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Inhibitors of SecA as Potential Antimicrobial Agents

Arpana S. Chaudhary

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INHIBITORS OF SECA AS POTENTIAL ANTIMICROBIAL AGENTS

by

ARPANA S. CHAUDHARY

Under the Direction of Prof. Binghe Wang

ABSTRACT

Protein translocation is essential for bacterial survival and the most important translocation mechanism in bacteria is the secretion (Sec) pathway. Thus targeting Sec pathway is a promising strategy for developing novel antibacterial therapeutics. We report the design, syntheses, mechanistic studies and structure-activity relationship analyses using HQSAR and 3-D QSAR Topomer CoMFA analyses of 4-oxo-5-cyano thiouracil derivatives. In summary, introduction of polar group such as –N₃ and linker groups such as –CH₂-O- enhanced the potency as well as logP and logS several fold.

We also report the discovery, optimization and structure-activity relationship study of 1,2,4-triazole containing pyrimidines as novel, highly potent antimicrobial agents. A number of inhibitors have been found to inhibit microbial growth at high nanomolar concentrations.

INDEX WORDS: SecA inhibitors, Sec pathway inhibition, Thiouracil, 1, 2,4-Triazole, Novel antibacterial therapeutics, Protein translocation inhibition
INHIBITORS OF SECA AS POTENTIAL ANTIMICROBIAL AGENTS

by

ARPANA S. CHAUDHARY

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

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2013
INHIBITORS OF SECA AS POTENTIAL ANTIMICROBIAL AGENTS

by

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August 2013
DEDICATION

I dedicate this work to my father, Sh. Prakash Chand Sagwal, who has been an immense source of inspiration, support and encouragement throughout my life. Without his guidance, I would not have achieved the goals I have. His belief in me gives me the strength to take on challenges with persistence and determination. He will always remain the source of motivation behind all my accomplishments.

This work is also dedicated to my mother, Smt. Sheela Sagwal. I cannot thank her enough for her love, dedication, adoring reprimands and light hearted beatings. The values she instilled in me during formative years have shaped me into a considerate and thoughtful person. She is the epitome of strength and spiritual learning for me. I credit her for whatever goodness I have in me.

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1. **DESIGN, SYNTHESSES AND MECHANISTIC STUDIES OF 4-OXO-5-CYANO THIOURACIL DERIVATIVES AS SEC A INHIBITORS**

Abstract: Protein translocation is essential for bacterial survival and the most important translocation mechanism in bacteria is the secretion (Sec) pathway. Thus targeting Sec pathway is a promising strategy for developing novel antibacterial therapeutics. In this chapter, we report the syntheses, evaluation and structure-activity relationship studies using HQSAR and 3-D QSAR Topomer CoMFA analyses. We designed and analyzed over 60 analogs of 4-oxo-5-cyano thioaracil derivatives based upon our previously reported core structure. In summary, introduction of polar group such as \(-N_3\) and linker groups such as \(-CH_2-O-\) enhanced the potency as well as \(\log P\) and \(\log S\) several fold. Apart from being potential antibacterial agents, SecA inhibitors can be indispensable tools for biologists to probe the mechanism of protein translocation via the SecA machinery in bacteria.

1.1 **Introduction**

1.1.1 **Why do we need NOVEL antibacterial agents?**

Ever since antibiotics were invented and referred to as ‘wonder-drugs’, they earned the reputation of a key defense mechanism for human survival against disease causing bacteria. However, the thriving occurrence of antibiotic resistance threatens a fit survival of human race and warns of impending epidemics. Looking into the chronology of resistance events, in the 1950’s *Staphylococcus aureus* resistant to last-line drugs tetracyclines and chloramphenicol appeared, and remained widespread throughout the world until the semi-synthetic penicillins and cephalosporins regained control of situation in the 1960’s. Introduced in early 1970’s, gentamicin and other aminoglycosides restrained the resistant bacteria until the late 1970’s when pathogens encoding aminoglycosides inactivating enzymes surfaced. In the early 1980’s three novel classes of antibiotics including carbapenems, fluoroquinolones and third generation cephalosporins exhibited an effective control against nearly all Gram-negative bacteria. In the last few
decades, the global emergence of extensively-drug resistant and pan-drug resistant bacterial strains has raised severe healthcare concerns.\textsuperscript{1} The US Center for Disease Control and Prevention (CDC) has classified “High Priority Antibiotic Resistant Bacteria” that include MRSA, *Clostridium difficile*, *Streptococcus pneumonia*, *Mycobacterium tuberculosis* (MDR, XDR), *Klebsiella species*, *Acinetobacter*, *Neisseria gonorrhoeae* and *Campylobacter and Salmonella*. Recently in the US, the dissemination of carbapenem-resistant *Enterobacteriaceae* (CRE) and its subtypes such as New Delhi metallo-β-lactamase resistant strains have raised added concerns due to the high mortality rates of these new strains.\textsuperscript{2}

As one can imagine, the widespread emergence of multi drug-resistant bacteria or superbugs has become a major public health concern in recent years. The urgent need for antimicrobial agents cannot be overstated and developing drugs with novel mechanism of action or new targets is more imperative than ever before.\textsuperscript{3} However, 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.\textsuperscript{3b} Last three decades have seen only three entirely new classes of antibiotics (linezolid,\textsuperscript{4} daptomycin\textsuperscript{5} and platensimycin\textsuperscript{6}) other than improved analogs of pre-existing antibiotic agents. The focus in the field is on the search for antimicrobials with new mechanisms of action and/or new targets instead of analog design along the lines of existing drugs.

\textbf{1.1.2 Is SecA an ideal target?}

SecA is an integral membrane protein, an ATPase and a protein-conducting channel across bacteria. It is the central driving force of the Sec machinery, is essential for bacterial viability and virulence, has no human counterparts and is highly conserved in sequence. In addition, the structure and function of the Sec machinery in bacteria have been extensively studied\textsuperscript{7} and a great deal of information about SecA is known through X-ray crystallography,\textsuperscript{7c,8} NMR spectroscopy,\textsuperscript{9} cryo-EM,\textsuperscript{7c,10} and other techniques.\textsuperscript{11} This makes SecA an ideal target for design and development of inhibitors.
1.1.3 Insights into Form, Function and utility of SecA as an ideal target

1.1.3.1 Structure of SecA

In order to achieve a good level of understanding for inhibitor design, it is important that we come to an adequate appreciation of the structural features of this protein such as various forms, conformational changes, and subunit complexation. SecA is the central component of the Sec machinery and is conserved in nearly all bacteria. It functions as an ATPase, assisting in the transport and folding of proteins\(^{12}\) and interacting with nearly all other components of the Sec machinery. In each \textit{E. coli} cell, there is relatively high concentration of SecA (8 µM),\(^{13}\) which corresponds to around 13,000 copies per cell if assuming a cellular volume of 2.75 µm\(^3\).\(^{14}\) SecA is a soluble protein and it localizes both to the cytosol and the cytoplasmic membrane.\(^{15}\) It is believed that most soluble SecA forms homo-dimers in the cytosol.\(^{13,16}\) This dimerization process is dynamic, and the dissociation constant is estimated at low µM range (\(K_d \sim 0.1 \mu M\)) previously.\(^{16a}\) A more accurate binding constant is determined to be 0.76 nM using an equilibrium technique and dual-color fluorescence-burst analysis (DCFBA).\(^{17}\) However, the sensitive nature of the dimerization process influenced by many factors such as salt, detergents or temperature has provided debatable results amongst a series of studies.\(^{18}\) Although there is a long-lasting debate about oligomerization of SecA in solution, it is the consensus that each protomer contains several sub-structural domains (Fig. 1.1). 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.\(^{19}\) 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 anti-parallel β strand (stem) connects PBD and NBD, and a bilobate globular domain (bulb).\(^{20}\) 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).
Figure 1.1. Three-dimensional structure of the *E. coli* SecA monomer

Table 1.1. 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><em>M. tuberculosis</em></td>
<td>1NL3&lt;sup&gt;3b&lt;/sup&gt;</td>
<td></td>
<td>1NKT (ADP, Mg&lt;sup&gt;2+&lt;/sup&gt;)</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>2IBM&lt;sup&gt;8d&lt;/sup&gt;</td>
<td>ADP</td>
<td></td>
</tr>
<tr>
<td><em>T. thermophiles</em></td>
<td>2IPC&lt;sup&gt;8c&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>2FSF&lt;sup&gt;21&lt;/sup&gt;</td>
<td></td>
<td>2FSG (ATP), 2FSH (AMP-PNP), 2FSI (ADP)</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>1M6N&lt;sup&gt;22&lt;/sup&gt;</td>
<td></td>
<td>1M74 (ADP, Mg&lt;sup&gt;2+&lt;/sup&gt;,SO&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;2-&lt;/sup&gt;)</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>3JV2&lt;sup&gt;23&lt;/sup&gt;</td>
<td>ADP, Mg&lt;sup&gt;2+&lt;/sup&gt;, peptide</td>
<td></td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>3DL8&lt;sup&gt;24&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>1TF5&lt;sup&gt;25&lt;/sup&gt;</td>
<td></td>
<td>1TF2 (ADP, Mg&lt;sup&gt;2+&lt;/sup&gt;)</td>
</tr>
<tr>
<td><em>T. maritima</em></td>
<td>3JUX&lt;sup&gt;26&lt;/sup&gt;</td>
<td>ADP, Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><em>T. maritima</em></td>
<td>3DIN&lt;sup&gt;27&lt;/sup&gt;</td>
<td>ADP, Mg&lt;sup&gt;2+&lt;/sup&gt;, BEF</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>3BXZ&lt;sup&gt;28&lt;/sup&gt;</td>
<td>ADP, Mg&lt;sup&gt;2+&lt;/sup&gt;, spermidine</td>
<td></td>
</tr>
</tbody>
</table>

The structure of SecA has been studied using X-ray crystallography (Table 1.1), tNMR spectroscopy,<sup>9a</sup> cryo-EM,<sup>27</sup> atomic force microscopy,<sup>27a</sup> small angle X-ray scattering<sup>11b</sup> and small angle neutron scattering (SANS).<sup>11a</sup> Most of these studies revealed a dimeric structure for SecA with one exception.<sup>28</sup> 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. 1.2A), while one has a parallel orientation (Fig. 1.2B).

