable soluble domains and lipid-specific domain of SecA.

There exist two forms of SecA in the membrane: SecA\textsubscript{S}, which is similar to the soluble form with 2 separable domains: N68 and C34, and the other SecA\textsubscript{M} with the N36 and M48 domains spanning the lipid membrane.\textsuperscript{70} Top: X-ray ribbon structure of EcSecA with N68 (Yellow) and C34 (Green).

### 1.1.3 SecA inhibitors

So far, only a limited number of SecA inhibitors have been reported.\textsuperscript{17} Some are isolated from natural sources (such as fungi and plant) and others are synthetic. The oldest known inhibitor of SecA is
the inorganic azide, which was first reported in 1891\textsuperscript{71} to possess antimicrobial activity. Later on\textsuperscript{72} the antimicrobial activity was attributed to its ability to inhibit SecA mediated protein translocation. However, azide also inhibits many other enzymes such as cytochrome c oxidase,\textsuperscript{73,74} superoxide dismutase,\textsuperscript{75} alcohol dehydrogenase,\textsuperscript{76} and ceruloplasmin.\textsuperscript{77} Therefore, it is hard to imagine how azide can be used as an antimicrobial for \textit{in vivo} applications.

1.1.3.1 SecA inhibitors from natural sources

Two natural products (1 and 2, Fig. 1.5) have been reported to possess modest antibacterial activities by inhibiting SecA. Compound 1(CJ-21058), isolated from an unidentified fungus (CL47745) broth, showed an IC\textsubscript{50} of 15$\mu$g/ml against SecA and an MIC of 5$\mu$g/ml against multi-drug resistant \textit{S. aureus} and \textit{Enterococcus faecalis}.\textsuperscript{78} Interestingly, CJ-21058 was found to be a close analog of Equisetin, a fungal toxin from white mold \textit{Fusarium equiseti}, previously isolated by the same group. Equisetin differs only in having a hydrogen atom at C-4 position of CJ-21058. It is also known to show a broad range of biological activities such as antibacterial, anti-HIV, cytotoxicity and DNA binding properties.\textsuperscript{79} The SecA inhibition activity of CJ-21058 was evaluated using malachite green assay for monitoring ATP hydrolysis using the SecA mediated translocation of proOmpA through the inner membrane vesicles bearing the heterotrimeric SecYEG complex. The striking structural similarities between CJ-21058 and Equisetin as well as their potent antibacterial properties point towards a possible correlation between antibacterial and SecA inhibitory activities of these compounds. A comparison of the common structural features between the two compounds clearly indicates that the methyl group at C-4 position (in CJ-21058) of hydrophobic bicyclic moiety seems to be contributing to the enhancement of antibacterial activity.

Another report of a natural SecA inhibitor came from antisense-based random screening of nearly 115,000 compounds against a sensitized strain of \textit{S. aureus} having an inducible SecA antisense construct. The strategy behind the antisense-based screening involves selectively expressing an antisense RNA, which can bind to the mRNA of the targeted gene, causing its degradation. This results in decreased translation, leading to lesser gene product formation and causing subsequent sensitization of the
weakened strains to the inhibitors of the gene product. A two-plate agar-based differential sensitivity assay was used to selectively screen compounds that target SecA, by comparison of growth inhibition of sensitized SecA antisense strain against wild-type control strain of *S. aureus*. A similar strategy was also used to establish that SecA is essential for *S. aureus* viability.\(^8^0\) Using this methodology, a *cis*-decalin secondary metabolite, pannomycin (2), extracted from a leaf litter fungus *Geomyces Pannorum*, was found to possess weak antibacterial activity against Gram-positive bacteria *B. subtilis* (MIC, 0.4 mM), *S. aureus* (MIC, 1.4 mM) and *E. faecalis* (MIC, 1.4 mM)\(^8^1\). Interestingly, a structural analogue of pannomycin named cissetin also contains a tetramic acid moiety (similar to CJ-21058) and shows a MIC of 10 µM against MRSA strains\(^8^2\). From a structural comparison of common features essential for activity amongst the natural SecA inhibitors (CJ-21058 and pannomycin), the authors speculated that the hydrophobic decalin core with a quaternary carbon atom and the tetramic acid moiety seem to be the minimum structure requirement for SecA inhibition. Although, the inhibitory potential of these natural compounds is only modest, they offer a promising starting point for structure optimization.

![Figure 1.5. Structures of SecA Inhibitors found in nature and their natural analogs](image)

1.1.3.2 Synthetic small-molecule inhibitors

In the field of drug discovery, a common approach to finding new chemical scaffolds active against a target of interest is computer based virtual screening (VS). The method includes both ligand-based and structure-based. There have been some efforts towards high throughput random screening and
structure-based virtual screening of chemical libraries to obtain new scaffolds for SecA inhibition. Researchers at Wyeth-Ayerst\textsuperscript{83} carried a random screening of compound libraries using cell-based assays. The idea was to employ a SecA-lacZ reporter fusion to identify compounds that enhance SecA expression through inhibition of protein secretion. SecA is known to autogenously regulate its expression under normal protein secretion, by binding to its own mRNA, blocking the translation active site and inhibiting its expression. In contrast, inhibition of secretion dissociates the SecA-mRNA complex and causes an up-regulation of SecA. With this approach, both synthetic and natural product libraries were screened for compounds that enhance SecA expression. A set of diverse structures showing modest antibacterial activities was obtained. For example, compound 5 showed a MIC\textsubscript{50} value of 2\mu g/ml for \textit{S. aureus} RN 8081. Compounds 5 and 7 (Fig. 1.6) share some common structural motifs such as the presence of an imino moiety and lipophilic characteristics. However, these compounds were also associated with high cell toxicity (>75% growth inhibition at 10\mu g/ml) due to membrane damage. The compounds reported induce the overexpression of SecA and function by inhibition of protein secretion, but their SecA inhibition activity was not tested. Thus it is not certain whether the mechanism of action is through SecA inhibition. No further efforts have been reported to explore their potential as SecA inhibitors possibly due to the associated high toxicity.

A series of thiazolo[4,5-d]pyrimidine derivatives\textsuperscript{84} were recently reported to inhibit Sec A. The compounds were obtained by using an \textit{in vitro} malachite green screening method employing recombinant

![Figure 1.6. Reported SecA inhibitors 5, 6\textsuperscript{83} and 7\textsuperscript{84}](image)
*E. coli* or *S. aureus* SecA. The compounds were also tested for inhibition of protein translocation using membrane vesicles containing overexpressed SecYEG and the *E. coli* preprotein AlkProPhoA(Cys-). The most potent compound 6 (Fig. 1.6) exhibited mixed-type inhibition with a high IC$_{50}$ value of 135 µM against EcSecA intrinsic ATPase and also showed minor effects on the translocation ATPase activity of SecA with IC$_{50}$ nearly 200 µM. Although, the compounds synthesized showed weak SecA and protein translocation inhibition, the thiazolo[4, 5-d] pyrimidine scaffold introduced by this report has the potential for further optimization to achieve high potency.

The Wang and Tai labs undertook the first rational approach towards discovering new SecA inhibitors based upon *in silico* screening of small molecule ligand libraries. Virtually screening of a library of nearly 115,000 compounds was conducted using structure-based methods against the *E. coli* SecA active site. Nearly 30 top ranked compounds were tested *in vitro* for inhibition of ATPase activity against *E. coli* N68. Two modest inhibitors were obtained (Figure 1.7, 8 and 9) showing IC$_{50}$ values around 100 µM. To obtain a sense of binding patterns and to aid in further structure optimization for analog synthesis, molecular docking of compounds 8 and 9 in the active site of *E. coli* SecA was conducted. The two modestly active compounds showed different structural features and somewhat varied binding orientations, which suggested that there is much room for structural optimization in order to further improve potency. Further optimization of the isoxazolcarboxamide series (8) was carried out with the focus being on the aryl group attached to the amide. In the second series (9), we started by testing different aryl structures flanking the central ring followed by 5-cyano-6-aryl-2-thiouracils derivatives. A further simplified ‘monomer” series of compounds was also prepared to understand the core structural need for inhibition. The compounds were tested *in vitro* using EcN68 SecA and the whole EcSecA. *In vivo* studies were done with a leaky mutant NR698 and wild type *E. coli* strain MC4100.
Two analogs Rose Bengal (RB) and erythrosine B (EB) were found to show strong inhibition on all three forms of SecA, SecA mediated protein translocation and antimicrobial activity (Table 1.3).

Table 1.3. Fluorescein analogs RB and EB and their inhibitory properties

<table>
<thead>
<tr>
<th></th>
<th>RB</th>
<th>EB</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vitro</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(IC$_{50}$/µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unregulated/truncated N68</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>Intrinsic</td>
<td>25</td>
<td>21</td>
</tr>
<tr>
<td>Membrane bound</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>Translocation</td>
<td>0.9</td>
<td>10</td>
</tr>
<tr>
<td>SecA mediated bacterial translocation</td>
<td>0.25</td>
<td>4</td>
</tr>
<tr>
<td><strong>In vivo</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(MIC$_{95}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> NR698 (leaky mutant)</td>
<td>3.1</td>
<td>250-500</td>
</tr>
<tr>
<td><em>B. Subtilis</em></td>
<td>3.1</td>
<td>250-500</td>
</tr>
<tr>
<td><em>E. coli</em> (Wild type)</td>
<td>&gt;1000</td>
<td>&gt;10,000</td>
</tr>
</tbody>
</table>

RB and EB showed IC$_{50}$ values of 0.5 µM and 2 µM respectively against truncated N68. As shown in the Table 3, both RB and EB show increasing IC$_{50}$ trends from translocation, membrane and intrinsic SecA. As expected, RB (25 µM) and EB (21 µM) show higher IC$_{50}$ values against intrinsic SecA than its unregulated counterpart. The IC$_{50}$ values for membrane bound SecA inhibition caused by RB and EB are 5 µM and 12 µM respectively. For the translocation ATPase RB shows IC$_{50}$ value of 0.9 µM and EB shows 10 µM. Protein translocation experiments using the oocyte model show that RB and EB
severely inhibit SecA-dependent *in vitro* protein translocation of precursor proOMpA into the membrane vesicles with IC$_{50}$ values of 0.25 µM and 4 µM respectively. As a validation of principle, the MIC values observed for Gram-negative bacteria (*E. coli*, >1 mM) are much higher than those obtained for Gram-positive (*B. subtilis*, 3.1µM) and the leaky mutant (*E. coli* NR698, 3.1µM), suggesting that outer cellular membrane in Gram-negative bacteria acts as a barrier to inhibitor permeability.

### 1.1.4 Future directions

With the urgent need of developing new antimicrobials with novel mechanisms of action, SecA has emerged as a promising target. There are several aspects to targeting SecA. **First**, the target, SecA, is very novel and has never been successfully explored for antimicrobial development. 86 Although the importance of the secretory machinery such as SecA is obvious in bacterial survival, work in this area has been very limited because of the high degree of difficulty and lack of lead compounds. **Second**, promising leads have been identified. **Third**, the concept of antimicrobial effects through inhibition of SecA has been demonstrated. **Fourth**, bacterial SecA is involved in virulence factors secretion, inhibition of which can greatly attenuate pathogenicity. 87-90 **Fifth**, SecA functions as a membrane protein that spans the bacterial membrane and forms a channel. 68,91 In Gram-positive bacteria, this means that the target may be directly accessible from the extracellular matrix. If this is true, one can envision the design of drugs that enter the target directly from the extracellular matrix and exert their effect without actually entering the cell. The advantage of such a situation is the avoidance of the effect of efflux transporters in bacteria, the expression of which is a major mechanism for the development of multi-drug resistance (MDR) 92-98. Given the wide-spread nature of efflux in bacteria and its importance in drug-resistance, such a finding by itself would be of extraordinary novelty and significance. We hope this review will help stimulate more research in this area so that new antimicrobials can be obtained by targeting SecA.
1.2 Results and Discussions

1.2.1 Chemistry

In our earlier virtual screening efforts, two hits, 8 (SEW-05929) and 9 (HTS-12302), were shown to have modest SecA inhibitory activities (IC\textsubscript{50} values of about 100 μM).\textsuperscript{99,100} Another hit 12, which was obtained from random screening, showed good SecA inhibitory activities (IC\textsubscript{50} values of about 45 μM). Our effort to search for potent SecA inhibitors started with the optimization of these three inhibitors (Figure 1.8). Since at the starting point of our studies, there were no other known SecA inhibitors except one natural product, for which the true inhibition mechanism was not known.\textsuperscript{101}

![Figure 1.8. Hit compounds and their derivatives](image)

Our optimization effort first started with the isoxazole carboxamide series (8) with the focus being on optimizing the aryl group attached to the amide. In this series, 14 analogs were synthesized. The synthesis began with conversion of halogenated benzaldehyde 17 to the corresponding oxime 18 (Scheme 1.1). Isoxazole acid 20 was prepared by reacting 19 with ethyl acetoacetate followed by hydrolysis.\textsuperscript{102} Subsequent coupling/amidation reactions using EDCI and DMAP gave the final isoxazole carboxamide derivatives 21a-n. In this series, there are amides of aniline compounds 21a,e,f,h,i,k,l, benzylamines 21b,m, secondary alkylamines 21c,d,j, and primary alkylamines 21g,n.
In optimizing the second series (9, Figure 1.8), we first started by testing different aryl structures flanking the central ring. In our initial effort, 6-chloro-2-mercaptobenzothiazole and 2-mercaptobenzoxazole derivatives were prepared by reacting potassium ethylxanthate 22 with 2,4-dichloroaniline 23 or substituted 2-aminophenol 24 (Scheme 1.2). Further, 5-cyano-6-aryl-2-thiouracils were prepared by condensation of an aldehyde with ethyl cyanoacetate and thiourea in the presence of piperidine.\textsuperscript{103} The symmetrical compounds 29a-g or 30a-i were obtained by reacting two equivalents of compounds 25a-g or 28a-i with p-xylylene dibromide in acetonitrile in the presence of K$_2$CO$_3$ (Scheme 3). One successful series of analogs was the 2,2'-(α,α'-xylene)sulfanediylbis-(6-aryl-5-cyano-4-oxopyrimidine) 30a-i (see below for biological results). For this series, we were interested in further simplifying the structure to understand the core structural need. Therefore, "monomer" series 31d,e,g-i was prepared by benzylation of compounds 28d,e,g-i with benzyl chloride, and the difference in activities between the "dimer" and "monomer" series was also studied.
Scheme 1.2. Synthesis of compounds 25a-g and 28a-i.

Reagents and conditions: (a) EtOH, reflux; (b) piperidine, EtOH, reflux

Scheme 1.3. Synthesis of compounds 29a-g, 30a-i and 31d,e,g-i.

Reagents and conditions: (a) K₂CO₃, CH₃CN, reflux

1.2.2 Biological evaluation

1.2.2.1 In vitro study

The synthesized compounds were first evaluated using EeN68 SecA, which is a truncated version without the C-terminal regulatory/inhibitory domain, by following procedures published earlier. Briefly,
ATPase activities were determined by the release of phosphate (Pi), which can be detected spectrophotometrically using malachite green. For compounds 21a-n, none of them showed improved activities over the original hit (8) or significant inhibition at 100 μM (Figure 1.9). Such results coupled with the weak activities of the original hit compound led to the decision of not pursuing this class of compounds any further.

Figure 1.9. Inhibitory effect of compounds 8 and 21a-n at 100 μM against EcN68 Sec A.

For analogs of 9, compounds 29a-g did not show significant improvement over the initial hit. However, the substituted thiouracils (30) showed very significant activities when screened at 100 and 30 μM (Figure 1.10). Those compounds that showed potent inhibition at 30 μM were further screened at 5 μM (Figure 1.11). Within the symmetrical compound series 30a-i, there are two substitution patterns: one with a phenyl ring substituted at the 4-position and the other with a phenyl ring substituted at the 3-position. The results showed that the 4-substituted analogs were more potent than the 3-substituted class, which was in turn slightly more potent than the ones without phenyl substituent. For example, the activities of the 4-methyl substituted (30c, Figure 1.10) was higher than the 3-methyl analog (30b, Figure 1.10), which was in turn higher than the un-substituted one (30a, Figure 1.10). With the initial indication that derivatives with a phenyl ring bearing a 4-substituent were more active, the subsequent effort was focused on optimizing this series of compounds. One approach adopted was to use relatively bulky alkyl groups at the 4-position. It turned out that these compounds were more potent than the corresponding 4-methyl substituted compounds. Among these compounds, those with an electron donating (e.g., methoxy)
substituent seemed to be less active than the un-substituted ones (e.g., \(30f < 30a\), Figure 1.10). At 5 \(\mu\)M, analogs with a halogen or aryl group substitution at the 4-position were more potent than the analogues with an alkyl substitution (e.g., \(30g,h,i > 30c,d,e\) Figure 1.11). For the examination of the difference between the “dimers” and “monomers”, \(S\)-benzyl-2-thiouracils analogues \(31d, e, g-i\) were also tested (Figure 1.12). First of all, both thiouracil-based “dimer” and “monomer” compounds showed more potent inhibition than the benzothiazole or benzoxazole compounds \(29a-g\).

![Figure 1.10. Inhibitory effect of compounds 30a-i at 30 \(\mu\)M against EcN68 Sec A.](image1)

![Figure 1.11. Inhibitory effect of compounds 30c-e, g-i at 5 \(\mu\)M against EcN68 Sec A.](image2)

However, the "dimer" series \(30d,e,g-i\) were more potent than the "monomer" series \(31d,e,g-i\), respectively. This higher potency for the “dimer” series seems to come from better fitting of the binding pocket of these compounds (see below). In the “monomer” series, it was observed that a large sized R’
group seemed to confer high potency (e.g., $31h > 31g \approx 31e > 31d$). However, when the substituented phenyl ring was replaced by a larger 1-naphthyl group, the activity seemed to decrease slightly.

![Inhibitory effect of compounds 31d, e, g-i at 30 μM against EcN68 Sec A.](image)

Figure 1.12. The inhibitory effect of compounds 31d, e, g-i at 30 μM against EcN68 Sec A.

We determined the IC$_{50}$ values of compound 30 g and 30 h since they showed the most potent activities of all the compounds screened at 5 μM. The result showed they had low micro molar inhibition (IC$_{50}$: 2 μM, Figure 1.13), which is 50-fold more potent than the hit compound 9 (IC$_{50}$: 100 μM).99

![Inhibitory curves of the two most potent compounds, 30g and 30h, against EcN68 Sec A.](image)

Figure 1.13. The inhibitory curves of the two most potent compounds, 30g and 30h, against EcN68 Sec A.

Inhibition tests using whole EcSecA gave similar results (30g IC$_{50}$: 20 μM, 30h IC$_{50}$: 50 μM and 31h IC$_{50}$: 60 μM, Figure 1.14), which suggest that the EcN68 inhibition assay is more sensitive than the whole SecA inhibition assay. This is understandable since EcSecA contains a regulatory domain, which is essentially an inhibitor.
1.2.2.2 In vivo study

The biological activities of “dimer” and “monomer” compounds \(30h\) and \(31h\) were assessed against leaky mutant NR698 and wild type \(E. coli\) strain MC4100 by determining the minimum inhibition concentration (MIC) (Figure 1.15). “Monomer” compound \(31h\) exhibited the most potent inhibition effects against NR698, whereas “dimer” compounds \(30h\) did not exhibit significantly antimicrobial activities. However, neither \(31h\) nor \(30h\) exhibited inhibition effects against wild type \(E. coli\) strain MC4100. Such results suggest that the permeability of \(30h\) against NR698 and \(31h\) against MC4100 might be a key factor and for \textit{in vivo} applications future studies should focus on low molecular weight compounds such as \(31h\) for structural optimization.
1.2.3 Computational modeling

In order to achieve a detailed understanding the binding mode between SecA and our compounds, \textit{in silico} modeling was conducted by using molecular simulation.\textsuperscript{104-107} Herein, the parent compound, HTS-12302 and the most active compound, 30g, were docked into the ATP site of SecA using DOCK 5.4. The docked complexes were then optimized by molecular mechanics and molecular dynamics simulation implemented in AMBER 8. Finally, the possible ligand-protein interactions were examined by following similar procedures we used in previous studies.\textsuperscript{99} After molecular simulation, compound HTS-12302 seems to bind SecA through interactions with Thr 104 by forming hydrogen bond and with Met 81, Phe 84, Gln 87, Gly 105, Glu 106, Gly 107, Lys 108, Thr 109, Leu 110, Gly 392 and Arg 509 through hydrophobic interactions (Figure 9). Compound 30g has a similar binding conformation and orientation, in which it seems to engage in more hydrogen bond interactions with Phe 84, Gln 87, Lys 108 and Glu 210. Moreover, compound 30g still bears hydrophobic interactions with Met 81, Thr 104, Gly 105, Glu 106, Gly 107, Leu 110 and Arg 509. Upon analysis of the structural features of these two compounds, it seems that the inclusion of the thiouracil moiety may contribute to the inhibitory activity because of more
hydrophobic interaction and hydrogen bonds when compared with lead compound HTS-12302. Such structural insights will play a very critical role in future design of potent SecA inhibitors and in further structural optimizations.

Figure 1.16. (A) The proposed docking conformation of HTS-12302 (white sticks) and compound 30g (green sticks) around SecA ATP-site; (B) The proposed schematic interactions of HTS-12302 with SecA; (C) The proposed schematic interactions of compound 30g with SecA

1.3 Experimental part

1.3.1 Chemistry

**General Chemical Methods.** All reagents and solvents were reagent grade or were purified by standard methods before use. Column chromatography was carried out on flash silica gel (Sorbent 230-400 mesh). TLC analysis was conducted on silica gel plates (Sorbent Silica G UV254). NMR spectra were recorded at $^1$H (400 MHz) and $^{13}$C (100 MHz) with a Bruker instrument. Chemical shifts ($\delta$ values) and coupling constants ($J$ values) are given in ppm and Hz, respectively, using TMS ($^1$H NMR) and solvents ($^{13}$C NMR) as internal standards.
General procedure for the preparation of isoxazole carboxamide derivatives (21a-n). Under N₂ atmosphere, a solution of an isoxazole carboxylic acid (6, 0.1 mmol), amine (0.12 mmol), EDCI (23 mg, 0.12 mmol), DMAP (14.7 mg, 0.12 mmol) and HOBr (27 mg, 0.2 mmol) in DMF (2.5 mL) was stirred at room temperature overnight. Then most of the solvent was removed under reduced pressure. To the residue was added 10 mL H₂O and 10 mL EtOAc. Then the aqueous solution was extract by EtOAc (20 mL × 2). The organic layer was subsequently washed with brine (20 mL). The crude compound was purified by flash chromatography on silica gel using hexane and EtOAc (9:1) as the mobile phase to give 7a-7n.

3-(2,6-Dichlorophenyl)-5-methyl-N-m-tolylisoxazole-4-carboxamide (21a). Yield 76%; ¹H NMR (CDCl₃) δ 1.70 (s, 3H), 2.86 (s, 3H), 6.74 (bs, 1H), 7.00-7.07 (m, 2H), 7.18 (td, 1H, J = 1.6 Hz, 7.2 Hz), 7.44 (dd, 1H, J = 6.4 Hz, 9.6 Hz), 7.50 (m, 2H), 7.90 (d, 1H, J = 8.0 Hz); ¹³C NMR (CDCl₃) δ 13.7, 16.9, 112.0, 123.0, 125.4, 127.0, 127.3, 128.3, 129.1, 130.6, 132.7, 135.3, 136.6, 155.7, 159.0, 176.4. HRMS-ESI (+): Calc. for C₁₈H₁₅N₂O₂Cl₂: 361.0511. Found: 361.0527 [M+H]⁺.

N-(3-Bromobenzyl)-3-(2,6-dichlorophenyl)isoxazole-4-carboxamide (21b). Yield 71%; ¹H NMR (CDCl₃) δ 2.27 (s, 3H), 2.84 (s, 3H), 6.85 (m, 3H), 7.11 (m, 2H), 7.48 (dd, 1H, J = 6.4 Hz, 9.6 Hz), 7.54 (m, 2H); ¹³C NMR (CDCl₃) δ 13.5, 21.7, 112.1, 117.1, 120.8, 125.8, 127.3, 129.0, 129.0, 132.7, 136.4, 137.2, 139.3, 155.9, 158.9, 175.7. HRMS-ESI (+): Calc. for C₁₈H₁₅N₂O₂Cl₂: 361.0511. Found: 361.0526 [M+H]⁺.

(3-(2,6-Dichlorophenyl)-5-methylisoxazol-4-yl)(morpholino)methanone (21c). Yield 81%; ¹H NMR (CDCl₃) δ 1.63 (s, 3H), 2.18 (s, 3H), 2.80 (s, 3H), 6.62 (bs, 1H), 6.82 (s, 1H), 6.92 (d, 1H, J = 8.4 Hz), 7.37 (dd, 1H, J = 6.4 Hz, 9.6 Hz), 7.44 (m, 2H), 7.66 (d, 1H, J = 8.4 Hz); ¹³C NMR (CDCl₃) δ 13.7, 16.9, 21.0, 112.0, 123.2, 127.4, 127.5, 128.6, 129.0, 131.2, 132.7, 135.3, 136.6, 155.7, 159.0, 176.2. HRMS-ESI (+): Calc. for C₁₉H₁₇N₂O₂Cl₂: 375.0667. Found: 375.0679 [M+H]⁺.

(3-(2,6-Dichlorophenyl)-5-methylisoxazol-4-yl)(piperidin-1-yl)methanone (21d). Yield 67%; ¹H NMR (CDCl₃) δ 2.24 (s, 3H), 2.78 (s, 3H), 6.70 (dd, 1H, J = 2.4 Hz, 8.4 Hz), 6.75 (bs, 1H), 7.12 (d, 1H, J = 2.4 Hz), 7.29 (d, 1H, 8.8 Hz), 7.43 (dd, 1H, J = 6.4 Hz, 9.6 Hz), 7.49 (m, 2H), 7.62 (m, 5H); ¹³C
NMR (CDCl$_3$) $\delta$ 13.6, 23.3, 111.9, 118.8, 120.1, 122.2, 127.2, 129.0, 132.8, 132.8, 136.3, 136.5, 139.0, 155.8, 158.8, 175.9. HRMS-ESI (+): Calc. for C$_{18}$H$_{14}$N$_2$O$_2$Cl$_2$Br: 438.9616. Found: 438.9633 [M+H]$^+$.  

3-(2,6-Dichlorophenyl)-N-(2,4-dimethylphenyl)-5-methylisoxazole-4-carboxamide (21e).
Yield 55%; $^1$H NMR (CDCl$_3$) $\delta$ 2.87 (s, 3H), 6.92-6.99 (m, 2H), 7.08 (t, 1H, $J = 7.6$ Hz), 7.20 (bs, 1H), 7.47 (dd, 1H, $J = 6.4$ Hz, 9.6Hz), 7.52 (m, 2H), 8.33 (td, 1H, $J = 1.6$ Hz, 8.0 Hz); $^{13}$C NMR (CDCl$_3$) $\delta$ 13.7, 111.8, 114.7, 114.9, 121.6, 124.7, 124.8, 124.8, 124.8, 126.3, 129.1, 132.8, 136.3, 153.4, 155.9, 158.8, 176.4. HRMS-ESI (+): Calc. for C$_{17}$H$_{12}$N$_2$O$_2$Cl$_2$F: 365.0260. Found: 365.0269 [M+H]$^+$.  