Figure 1.2. Space filling models of SecA dimers are shown. 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

1.2 SecA functioning

The Sec pathway is the major pre-protein translocation machinery in bacteria (Fig. 1.3). It includes a Sec YEG translocon, an association complex of three embedded proteins Sec Y, Sec E and Sec G, serv-
ing as molecular receptor for an energy powering ATPase, termed SecA. This SecA in complexation with the preprotein’s signal sequence and SecB chaperone binds to the SecYEG, becomes activated for ATP hydrolysis and powers the preprotein movement through the translocon to the periplasm.\(^{20a, 31}\) Details of the SecA-mediated secretion process have been discussed elsewhere.\(^{21, 32}\) Briefly, the prevailing view of transport process has the following events (Fig. 1.3)\(^{33}\) (1) SecB binds the preprotein and then SecA,\(^9\) (2) the pre-protein bound SecA sees and binds to the lateral gate SecY\(^{25}\) of the SecYEG hetero-trimeric complex; (3) electrostatic gate opening\(^{34}\) occurs due to allosteric regulation; (4) ADP is released from the SecA motor domains\(^{9b, 34-35}\) and SecA C-terminal domain mediated suppression relieves; (5) SecA acquires the translocation ATPase activity,\(^{36}\) and (6) SecYEG channel loosens\(^{37}\) 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.

![Figure 1.3. Protein translocation machinery in bacteria](image)

Despite the fact that several structural and biochemical studies have been done over the last two decades, the exact mechanism of SecA-mediated protein translocation is unknown. Some studies have
concluded that SecA functions as a dimer\textsuperscript{16b, 38} while other studies propose that monomeric SecA is the key component in protein translocation.\textsuperscript{24, 39} Meanwhile, both monomeric\textsuperscript{24, 39a, 40} and dimeric\textsuperscript{38a, 38c, 40-41} SecA was found to bind SecYEG. Therefore, another attempt to reveal the function of SecA in protein translocation took place. In 2011, a reciprocating piston model (Fig. 1.4) was proposed by Driessen and co-workers\textsuperscript{18} as another SecA-dependent translocation pathway model. It divides the whole translocation process into two parts. The initiation of protein translocation includes steps 1-4 and the translocation cycle is illustrated in steps 5-9. First, a SecA homodimer binds to the Sec- YEG channel (Step 1)\textsuperscript{38a, 38c} initiating the ATPase activity by changing the conformation of SecA (Step 2).\textsuperscript{34, 36b} Within the dimer, one SecA is anchored to SecYEG and the PBD of this protomer showing a clamp-like structure caused by a dramatic conformational change.\textsuperscript{24} The other protomer, in the meantime, only interacts with SecYEG bound SecA.\textsuperscript{17} In the cytosol, homotetrameric chaperone protein SecB binds to the mature region of newly synthesized preproteins keeping them partially unfolded and transfers them to SecA. SecB interacts with both C-terminal of the SecA dimer separately (step 3).\textsuperscript{42} Following, ATP binds with SecA and triggers SecB release, and the signal sequence and the adjacent mature region of the preprotein are pushed into the SecYEG channel forming a hairpin-like structure (Step 4).\textsuperscript{43} Now the initiation part of protein translocation is complete. The second part of the SecA mediated protein translocation starts from Step 5.

During this step, one of the dimeric SecA protomer powered by ATP hydrolysis dissociates from the preprotein and the one SecYEG bound SecA. The remaining SecA prevents backsliding of the polypeptide chain in the SecYEG channel. In addition, an assumed de-insertion step (Step 6), which is accelerated by the proton motive force (PMF),\textsuperscript{44} is led by the conformational change of SecYEG bound SecA during ATP hydrolysis. SecA from the soluble SecA pool in cytosol re-binds to the preprotein (Step 7) and to the remaining SecA protomer. This re-binding of preprotein segment and subsequent SecYEG bound SecA causes compression of the polypeptide chain and forces it into the channel.\textsuperscript{7b, 45} This ATP-independent translocation is responsible for the insertion of a 2 to 2.5 kDa peptide segment (Step 8).\textsuperscript{46} Then, ATP binding, insertion of SecA into SecYEG and translocation of another 2 to 2.5 kDa peptide
segment occurred (Step 9).\textsuperscript{24, 33a, 33b} Repeating Steps 5-9 leads translocation of 4-5 kDa or 25-30 amino acids in each cycle\textsuperscript{77, 46} until the preprotein is fully transported.

![Diagram](image)

Figure 1.4. A schematic model of the preprotein secretion pathway in bacteria. Modified from Segers \textit{et al} \textit{Chem. Biol.}, 2011, 18, 685-698 with copyright permission from Elsevier\textsuperscript{47}

1.2.1.1 \textit{SecA functions as a protein-conducting channel in the membrane}

SecA exists in 2 forms: a soluble and a membrane form. The membrane SecA interacts with phospholipids and integrates into membranes. The previously mentioned model for protein translocation views SecYEG as the core of the protein conducting channel in the membrane with SecA as a motor to transport proteins across membranes (Fig. 1.4, Wickner’s Model).\textsuperscript{33a} However in a major breakthrough that suggests an alternative model, it has been found that SecA alone in liposomes can function as a protein-conducting channel to promote protein translocation and ionic-channel activity\textsuperscript{48} (Fig. 1.5, Tai model). This is strongly supported by earlier findings that SecA can integrate into the membranes in two forms, SecA\textsubscript{M} and SecA\textsubscript{S} \textsuperscript{49} (Fig. 1.6), and can form ring-like pores structures in lipids\textsuperscript{27a, 27c, 50} that provide the physical basis for the protein-conducting channel. The finding that SecA functions as a protein-conducting channel as well as ATPases in the membrane strongly supports the notion that SecA is accessible from the extracellular matrix by inhibitors.
The Tai model: SecA dimer alone as the protein conducting-channel to promote protein translocation and ion-channel activity. There exist two forms of integral SecA in the membranes, SecA\textsubscript{S} has the same conformation as in soluble form, and SecA\textsubscript{M} is specific for lipids.

1.2.1.2 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 form 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 IRA\textsubscript{1}, 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 PBD-NBD interface causes a large rotation of the bulb domain, which drives trapping of the first amino-terminal segment of mature pre-protein do-
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, and brings subsequent conformational changes that cause 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.

### Assays for testing SecA inhibition

Developing assaying strategies that are robust, reliable and accurate to the physiological target is crucial to the discovery of novel inhibitors. For the 104 kDa, dimeric-membrane protein SecA, the task is even more challenging due to the existence of multiple forms of physiological SecA functional within the bacteria. As discussed above, SecA exists in a regulated form (with regulatory C-terminal), which becomes unregulated (C-domain truncated) upon membrane integration. The unregulated form serves as the transporter while bound to SecYEG translocon. Also, SecA functions as an ATPase in either solution or membrane-bound form. It becomes important to emphasize that because of the involvement of different SecA forms operating inside the cell to bring about protein translocation, inhibition studies need to involve different assay techniques in order to achieve a thorough understanding of the inhibition. Understandably, it is highly likely that different inhibitors may exhibit varying affinities for the different forms of SecA.
Presently, there are several ways to test SecA inhibitory activities. First, the ATPase activity can be examined using different forms of SecA in solution, such as intrinsic SecA (regulated ATPase), truncated SecA without the C-terminal inhibitory sequence in solution (e.g., EcN68, unregulated ATPase), SecA in membrane (membrane ATPase), and SecA complexed with SecYEG in membrane (translocation ATPase). Assaying inhibition of each form of SecA provides a partial understanding of the targeted SecA form, however these assays when combined together, can be used to obtain a reliable and more accurate understanding of the actual form/stage of protein translocation inhibited. Finally, the antimicrobial growth assay is used to correlate the enzyme inhibitory activity to bacterial growth inhibition. It is important to emphasize that SecA inhibition studies do need to involve all different methods in order to achieve a thorough understanding of the ability for these inhibitors to inhibit SecA ATPase. The following section provides a detailed discussion of these assays.

1.2.1.3.1 ATPase assay

All ATPase assays rely on the determination of free inorganic phosphate formed as a result of ATP hydrolysis. In 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 and 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 and in live bacteria. Another assay method uses truncated SecA with only the catalytic domain (N68) in solution (e.g., EcSecA-N68, unregulated ATPase, Figure 1.6). 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 membrane SecA assays. 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, which is located at the interface of the SD and the IRA1 domains (Fig.
1.1. 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.\textsuperscript{56,61} Therefore, using this mutated W775A EcSecA allows the benefit of the full-length enzyme without the intrinsic inhibition effect of the C-terminal domain.\textsuperscript{32h}

Figure 1.6. 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{[55]} Top: X-ray ribbon structure of EcSecA with N68 (Yellow) and C34 (Green).