N-(3-Chlorophenyl)-3-(2,6-dichlorophenyl)-5-methylisoxazole-4-carboxamide (21f).
Yield 42%; $^1$H NMR (CDCl$_3$) $\delta$ 2.79 (s, 3H), 6.81 (bs, 1H), 6.84 (m, 1H), 6.98 (m, 1H), 7.09 (t, 1H, $J = 8.0$ Hz), 7.30 (t, 1H, $J = 1.6$ Hz), 7.45(dd, 1H, $J = 6.4$ Hz, 9.6 Hz), 7.50 (m, 2H); $^{13}$C NMR (CDCl$_3$) $\delta$ 90.2, 113.8, 128.2, 128.6, 132.0, 158.2, 160.2, 176.0. HRMS-ESI (+): Calc. for C$_{17}$H$_{12}$N$_2$O$_2$Cl$_3$: 380.9964. Found: 380.9962 [M+H]$^+$.  

N-Cyclohexyl-3-(2,6-dichlorophenyl)-5-methylisoxazole-4-carboxamide (21g).
Yield 59%; $^1$H NMR (CDCl$_3$) $\delta$ 2.83 (s, 3H), 6.83 (bs, 1H), 6.87 (m, 1H), 6.98 (m, 1H), 7.42 (dd, 1H, $J = 2.8$ Hz, 6.8 Hz), 7.50 (dd, 1H, $J = 6.4$ Hz, 9.6 Hz), 7.55 (m, 2H); $^{13}$C NMR (CDCl$_3$) $\delta$ 13.6, 111.7, 116.8, 117.0, 119.6, 119.7, 121.4, 121.6, 122.4, 127.1, 129.1, 132.9, 133.8, 136.3, 154.0, 155.7, 156.4, 158.9, 176.1. HRMS-ESI (+): Calc. for C$_{17}$H$_{11}$N$_2$O$_2$FCl$_3$: 398.9870. Found: 398.9885 [M+H]$^+$.  

3-(2,6-Dichlorophenyl)-5-methyl-N-o-tolylisoxazole-4-carboxamide (21h).
Yield 79%; $^1$H NMR (CDCl$_3$) $\delta$ 0.83 (m, 2H), 1.04 (m, 1H), 1.19-1.32 (m, 4H), 1.39 (m, 1H), 2.02 (m, 2H), 2.73 (s, 3H), 3.74 (m, 1H), 5.02 (m, 1H), 7.39 (dd, 1H, $J = 6.4$ Hz, 9.6 Hz), 7.45 (m, 2H); $^{13}$C NMR (CDCl$_3$) $\delta$ 13.3, 24.1, 25.5, 32.5, 47.4, 112.0, 127.8, 128.7, 132.4, 136.3, 156.1, 159.9, 174.7. HRMS-ESI (+): Calc. for C$_{17}$H$_{19}$N$_2$O$_2$Cl$_2$: 353.0824. Found: 353.0838 [M+H]$^+$.  

N-(4-Bromo-3-methylphenyl)-3-(2,6-dichlorophenyl)-5-methylisoxazole-4-carboxamide (21i).
Yield 82%; $^1$H NMR (CDCl$_3$) $\delta$ 0.28 (m, 2H), 0.65-0.70 (m, 4H), 0.81 (d, 2H, $J = 6.8$ Hz), 0.94 (m, 2H), 1.16-1.25 (m, 2H), 1.31-1.44 (m, 4H), 1.49-1.58 (m, 4H), 1.75 (m, 2H), 3.63 (m, 1H), 4.10 (m, 1H), 7.39-7.51 (m, 5H); $^{13}$C NMR (CDCl$_3$) $\delta$13.2, 13.4, 22.2, 22.3, 29.6, 29.8, 31.5, 31.9, 32.9, 33.6, 44.3, 48.3,
3-(2,6-Dichlorophenyl)-5-methylisoxazol-4-yl)(thiomorpholino)methanone (21j). Yield 85%;

\[\text{\(^1\text{H NMR (CDCl}_3\text{ \(\delta\)}\text{ \(1.34\) (bs, 4H), 1.52 (m, 2H), 2.56 (s, 3H), 3.38 (bs, 4H), 7.32 (m, 1H), 7.40 (m, 2H);}\]\]

\[\text{\(\text{^{13}\text{C NMR (CDCl}_3\text{ \(\delta\)}\text{ \(12.4, 24.4, 25.9, 113.6, 127.7, 128.4, 131.5, 135.9, 157.5, 161.6, 169.6. HRMS-ESI (+): Calc. for }\text{C}_{18}\text{H}_{21}\text{N}_2\text{O}_2\text{Cl}_2: 367.0980. Found: 367.0991 [M+H]^+.}\)\]}

3-(2,6-Dichlorophenyl)-N-(2-fluorophenyl)-5-methylisoxazole-4-carboxamide (21k). Yield 84%;

\[\text{\(\text{\(\text{^1H NMR (CDCl}_3\text{ \(\delta\)}\text{ \(2.52\) (s, 3H), 3.36 (bs, 8H), 7.30 (dd, 1H, }\text{\(J\)}\text{ = 6.4 Hz, 9.6Hz), 7.37 (m, 2H);}\]\]

\[\text{\(\text{\(\text{^{13}\text{C NMR (CDCl}_3\text{ \(\delta\)}\text{ \(12.6, 66.7, 112.8, 127.4, 128.6, 131.8, 135.9, 157.3, 161.9, 170.4. HRMS-ESI (+): Calc. for }\text{C}_{15}\text{H}_{15}\text{N}_2\text{O}_3\text{Cl}_2: 341.0460. Found: 341.0466[M+H]^+.}\)\]}

N-(3-Chloro-4-fluorophenyl)-3-(2,6-dichlorophenyl)-5-methylisoxazole-4-carboxamide (21l). Yield 72%;

\[\text{\(\text{\(\text{\(\text{^1H NMR (CDCl}_3\text{ \(\delta\)}\text{ \(2.30\) (bs, 4H), 2.52 (s, 3H), 3.62 (bs, 4H), 7.30 (dd, 1H, }\text{\(J\)}\text{ = 6.4 Hz, 9.6Hz), 7.37 (m, 2H);}\]\]

\[\text{\(\text{\(\text{^{13}\text{C NMR (CDCl}_3\text{ \(\delta\)}\text{ \(12.6, 66.7, 113.0, 127.4, 128.6, 131.9, 135.9, 157.2, 162.2, 170.4. HRMS-ESI (+): Calc. for }\text{C}_{15}\text{H}_{15}\text{N}_2\text{O}_2\text{SCl}_2: 357.0231. Found: 357.0237 [M+H]^+.}\)\]}

N-(2-Bromobenzyl)-3-(2,6-dichlorophenyl)-5-methylisoxazole-4-carboxamide (21m). Yield 73%;

\[\text{\(\text{\(\text{\(\text{^1H NMR (CDCl}_3\text{ \(\delta\)}\text{ \(2.73\) (s, 3H), 4.37 (d, 2H, }\text{\(J\)}\text{ = 6.0 Hz), 5.62 (bs, 1H), 7.04 (m, 1H), 7.16 (m, 2H), 7.26-7.33 (m, 3H), 7.38 (d, 1H, }\text{\(J\)}\text{ = 8.0 Hz);}\]\]

\[\text{\(\text{\(\text{^{13}\text{C NMR (CDCl}_3\text{ \(\delta\)}\text{ \(13.4, 43.9, 111.6, 123.9, 127.3, 127.9, 128.8, 129.5, 130.6, 132.3, 132.8, 136.2, 136.9, 156.1, 160.6, 175.2. HRMS-ESI (+): Calc. for }\text{C}_{18}\text{H}_{14}\text{N}_2\text{O}_2\text{Cl}_2Br: 438.9616. Found: 438.9627 [M+H]^+.}\)\]}

General procedures for the preparation of and 2-mercaptobenzoxazole (25b-g). To a solution of a substituted 2-aminophenol (3 mmol) was added potassium ethylxanthate (484 mg, 3 mmol) in
absolute ethanol (10 mL). The resulting mixture was heated under reflux overnight and then cooled to room temperature. The precipitate was dissolved in H₂O (10 mL) and washed with ethyl acetate (10 mL × 3) and the aqueous solution was then neutralized to pH = 5 by slow addition of glacial acetic acid. Then the product precipitated (crystallized) out to give 25b-g.

2-Mercaptobenzoazoxide (25b). Yield 52%; ¹H NMR (DMSO-d₆) δ 7.29 (m, 3H), 7.53 (d, 1H, J = 7.6 Hz), 13.90 (bs, 1H); ¹³C NMR (DMSO-d₆) δ 110.0, 110.5, 123.7, 125.1, 131.2, 148.1, 180.1. HRMS-ESI (+): Calc. for C₇H₆NOS: 152.0170. Found: 152.0170 [M+H]⁺.

2-Mercapto-5-methylbenzoazoxide (25c). Yield 45%; ¹H NMR (DMSO-d₆) δ 2.37 (s, 3H), 7.05 (m, 2H), 7.34 (m, 1H), 13.73 (bs, 1H); ¹³C NMR (DMSO-d₆) δ 110.0, 110.5, 123.7, 125.1, 131.2, 148.1, 180.2. HRMS-ESI (+): Calc. for C₈H₈NOS: 166.0327. Found: 166.0333 [M+H]⁺.

2-Mercapto-6-methylbenzoazoxide (25d). Yield 64%; ¹H NMR (DMSO-d₆) δ 2.39 (s, 3H), 7.10 (d, 1H, J = 7.6 Hz), 7.16 (t, 1H, J = 7.6 Hz), 7.31 (d, 1H, J = 7.6 Hz), 13.95 (bs, 1H); ¹³C NMR (DMSO-d₆) δ 16.1, 107.2, 121.1, 123.6, 126.0, 130.4, 147.9, 180.1. HRMS-ESI (+): Calc. for C₈H₈NOS: 166.0327. Found: 166.0333 [M+H]⁺.

2-Mercapto-3-nitrobenzoazoxide (25e). Yield 14%; ¹H NMR (DMSO-d₆) δ 7.44 (t, 1H, J = 8.0Hz), 7.91 (d, 1H, J = 8.0 Hz), 8.06 (d, 1H, J = 8.0 Hz); ¹³C NMR (DMSO-d₆) δ 115.8, 119.8, 123.4, 128.0, 131.4, 149.8, 181.7. HRMS-ESI (+): Calc. for C₇H₅N₂O₃S: 197.0021. Found: 197.0013 [M+H]⁺.

2-Mercapto-5-nitrobenzoazoxide (25f). Yield 25%; ¹H NMR (DMSO-d₆) δ 7.17 (d, 1H, J = 8.8 Hz), 7.95 (d, 1H, J = 2.4 Hz), 7.99 (dd, 1H, J = 2.4 Hz, 8.8 Hz); ¹³C NMR (DMSO-d₆) δ 101.8, 112.3, 119.8, 139.7, 150.3, 153.2, 188.4. HRMS-ESI (+): Calc. for C₇H₅N₂O₃S: 197.0021. Found: 197.0018 [M+H]⁺.

2-Mercapto-5-chlorobenzoazoxide (25g). Yield 36%; ¹H NMR (DMSO-d₆) δ 7.30 (d, 2H, J = 7.6 Hz), 7.53 (d, 1H, J = 8.0 Hz), 14.04 (bs, 1H); ¹³C NMR (DMSO-d₆) δ 110.3, 111.2, 123.5, 129.3, 132.6, 147.0, 180.8. HRMS-ESI (+): Calc. for C₇H₅NOSCl: 185.9780. Found: 185.9789 [M+H]⁺.

**General procedures for the preparation of 2-thiouracils 28a-i.** To a solution of an aldehyde (RCHO, 10 mmol), ethyl cyanoacetate (1.0 mL, 10 mmol), and thiourea (0.76 g, 10 mmol) in absolute
ethanol (50 mL) was added piperidine (2.0 mL, 20 mmol); the mixture was heated under reflux overnight and then cooled to room temperature. The precipitate was dissolved in 0.5M NaOH (20 mL) and washed with ethyl acetate (10 mL × 3). The aqueous solution was then neutralized to pH = 2 by slow addition of 1M HCl. Then the product precipitated (crystallized) out to give 28a-i.

**5-Cyano-6-phenyl-2-thiouracil (28a).** Yield 67%; \(^1\)H NMR (DMSO-\(d_6\)) \(\delta\) 7.62 (m, 5H), 13.19 (s, 1H), 13.32 (bs, 1H); \(^{13}\)C NMR (DMSO-\(d_6\)) \(\delta\) 90.2, 113.8, 128.2, 128.6, 132.0, 158.2, 160.2, 176.0. MS-ESI (+): 252.0 [M+Na]

**5-Cyano-6-(3-tolyl)-2-thiouracil (28b).** Yield 31%; \(^1\)H NMR (DMSO-\(d_6\)) \(\delta\) 2.39 (s, 3H), 7.46 (m, 4H), 13.17 (s, 1H), 13.26 (bs, 1H); \(^{13}\)C NMR (DMSO-\(d_6\)) \(\delta\) 20.9, 90.6, 114.7, 125.9, 128.4, 129.1, 129.2, 132.8, 137.9, 158.5, 160.9, 176.2. HRMS-ESI (+): Calc. for C\(_{12}\)H\(_{10}\)N\(_3\)OS: 244.0545. Found: 244.0555 [M+H]

**5-Cyano-6-(4-tolyl)-2-thiouracil (28c).** Yield 43%; \(^1\)H NMR (DMSO-\(d_6\)) \(\delta\) 2.45 (s, 3H), 7.41 (d, 2H, \(J = 7.6\) Hz), 7.61 (d, 2H, \(J = 8.4\) Hz); \(^{13}\)C NMR (DMSO-\(d_6\)) \(\delta\) 12.2, 81.7, 105.9, 118.4, 120.1, 121.2, 135.3, 150.9, 153.2, 168.2. HRMS-ESI (+): Calc. for C\(_{13}\)H\(_{12}\)N\(_3\)OS: 258.0701. Found: 258.0702 [M+H]

**5-Cyano-6-(4-ethylphenyl)-2-thiouracil (28d).** Yield 25%; \(^1\)H NMR (DMSO-\(d_6\)) \(\delta\) 1.22 (t, 3H, \(J = 7.6\) Hz), 2.71 (q, 2H, \(J = 7.6\) Hz), 7.42 (d, 2H, \(J = 8.0\) Hz), 7.61 (d, 2H, \(J = 8.0\) Hz), 13.15 (bs, 1H); \(^{13}\)C NMR (DMSO-\(d_6\)) \(\delta\) 15.2, 28.1, 90.3, 114.8, 126.6, 127.9, 128.9, 148.6, 158.5, 160.9, 176.2. HRMS-ESI (+): Calc. for C\(_{14}\)H\(_{14}\)N\(_3\)OS: 272.0858. Found: 272.0867 [M+H]

**5-Cyano-6-(4-isopropylphenyl)-2-thiouracil (28e).** Yield 37%; \(^1\)H NMR (DMSO-\(d_6\)) \(\delta\) 1.24 (d, 6H, \(J = 6.8\) Hz), 3.00 (septet, 1H, \(J = 6.8\) Hz), 7.46 (d, 2H, \(J = 8.0\) Hz), 7.62 (d, 2H, \(J = 8.0\) Hz), 13.15 (bs, 2H); \(^{13}\)C NMR (DMSO-\(d_6\)) \(\delta\) 23.6, 33.5, 90.3, 114.9, 126.5, 126.7, 129.0, 153.1, 158.6, 160.8, 176.2. HRMS-ESI (+): Calc. for C\(_{14}\)H\(_{14}\)N\(_3\)OS: 272.0858. Found: 272.0867 [M+H]

**5-Cyano-6-(4-methoxyphenyl)-2-thiouracil (28f).** Yield 21%; \(^1\)H NMR (DMSO-\(d_6\)) \(\delta\) 3.86 (s, 3H), 7.12 (d, 2H, \(J = 8.8\) Hz), 7.68 (d, 2H, \(J = 8.8\) Hz), 13.13 (bs, 2H); \(^{13}\)C NMR (DMSO-\(d_6\)) \(\delta\) 55.7, 89.9, 114.0, 115.2, 121.1, 131.0, 158.8, 160.6, 162.5, 176.3. HRMS-ESI (+): Calc. for C\(_{12}\)H\(_{10}\)N\(_3\)O\(_2\)S: 260.0494. Found: 260.0496 [M+H]

5-Cyano-6-(4-bromophenyl)-2-thiouracil (28g). Yield 39%; $^1$H NMR (DMSO-$d_6$) $\delta$ 7.63 (d, 2H, $J = 8.4$ Hz), 7.80 (d, 2H, $J = 8.4$ Hz), 13.21 (s, 1H), 13.37 (bs, 1H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 91.1, 114.6, 125.9, 128.5, 130.9, 131.6, 158.4, 160.0, 176.2. HRMS-ESI (+): Calc. for C$_{13}$H$_7$N$_3$OSBr: 307.9493. Found: 307.9504 [M+H]$^+$. 

5-Cyano-6-(biphenyl-4-yl)-2-thiouracil (28h). Yield 75%; $^1$H NMR (DMSO-$d_6$) $\delta$ 7.45 (t, 1H, $J = 7.2$ Hz), 7.53 (t, 2H, $J = 7.2$ Hz), 7.78 (d, 4H, $J = 8.4$ Hz), 7.89 (d, 2H, $J = 8.4$ Hz) 13.19 (s, 1H), 13.35 (bs, 1H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 90.6, 114.8, 126.6, 127.0, 128.1, 128.4, 129.2, 129.6, 138.7, 143.7, 158.5, 160.5, 176.2. HRMS-ESI (+): Calc. for C$_{17}$H$_{12}$N$_3$OS: 306.0701. Found: 306.0714 [M+H]$^+$. 

5-Cyano-6-(1-naphthyl)-2-thiouracil (28i). Yield 22%; $^1$H NMR (DMSO-$d_6$) $\delta$ 7.74 (d, 4H, $J = 6.8$ Hz), 7.99 (dd, 1H, $J = 6.0$ Hz, 6.4 Hz), 8.06 (dd, 1H, $J = 6.0$ Hz, 6.4 Hz, ), 8.15 (d, 1H, $J = 8.4$ Hz), 13.11 (s, 1H), 13.46 (bs, 1H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 93.1, 114.5, 124.7, 125.2, 126.8, 127.2, 127.5, 128.3, 128.5, 129.5, 131.2, 132.8, 158.7, 161.1, 176.8. HRMS-ESI (+): Calc. for C$_{15}$H$_{10}$N$_3$OS: 280.0545. Found: 280.0554 [M+H]$^+$. 

General procedure for the preparation of 2,2'-(α,α'-Xylene)bis(sulfanediyl)bisbenzothiazole (29a), 2,2'-((α,α'-Xylene)bis(sulfanediyl)bisbenzoxazole (29b-g), and 2,2'-(α,α'-Xylene)bis(sulfanediyl)bis-4-oxopyrimidine (30a-i). To a solution of the 2-mercaptobenzothiazole, 2-mercaptobenzoxazole, or 2-thiouracil derivatives (25a-g or 28a-h, 0.1 mmol) and α, α'-xylenedibromide (12 mg, 0.045 mmol) in acetonitrile (2.5 mL) was added K$_2$CO$_3$ (42 mg, 0.3 mmol). The mixture was heated under reflux overnight and then cooled to room temperature. The liquid was removed on a rotavapor and the residue was washed with 0.5M NaOH (20 mL). Then the white solid residue was dried in vacuum oven at 40 °C overnight to give 29a-g or 30a-h.

2,2'-(α,α'-Xylene)bis(sulfanediyl)bis-(6-chlorobenzothiazole) (29a). Yield 84%; $^1$H NMR (DMSO-$d_6$) $\delta$ 4.62 (s, 4H), 7.45 (s, 4H), 7.47 (dd, 2H, $J = 2.0$ Hz, 8.4 Hz), 7.84 (d, 2H, $J = 8.4$ Hz), 8.11 (d, 2H, $J = 2.0$ Hz); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 36.3, 120.8, 121.7, 126.2, 128.7, 135.4, 135.9, 151.0, 166.6. HRMS-ESI (+): Calc. for C$_{22}$H$_{15}$N$_2$S$_4$Cl$_2$: 504.9495. Found: 504.9499 [M+H]$^+$. 


2,2′-(a,a′-Xylene)bis(sulfanediyl)bis(benzoxazole) (29b). Yield 35%; \(^1\)H NMR (DMSO-\(d_6\)) \(\delta 4.60 (s, 4H), 7.34 (m, 4H), 7.49 (m, 4H), 7.65 (m, 4H); \(^{13}\)C NMR (DMSO-\(d_6\)) \(\delta 35.1, 110.2, 118.3, 124.3, 124.6, 129.2, 136.1, 141.2, 151.3, 163.8. HRMS-ESI (+): Calc. for C\(_{22}\)H\(_{17}\)N\(_2\)O\(_2\)S\(_2\): 405.0731. Found: 405.0732 [M+H]\. 

2,2′-(a,a′-Xylene)bis(sulfanediyl)bis-(5-methylbenzoxazole) (29c). Yield 57%; \(^1\)H NMR (DMSO-\(d_6\)) \(\delta 2.40 (s, 6H), 4.57 (s, 4H), 7.12 (d, 2H, \(J = 8.0\) Hz), 7.46 (m, 8H); \(^{13}\)C NMR (DMSO-\(d_6\)) \(\delta 20.9, 35.1, 109.6, 118.3, 125.1, 129.2, 134.0, 136.2, 141.4, 149.5, 163.6. HRMS-ESI (+): Calc. for C\(_{24}\)H\(_{21}\)N\(_2\)O\(_2\)S\(_2\): 433.1044. Found: 433.1041 [M+H]\. 

2,2′-(a,a′-Xylene)bis(sulfanediyl)bis-(6-methylbenzoxazole) (29d). Yield 86%; \(^1\)H NMR (DMSO-\(d_6\)) \(\delta 2.49 (s, 6H), 4.59 (s, 4H), 7.18 (m, 4H), 7.43 (d, 2H, \(J = 7.6\) Hz), 7.49 (s, 4H); \(^{13}\)C NMR (DMSO-\(d_6\)) \(\delta 16.0, 35.2, 107.5, 124.0, 125.1, 128.3, 129.3, 136.2, 140.4, 151.0, 162.6. HRMS-ESI (+): Calc. for C\(_{24}\)H\(_{21}\)N\(_2\)O\(_2\)S\(_2\): 433.1044. Found: 433.1050 [M+H]\. 

2,2′-(a,a′-Xylene)bis(sulfanediyl)bis-(4-nitrobenzoxazole) (29e). Yield 61%; \(^1\)H NMR (DMSO-\(d_6\)) \(\delta 4.66 (s, 4H), 7.50 (t, 4H, \(J = 8.0\) Hz), 7.52 (s, 4H), 8.01 (d, 2H, \(J = 8.0\) Hz), 8.08 (d, 2H, \(J = 8.0\) Hz); \(^{13}\)C NMR (DMSO-\(d_6\)) \(\delta 35.3, 115.6, 119.8, 123.5, 128.8, 135.4, 136.9, 152.6, 168.1. HRMS-ESI (+): Calc. for C\(_{22}\)H\(_{15}\)N\(_4\)O\(_6\)S\(_2\): 495.0433. Found: 495.0438 [M+H]\. 

2,2′-(a,a′-Xylene)bis(sulfanediyl)bis-(6-nitrobenzoxazole) (29f). Yield 81%; \(^1\)H NMR (DMSO-\(d_6\)) \(\delta 4.67 (s, 4H), 7.53 (s, 4H), 7.85 (d, 2H, \(J = 8.4\) Hz), 8.27 (dd, 2H, \(J = 2.0\) Hz, 8.4 Hz), 8.62 (d, 2H, \(J = 2.0\) Hz); \(^{13}\)C NMR (DMSO-\(d_6\)) \(\delta 35.4, 106.9, 118.2, 121.0, 129.4, 135.9, 143.9, 146.6, 150.6, 170.0. HRMS-ESI (+): Calc. for C\(_{22}\)H\(_{15}\)N\(_4\)O\(_6\)S\(_2\): 495.0433. Found: 495.0438 [M+H]\. 

2,2′-(a,a′-Xylene)bis(sulfanediyl)bis-(5-chlorobenzoxazole) (29g). Yield 49%; \(^1\)H NMR (DMSO-\(d_6\)) \(\delta 4.66 (s, 4H), 7.36 (d, 2H, \(J = 2.0\) Hz, 8.8 Hz), 7.48 (s, 4H), 7.67 (d, 2H, \(J = 8.8\) Hz), 7.76 (d, 2H, \(J = 2.0\) Hz); \(^{13}\)C NMR (DMSO-\(d_6\)) \(\delta 35.2, 111.5, 118.1, 124.3, 129.0, 129.3, 136.0, 142.5, 150.1, 165.9. HRMS-ESI (+): Calc. for C\(_{22}\)H\(_{15}\)N\(_2\)O\(_2\)S\(_2\)Cl\(_2\): 472.9952. Found: 472.9974 [M+H]\. 

2,2′-(a,a′-Xylene)bis(sulfanediyl)bis-(6-phenyl-5-cyano-4-oxopyrimidine) (30a). Yield 80%; \(^1\)H NMR (DMSO-\(d_6\)) \(\delta 4.26 (s, 4H), 7.31 (s, 4H), 7.44 (m, 6H), 7.77 (m, 4H); \(^{13}\)C NMR (DMSO-\(d_6\)) \(\delta\)
33.6, 88.8, 119.9, 127.8, 128.6, 129.3, 137.2, 137.7, 166.8, 170.3, 171.5. HRMS-ESI (-): Calc. for C_{30}H_{19}N_{6}O_{2}S_{2}: 559.1011. Found: 559.0989 [M-H]-.

2,2'-((\alpha,\alpha')-Xylene)bis(sulfanediyl)bis-(6-(3-tolyl)-5-cyano-4-oxopyrimidine) (30b). Yield 62%; ¹H NMR (DMSO-\textit{d}_6) δ 2.35 (s, 6H), 4.24 (s, 4H), 7.31 (m, 8H), 7.54 (s, 4H); ¹³C NMR (DMSO-\textit{d}_6) δ 21.0, 33.6, 88.9, 120.1, 125.3, 127.9, 128.6, 128.8, 130.2, 137.2, 137.8, 167.2, 170.5, 171.6. HRMS-ESI (-): Calc. for C_{32}H_{23}N_{6}O_{2}S_{2}: 587.1324. Found: 587.1348 [M-H]-.

2,2'-((\alpha,\alpha')-Xylene)bis(sulfanediyl)bis-(6-(4-tolyl)-5-cyano-4-oxopyrimidine) (30c). Yield 80%; ¹H NMR (DMSO-\textit{d}_6) δ 2.36 (s, 6H), 4.25 (s, 4H), 7.26 (d, 4H, \textit{J} = 8.0 Hz), 7.32 (s, 4H), 7.68 (d, 4H, \textit{J} = 8.0 Hz); ¹³C NMR (DMSO-\textit{d}_6) δ 21.0, 33.7, 88.6, 120.2, 128.1, 128.9, 134.9, 137.4, 139.4, 166.9, 170.7, 171.7. HRMS-ESI (-): Calc. for C_{32}H_{23}N_{6}O_{2}S_{2}: 587.1324. Found: 587.1306 [M-H]-.

2,2'-((\alpha,\alpha')-Xylene)bis(sulfanediyl)bis-(6-(4-ethylphenyl)-5-cyano-4-oxopyrimidine) (30d). Yield 96%; ¹H NMR (DMSO-\textit{d}_6) δ 1.21 (t, 6H, \textit{J} = 7.6 Hz), 2.65 (q, 4H, \textit{J} = 7.6 Hz), 4.26 (s, 4H), 7.29 (d, 4H, \textit{J} = 8.0 Hz), 7.32 (s, 4H), 7.70 (d, 4H, \textit{J} = 8.0 Hz); ¹³C NMR (DMSO-\textit{d}_6) δ 15.5, 28.1, 33.7, 88.6, 120.3, 127.5, 128.2, 128.9, 135.2, 137.4, 145.6, 166.9, 170.7, 171.7. HRMS-ESI (-): Calc. for C_{34}H_{27}N_{6}O_{2}S_{2}: 615.1637. Found: 615.1613 [M-H]-.