1.2.1.3.2 Protein-channel activity

The protein/ion channel activity assay developed in Tai lab\textsuperscript{48,62} is a semi-physiological assay for electrophysiological measurement of protein-channel activity in the oocytes. It truly mimics the physiological conditions, is easy to use, requires nanogram amounts of materials and studies the translocation by exogenous SecA through membrane vesicles/liposomes embedded in individual oocytes. Due to their large size, 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{62} The procedure involves using \textit{E. coli} total lipids (dried, re-suspended in 150 mM KCl, and sonicated in ice water bath, 3-5 mins), liposomes and oocytes (obtained from live frog
Xenopus laevis, Xenopus Express, Inc.). The sample mixture is injected into dark pole site of oocytes using Nanoject II injector and the ion current is recorded three hours after the injection. The amount for each component is 120 ng liposomes, 120 ng SecA, 14 ng proOmpA, 2 mM ATP, and 1 mM Mg$^{++}$ component in the oocytes, based upon the average volume of 500 nl for each oocyte. It is important to emphasize that the results from the truncated SecA assay and the electrophysiology oocyte assays seem to parallel that of antimicrobial results, which adds further support to its near physiological nature.

1.2.2 Reported SecA inhibitors and screening strategies

The suitability of SecATPase as an ideal target for the development of antibacterial agents has been increasingly recognized$^{63}$ and thus there have been efforts in developing SecA inhibitors. Table 1.2 provides a comparative summary of the various inhibitors that have been reported and the screening strategies employed therein. Prior to the recent efforts, inorganic azide (1) (Table 1.2, Entry 1) was the only known SecA inhibitor and was reported to possess antibacterial activity in 1891.$^{64}$ Decades later, its antibacterial activity was attributed to inhibition of SecA mediated protein translocation both in vitro and in vivo.$^{65}$ Sodium azide resistance in E. coli and B. subtilis has been mapped on the SecA gene$^{66}$ and it is also known to inhibit the translocase form of SecA in E. coli, albeit with a high IC$_{50}$ of 5 mM. Although, no effect on the intrinsic or membrane-bound SecA functioning has been observed. The SecA binding mechanism of sodium azide has been extensively probed,$^{33b, 46b, 67}$ which point towards a possible inhibition mechanism involving the stabilization of membrane-inserted, ADP bound form of SecA during protein translocation. Nevertheless, sodium azide is least likely to hold strong grounds as a promising SecA inhibitor given its human toxicity and non-specificity for a diverse array of other enzymes, which includes mitochondrial ATPase,$^{68}$ cytochrome c oxidase,$^{69}$ superoxide dismutase,$^{70}$ alcohol dehydrogenase,$^{71}$ and ceruloplasmin.$^{72}$

Later on, through virtual screening, random screening, and natural product isolation, inhibitors of various structural classes have been identified. A random screening of natural and synthetic compound
libraries using a SecA-LacZ reporter assay\textsuperscript{73} was conducted in 2000. 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. On the other hand, inhibition of secretion dissociates the SecA-mRNA complex and causes an up-regulation of SecA. The strategy was to identify compounds that enhance SecA expression through inhibition of protein secretion caused due to SecA inhibition. An imino-containing lipophilic compound (2) (Table 1.2, Entry 2) shows the lowest IC\textsubscript{50} value of 6.2 \(\mu\)M for \textit{S. aureus} RN 8081. A key disadvantage associated with the mentioned class of compounds is their deleterious effects on the cell membrane and the resulting high cell toxicity, which seems to have led to discontinued efforts.

CJ-21058 (3) (Table 1.2, Entry 3) a natural product isolated from CL47745 (unidentified fungus) was found to inhibit translocation SecATPase with IC\textsubscript{50} of 38.4 \(\mu\)M and showed growth inhibition of multi-drug resistant \textit{S. aureus} and \textit{Enterococcus faecalis} with MIC\textsubscript{50} value of 12.9 \(\mu\)M.\textsuperscript{74} The screening strategy was to monitor the effect of inhibitors on the SecA catalyzed translocation of proOmpA through membrane vesicles bearing SecYEG. Malachite green was used to quantify the ATP consumption by energy-motor SecA during the translocation process. However, no effect of these compounds was observed on the inhibition of \textit{S. pyogenes} and \textit{E. coli} growth.

Another report of a natural product SecA inhibitor employed an antisense-based screening method. The strategy involved using an antisense mRNA to bind selectively to mRNA regions encoding SecA, thereby blocking its translation. This decreased translation caused a subsequent bacterial sensitization towards SecA inhibitors. A two-plate agar based differential sensitivity assay including the antisense sequence RNA (AS-RNA) sensitized strain and the wild type controls were used to compare the growth inhibition. Pannomycin (4) (Table 1.2, Entry 4) obtained from the extract of a leaf-litter fungus \textit{Geomyces Pannorum} was shown to possess antibacterial activity\textsuperscript{75} against \textit{B. subtilis} (IC\textsubscript{50} = 0.4 mM), \textit{S. aureus} (IC\textsubscript{50} = 1.4 mM), \textit{E. faecalis} (IC\textsubscript{50} = 1.4 mM), although no \textit{in vitro} SecA inhibition was reported.
Our initial efforts led to the discovery of the first ever SecA inhibitors. An in silico screening of small molecule ligand libraries (~115,000 compounds) was carried out using structure-based virtual screening methods against the E. coli SecATPase. The top ranked compounds were further tested for in vitro inhibition of ATPase activity against E. coli N68. Two modest inhibitors that were obtained (Scheme 1, 11 and 12) showed IC_{50}s in the range of 100 µM.\textsuperscript{63a} For a rationally guided design, the two hits obtained were docked into the enzyme active site and binding interactions were examined to aid in structure optimization for analog syntheses. A further round of structure optimization of the isoxazolecarboxamide series 11 was carried out with the focus laid on the aryl group attached to the amide. In the second series 12, we started by testing different aryl structures flanking the central ring followed by 5-cyano-6-aryl-2-thiouracils derivatives 5 and 6 (Scheme 1 and Table 1.2, Entry 5). A 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.\textsuperscript{63b} Bacterial growth inhibition studies were done with a leaky mutant NR698 and wild type E. coli strain MC4100. The result showed that dimer series compound 5 shows low micromolar inhibition (IC_{50} = 2 µM), which is 50-fold more potent than the hit compound 11 (IC_{50} = 100 µM). For growth inhibition, the ‘monomer’ compound 6 exhibited the most potent inhibition against NR698 (IC_{50} = 20 µM), whereas ‘dimer’ compound 5 did not exhibit significantly antimicrobial activities. However, neither 5 nor 6 showed inhibition effects against wild type E. coli strain MC4100. Such results suggested that the permeability of 5 against NR698 and 6 against MC4100 might be a key factor.\textsuperscript{76} Currently, low molecular weight inhibitors are being explored to aid better membrane permeability and improved antibacterial growth inhibition.

Since our publication, another report of a series of thiazolo [4, 5-d] pyrimidine derivatives (7) (Table 1.2, Entry 6) as SecA inhibitors has been reported.\textsuperscript{63c} Their screening strategy involved using an in vitro malachite green method employing recombinant E. coli or S. aureus SecA. The compounds were also tested for inhibition of protein translocation using E. coli preprotein AlkProPhoA(Cys-)3 translocation through SecYEG overexpressed in membrane vesicles. Compound 7 was reported to have an IC_{50} value of 135 µM against EcSecA intrinsic ATPase and 200 µM for translocation SecATPase.
As discussed above, the question of which forms of reported SecA structures are truly physiological and functionally dominant still remains to be answered. From a drug discovery perspective, designing inhibitors targeting specific SecA forms can be efficiently pursued if we could link the oligomerization states such as monomer-dimer equilibria to protein secretion events. The issue of oligomerization of SecA during catalysis and in assembly with the translocase remains crucial for medicinal chemists to design selective inhibitors. For a rational drug design, this raises several issues, such as how do different SecA forms respond to inhibitors? Which could be the best SecA form to target? In our recent report,\textsuperscript{77} we (Wang and Tai labs) approached this issue by using assay techniques to individually assess inhibition for different SecA forms.

A diverse array of assaying techniques were designed and optimized to assess the inhibition abilities of Rose Bengal (RB) (8) and erythrosine B (EB) (9) (Table 1.2, Entry 7). The inhibition assays involved truncated/unregulated SecA, regulated SecA (intrinsic, membrane-bound and translocase), SecA-mediated protein translocation and the antimicrobial growth inhibition using leaky mutant EcN68 and wild type strains.\textsuperscript{77} As seen there are large differences in the sensitivity shown by different forms of Sec-ATPases towards inhibitors, which could largely be due to the effect of conformational changes affecting the accessibility of the inhibitors. The intrinsic SecA which is the native and regulated form shows higher IC\textsubscript{50} values for RB (25 µM) and EB (21 µM). The membrane-bound form shows IC\textsubscript{50} of 5 µM for RB and 12 µM for EB. The translocation ATPase shows IC\textsubscript{50} of 0.9 µM and 10 µM for RB and EB inhibition, respectively.

The effect of inhibitors on SecA-dependent protein translocation of precursor proOMpA into the membrane vesicles was also studied. Both RB and EB were found to inhibit its translocation with IC\textsubscript{50} values of 0.25 µM and 4 µM respectively. We know that Sec dependent protein translocation maintains normal physiology and is crucial for viability; therefore the effect of inhibitors on bacterial growth was studied. The MIC values observed for Gram-negative bacteria (\textit{E. coli} > 1 mM) are much higher than
gram positive (*B. subtilis*, 3.1 µM) and the leaky mutant (*E. Coli* NR698, 3.1 µM), suggesting that cellular membrane acts as a barrier to inhibitor permeability.