2,2'-((\alpha,\alpha')-Xylene)bis(sulfanediyl)bis-(6-(4-isopropylphenyl)-5-cyano-4-oxopyrimidine) (30e). Yield 87%; ¹H NMR (DMSO-\textit{d}_6) δ 1.25 (d, 12H, \textit{J} = 7.2 Hz), 2.98 (septet, 2H, \textit{J} = 7.2 Hz), 4.52 (s, 4H), 7.36 (s, 4H), 7.38 (d, 4H, \textit{J} = 8.4 Hz), 7.87 (d, 4H, \textit{J} = 8.0 Hz); ¹³C NMR (DMSO-\textit{d}_6) δ 22.7, 32.7, 33.7, 91.8, 114.9, 125.8, 128.2, 128.4, 132.2, 135.3, 152.1, 161.1, 165.3, 166.4. HRMS-ESI (-): Calc. for C_{36}H_{31}N_{6}O_{2}S_{2}: 643.1950. Found: 643.1943 [M-H]-.

2,2'-((\alpha,\alpha')-Xylene)bis(sulfanediyl)bis-(6-(4-methoxyphenyl)-5-cyano-4-oxopyrimidine) (30f). Yield 46%; ¹H NMR (DMSO-\textit{d}_6) δ 3.81 (s, 6H), 4.28 (s, 4H), 6.97 (d, 4H, \textit{J} = 8.8 Hz), 7.30 (s, 4H), 7.82 (d, 4H, \textit{J} = 8.8 Hz); ¹³C NMR (DMSO-\textit{d}_6) δ 33.7, 55.3, 88.2, 113.5, 120.4, 128.9, 130.0, 137.4, 160.6, 166.3, 170.6, 171.4. HRMS-ESI (-): Calc. for C_{32}H_{23}N_{6}O_{4}S_{2}: 619.1222. Found: 619.1230 [M-H]-.

2,2'-((\alpha,\alpha')-Xylene)bis(sulfanediyl)bis-(6-(4-bromophenyl)-5-cyano-4-oxopyrimidine) (30g). Yield 95%; ¹H NMR (DMSO-\textit{d}_6) δ 4.25 (s, 4H), 7.31 (s, 4H), 7.67 (d, 4H, \textit{J} = 8.4 Hz), 7.72 (d, 4H, \textit{J} =
8.8 Hz); $^{13}$C NMR (DMSO-$d_6$) δ 33.7, 88.9, 119.9, 123.3, 128.9, 130.2, 131.1, 136.8, 137.3, 165.9, 170.2, 171.8. HRMS-ESI (-): Calc. for C$_{30}$H$_{18}$Br$_2$N$_6$O$_2$S$_2$: 714.9221. Found: 714.9213 [M-H].

2.2'-($\alpha,\alpha'$-Xylene)bis(sulfanediyl)bis-(6-(biphenyl-4-yl)-5-cyano-4-oxopyrimidine) (30h). Yield 68%; $^1$H NMR (DMSO-$d_6$) δ 4.28 (s, 4H), 7.35 (s, 4H), 7.40 (t, 2H, $J = 7.2$ Hz), 7.49 (t, 4H, $J = 7.6$ Hz), 7.75 (m, 8H), 7.88 (d, 4H, $J = 8.4$ Hz); $^{13}$C NMR (DMSO-$d_6$) δ 33.7, 88.8, 120.3, 126.3, 126.8, 127.8, 128.7, 128.9, 136.7, 137.4, 139.5, 141.3, 166.4, 170.4, 171.8. HRMS-ESI (-): Calc. for C$_{42}$H$_{27}$N$_6$O$_2$S$_2$: 711.1637. Found: 711.1661 [M-H].

2.2'-($\alpha,\alpha'$-Xylene)bis(sulfanediyl)bis-(6-(1-naphthyl)-5-cyano-4-oxopyrimidine) (30i). Yield 92%; $^1$H NMR (CD$_3$OD) δ 4.37 (s, 4H), 7.32 (s, 4H), 7.44-7.57 (m, 8H), 7.74 (d, 2H, $J = 8.0$ Hz), 7.91 (d, 2H, $J = 8.2$ Hz), 7.96 (dd, 2H, $J = 2.0$ Hz, 7.0 Hz); $^{13}$C NMR (CD$_3$OD) δ 35.7, 94.1, 119.1, 126.2, 126.5, 127.4, 127.8, 127.9, 129.5, 130.3, 130.9, 132.0, 135.2, 136.6, 138.5, 171.6, 174.0, 175.0. HRMS-ESI (-): Calc. for C$_{38}$H$_{23}$N$_6$O$_2$S$_2$: 659.1324. Found: 659.1343 [M-H].

General procedure for the preparation of S-benzyl-2-thiouracils (31d, e, g-i). To a solution of the 2-thiouracil derivatives (24d, e, g-i, 2 mmol) and benzylchloride (253 mg, 2 mmol) in acetonitrile (10 mL) was added K$_2$CO$_3$ (829 mg, 6 mmol). The mixture was heated under reflux for 8 h and then cooled to room temperature. The liquid was removed on a rotavapor, and the residue was washed by H$_2$O (20 mL). Then the solid was dried in a vacuum oven at 40 ºC overnight to give 31d, e, g-i.

S-Benzyl-5-cyano-6-(4-ethylphenyl)-2-thiouracil (31d). Yield 28%; $^1$H NMR (DMSO-$d_6$) δ 1.27 (t, 3H, $J = 7.6$ Hz), 2.71 (q, 2H, $J = 7.6$ Hz), 4.40 (s, 2H), 7.21 (m, 1H), 7.28 (m, 4H), 7.41 (d, 2H, $J = 7.2$ Hz), 7.74(d, 2H, $J = 8.4$ Hz); $^{13}$C NMR (DMSO-$d_6$) δ 16.0, 29.8, 36.0, 90.6, 120.1, 128.2, 129.2, 129.4, 129.8, 130.1, 136.0, 139.8, 148.2, 170.2, 174.9. HRMS-ESI (+): Calc. for C$_{20}$H$_{18}$N$_3$OS: 348.1171. Found: 348.1185 [M+H].

S-Benzyl-5-cyano-6-(4-isopropylphenyl)-2-thiouracil (31e). Yield 40%; $^1$H NMR (DMSO-$d_6$) δ 1.23 (d, 6H, $J = 6.8$ Hz), 2.94 (septet, 1H, $J = 6.8$ Hz), 4.30 (s, 2H), 7.22 (t, 1H, $J = 6.8$ Hz), 7.31 (m, 4H), 7.40 (d, 2H, $J = 7.2$ Hz), 7.73 (d, 2H, $J = 7.6$ Hz); $^{13}$C NMR (DMSO-$d_6$) δ 23.7, 33.3, 33.8, 88.7, 120.3,
35

126.0, 126.7, 128.2, 128.3, 128.8, 135.3, 139.0, 150.2, 166.8, 170.5, 171.5. HRMS-ESI (+): Calc. for C_{21}H_{20}N_{3}O_{3}: 362.1327. Found: 362.1335 [M+H]^+.

**S-Benzyl-5-cyano-6-(4-bromophenyl)-2-thiouracil (31g).** Yield 33%; ¹H NMR (DMSO-d_6) δ 4.27 (s, 2H), 7.22 (t, 1H, J = 7.2 Hz), 7.29 (t, 2H, J = 7.2 Hz), 7.39 (d, 2H, J = 7.2 Hz), 7.67 (d, 2H, J = 8.4 Hz), 7.71 (d, 2H, J = 8.4 Hz); ¹³C NMR (DMSO-d_6) δ 33.8, 88.9, 120.0, 123.2, 126.7, 128.3, 128.9, 130.2, 131.1, 136.9, 139.0 165.8, 170.1, 171.8. HRMS-ESI (+): Calc. for C_{18}H_{13}N_{3}OSBr: 397.9963. Found: 397.9950 [M+H]^+.

**S-Benzyl-5-cyano-6-(biphenyl-4-yl)-2-thiouracil (31h).** Yield 37%; ¹H NMR (DMSO-d_6) δ 4.32 (s, 2H), 7.23 (t, 1H, J = 7.6 Hz), 7.31 (t, 2H, J = 7.6 Hz), 7.40 (m, 3H), 7.50 (t, 2H, J = 7.6 Hz), 7.74 (d, 2H, J = 8.0 Hz), 7.77 (d, 2H, J = 8.4 Hz), 7.90 (d, 2H, J = 8.0 Hz); ¹³C NMR (DMSO-d_6) δ 33.8, 89.1, 126.4, 126.8, 127.9, 128.3, 128.8, 128.9, 129.0, 136.6, 139.0, 139.4, 141.4, 166.4, 169.7, 171.3. HRMS-ESI (+): Calc. for C_{24}H_{18}N_{3}OS: 396.1171. Found: 396.1187 [M+H]^+.

**S-Benzyl-5-cyano-6-(1-naphthyl)-2-thiouracil (31i).** Yield 43%; ¹H NMR (DMSO-d_6) δ 4.26 (s, 2H), 7.23 (t, 1H, J = 7.2 Hz), 7.29 (t, 2H, J = 7.2 Hz), 7.39 (d, 2H, J = 7.2 Hz), 7.55 (m, 5H), 7.78 (d, 1H, J = 8.0 Hz), 7.99 (t, 2H, J = 6.8 Hz); ¹³C NMR (DMSO-d_6) δ 33.8, 92.4, 119.3, 125.2, 125.4, 126.0, 126.2, 126.4, 126.8, 128.2, 128.3, 128.9, 130.1, 133.1, 135.9, 138.9, 168.7, 167.6, 171.4. HRMS-ESI (+): Calc. for C_{22}H_{16}N_{3}OS: 370.1014. Found: 370.1015 [M+H]^+.

1.3.2 Biological evaluation

**General in vitro biological methods:** EcN68, the N-terminal fragment of SecA from *E. coli* without the C-terminal regulatory domain, and EcSecA, the full length SecA from *E. coli*, were over-expressed from pIMBB-8\(^{108}\) and pT7-SecA,\(^{109}\) respectively, and purified as described.\(^{110,111}\) EcN68 was used for screening because it has higher intrinsic activity and is more sensitive to inhibitors.

All potential inhibitors were dissolved in 100% DMSO. The ATPase activity was determined by the release of phosphate (Pi) detected by malachite green as described\(^{14}\) in a modified procedure\(^{102}\) and in the presence of 10% DMSO. Inhibitory effect was determined by the percentage of the remaining ATPase
activity as compare to the controls without test compounds. Briefly, 50 µL reaction mixture was prepared so that it contained 2.25 µg N68 or 5 µg SecA, 2 mM ATP, 50 mM Tris-HCl (pH 7.6), 20 mM KCl, 20 mM NH₄Cl, 1 mM DTT, and 2 mM Mg(OAc)₂. Reactions took place at 40 °C for 20 min (for N68) or 40 min (for SecA) then were stopped by adding 800 µL of malachite green and then 100 µL of 34% citric acid within 1 min. The mixtures were incubated at room temperature for 40 min and then the absorption at 660 nm was measured. All assays were done at least in triplicate, and the results were presented as bar graphs with standard error of the mean.

**General in vivo biological methods:** Log-phase growing cells (O.D. 600nm~ 0.5 to 1.0) were diluted to an absorbance of 0.05 at O.D. 600 nm, added with indicated compounds, and followed by culturing in an Eppendorf Thermomixer R (Brinkmann instruments, Inc.) at 37 °C,1050 rpm for 10 to 12 hours. All cultures contain 5% DMSO with a final volume of 100 µl. All tested compounds were dissolved in 100% DMSO (Sigma).

Bacterial strain: NR698 (MC4100 imp4213)¹¹² with increased outer membrane permeability. MC4100, an *Ecoli* K-12 wild-type strain.¹¹³

### 1.3.3 Computational analysis

**Molecular simulation of ligand-SecA complexes.** The 3D structures for these compounds were refined using the PM3 method in the MOPAC 7 program¹¹⁴ and assigned with AM1-BCC partial charges¹¹⁵-¹¹⁷ by the QuACPAC program. All partial charges on the atoms of the homology model were derived from AMBER 8 parameters. Docking of the ligands into SecA around the active site (included residues Gly80, Mse81, Arg82, His83, Phe84, Gln87, Arg103, Thr104, Gly105, Glu106, Gly107, Lys108, Thr109, Leu110, Arg138, Asp209, Glu210, Arg509 and Gln578) was performed by using DOCK 5.4.¹¹⁸ After docking, MD simulations were conducted with the ligand-receptor complexes following similar procedures we reported before.¹⁰⁴-¹⁰⁷ In brief, the docked complexes were solvated by using the TIP3P water model,¹¹⁹ subjected to 500-steps of molecular mechanics minimization and molecular dynamics simulations at 300 K for 1.0 ns using the SANDER module in the AMBER 8 program.¹²⁰ The resulting
structures were then analyzed using PyMOL 1.0, HBPLUS 3.06 and Ligplot 4.22 to identify spec-
specific contacts between ligands and SecA. During the computation, molecular docking (DOCK 5.4),
binding analysis (HBPLUS 3.06 and Ligplot 4.22) and visualization (PyMOL 1.0) were carried out on a
Xeon-based Linux workstation. Molecular mechanics calculations and molecular dynamics simulations
(AMBER 8) were performed on URSA, a 160-processor computer based on the Power5+ processor and
IBM’s P series architecture.

1.4 Conclusion

Through optimization of two hit compounds 8 (SEW-05929) and 9 (HTS-12302) identified from
virtual screening and 12 identified from random screening, we have found a series of thiouracil
derivatives that are much more potent than the primary hits. The two most potent compounds, 30g and
30h, are 50-fold more active than the hit compounds. Results of antimicrobial tests suggest that future
work should focus on low molecular weight analogs of 31h for in vivo applications. These compounds are
the first in its class and should be very useful as research tools in studying bacterial protein transport. The
new inhibitory structural features identified should also be very useful for further structural optimization
in search of even more potent inhibitors as potential antimicrobial agents.

1.5 Acknowledgements

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MECHANISTIC STUDIES OF 1,2,4,5-TETRAZINE CLICK CHEMISTRY AND ITS APPLICATION IN DNA POSTSYNTHETIC FUNCTIONALIZATION

Abstract: This chapter is mainly based on my publications: Chem. Commun. in 2012, from page 1736-1738. Post synthetic modification is an important and efficient way of DNA functionalization especially in DNA aptamer selection. In this research, the feasibility of norbornene (Neo) modified thymidine triphosphate incorporation was described. Besides, substituted tetrazines have been found to undergo facile inversed electron demand Diels-Alder reactions with “tunable” reaction rates. This finding paves the way to utilize tetrazine conjugation reactions for not only DNA but also other labeling work.

2.1 Introduction

DNA molecule is not only served as genomic material but also has structural purpose which is used in nanosensing,\textsuperscript{124} nanocomputing,\textsuperscript{125} aptamer selection,\textsuperscript{126-128} and reaction encoding.\textsuperscript{129,130} Among these applications, aptamer selection is one of our lab’s long term interests. DNA aptamer is a DNA molecule that forms a certain 3-D structure, which could bind to specific targets with high affinity (K\textsubscript{d} in the nanomolar to micromolar range). These targets include small molecule, protein and even cell. Finding an aptamer is normally achieved through \textit{in vitro} selection process termed SELEX (Systematic Evolution of Ligands by Exponential Enrichment). Typically in this process, DNA single strands from the combinatorial library bind with the target are enriched and separated from the unbinding or weak binding strands, which are subjected to polymerase chain reaction (PCR) and next round of selection.

Glycans, complex sugar chains and the important role of protein glycosylation, have been drawing tremendous amount of attentions nowadays. Proteins on cell surface are usually heavily glycosylated, which indicating the importance of these post-translational modifications in cell signaling, cell-cell interactions and the immune response, for instance.\textsuperscript{131} Altered protein glycosylation patterns are implicated in a number of diseases such as diabetes\textsuperscript{132} and cancer.\textsuperscript{133,134} Besides, glycan biomarkers have been identified from many other diseases.\textsuperscript{135-138} However, as a post-translational process, glycosylation is by definition nontemplated, in other word, glycans on glycoproteins are indirect gene products. Besides,
unlike phosphorylation, tens to hundreds of different glycan attachments can be found on one protein makes probing and differentiation exponentially more difficult.

As mentioned above, high affinity and specificity make aptamers possible tools for studying glycosylation and diagnose and therapy of glycosylation-related diseases. Boronic acid is well-known by its strong interactions with diols, alcohols, and nucleophiles (Scheme 2.1). Therefore, considering the intrinsic affinity of boronic acid to carbohydrates, incorporation of the boronic acid moiety into DNA could lead the discovery of new aptamers against carbohydrates, glycoproteins, and glycolipids. Furthermore, boronic acid modified DNA aptamer would differentiate the glycosylation patterns.

Scheme 2.1. Illustration of boronic acid-diol interaction

Based on the hypothesis that boronic acid modified DNA would have enhanced recognition of carbohydrate, our group has previously reported the design and synthesis of boronic acid modified thymidine analogues. For example, first generation boronic acid modified thymidine triphosphate (B-TTP Figure 2.1), which was incorporated into DNA through PCR, demonstrated the feasibility of boronic acid modified DNA aptamer selection for glycoprotein. The second generation boronic acid modified TTP that changes fluorescent properties upon binding to sugar allows incorporated DNA to sense target without any additional reporting groups. As we reported earlier, B-TTP with a naphthalimide moiety, named as NB-TTP (Figure 2.1), has also been successfully synthesized and
introduced into DNA. The NB-TTP modified DNA showed fluorescence intensity change upon sugar addition.

Figure 2.1. Incorporation of boronic acid moiety into DNA through pre-installed B-TTP

The second stage of applications of boronic acid (BA) modified DNA aptamer involves large scale production. Therefore, solid-phase synthesis of BA modified DNA was explored by our group as well. Owning to the difficulty of preparing the BA “pre-installed” phosphoramidite building blocks, we turned our head to use DNA postsynthetic functionalization methods. Since click chemistry,\textsuperscript{168,169} which is developed by Sharpless, is compatible with DNA molecule,\textsuperscript{170} and extensive studies of post-synthesis modifications of DNA using click chemistry,\textsuperscript{171} we firstly decided to use the click chemistry of copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction. However, BA moiety, especially aryl BA, was degraded in the presence of Cu(I).\textsuperscript{171,172} Though we found addition of fluoride will stabilize BA,\textsuperscript{173} BA degradation is still not acceptable to a degree in DNA modification where high yield at each nucleotide site is very important.

Copper free click chemistry is playing an increasingly important role in a variety of areas of research,\textsuperscript{172,174-180} including bioorthogonal labeling.\textsuperscript{181-185} Critical to this field is the availability of
reactions and reagents that allow for facile and specific reactions. Many reagents have been identified that serve this purpose very well in different situations. These include difluorinated cyclooctyne (DIFO), dibenzocyclooctyne (DIBO), aza-dibenzocyclooctyne (DIBAC), and biarylazacyclooctynone (BARAC). We then took advantage of DIFO click tool and converted it to the phosphoramidite building block for DNA post-synthetic BA modifications.

Scheme 2.2. Solid-phase synthesis of boronic acid modified DNA through copper-free post-synthesis modification

As showed in Scheme 2.2, phosphoramidite 37 was used in solid phase synthesis, which gave a “handle” to DNA molecule 38. This handle was later clicked with BA 39 to obtain modified DNA product 40. This was the first demonstration that BA can be successfully introduced into DNA through post-synthesis. Nevertheless, all these approaches used one type of reactions: 1,3-dipolar cycloaddition involving an azido and an alkynyl group. However, this field needs more such reactions in its arsenal for reasons of multiplexing, labeling diversity, biocompatibility under different conditions, and compatibility with other functional groups as well reaction conditions for reagent preparation. In addition, rate difference among various click reactions can also be exploited for selective labeling. Last but not the least, due to lack of symmetry, multiple regioisomers were generated (40a and 40b Scheme 2.2). Thus
having click reactions with sufficiently different reaction rates and single isomer product will be very use-
useful in DNA post-synthetic modification.

Fox$^{187,192,193}$ and Weissleder$^{178}$ have developed a reaction using tetrazine and a strained trans-
alkene. The reaction is very fast with a second order rate constant of 2000 to 22000 M$^{-1}$s$^{-1}$, making it very
useful for biolabeling applications. However, the asymmetric reaction product makes it hard to be used in
DNA aptamer selection work where single isomer modification productivity is required. Recently,
Jäschke reported a bioorthogonal pair, tetrazine-norbornene (Figure 2.2), which is successfully utilized in
solid phase synthesis of modified DNA for post-synthesis.$^{194}$ This first example intrigued us, and
therefore we used this in our enzymatic approach to prepare DNA molecule with a handle for later
manipulations. However slow reaction rates along with asymmetric productivity made the tetrazine-
norbornene pair unacceptable in BA modified DNA aptamer selection.

![Illustration of Solid-phase synthesis of modified DNA through copper-free post-synthesis modification involving inverse-electron demand Diels-Alder reaction](image)

Figure 2.2. Illustration of Solid-phase synthesis of modified DNA through copper-free post-synthesis modification involving inverse-electron demand Diels-Alder reaction

In our DNA labeling work,$^{167,195,196}$ we are interested in developing fast click reactions with
tunable reaction rates and sympatric product that can be easily obtained. We thus turned to another type of
inverse-electron demand Diels-Alder reactions (IEDDA) involving an alkyne and tetrazine for several
reasons. First, Schuster$^{197}$ has demonstrated that un-substituted tetrazine can react very quickly with
alkenes and alkynes. Second, many strained alkynes have been reported, giving us a set of “tools” for the
intended studies. Third, unlike azido compounds, tetrazine’s reactivity can be tuned by manipulating its
electron deficiency through the introduction of functional groups at the 3,6-position. In our case, we need
reactions to be fast enough so that at 10 µM concentrations, the half-life is no more than 2 h. This
requires the second order rate constant to be higher than $20 \text{ M}^{-1} \text{s}^{-1}$ at ambient temperature. This rate is higher than that of most strained promoted azido-alkyne cycloadditions (SPAAC) reported thus far.

The reaction between un-substituted tetrazine and cyclooctyne has been reported with a stated second order rate constant of $27.1 \text{ M}^{-1} \text{s}^{-1}$. However the highly volatile 1,2,4,5-tetrazine is not suitable for any labeling work.\cite{197} In our initial studies, we found the second order rate constant for the reaction between cyclooctyne (50) and diphenyltetrazine (51) to be $0.07 \text{ M}^{-1} \text{s}^{-1}$ (Figure 2.5). This rate is similar to that of a typical SPAAC reaction\cite{180} and is slower than what we need. Thus, we undertook the effort to explore ways to enhance the reaction rates by performing modifications guided by computational work. In the present research, synthesis of norbornene modified TTP analog (Neo-TTP 48, Scheme 2.3), enzymatic incorporation the Neo-TTP into DNA, post-synthetic modification, and a new click reaction with tunable reaction rates are reported.

2.2 Results and discussions

2.2.1 Synthesis of Norbornene-modified deoxyuridine (Neo-TTP)

Scheme 2.3. Synthetic route of Neo-TTP 48
Neo-TTP (48) was prepared in only 4 steps. Firstly, we followed literature to get compound 47 in 3 steps.\textsuperscript{194,198} Then the triphosphorylation was accomplished by the classical one-pot three-step method\textsuperscript{199} (Scheme 2.3) to provide Neo-TTP (48) in 9\% overall isolated yield, which was then used for enzymatic incorporation.

2.2.2 Enzymatic incorporation and post-synthesis labeling of Neo-TTP

The incorporation of Neo-TTP into DNA was studied through primer extension. Specifically, the excrement was conducted by using a short sequence of 21-mer oligonucleotide 5’-GGTTCCACCAGCAACCCGCTA-3’ as the template, a 14-mer oligonucleotide 5’-TAGCGGGTTGCTGG-3’ as the primer, Neo-TTP as the TTP analog and the Klenow fragment as the enzyme (Figure 2.3). This type of experiment has been successfully used in our previous incorporation studies of different functionalized DNA.\textsuperscript{163,164,200} Klenow fragment without 3’-5’ exo-nuclease activity was used to avoid cleavage of the template. Since the primer was designed in such a way that the first elongated nucleotide would be a thymidine triphosphate, there are two possible scenarios in the experiment: either full extension or no extension at all. In order to study the DNA products polyacrylamide gel electrophoresis (PAGE) was used. As shown in Figure 2.4, negative control without
dTTP (lane 1) showed no extension, on the other hand, positive control with Klenow fragment and natural dNTPs only (Lane 2) and primer extension product using Neo-TTP in place of dTTP (Lane 3) gave a similar full-length DNA band. Such results indicated that Neo-TTP could be recognized by the enzyme and therefore incorporated into DNA as natural dTTP.

Figure 2.4. Primer extension using Neo-TTP catalyzed by Klenow fragment (3’-5’ exo-) and post-synthetic modification using “click” reagent 49, 20% PAGE analysis

The feasibility of the post-synthetic modification of Neo-TTP incorporated DNA was then explored. Compound 49 synthesized by our group (not published result) was used as the reporter for labeling the Neo-DNA. In Figure 2.4, after treating the primer extension product with 49 (1 mM, final concentration (50 equiv.), only Neo-DNA (lane 5-8) but not the natural DNA (lane 4) showed the post-synthesis product as expected. The results also showed the modification was done in 2 hours.

2.2.3 Development of new click reaction involving 1,2,4,5-tetrazine and cyclooctynes
Figure 2.5. Strain-promoted [4+2] cycloadditions of 1,2,4,5-tetrazine 51 and cyclooctynes 53.

Tetrazine: 1 mM, Alkyne: 10 mM in dried MeOH

The reaction between cyclooctyne (50) and a substituted tetrazine (51) leads to significant changes in the UV-vis spectrum of tetrazine due to its conversion to a 1,2-diazine product (Figure 2.5). Hence, reaction can be easily monitored. We first studied the reaction between cyclooctyne (50) and 3,6-diphenyl-1,2,4,5-tetrazine (51) in dry methanol as a reference. The rate constant for the reaction between diphenyltetrazine and cyclooctyne was found to be 0.07 M⁻¹s⁻¹ (Table 2.1), which is far smaller than that of the reaction between unsubstituted tetrazine and cyclooctyne. Since the reaction between 1,2,4,5-tetrazines and an octyne depends on the LUMO_diene-HOMO_phil gap, we were interested in exploring ways to lower the LUMO of the diene or elevate the HOMO of the dienophile in order to facilitate the reaction. In such a case, it is reasonable to expect that electron donating substituents, as well as high strain energy will increase the HOMO energy of the dienophiles, and electron withdrawing substituents will decrease the LUMO energy of the diene, leading to a decrease in the LUMO_diene-HOMO_phil gap and consequently an increase in the reaction rate.

<table>
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<tr>
<th>Reaction</th>
<th>Rate Constant</th>
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<tr>
<td>50 + 51</td>
<td>(7.0±0.7) x 10² M⁻¹s⁻¹</td>
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<tr>
<td>50 + 53</td>
<td>3.3±0.4 M⁻¹s⁻¹</td>
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We then conducted quantum mechanics (QM) calculation using known strained alkynes and trans-cyclooctene and some modified tetrazines. From Figure 2.6, one can see that relative to cyclooctyne (51), the HOMO of BCN\textsuperscript{204} (53) is 1.5 kcal/mol higher, giving this a chance to have increased reactivity. Installing electron withdrawing groups on cyclooctynes decreases the HOMO energy substantially. For example, computational results indicate that the HOMO energy decreases by 6.1 kcal/mol for DIFO\textsuperscript{186} (58) and 11.5 kcal/mol for fluorocyclooctyne\textsuperscript{205} (59) relative to cyclooctyne. Correspondingly, di-pyridine substituted 1,2,4,5-tetrazine 55 lowers the LUMO energy by 2.3 kcal/mol relative to 3,6-diphenyl-1,2,4,5-tetrazine 51 (Figure 2.6). Such calculations suggest that the BCN-tetrazine (55) pair could have significantly improved reactivity.