In a recent study, SecA inhibitors were reported for a citrus plant bacterium *Candidatus Liberibacter asiaticus* (Las) responsible for causing Huanglongbing citrus disease (Table 1.2, Entry 8).[^5] SecA homology model of Las SecA was derived from *E. coli* SecA and used for virtual screening studies. The 20 hits obtained were tested for SecA ATPase activity inhibition using malachite green spectrophotometric method and antimicrobial growth inhibition was studied for *A. tumefaciens*. The most potent SecA inhibitor obtained was 10 with IC$_{50}$ of 0.25 µM. This is the first example of inhibitors targeting Sec-ATPase of a plant infecting bacteria and its potential for SecATPase inhibition of animal infecting bacteria is worth exploring.

Table 1.2. A comparative account of various SecA inhibitors and the assays

<table>
<thead>
<tr>
<th>Entry</th>
<th>Structure and ID</th>
<th>Inhibition constants (IC$_{50}$s) and assay used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NaN$_3$(1)</td>
<td><em>Translocation</em> EcSecA: 5 mM</td>
</tr>
<tr>
<td></td>
<td>Inorganic azide$^{65}$ (1990)</td>
<td><em>Intrinsic and Membrane-bound SecATPase</em>: No effect</td>
</tr>
<tr>
<td>2</td>
<td><img src="image" alt="Imino-containing molecule" /></td>
<td><em>Antibacterial</em>: 6.2 µM (<em>S. aureus</em> RN 8081)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Assay</em>: SecA-lacZ reporter fusion to identify compounds that enhance SecA expression through inhibition of protein secretion</td>
</tr>
<tr>
<td>3</td>
<td><img src="image" alt="CJ-21058" /></td>
<td><em>Translocation SecATPase</em>: 38.4 µM</td>
</tr>
<tr>
<td></td>
<td>CJ-21058 (3)$^{74}$</td>
<td><em>Antibacterial</em>: 12.0 µM (<em>MRSA, Enterococcus faecalis</em>)</td>
</tr>
<tr>
<td></td>
<td>Source: CL47745 (unidentified fungus) (2002)</td>
<td><em>Assay</em>: Monitoring ATP hydrolysis using the SecA mediated translocation of proOmpA through the inner membrane vesicles bearing the heterotrimeric SecYEG complex</td>
</tr>
</tbody>
</table>
### 4

**Pannomycin (4)**

Source: *Geomyces Pannorum* (Fungus) (2009)

| Antibacterial: | 
| B. subtilis: 0.4 mM |
| S. aureus: 1.4 mM |
| E. faecalis: 1.4 mM |

**Assay:** Antisense-based screening strategy

### 5

**5-cyano-6-aryl-2-thiouracils derivatives**

Source: Synthetic (2010)

- **Intrinsic SecATPase (EcN68):** 2 µM (5)
- **Antibacterial (EcNR698):** 20 µM (6)

**Assay:** ATPase Malachite green assay

### 6

**Thiazolo [4,5-d] pyrimidine derivatives (7)**

Source: Synthetic (2011)

- **Intrinsic SecATPase:** 135 µM
- **Translocation SecATPase:** 200 µM
- **E. coli or S. aureus SecA**

**Assay:**
1. ATPase Malachite green assay
2. Protein translocation using membrane vesicles containing overexpressed SecYEG and the *E. coli* preprotein AlkProPhoA(Cys-)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th>SecA type</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vitro</strong> (IC₅₀/μM)</td>
<td>Unregulated/truncated N68</td>
<td>0.5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intrinsic ATPase</td>
<td>25</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Membrane bound ATPase</td>
<td>5</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Translocation ATPase</td>
<td>0.9</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td><strong>In vivo</strong> (MIC₉₅)</td>
<td>E. coli NR698 (leaky mutant)</td>
<td>3.1</td>
<td>250-500</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. subtilis168</td>
<td>3.1</td>
<td>250-500</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E. coli (Wild type)</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td></td>
</tr>
</tbody>
</table>

**RB analog (8)**

**EB analog (9)**

**Intrinsic SecATPase inhibition:** 0.25 μM (Candidatus Liberibacter asiaticus)

**Antibacterial:** 0.76 mM (A. tumefaciens)

**Assay:** ATPase Malachite green assay

**Indole derivative (10)**

Source: Synthetic (2012)
We have continued our structural optimization efforts on the core scaffold, to further explore the structural requirements for strong and selective SecA inhibition and enhanced drug-likeness. Our structure optimization has successfully achieved an overall increase of 50-fold in enzymatic inhibition and nearly 10-fold in antimicrobial inhibition with improvement in calculated physicochemical properties such as logP and logS.
1.3 Our Rationale

To begin with, we reasoned that the two most potent compounds we obtained previously (5 and 6, Scheme 1.1 and Table 1.2) were highly hydrophobic, poorly water soluble and showed low membrane permeability. As the core scaffold (optimized hit) was promisingly potent, we set out to design compounds with modifications around the core structure. Our design was guided using experimental assessment of potency along with medicinal chemistry intuition for improving pharmaceutical properties. Our focus remained on enhancing potency, membrane permeability (logP) and solubility (logS) possibly with low MW compounds. Keeping this in mind, 65 novel compounds were synthesized by introducing functional groups such as PEG and triazole linkers, charged polar groups (COOH, B(OH)$_2$, NH$_2$, OH) and uncharged polar groups (N$_3$, COOR, OR, NHR, NHOH).

1.4 Chemistry

1.4.1 Syntheses of inhibitors for enzyme and bacterial growth inhibition studies

The synthetic procedures are summarized in Schemes 1.2 and 1.3. The methods for obtaining the core structure have been reported previously.$^{63b}$ Briefly, 5-cyano-6-aryl-2-thiouracils (14a-d and 20a-c) were prepared by condensation of an appropriate aldehyde with ethyl cyanoacetate and thiourea in the presence of piperidine. This was followed by the benzylation/alkylation in the presence of potassium carbonate in acetonitrile or NaOH/THF to obtain compounds 15a-p (Scheme 1.2) and 21a-o (Scheme 1.3). Compound 16 was obtained from 15a using POCl$_3$ to introduce a -Cl group, which was then subjected to various amines/nucleophiles to obtain series 17a-j. The compounds containing azido moiety were further subjected to click coupling (Scheme 1.3) with different alkynes in order to obtain a new series having 1, 2, 3 triazole linkers (18a-g). The syntheses were carried out using the standard Cu-catalyzed click reactions. Also, in order to study the amide bond interactions, N and O-alkylations were done on 15a using the corresponding alkyl/aryl halides under basic conditions to obtain 19a-c (Scheme 1.3).
Scheme 1.2. Syntheses of series 2 and 4 with various functional group modifications (R₁-R₇)

Reagents and conditions: a) R₂-Br, K₂CO₃, CH₃CN or NaOH, THF:H₂O (3:1), rt, 8-16 h, 65-80%; b) POCl₃, 80°C, 2-3 h, 65-80%; c) R₂-NH₂/R₂-OH, CH₃CN:DMF (3:1), rt, 6-12 h, 65-85%.

Scheme 1.3. Syntheses of series 5, 6, 7 and 8 with various functional group modifications (R₈-R₁₁)

Reagents and conditions: a) R₂-CH, sodium ascorbate, CuSO₄, H₂O, DMF, rt, 16 h, 65-80%; b) R₂-X, K₂CO₃, CH₃CN:DMF, rt, 6-9 h, 70-80%; c) R₂-Br, K₂CO₃, CH₃CN or NaOH, THF:H₂O (3:1), rt, 8-16 h, 65-70%.
1.4.2 Syntheses of probes for preliminary mechanistic studies and target validation

Figure 1.7. Structure of pyrimidine analogs conjugated to biotin or immobilized on beads

1.4.2.1 Synthesis of biotinylated probe 24

Scheme 1.4. Synthesis of compound 24

Reagents and conditions: a) NHS, EDCI, DMF, rt, 16 h; b) 15o, TEA, DMF, rt, 8h, 75%

Scheme 1.4 depicts the synthesis of compound 24 in two steps. First, compound 23 (NHS-activated biotin) was obtained by reacting biotin with EDCI and NHS in DMF. The crude product thus obtained (23) was further reacted with 15o, in the presence of triethylamine in DMF, to obtain 24 in good yields. Compounds 25 and 26 were synthesized by reacting 15o and 17g with agarose beads as described in experimental section.
1.5 Biological Evaluation

1.5.1.1 Enzyme and bacterial growth inhibition assays

The enzyme inhibition studies were done using EcN68SecA, a truncated form lacking the C-terminal inhibitory domain. The literature colorimetric malachite green ATPase assay was used to measure the release of free inorganic phosphate. The growth inhibition activities of the compounds were assessed against a leaky mutant NR698 of E. coli by determining the minimum inhibition concentrations (MIC).

1.6 Results and Discussion

For initial screening, compounds were evaluated at a concentration of 25 µM. Those showing more than 50% inhibitions were further screened at 5 µM for EcN68SecA. A total of 23 compounds show IC_{50} values below 5 µM as shown in Figure 1.8.

![Figure 1.8](image.png)

Figure 1.8. Compounds screened at a concentration of 5 µM for EcN68SecA inhibition

In series 15, effect of modifications on rings A and D was studied (Scheme 1.2). The enzymatic inhibition results for 15a-15p show that modifications on ring D with groups such as −N_{3} (15a, IC_{50} = 3.8
µM; 15h, IC₅₀ = 3.5 µM), -COOME (15e, IC₅₀ = 6.0 µM) and -COOEt (15i, IC₅₀ = 8.5 µM) enhance the inhibition activity as compared to the lead (IC₅₀ = 12 µM). On the other hand, introduction of polar, highly ionizable groups such as –COOH (15d, IC₅₀ = 30 µM), -B(OH)₂ (2c, IC₅₀ = 60 µM) and –NH₂ (15b, IC₅₀ > 25 µM) at the p-position causes the inhibition potency to drop by several fold. Of all the modifications done in series 15, the one showing the most potent enzyme inhibition is the p-azido substituted compound (IC₅₀ = 3.8 µM). This led us to explore the significance of –N₃ position, resulting in synthesis of the m-N₃ substituted analog 15j. A comparison of the IC₅₀ values of 15a (IC₅₀ = 3.8 µM) and m-N₃ substituted 15j (IC₅₀ > 200 µM) shows a drastic decrease in inhibition potency. It seems that p-substitution of at least the azido moiety is crucial for enzyme inhibition activity.

![Inhibition on the ATPase activity of EcSecA N68](image)

Figure 1.9. IC₅₀ values for compounds 21a, 21j and 21k against EcSecA N68

In the same series, some early modifications done at ring A with functional groups such as –OMe, -Me,-OH, and -NHBoc (Scheme 1.2) did not show any improved inhibition over 15a. This observation was in agreement with our previously published results⁶³b where we established that biphenyl moiety works the best by itself over several other modifications such as introduction of halogens and various alkyl groups on the phenyl ring. Our general observation from modifications on ring A is that polar groups
are not very well tolerated and so far the biphenyl rings works best by itself, perhaps providing just the right combination of bulkiness and hydrophobicity.

Next, we wanted to obtain an understanding of 4-oxo-5-cyano thiouracil ring role in inhibition, mainly the importance of the amide bond. Therefore, in series 17, first carbonyl oxygen was replaced with different amines with or without the –N_3 group at the R_6 position (17a-j). Because of a good understanding of the –N_3 modification at p-position on ring D showing better potency than other functional groups, our further modifications were concentrated on keeping the –N_3 moiety intact at this position. Amongst series 17, compound 17a and 17d show IC_{50} values of 4.7 µM and 3.5 µM respectively. However, 17a with –NHOH group shows a better MIC value of 20.5 µM as compared to 17d, which shows MIC > 250 µM. The modifications done in compounds 17h (-NHCH₂CH₂COOH), 17i (-NHNH₂), and 17j (-NHCH₂CHCH) caused significant drops in the potency. Also, PEG linkers were introduced in 4e, 4f and 17g to enhance water solubility. Analog 17g (MIC = 12.5 µM) having a PEG linker with a free amine shows an MIC value, which is the same as that of 15a, although the enzyme inhibition potency for 17g (IC_{50}, 17g = 25 µM) is lower than that of 15a. A comparison of 17g with 17e and 17f (MIC > 250 µM) reveals that a terminal free –NH₂ group equipped with a PEG linker contribute to improved antimicrobial inhibition.

In series 18, 1, 2, 3-triazoles having various functional groups were introduced on ring D (Scheme 1.3). The idea was to enhance the water solubility and drug-likeness by introducing a polar, nitrogen rich triazole moiety equipped with -H bond donors and acceptors. The results reveal that compounds 18a (IC_{50} = 2.5 µM) and 18b (IC_{50} = 3.0 µM), having a phenyl and p-NH₂-phenyl respectively at position R_8 on the triazole moiety show better enzyme inhibition potency than 15a. Other modifications such as –COOH, -CH₂OOCCH₃, -CH₂CH₂COOH, -CH₂COOH, -CH₂OH in 18c-g respectively, contribute moderately (IC_{50s}, 18c = 6.5 µM, 18d = 7.5 µM, 18e = 8.0 µM, 18f = 10 µM, 18g = 8.0 µM) to enzymatic inhibition. However, there was a general trend throughout series 18, having marked decreases in antimicrobial inhibition with MICs > 100 µM. This led us to conclude that introduction of a triazole moiety with various func-
tional groups drastically diminishes the antimicrobial potency, despite 18a and 18b showing slightly improved enzymatic inhibition over 15a. Following such observations, we discontinued this series because adding bulk on ring D was adversely affecting the antimicrobial potency.

To further study the role of amide bond in the enzyme inhibition, N- and O-modifications were achieved in 19a-c. In 19a, methylation at the N-position led to a large drop in potency with an IC$_{50}$ value of 60 µM as compared to 19a (IC$_{50}$ = 3.8 µM). It is possible that the N-H bond could be contributing as H-bond donor to the enzyme binding site and thus its absence causes decreased activity. A structural comparison of 19b to 17d reveals the only difference being –OMe (IC$_{50}$ = 5 µM) and -NHMe (IC$_{50}$ = 3.8 µM) respectively in place of a carbonyl group in 15a (IC$_{50}$ = 3.8 µM). Despite the amide modifications being well-tolerated for enzymatic inhibition, they all were accompanied by loss of antimicrobial potency (MIC > 250 µM). Hence, amide bond was retained as such in further modifications, in order to preserve the balance of enzymatic and bacterial growth inhibition of our compounds.

At this point, compound 15a was our best candidate for further modifications. With the p-N$_3$ modification being beneficial on ring D, enhancing the potency and permeability several fold, next we focused our attention on the rigid biphenyl structure (rings A and B). Our initial modifications indicated less tolerance for polar moieties and introduction of –OMe, COOMe, OH groups dropped the potency several fold. So, we thought to ease out its rigidity in order to make it sufficiently flexible for a better fit and also introduce groups that would help the solubility. The introduction of linkers such as –CH$_2$O, -O-, -C≡C- would give us a clear understanding of the ring’s preference to stay rigid (biphenyl), Linear (alkyne), restricted (-O-) or a relatively free rotation (-CH$_2$O). The results obtained were remarkable in understanding the biphenyl preference. Table 1.3 shows a comparison of the effect of linkers and various R$_{10}$ substituents on enzyme and growth inhibition. The IC$_{50}$ values of compounds 21a (-CH$_2$O-), 21i (-O-) and 21l (-C≡C-) are 2.4 µM, 2.2 µM, and 1.4 µM respectively. When compared to 15a, these modifications show slight increase in enzyme inhibition; however growth inhibition remains particularly interesting for these compounds. Compounds 21a and 21i show good antimicrobial inhibition with MICs of 12.5 µM and 18.2
µM respectively, however compound 21l does not show any antimicrobial inhibition up to 250 µM. Compound 21l has poor aqueous solubility (log S = -4.07), which is much lower even when compared to the 6 (log S = -3.31) or 21a and 21i (Table 1.3). Compound 21a and 21e show significantly improved logP (0.60 and 1.78 respectively) and log S (-2.08 and -2.59 respectively) over 15a (1.28, -2.91 respectively) and compound 6. Figure 1.10 shows a summary of the structure-activity relationship study of 4-oxo-5-cyano-thiouracil analogs.

Table 1.3. A comparison to show the effect of different linkers on enzymatic and bacterial growth inhibition for series 21

<table>
<thead>
<tr>
<th>ID</th>
<th>R_{10}</th>
<th>-Y-</th>
<th>EcSecAN68 IC_{50} (µM)</th>
<th>E. coli NR698 MIC (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21a</td>
<td>-p-N_2-Bn</td>
<td>-CH_2O-</td>
<td>2.4</td>
<td>12.5</td>
</tr>
<tr>
<td>21b</td>
<td>-p-COOMe-Bn</td>
<td>-CH_2O-</td>
<td>2.0</td>
<td>&gt;250</td>
</tr>
<tr>
<td>21c</td>
<td>-p-OMe-Bn</td>
<td>-CH_2O-</td>
<td>&gt;25</td>
<td>&gt;250</td>
</tr>
<tr>
<td>21d</td>
<td>-p-Br-Bn</td>
<td>-CH_2O-</td>
<td>2.8</td>
<td>12.5</td>
</tr>
<tr>
<td>21e</td>
<td>-o-Br-Bn</td>
<td>-CH_2O-</td>
<td>2.5</td>
<td>8.8</td>
</tr>
<tr>
<td>21f</td>
<td>-Cyhexyl</td>
<td>-CH_2O-</td>
<td>4.6</td>
<td>16.7</td>
</tr>
<tr>
<td>21g</td>
<td>-(3,5-OMe-Bn)</td>
<td>-CH_2O-</td>
<td>2.0</td>
<td>&gt;250</td>
</tr>
<tr>
<td>21h</td>
<td>-(3,5-Me-Bn)</td>
<td>-CH_2O-</td>
<td>5.0</td>
<td>18.8</td>
</tr>
<tr>
<td>21i</td>
<td>-p-N_2-Bn</td>
<td>-O-</td>
<td>2.2</td>
<td>18.2</td>
</tr>
<tr>
<td>21j</td>
<td>-p-COOMe-Bn</td>
<td>-O-</td>
<td>2.8</td>
<td>8.0</td>
</tr>
<tr>
<td>21k</td>
<td>-p-OMe-Bn</td>
<td>-O-</td>
<td>3.8</td>
<td>12.5</td>
</tr>
<tr>
<td>21l</td>
<td>-p-N_2-Bn</td>
<td>-C≡C-</td>
<td>1.4</td>
<td>&gt;250</td>
</tr>
<tr>
<td>2m</td>
<td>-p-COOMe-Bn</td>
<td>-C≡C-</td>
<td>2.0</td>
<td>&gt;250</td>
</tr>
<tr>
<td>21n</td>
<td>-p-OMe-Bn</td>
<td>-C≡C-</td>
<td>1.8</td>
<td>&gt;250</td>
</tr>
<tr>
<td>21o</td>
<td>-(3,5-OMe-Bn)</td>
<td>-C≡C-</td>
<td>5.0</td>
<td>&gt;250</td>
</tr>
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</table>
Table 1.4. A comparison of logP and logS