<table>
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<tr>
<th>BCN</th>
<th>2.0±0.3 M\textsuperscript{-1}s\textsuperscript{-1}</th>
<th>44.8±4.9 M\textsuperscript{-1}s\textsuperscript{-1}</th>
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<tr>
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<td>40.9±13.8 M\textsuperscript{-1}s\textsuperscript{-1}</td>
<td>44.8±4.9 M\textsuperscript{-1}s\textsuperscript{-1}</td>
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Table 2.1. Second order rate constants of cyclooctynes with tetrazines.
Indeed, when we tested the reactivity of diphenyltetrazine (51) with 53 at a 1:10 ratio (tetrazine: 1 mM; alkyne: 10 mM), the reaction finished within 10 minutes, with a second order rate constant of 3.3 M⁻¹s⁻¹ (Figure 2.5). This represents a 47-fold improvement in reaction rate compared to the reaction between cyclooctyne and diphenyltetrazine. To further accelerate the reaction, two electron-withdrawing groups were attached to the tetrazine ring to give 55. The second order rate constant for the reaction between 3,6-di-2-pyridyl-1,2,4,5-tetrazine (55) and alkyne (53) in dry MeOH at ambient temperature was found to be 44.8 M⁻¹s⁻¹, which represents a 640-fold improvement in reaction rate over the tetrazine-cyclooctyne pair. On the other hand, 58 and 59 did not react with tetrazine 55 to an appreciable degree, as expected. To achieve a deeper understanding of the improved reaction rates, Density Functional Theory (DFT) calculations at the 6-31G** level of theory were performed to examine the possible transition state(s) and activation energies. Schematic representation of the energy profiles for the tetrazine alkyne reactions are shown in Figure 2.7. All of the QM energies are in kcal/mol and are relative to the reactants (50 and 51 at the top of Figure 2.7, and 53 and 51 at the bottom of Figure 2.7). The activation energy for the first step of the IEDDA reaction between 51 and 53 via transition state TS1’ is 17.6 kcal/mol as compared to 23.3 kcal/mol for the reaction between cyclooctyne 50 and tetrazine 51. The computational results support the idea that the increase HOMO energy in BCN (53) compared with cyclooctyne (50) translates into lowered activation energy and thus increased reaction rate. The intermediate IN1 from the first step of the 50-51 reaction pair quickly loses nitrogen gas to yield product 52 by passing through a low activation barrier of 8.6 kcal/mol, with the reaction being strongly exergonic by -91.4 kcal/mol. Because of the low barrier for the second step, the reaction rate is entirely controlled by the first step of the reaction. For the reaction between BCN (53) and tetrazine 51, the situation is similar except that the conversion of the intermediate IN1’ to the final product has almost no activation barrier, with the overall reaction being strongly exergonic by -88.1 kcal/mol. All these results indicate that the IEDDA reaction and the subsequent elimination reaction take place spontaneously and irreversibly toward the pyridazine products.
Figure 2.7. Schematic representations (energy vs. reaction coordinate) of the reaction between diphenyl
tetrazine and strained alkynes.
With these exciting findings and resulting tunable reaction rates, we turned our attention to developing the chemistry needed for bioconjugation. In order to do so, a “handle” need to be installed on the tetrazine system. Therefore 4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzoic acid\(^\text{206}\) was prepared. As a model, isopropyl amine was chosen for conjugation with the benzoyl chloride tetrazine derivative. The reaction between isopropyl amide tetrazine \(\text{56}\) and \(\text{53}\) gave a second order rate constant of \(40.9 \text{ M}^{-1}\text{s}^{-1}\), with half-lives of 24 seconds and 0.68 hour at mM and 10 \(\mu\text{M}\) concentrations, respectively. This is among the fastest click reactions, and can be used for a variety of labeling work.

2.3 Conclusion

In summary, norbornene modified TTP was successfully synthesized and incorporated into DNA through enzymatic primer extension. However slow reaction rate is not acceptable for DNA aptamer selection process. Therefore a new inverse electron-demand Diels-Alder reaction involving 1,2,4,5-tetrazine and cyclooctynes with tuned reaction rate was developed. The facile conjugation was also demonstrated. The reaction rates found are among the fastest involving a tetrazine and alkyne. Furthermore, the wide range of reaction rates (over 64-fold difference) that can be achieved suggest possibilities of using such chemistry for mutiplexing click labeling. This work paves the way to utilize BCN-tetrazine conjugation reactions for not only DNA but also other labeling work.

2.4 Experimental part

2.4.1 Synthesis

Materials and Methods. All reagents and solvents were reagent grade or were purified by standard methods before use. Column chromatography was carried out on flash silica gel (Sorbent 230–400 mesh). TLC analysis was conducted on silica gel plates (Sorbent Silica G UV254). NMR spectra were recorded at \(^1\text{H}\) (400 MHz) and \(^{13}\text{C}\) (100 MHz) on a Bruker instrument. Chemical shifts (\(\delta\) values) and coupling constants (\(J\) values) are given in ppm and hertz, respectively, using solvents (\(^1\text{H}\) NMR, \(^{13}\text{C}\) NMR) as the internal standard. DIFO \(\text{(58)}\),\(^{186}\)
methyl 1-fluorocyclooct-2-ynecarboxylate (59), \(^{205}\) compound 50, \(^{3}\) compound 51, \(^{5}\) compound 53, \(^{4}\) and compound 55\(^{6}\) were synthesized according to literature procedures.

**Preparation of Neo-TTP**

**Preparation of Cyclooctyne 50.** \(^{207}\)

Scheme 2.4. Reagents and conditions for the synthesis of cyclooctyne 50

**Cyclooctyne (50):** \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 2.14 (s, 4H), 1.83 (s, 4H), 1.60 (s, 4H). \(^{13}\)C NMR (CDCl\(_3\)) \(\delta\) 94.6, 34.6, 29.8, 21.0.

**Preparation of \((1R,8S,9S)-Bicyclo[6.1.0]non-4-yn-9-ylmethanol (endo-53).** \(^{204}\)

Scheme 2.5. Reagents and conditions for the synthesis of \((1R,8S,9S)-bicyclo[6.1.0]non-4-yn-9-ylmethanol (endo-53)\)

\((1R,8S,9S)-Bicyclo[6.1.0]non-4-yn-9-ylmethanol (endo-53):** \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 3.66 (d, \(J = 8.0\) Hz, 2H), 2.28-2.14 (m, 6H), 1.55-1.54 (m, 2H), 1.31-1.25 (m, 1H), 0.89-0.85 (m, 2H). \(^{13}\)C NMR (CDCl\(_3\)) \(\delta\) 98.9, 59.7, 29.0, 21.5, 21.3, 20.0.

**Preparation of 3,6-diphenyl-1,2,4,5-tetrazine 51.** \(^{208}\)
Scheme 2.6. Reagents and conditions for the synthesis of 3,6-diphenyl-1,2,4,5-tetrazine 51.

3,6-Diphenyl-1,2,4,5-tetrazine (51): $^1$H NMR (CDCl$_3$): δ 8.65 (t, $J = 6.0$ Hz, 4H), 7.65-7.59 (m, 6H). $^{13}$C NMR (CDCl$_3$) δ 164.0, 132.7, 131.8, 129.3, 128.0.

Preparation of 3,6-di(pyridin-2-yl)-1,2,4,5-tetrazine 55.$^{209}$

Scheme 2.7. Reagents and conditions for synthesis of 3,6-di(pyridin-2-yl)-1,2,4,5-tetrazine 55

3,6-Di(pyridin-2-yl)-1,2,4,5-tetrazine (55): $^1$H NMR (CDCl$_3$): δ 8.99 (d, $J = 4.8$ Hz, 2H), 8.76 (d, $J = 8$ Hz, 6H), 8.04-7.99 (m, 2H), 7.60-7.57 (m, 2H). $^{13}$C NMR (CDCl$_3$) δ 163.8, 151.0, 150.0, 137.4, 126.5, 124.5.

Preparation of N-isopropyl-4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin3-yl) benzamide 56.$^{210}$
Scheme 2.8. Reagents and conditions for the synthesis of \( N \)-isopropyl-4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzamide 56

4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzoic acid (74)\( ^7 \): To a solution of 2-pyrimidinecarbonitrile (935 mg, 8.9 mmol) in 30 mL ethanol, 4-cyanobenzoic acid (1.96 g 13.3 mmol) was added, followed by addition of hydrazine monohydrate (5.5 mL). Then the solution was heated under reflux for 20 h with stirring. After cooling to r.t., the solid was collected by filtration and washed with acetone (2 × 50 mL). The dihydrotetrazine without carboxyl group (71) went into acetone. The remaining solid was added acetic acid (10mL) followed by an aqueous solution of NaNO\(_2\) (2.76g, 40 mmol) at 10 °C. The purple colored tetrazine was collected and washed with water (3 × 10 mL). The solid was added into nearly boiled DMF and kept at this temperature for another 5 min. The DMF solution was filtered while it was hot. The filtrate, which contained mono-carboxyl substituted product, was collected and dried under vacuum to
give a purple product (300 mg, 12%). The benzoic acid tetrazine was used for next step without further purification.

*N*-Isopropyl-4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzamide (56): To a suspension of 4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzoic acid (74, 60 mg, 0.214 mmol) in 5 mL DCM, thionyl chloride (187 µL, 257 mmol) was added. This mixture was refluxed for 20 h with stirring, at which point TLC indicated completion of the reaction. The solution was cooled to r.t. and dried under vacuum. The residue was suspended in dry 3 mL DCM, then propan-2-amine (55 µL, 0.64 mmol) was added and the resulting mixture was stirred at r.t. for 4 h. DCM was removed in vacuum and the reaction mixture was purified by silica gel column chromatography (DCM: MeOH, 20:1) to give a purple solid product (40 mg, 59 %). $^1$H NMR (CDCl₃): δ 9.14 (d, $J = 2.0$ Hz, 2H, 4-Pyr-H, 6-Pyr-H), 8.78 (d, $J = 4.0$ Hz, 2H, 2’-Ph-H, 6’-H), 8.00 (d, $J = 4.0$ Hz, 2H, 3’-Ph-H, 5’-Ph-H), 7.60 (br, 1H, 5-Pyr-H), 6.17 (d, $J = 4.0$ Hz, 1H, -NH), 4.34-4.32 (m, 1H, -CH-NH-), 1.32 (s, 3H, -CH₃), 1.31 (s, 3H, -CH₃); $^{13}$C NMR (CDCl₃) δ 165.7, 164.0, 163.1, 159.4, 158.4, 139.2, 133.7, 128.9, 127.8, 122.6, 42.2, 22.8. MS calcd. For C₁₆H₁₅N₇O [M+H]$^+$ 322.1, found 322.3

2.4.2 Enzymatic incorporation and post-synthesis labeling

2.4.2.1 General procedure for Klenow fragment primer extension using dTTP or Neo-TTP

The reaction mixtures of a final volume of 50 µL contained 21-mer template (5’-GGTTCCACCAG-CAACCCGCTA-3’, 20 µM), 14-mer primer (5’-TAGCGGGTTGCTGG-3’, 20 µM), 10 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl₂, 1 mM Dithiothreitol at pH 7.9, dATP, dCTP, dGTP, dTTP or Neo-TTP at concentrations of 200 µM and 0.5 U µL⁻¹ Klenow fragment. The prepared reaction solution was incubated at 25 °C for 30 min. The primer extension products were analyzed by 20% PAGE.
2.4.2.2 Post-synthesis labeling of the primer extension products Neo-DNA

The primer extension product was purified using Millipore Amicon 3 kDa spin column and react with 30 μL of 2 mM 49 in 1 × PBS buffer. The reaction was allowed to stand at 37 °C for 1 h, 2h, 4h and overnight. The negative control experiment was performed following the same procedure. The resulting post-synthetic modified DNA products were purified with Millipore Amicon 3 kDa spin column and analyzed by 20% PAGE.

2.4.3 Characterization of cyclization products

General procedure for strain-promoted inverse electron demand Diels-Alder reactions with 1,2,4,5-tetrazine.

To a solution of tetrazine (51, 55, 56) in CH₂Cl₂ (1 mL), alkyne (50, 53) in CH₂Cl₂ (1 mL) was added. Reactions were stirred at room temperature for 5 to 30 min. The progress of the reaction was monitored by TLC. Upon completion, the reaction mixture was directly loaded on the flash column chromatography for purification.

**Compound 52**

![Compound 52](image)

Purified by eluting with DCM:MeOH (10:1, R_f = 0.65) (white solid, 91%).

¹H NMR (CDCl₃) δ 7.55-7.47 (m, 10H, Ph-H), 2.83-2.82 (m, 4H, -CH₂-C=C-), 1.60 (br, 4H, -CH₂-), 1.43 (br, 4H, -CH₂-). ¹³C NMR (CDCl₃) δ 161.1, 139.0, 138.2, 129.1, 128.3, 128.2, 30.3, 27.1, 25.9. MS calcd. For C₂₂H₂₂N₂ [M+H]^+ 315.2, found 315.3

**Compound 54**

![Compound 54](image)

Purified by eluting DCM:MeOH (10:1, R_f = 0.55) (white solid 88%). ¹H NMR (CDCl₃) δ 7.48-7.41 (m, 10H, Ph-H), 3.63 (d, J = 3.6 Hz, 2H, -CH₂-OH), 2.93-2.85 (m, 2H, -CH₂-C=C-), 2.75 (br, 2H, -CH₂-C=C-),
2.50 (br, 1H, -CH-CH2OH), 2.18-2.15 (br, 2H, -C-CH-C-), 1.43-1.42 (br, 2H, -CH2-), 1.13 (br, 2H, -CH2-). $^{13}$C NMR (CDCl$_3$) δ 160.8, 140.5, 137.9, 129.2, 128.4, 128.2, 58.8, 28.2, 24.3, 22.9, 20.4. MS calcd. For C$_{26}$H$_{23}$NO$_3$ [M+H]$^+$ 357.2, found 357.3.