<table>
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<th>ID</th>
<th>logP</th>
<th>logS</th>
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<tbody>
<tr>
<td>6</td>
<td>2.33</td>
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<tr>
<td>15a</td>
<td>1.28</td>
<td>-2.91</td>
</tr>
<tr>
<td>21a</td>
<td>0.60</td>
<td>-2.08</td>
</tr>
<tr>
<td>21e</td>
<td>1.78</td>
<td>-2.59</td>
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<tr>
<td>21i</td>
<td>0.68</td>
<td>-2.43</td>
</tr>
<tr>
<td>21l</td>
<td>1.88</td>
<td>-4.07</td>
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Table 1.5. Comparison of top ranked inhibitors for enzyme and growth inhibition with compound 6

<table>
<thead>
<tr>
<th>ID</th>
<th>EcSecAN68 IC₅₀ (µM)</th>
<th>E. coli NR698 MIC (µM)</th>
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</thead>
<tbody>
<tr>
<td>6</td>
<td>12.1</td>
<td>59.7</td>
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<td>17a</td>
<td>4.7</td>
<td>20.5</td>
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<tr>
<td>21a</td>
<td>2.4</td>
<td>12.5</td>
</tr>
<tr>
<td>21d</td>
<td>2.8</td>
<td>12.5</td>
</tr>
<tr>
<td>21e</td>
<td>2.5</td>
<td>8.8</td>
</tr>
<tr>
<td>21f</td>
<td>4.6</td>
<td>16.7</td>
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<tr>
<td>21h</td>
<td>&lt;5</td>
<td>18.8</td>
</tr>
<tr>
<td>21i</td>
<td>2.2</td>
<td>18.2</td>
</tr>
<tr>
<td>21j</td>
<td>2.8</td>
<td>8.0</td>
</tr>
<tr>
<td>21k</td>
<td>3.8</td>
<td>12.5</td>
</tr>
</tbody>
</table>

Figure 1.10. A summary of structure-activity relationship of 4-oxo-5-cyano-thiouracil analogs

- Spacers enhance antimicrobial potency
- Increased length or flexibility better
- Rigidity not good
- Phenyl at p-position works best
- Overall hydrophobicity preferred
- Polar groups not tolerated
- Polar ionizable well tolerated
- Carbonyl not crucial
- H-bond donors/acceptors good
- NH crucial for activity
- p-position very crucial for -N₃ group
- No activity for m-N₃ analog
- Triazole modifications good for enzymatic inhibition, poor for antimicrobial growth
- Polar (un-ionizable) > non-polar > polar (ionizable)
1.7 Preliminary mechanistic and target validation studies:

We used 15a as a representative inhibitor of 4-oxo-5-cyano thiouracil class to evaluate 1) ion-channel inhibition, 2) bactericidal and bacteriostatic inhibition against multi-drug resistant (MRSA and B. anthracis) bacterial strains, 3) response of efflux pumps, 4) effect on the virulence factors (bacterial toxins) production, and 5) SecA binding. We also conducted preliminary target evaluation using probes 24, 25 and 26 to perform 6) whole cell pull-down assays. In summary, results indicate that 15a inhibits secretion of virulence factors, remains largely unaffected by multi-drug resistance causing efflux pumps and pulls out SecA from the whole-cell lysates. These studies validate SecA as the target, and establish that 15a inhibits the production of virulence factors in bacteria.

1.7.1 Inhibition of S. aureus and B. anthracis SecA

SecA1 and SecA2 are the two SecA homologues identified in S. aureus and B. anthracis. SecA1 is important for housekeeping while SecA2 is required for virulence factor secretion such as adhesion-SraP in S. aureus and two S-layer proteins secretion in B. anthracis. The ATPase inhibition activity of 15a was analyzed against SecA1 and SecA2 for both S. aureus and B. anthracis, as shown in Table 1.6.

<table>
<thead>
<tr>
<th>Assays</th>
<th>Proteins</th>
<th>IC₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATPase activity</td>
<td>BaSecA2</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>SaSecA2</td>
<td>13</td>
</tr>
<tr>
<td>Ion-channel activity</td>
<td>SaSecA1</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>BaSecA1</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Table 1.6 15a induced inhibition of SecA homologues

Next, SecA-liposomes ion-channel activity assays (in oocytes) was done to evaluate the effect of 15a on membrane embedded SecA1, for both SaSecA1 and BaSecA1. This assay is a sensitive semi-
physiological assay developed in Tai lab. As shown in Table 1.6, 15a shows potent inhibition against ion-channel activity of SaSecA1-liposomes (IC$_{50}$ = 2 μM) and BaSecA1-liposomes (IC$_{50}$ = 2.8 μM) in the oocytes. These results obtained from enzyme inhibition and ion-channel activity assays indicate that 15a inhibits the protein translocation through SecA inhibition.

1.7.2 Antimicrobial activities for MRSA strains and B. anthracis Sterne

We tested the bacteriostatic inhibition effects of 15a and 17a on microbial growth (Table 1.7) of MRSA strains (N315 and Mu50), a clinically isolated S. aureus ATCC 6538, and B. anthracis Sterne. Both inhibitors show promising bacteriostatic effects. B. anthracis sterne is more sensitive with MIC$_{95}$ (4-5 μM) and S. aureus strains with MIC$_{95}$ 10-25 μM. In addition, bactericidal effects of 15a and 17a were also studied for S. aureus 6538, S. aureus Mu50 and B. anthracis sterne. As shown in Figure 1.11, 15a at 10 μM kills two log numbers of B. anthracis sterne in one hour, while 25 μM kills three log numbers of the other strains.

<table>
<thead>
<tr>
<th>Strains</th>
<th>15a MIC$_{50}$</th>
<th>15a MIC$_{95}$</th>
<th>17a MIC$_{50}$</th>
<th>17a MIC$_{95}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. anthracis Sterne</td>
<td>4</td>
<td>5</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>S. aureus 6538</td>
<td>12</td>
<td>15</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>S. aureus Mu50</td>
<td>23</td>
<td>25</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>S. aureus N315</td>
<td>9</td>
<td>25</td>
<td>9</td>
<td>18</td>
</tr>
</tbody>
</table>

Table 1.7. Bacteriostatic effects of 15a and 17a for S. aureus and B. anthracis
1.7.3 Effect of efflux pumps on antimicrobial potency

EcSecA is known to exist in both membrane and soluble forms,\textsuperscript{81} and constitutes ring-like structures upon interaction with anionic phospholipids.\textsuperscript{10a, 82} It has also been demonstrated to export prOmpA across liposomes\textsuperscript{83} which suggests that SecA could very well be involved in forming a protein conducting channel spanning the entire membrane. Thus, SecA inhibitors may be able to access SecA through extracellular matrix, exerting their inhibitory effects without entering cellular membrane. This could bypass the effect of efflux transporters, thereby overcoming the drug resistance issue. Drug efflux pumps are a major cause of multi-drug resistance in bacteria, therefore in Gram-positive bacteria, targeting SecA might be able to bypass the effect of efflux pumps. NorA and MepA are two major efflux pumps in \textit{S. aureus}. In this study (Table 1.8), we investigated the effect of overexpression (NorA\textsuperscript{+++} and MepA\textsuperscript{+++}) or deletion (NorA\textsuperscript{−} and MepA\textsuperscript{−}) of these efflux pumps, implicated in drug resistance, on bacteriostatic and/or bactericidal properties of inhibitors. Both, 15a and 17a were used as representative inhibitors. Table 1.8 shows the bacteriostatic effects of 15a and 17a on the \textit{S. aureus} mutants. Deletion or overexpression of NorA or MepA has not resulted in significant changes in the potency of inhibitors.
Bacteriostatic effects against *S. aureus* efflux strains

<table>
<thead>
<tr>
<th>ID</th>
<th>Strains</th>
<th>WT</th>
<th>NorA−</th>
<th>NorA+++</th>
<th>MepA−</th>
<th>MepA+++</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>8325-4</td>
<td>K1758</td>
<td>K2361</td>
<td>K2908</td>
<td>K2068</td>
</tr>
<tr>
<td>15a</td>
<td>MIC₅₀ (μM)</td>
<td>22</td>
<td>25</td>
<td>23</td>
<td>30</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>MIC₉₅ (μM)</td>
<td>64</td>
<td>50</td>
<td>40</td>
<td>75</td>
<td>50</td>
</tr>
<tr>
<td>17a</td>
<td>MIC₅₀ (μM)</td>
<td>27</td>
<td>33</td>
<td>21</td>
<td>32</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>MIC₉₅ (μM)</td>
<td>50*</td>
<td>&gt;50</td>
<td>50*</td>
<td>&gt;50</td>
<td>50*</td>
</tr>
</tbody>
</table>

Table 1.8. Bacteriostatic effects of 15a and 17a against *S. aureus* efflux strains

One would expect to see a drastic decrease in antimicrobial potency of 15a, if efflux pumps pumped out the inhibitors and *vice versa*. Neither deletion nor overexpression of NorA and MepA has significantly affected the bacteriostatic properties of 15a and 17a. There are only small changes, NorA− and MepA− slightly lowered bactericidal effects, compared to wild type, while over-expression shows slight increase in bactericidal properties. Thus, there is no marked influence of efflux pumps, on the bactericidal properties of our compounds. This evidence further supports our hypothesis, that 4-oxo-5-cyano thiouracil inhibitors may be able to access the transmembrane SecA from the periplasm.

1.7.3.1 Inhibition on secretion of *B. anthracis* toxins

15a inhibits the secretion of lethal factor, edema factor, and protective antigen toxins in *B. anthracis* as shown in Figure 1.12, at a much lower concentration than that required for *S. aureus*.

![Figure 1.12. Inhibition on the secretion of specific toxins of *B. anthracis* Sterne](image)
15a was added to the mid-log phase of *B. anthracis* sterne at OD600 ≈ 3.2 ml culture was collected after 1, 3 and 5 hrs. Then the supernatant and cell pellet were separated. 200 μl supernatant was added to 50 μl of 5X sample buffer and 40 μl was loaded into each lane. Specific antibodies were used to detect LF, EF, and PA.

These results establish that 15a is able to significantly reduce the bacterial virulence, most likely by inhibiting the in vivo functions of SecA1 in *S. aureus* and *B. anthracis*. Thus, it appears that SecA targeting inhibitors function by bringing down the bacterial virulence, caused by inhibiting the maturation of virulence causing proteins.

1.7.4 Trypsin digestion of SecA: 15a alters the digestion pattern

In order to further validate SecA as the target of our inhibitors, we investigated the SecA-inhibitor binding interactions by monitoring the difference in trypsin digestion. Ligands bound to proteins are known to cause protein stabilization, leading to conformational changes and subsequently affecting the protease digestion. Figure 1.13 (left panel) shows the effect of 15a (80 μM) on EcSecAN68 digestion pattern. First, separate additions of ATP and 15a lead to different digestion patterns; when the two are added together, the digestion is similar in pattern to only 15a. No significant effects of UV induced azido-protein cross-linking were seen. Besides, 15a also changes the trypsin digestion of SecA homologs SaSecA1 and SaSecA2 (Fig. 1.13, right panel). These results indicate that SecA is indeed the biological target of 15a.
10 μg of SaSecA1 and SaSecA2, respectively, was incubated at 25°C for 10 min with or without 15a. Then 1.2 μg of trypsin was added and the mixture was incubated on ice for 25 min. 15% SDS-PAGE were run.

### 1.7.5 Pull down assay

We performed pull-down assays to further validate SecA as the target. For this purpose, a PEG linker was used to attach 15a to biotin (24) and agarose beads (25 and 26). Western blots analysis was done and EcSecA antibodies were used to probe for SecA. Results shown in in Figure 1.14, clearly demonstrate the presence of SecA in E.coli and S. aureus lysate pull-downs respectively, with the corresponding negative controls showing no detection of SecA protein. These results further validate that 4-oxo-5-cyano thiouracil derivatives target SecA in bacteria, as shown by the studies done using the representative compound 15a.
Figure 1.14. Pull down results from *E. coli* MC4100 whole-cell lysate (top panel). Pull down results from *S. aureus* whole-cell lysate (bottom panel).

### 1.8 Computational studies

#### 1.8.1.1 Molecular docking

To get an insight into the binding modes of our inhibitors, we used molecular docking to analyze the possible binding pocket of our top 20 active ligands using Surflex-docking method in Sybyl-X 2.0. Our previously published results show the core structure binds to the ATPase binding pocket of SecA. Figure 1.15 shows the top 20 active ligands in the ATP binding pocket.
Figure 1.15. Top 20 active compounds docked in the active site of SecATPase

Figure 1.16. Compound 21a is shown in the binding pocket of SecA. Enzyme residues are color coded, blue: hydrophilic, brown: hydrophobic. 21a is colored by atom type and ATP is seen to the bottom right.

We investigated the binding modes of our compounds and Figure 1.16 depicts one of the most potent compound 21a, in the ATPase binding pocket of SecA interacting with residues at the interface of chain A and B. The azido group is seen at a distance of 6.5 Å from A/ATP900 and points towards hydrophilic residues including A/ASP512, A/ARG509, A/GLY510 and A/THR511. The change in torsion an-
gle caused by the –O atom between the two phenyl groups allows the phenoxy group to rest in a pocket, away from B/THR511, B/GLY510 and B/GLU487 residues.

1.8.1.2 *HQSAR (2D-QSAR) and Topomer CoMFA (3D-QSAR) models*

HQSAR or molecular fingerprinting is a method that represents any particular molecule into a unique string of numbers called bins. The bins are mathematical representations of unique fragments within a particular molecule that are allocated by a cyclic redundancy algorithm. For 2D-QSAR studies, we used the Hologram representation package in Sybyl-X 2.0. The training (n = 45) and test set (n = 5) molecules were fragmented in size range of four to seven atoms and various hologram lengths consisting of 97, 151, 199, 257, 307, 353, 401 bins were used. The best model was obtained from 97-bin length and was selected based upon the least standard error value ($r^2 = 0.781$, $q^2 = 0.539$, std. error = 0.289).

Figure 1.16 shows the individual atomic contributions for compound 21a in the training set. The yellow and green represent positive contributions to the activity and white are intermediate, mainly due to being invariant in the training set. Colors at the red end of the spectrum (red, red-orange, orange) reflect poor contributions and are not seen in 21a.

Figure 1.17. HQSAR generated individual atomic contributions for 21a. Green and yellow represent favorable contribution. (Contributions: white: -0.042854667 to 0.047735, yellow: 0.047735 to 0.0716025, green: 0.1193375 and above).
We used HQSAR and Topomer CoMFA to derive a global QSAR analysis using the enzyme inhibition values as dependent variables. Topomer CoMFA methodology is used as a tool to visualize steric and electrostatic interactions of the ligands and can also be used for R-group virtual screening to help guide the design of new compounds. A training set of 45 inhibitors was used for deriving the two QSAR models and an external validation using a test set of five compounds was done to analyze the predictive power of both models. Our test set compounds represented different structural properties that are incorporated within our training set compounds. The \( q^2 \) value is considered a good measure of internal inconsistencies in the model and CoMFA models have been suggested to have \( q^2 > 0.3 \), although a high \( q^2 \) value does not necessarily indicate good predictability. External validation, as described above, is an effective method for testing the predictability of model because the compounds are not presented to the program in the training set.

Figure 1.18. Topomer representations of compound 15a. Thiouracil biphenyl fragment on the left and azido benzyl on the right with overlaid steric and electrostatic contours from the Topomer CoMFA model of EcSecAN68 inhibition. (Green and yellow contours indicate the favored and disfavored steric interactions. Red and Blue contours represent the favored negative and positive electrostatic environment respectively)

Figure 1.17 shows CoMFA PLS contour maps for the steric and electrostatic fields of compound 15a. Green and yellow contours represent favorable and unfavorable steric tolerance respectively, whereas red and blue represents a preference for electronegative and electropositive groups respectively. Our
model shows an overall good correlation with topomer CoMFA training set ($r^2 = 0.789$, $q^2 = 0.428$) and HQSAR ($r^2 = 0.781$, $q^2 = 0.539$). Figures 1.18 and 1.19 show the graphic results for experimental vs predicted PIC$_{50}$ values for HQSAR and Topomer CoMFA respectively. Also, Table 1.9 shows the test set compounds with predictions using HQSAR and CoMFA models; in close correlation with each other and the experimental values. Compound 21n shows higher residual values than expected. The reason for this could be structural dissimilarity of 21n due to relatively small dataset. Nevertheless, the overall correlation of $r^2$ and $q^2$ values in both HQSAR and Topomer CoMFA models is good and the contour plots are in agreement with the experimental observations.

A structural interpretation of the contour for thiouracil biphenyl topomer indicates major steric contributions to the activity with a bulky phenyl at $p$-position (green contour). Yellow contour represents unfavorable steric clashes on the phenyl moiety as indicated by experimental results with groups such as –NHBoc (15k), -COOMe (15m and 15n) with IC$_{50s} > 25$ µM. The electronegative -CN and amide -NH bond contribute favorably to the activity with the red contour showing a preference for the negative charge. The potency drastically goes down upon methylation of –NH to –NMe (19a, IC$_{50} = 60$ µM), which is clearly demonstrated by the yellow and red contours at –NH showing its preference for non-bulky and negatively charged substituents. Favorable modifications at carbonyl group of amide with –NHMe (17d, IC$_{50} = 3.5$ µM and –OMe (19b, IC$_{50} < 5$ µM) are shown in blue contour for electropositive groups well-tolerated. For the benzyl azido topomer, a preference is clearly indicated for the bulky groups at $p$-position, which stands true to our observation of triazole substituted groups showing improved enzymatic inhibition over 15a (5a and 5b, IC$_{50} = 2.5$ and 3 µM respectively). We know from experimental observation that –COOH and –B(OH)$_2$ (15c, IC$_{50} = 30$ µM; 15d, IC$_{50} = 60$ µM) substitutions bring down the activity drastically and the blue contour map in the region show a preference for electropositive groups. Yellow contours represent steric bulk is not good at the 3-position (15j, IC$_{50} >100$ µM, $m$-substituted azido group) and a preference for electronegative group at position 2 (21e, IC$_{50} = 2.5$ µM). Topomer CoMFA models built from available structure and activities for 45 molecules in the training set.
are in good coherence with experimental results (Figure 1.20, Table 1.10) coupled with statistically good $q^2$ values and good predictability for the test set compounds (Table 1.9).