**Compound 76**

Purified by eluting DCM:MeOH (10:1, $R_f = 0.54$) (white solid 90%). $^1$H NMR (CDCl$_3$) δ 8.66 (d, $J = 2.2$ Hz, 2H, 6-Py-H), 7.84-7.79 (m, 4H, 3-Py-H, 4-Py-H), 7.33-7.30 (m, 2H, 5-Py-H), 3.01-2.98 (m, 4H, -CH=C=C-), 1.72 (br, 4H, -CH$_2$-), 1.38 (br, 4H, -CH$_2$-). $^{13}$C NMR (CDCl$_3$) δ 158.7, 156.9, 148.4, 140.9, 136.6, 124.8, 123.1, 30.3, 26.4, 25.9. MS calcd. For C$_{20}$H$_{20}$N$_4$ [M+H]$^+$ 317.2, found 317.2.

**Compound 77**

Purified by eluting DCM:MeOH (10:1, $R_f = 0.35$) (white solid 89%). $^1$H NMR (CDCl$_3$) δ 8.72 (d, $J = 2.0$ Hz, 2H, 6-Py-H), 7.95-7.85 (m, 4H, 3-Py-H, 4-Py-H), 7.39-7.36 (m, 2H, 5-Py-H), 3.72 (d, $J = 3.6$ Hz, 2H, -CH$_2$-C-OH), 3.06-3.05 (m, 4H, -CH$_2$-C=C-), 2.39-2.36 (br, 2H, -CH$_2$-), 1.62 (br, 2H, -CH$_2$-), 1.10 (br, 3H, -CH-, -CH$_2$-). $^{13}$C NMR (CDCl$_3$) δ 159.1, 157.0, 148.7, 142.2, 136.8, 125.0, 123.3, 59.5, 27.8, 24.4, 22.7, 19.7. MS calcd. For C$_{22}$H$_{22}$N$_4$O [M+H]$^+$ 359.2, found 359.3.

**Compound 78**

Purified by eluting DCM:MeOH (10:1, $R_f = 0.32$) (white solid 82%). $^1$H NMR (CDCl$_3$) δ 8.94 (d, $J = 2.4$ Hz, 2H, 6-Pyr-H, 6-Pyr-H) 7.86 (d, $J = 4.2$ Hz, 2H, 2'-Ph-H, 6'-Ph-H), 7.53 (d, $J = 4.0$ Hz, 2H, 3'-Ph-H, 5'-Ph-H), 7.42 (t, $J = 5.0$ Hz, 1H, 5-Pyr-H), 6.25 (d, $J=3.8$ Hz, 1H, -NH), 4.35-4.26 (m, 1H, -NH-CH-), 3.72 (d, $J = 3.6$ Hz, 2H, -CH$_2$-C-OH),
2.99-2.89 (m, 2H, -CH$_2$-C=C-), 2.83-2.78 (m, 2H, -CH$_2$-C=C-), 2.32-2.31 (br, 1H, -CH-), 2.19-2.17 (br, 1H, -CH-), 1.84 (br, 1H, -CH-), 1.59-1.56 (br, 2H, -CH$_2$-), 1.29 (s, 3H, -CH$_3$), 1.27 (s, 3H, -CH$_3$), 1.15 (br, 2H, -CH$_2$-). $^{13}$C NMR (CDCl$_3$) δ 166.4, 164.8, 161.0, 158.0, 157.2, 141.2, 141.0, 140.5, 135.2, 129.4, 127.0, 120.4, 59.2, 42.0, 28.0, 24.1, 22.8, 21.0, 19.7, MS calcd. For C$_{26}$H$_{29}$N$_5$O$_2$ [M+H]$^+$ 444.2, found 444.3.

2.4.4 Kinetics measurements of 51, 55 and 56 with 50 or 53.

UV/Vis kinetic measurements: Separate solutions of pure tetrazines 51, 55, 56 and pure alkynes 50, 53 (>95-98% by $^1$H-NMR) were prepared in HPLC-grade MeOH at 297 K. The stability of 51, 55, and 56 in MeOH was examined by monitoring its absorption maximum at 295 nm. Solutions containing 51, 55, 56 (25 uM) and 10-18 fold excess of an alkyne (50 or 53) were pipetted into quartz cuvettes for UV measurements and thoroughly mixed. All kinetic runs were triple triplicates. Curve fitting was operated in Prism3 software.

A) Tetrazine 51 with alkyne 50

\[
\begin{align*}
\text{Ph-tetrazine click reaction} \\
\text{Ph-tetrazine: 25 uM} \\
\text{Alkyne: 450 uM}
\end{align*}
\]

\[
\begin{align*}
&k_1 = (3.15 \pm 0.3) \times 10^{-5} \text{s}^{-1} \\
&k_2 = k_1 / 450 \text{ uM} = (7.0 \pm 0.7) \times 10^{-2} \text{ M}^{-1} \text{s}^{-1}
\end{align*}
\]

Figure 2.8. Reaction rate between Ph-tetrazine 51(25 uM) and alkyne 50(450 uM).

B) Tetrazine 55 with alkyne 50
Figure 2.9. Reaction rate between Py-tetrazine 55 (25 uM) and alkyne 50 (250 uM, 300 uM, 350 uM, 400 uM, 450 uM).

Second order rate constant $k_2 = 2.0 \pm 0.3 \text{ M}^{-1}\text{s}^{-1}$

C) Tetrazine 51 with alkyne 53
Figure 2.10. Reaction rate between Ph-tetrazine 51 (25 uM) and alkyne 53 (250 uM, 300 uM, 350 uM, 400 uM, 450 uM).

Second order rate constant $k_2 = 3.3 \pm 0.4 \text{ M}^{-1}\text{s}^{-1}$

D) Tetrazine 55 with alkyne 53

![Chemical Reaction Diagram]

Figure 2.11. Reaction rate between Py-tetrazine 55 (25 uM) and alkyne 53 (250 uM, 300 uM, 350 uM, 400 uM, 450 uM).

Second order rate constant $k_2 = 44.8 \pm 4.9 \text{ M}^{-1}\text{s}^{-1}$

E) Tetrazine 56 with alkyne 53

![Chemical Reaction Diagram]
Figure 2.12. Reaction rate between IsoP-tetrazine 56 (25 uM) and alkyne 53 (250 uM, 300 uM, 350 uM, 400 uM, 450 uM).

Second order rate constant \( k_2 = 40.9 \pm 13.8 \text{ M}^{-1}\text{s}^{-1} \)

### 2.4.5 Computational detail

All calculations were performed using the Gaussian 03 program. Initial geometry optimizations were carried out at the AM1 semiempirical level. DFT calculations were carried out with use of the B3LYP with the standard 6-31G** basis set. The stationary points were characterized by frequency calculations to verify that TSs had one and only one imaginary frequency.
2.5 Acknowledgements

I would like to specially thank Danzhu Wang for the kinetic study work and synthesis of compound 49, Hanjing Peng for the PAGE analysis, Dr. Chaofeng Dai for the synthetic analysis, Dr. Siming Wang and Dr. Lifang Wang for the MS work.
3 CONCLUSIONS

In summary, as the very first group who found a series of SecA inhibitors that showed novel antimicrobial mechanism, we herein designed and synthesized a series of analogues based on the structures of two lead inhibitors identified through virtual screening and one lead identified from random screening. The best inhibitors showed \textit{in vivo} IC$_{50}$ against bacteria from low $\mu$M to high nM range. These compounds are the first in its class and should be very useful as research tools in studying bacterial protein transport. The new inhibitory structural features identified should also be very useful for further structural optimization in search of even more potent inhibitors as potential antimicrobial agents with novel mechanisms of action.

Postsynthetic modification provides an important way for DNA modifications. In the present research, Neo-TTP was successfully synthesized and incorporated into DNA, which enriched the toolbox of DNA postsynthetic functionalization. Further on the way of finding novel ways for rapid and site-specific post-synthesis modification of DNA without the issue of regioisomers, we described a new inverse electron-demand Diels-Alder reaction involving BCN-tetrazine pair with tunable reaction rates. Such reaction with the wide range of reaction rates (over 64-fold difference) that can be achieved suggests possibilities of multiplexing click labeling. This work paves the way to utilize BCN-tetrazine conjugation reactions for not only DNA but also other labeling work.
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(100) In our earlier screening, these two compounds were found to have IC50 values of about 30 μM. However, upon more rigorous studies, 2 was found to have IC50 of 100 μM. 1 showed similar inhibition activities as 2, but started having solubility problems when approaching 100 μM.


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APPENDICES

Appendix A. $^1$H and $^{13}$C Spectra of compounds in chapter 1

![Spectra Diagram]
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<tr>
<td>NUC1</td>
<td>13C</td>
</tr>
<tr>
<td>f1</td>
<td>8,00 usec</td>
</tr>
<tr>
<td>f2</td>
<td>16,00 usec</td>
</tr>
<tr>
<td>PL1</td>
<td>-3,00 db</td>
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<tr>
<td>SF01</td>
<td>100,6226298 MHz</td>
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#### CHANNEL f2

<table>
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<th>Parameter</th>
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<tbody>
<tr>
<td>NUC2</td>
<td>1H</td>
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<tr>
<td>P3</td>
<td>12,28 usec</td>
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<tr>
<td>f4</td>
<td>24,77 usec</td>
</tr>
<tr>
<td>PA002</td>
<td>70,00 usec</td>
</tr>
<tr>
<td>PL1</td>
<td>-1,00 db</td>
</tr>
<tr>
<td>PL12</td>
<td>-4,00 db</td>
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<tr>
<td>SF02</td>
<td>400,1314809 MHz</td>
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#### F2 - Processing parameters

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<td>ET</td>
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<tr>
<td>F</td>
<td>100,6129212 MHz</td>
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<tr>
<td>COM</td>
<td>EM</td>
</tr>
<tr>
<td>SNU</td>
<td>0</td>
</tr>
<tr>
<td>L3</td>
<td>1,00 Hz</td>
</tr>
<tr>
<td>CS</td>
<td>0</td>
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<tr>
<td>C</td>
<td>1,40</td>
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No title

Current Data Parameters
NAME: SecAO-5-Me-H
EXPNO: 1
PROCNO: 1

P2 - Acquisition Parameters
Date: 20160801
Time: 14.49
INSTRUM: spect
PROBID: 5 mm PARRO H8-
HUPTOC: zg30
TD: 65336
SOLVENT: DMSO
NS: 16
DS: 2
SWH: 6278.144 Hz
FDNRES: 0.126314 Hz
AQ: 3.9584243 sec
RD: 322.5
DW: 60.400 usec
DE: 7.00 usec
TE: 298.2 K
DI: 1.00000000 sec
MCRENT: 0.00000000 sec
MCRWX: 0.01500000 sec

= CHANNEL f1 =
NUC1: 1H
F1: 12.82 usec
PL1: 0.00 dB
SF01: 400.1324710 MHz

P2 - Processing parameters
SI: 52768
SF: 400.1300018 MHz
WCM: FM
SSB: 0
LB: 0.30 Hz
GB: 0
PC: 1.40
No title

---

**Current Data Parameters**

<table>
<thead>
<tr>
<th>NAME</th>
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<tbody>
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<td>PROCNO</td>
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**F1 - Acquisition Parameters**

<table>
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<tbody>
<tr>
<td>Time</td>
<td>17.20</td>
</tr>
<tr>
<td>INSTRUM</td>
<td>spect</td>
</tr>
<tr>
<td>PROBD</td>
<td>5 mm PARRO R8-</td>
</tr>
<tr>
<td>PULPROG</td>
<td>zg30</td>
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<tr>
<td>TD</td>
<td>65p36</td>
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<tr>
<td>SOLVENT</td>
<td>D20</td>
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<tr>
<td>NS</td>
<td>16</td>
</tr>
<tr>
<td>US</td>
<td>2</td>
</tr>
<tr>
<td>SWH</td>
<td>8278.146 Hz</td>
</tr>
<tr>
<td>FIDRES</td>
<td>0.126314 Hz</td>
</tr>
<tr>
<td>AQ</td>
<td>3.9584243 sec</td>
</tr>
<tr>
<td>RG</td>
<td>228.1</td>
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<tr>
<td>DW</td>
<td>60.400 usec</td>
</tr>
<tr>
<td>DE</td>
<td>7.000 usec</td>
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<tr>
<td>TE</td>
<td>323.3 K</td>
</tr>
<tr>
<td>D1</td>
<td>1.00000000 sec</td>
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<tr>
<td>MCREST</td>
<td>0.00000000 sec</td>
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<tr>
<td>MCXW</td>
<td>0.01500000 sec</td>
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**F1 - Processing parameters**

| SI     | 52768       |
| SF     | 400.1300058 MHz |
| WCM    | TM          |
| SBS    | 0           |
| LB     | 0.30 Hz     |
| GS     | 0           |
| PC     | 1.40        |

---

![Chemical Structure](image-url)
Appendix B. $^1$H and $^{13}$C Spectra of compounds in chapter 2
DZ-III-78-Py-Tetrazine-alkyne-1H

Current Data Parameters
PNAME:  nz-III-78
EXPN0:  1
PROCNO:  1

F2 - Acquisition Parameters
Date:  20110718
Time:  9.49
INSTRUM:  spect
PROBNO:  5 mm PABBO BB-
PULPROG:  zg30
TD:  65536
SOLVENT:  CDCl3
MS:  16
DS:  2
SWH:  823.685 Hz
FIDRES:  0.125483 Hz
AQ:  3.904639 sec
RG:  32
DW:  60.800 usec
DE:  6.50 usec
TF:  0 K
D1:  1.00000000 sec

---------- CHANNEL f1 ----------
MUC1:  1H
F1:  13.50 usec
PLW1:  16.00000000 W
SPOL:  400.1424710 MHz

F2 - Processing parameters
SI:  65536
SF:  400.140111 MHz
WDW:  EM
SSB:  0
LB:  0.30 Hz
GB:  0
FC:  1.00
DZ-III-80-Ph-tetrazine-alkyne with 3 ring-1H
DZ-III-79-isop-Tetrazine-alkyne with 3 ring-C13