Table 1.9. External cross-validation to test HQSAR and Topomer CoMFA models’ predictability (n = 5)

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<th>No.</th>
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<th>Exp. PIC$_{50}$</th>
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Figure 1.19. Graphic plots for experimental vs predicted PIC$_{50}$ values for the training (cyan) and test set (red) for HQSAR model
Figure 1.20. Graphic plots for experimental vs predicted PIC\textsubscript{50} values for the training (cyan) and test set (red) for Topomer CoMFA 3D-QSAR model

1.9 Conclusions

Scheme 1.5 summarizes the structure optimization efforts and the sites of modifications on the optimized hit (as depicted by arrows) are also shown. Structure optimization of this novel class of SecA inhibitors has led to nearly 50-fold improvement in enzyme inhibition over the hit and 10-fold improvement in antimicrobial potency. Besides, improvement in calculated physicochemical properties such as logP and aqueous solubility (logS), have added more drug-like character to these inhibitors. The computational tools such as molecular docking, 2D and 3D-QSAR analyses allowed for a deeper understanding and valuable insights into structure-activity relationship of 4-oxo-5-cyano thiouracil inhibitors.
The mechanistic studies establish that 15a shows promising inhibition effect on ATPase activities of SecA homologues, as well as bacteriostatic and bactericidal effects on B. anthracis sterne and drug-resistant S. aureus strains. It also inhibits the secretion of virulence causing toxins in B. anthracis and thus shows the potential of reducing virulence dramatically. Virulence-targeting therapies are not sufficient to combat infection, but targeting SecA homologues does not only reduce virulence, it also decreases bacterial viability. This dual ability depicted by 4-oxo-5-cyano thiouracil inhibitors, significantly increases the chances of controlling infection and reducing the occurrence of drug resistance.

Partial trypsin digestion studies establish that SecA binding causes changes in cleavage patterns of SecA digestion. This is due to altered trypsin accessibility of SecA cleavage sites, which could be due to conformational changes induced upon inhibitor binding.
Figure 1.21 shows a schematic summary of 15a evaluation. Pull-down studies indicate SecA as a major target for 15a. However, other proteins were also pulled down including D-ribosomal binding protein, tryptophanase, trp repressor binding protein, glutamate dehydrogenase, acyl-CoA carboxylase, acyl-CoA dehydrogenase, and enoyl-(acyl-carrier protein) reductase. Although, having multiple targets inside bacteria can be a possibility, but when we analyze pull down assay results, we have to consider the limitations involved in affinity chromatography. Small-molecule binding to targets is defined by its structural attributes; here we have modified our small molecules with long PEG linkers conjugated to beads or biotin. Understandably, this influences the binding ability, selectivity and bioactivity of these molecules, resulting in possible nonspecific binding with non-target proteins. Therefore, further studies are needed to evaluate whether inhibition on those proteins could result in the antimicrobial phenotype of those inhibitors.

Also, the proteins identified from pull down assay might not be essential for bacteria, except for the known SecA and enoyl-(acyl-carrier protein) reductase. Besides, enoyl-(acyl-carrier protein) reductase is an attractive target for developing antimicrobials as it is a key enzyme of the type II fatty acid synthesis pathway.
We need to further investigate whether 4-oxo-5-cyano thiouracil inhibitors could inhibit enoyl-(acyl-carrier protein) reductase activity. It would be an exciting discovery if a single small molecule could target two essential and specific bacterial proteins. Currently, our efforts are focused on further improvement of selectivity and potency for these inhibitors.

1.10 Acknowledgements

I would like to thank Dr. Jinshan Jin and Dr. Hsiuchin Yang (Prof. P.C. Tai’s lab, Department of Biology, Georgia State University) for the biological studies of compounds. I also want to thank Dr. Siming Wang and Dr. Lifang Wang for the MS analyses.

1.11 Experimental

The following section discusses the various experimental details.

1.12 Material and Methods

1.12.1 Bacterial strains and culture condition

*S. aureus* strains ATCC 35556 and ATCC 6538 were obtained from the American Type Culture Collection. Various *S. aureus* strains Mu50, Mu3, and N315 were generously provided by Dr. Chung-Dar Lu laboratory at Georgia State University. *S. aureus* strains 8325-4, K1758 (NorA−), K2361 (NorA+), K2908 (MepA−), K2068 (MepA++) were kindly provided by Dr. GW Kaatz at Wayne State University School of Medicine. All strains were grown on (LB) agar plates or broth at 37°C.

1.12.2 SecA-liposomes ion-channel activity assays in the oocytes

The procedure for preparation of liposomes has been described. *E. coli* lipids (Avanti) were dried and re-suspended in 150 mM KCl, and then sonicated in ice water bath for 3-5 min, till the solution was clear. Liposomes were stored at -80 °C and thawed just before use. The oocytes were obtained from live frog Xenopus laevis (Xenopus Express, Inc) and injected with sample mixtures as described previously. Nanoject II injector (Drummond Scientific Co., Broomall, PA) was used to inject 50 nl sample into dark
pole site of oocytes. The amount for each component is 120 ng liposomes, 120 ng SecA, 14 ng proOmpA, 2 mM ATP, and 1 mM Mg++. The effective concentration of component in the oocytes was based on the average volume of oocytes of 500 nl. The ion current was recorded three hours after injection. Figure 2.3 shows a schematic procedure of the ion-channel activity assay.

1.12.3 Bacterial growth inhibition assay

For each experiment, a 12 ml culture tube was used to inoculate a single colony of bacteria in 3 ml of LB medium. The colony was grown at 37°C to an OD$_{600}$ of ~ 0.5. This was followed by dilution of 300 μl of the culture into 3 ml LB medium. Then, 97.5 μl of the cell culture was aliquoted and transferred to 1.5 ml eppendorf tube. To this tube was added 2.5 μl of SecA inhibitor at different concentrations and was left to incubate for 14 hr at 37°C in a thermo-mixer at (1000 rpm). Finally, OD$_{600}$ was measured to determine the inhibition effect.

1.12.4 Bactericidal effect determination

For each experiment, a 12 ml culture tube was used to inoculate a single colony of bacteria in 3 ml of LB medium. The colony was grown at 37 °C to an OD$_{600}$ of about 0.5, then 97.5 μl of the cell culture was aliquoted and transferred to 1.5 ml eppendorf tube. To this tube was added 2.5 μl of SecA inhibitor at different concentrations and was left to incubate for 1-2 hr, (1000 rpm) at 37°C. Finally, it was diluted with deuterated H$_2$O and 150 μl of the culture was spread on to LB plates.

1.12.5 Virulence factor secretion

The inhibitors were added to the culture of S. aureus Mu50 or B. anthracis sterne at the mid-log phase of growth. OD$_{600}$ was used to monitor the bacteria growth and the culture was collected after treatment with inhibitors at different time points. For the S. aureus culture, the supernatant and cell pellet were separated using both centrifugation and syringe disk filter (0.45 μm) purchased from Fisher scientific. However, the supernatant of B. anthracis was separated from pellet using only the disk filter (0.45 μm). The toxins were detected using toxin-specific antibodies in western blot assay. The antibodies were pur-
chased from Abcam (\textit{S. aureus} enterotoxin B, \textit{B. anthracis} lethal factor (LF), \textit{B. anthracis} edema factor (EF), and \textit{B. anthracis} protective antigen (PA), Acris-antibodies (\textit{S. aureus} toxin shock syndrome toxin-1 (TSST-1)) and Sigma-Aldrich (\textit{S. aureus} \alpha-hemolysin).

1.12.6 Pull down assay

\textit{E. coli} MC4100 and \textit{S. aureus} Mu50 were grown in LB medium overnight at 37°C. Bacterial cells were harvested using centrifugation and then washed with binding buffer (0.1 M K$_3$PO$_4$, pH 7.2). Cell pellets thus obtained were re-suspended in binding buffer, containing a cocktail of protease inhibitors. Cell pellets were run through french press at 10,000 psi and the unbroken cells were separated by centrifugation at 5,000 rpm. Agarose beads (purchased from invitrogen) and pierce streptavidin agarose resins (purchased from Thermo-scientific) were used for pull down. For pull-down with agarose beads, the whole cell lysates were mixed with beads, and was left on a circular shaker at 4°C for 1 h, in the presence/absence of inhibitors, followed by rolling in shaker at rt for 10 hr. For streptavidin beads, the whole cell lysates were pretreated with beads at 4°C for 1 hr and then centrifuged for 2 min at 500 rpm. The supernatant was treated as above. Both agarose beads and streptavidin beads were separated from cell lysates by centrifugation at 500 rpm for 2 min. Then, beads were washed with binding buffer for 5 times. After washing, beads were mixed with binding buffer and SDS sample buffer, and then boiled for 30 min.

1.12.7 Computational studies

The QikProp module in Maestro v9.3.518 (Schrodinger) software was used for physicochemical property (log\textit{P}, log\textit{S}) calculations.

The computational studies including molecular docking, HQSAR, Topomer CoMFA was done using SYBYL-X 2.0 by following the standard procedures provided in the manual. HQSAR was run from SYBYL using default parameters: hologram lengths (97, 151, 199, 257, 307, 353 and 401), fragment atom count (4-7 atoms) were used. The best model was selected on the basis of the least standard error. For Topomer CoMFA, the two-piece fragmentation was done followed by automated PLS analysis, fragment alignment and activity predictions. SYBYL generated statistical parameters such as $q^2$, $r^2$, number of
components. Standard errors of prediction were recorded for both sets and steric and electrostatic contour maps were generated. A training set (n = 45) and test set (n = 5) were used for model building and cross-validation respectively for both HQSAR and Topomer CoMFA 3-D models. Table shows the structures and Test and training set predictions.

For molecular docking, surflex-dock method was used to dock top 20 active ligands into SecA crystal structure (PDB ID: 2FSG) using automatic docking. Standard parameters given in SYBYL X 2.0 were used for energy minimization of SecA crystal structure. Protein preparation was done using AMBER7 FF99 force field and the default parameters in surflex-Dock (SFXC). A maximum of 20 docking poses for each molecule were generated.

Table 1.10. A comparison of experimental and predicted PIC$_{50}$ values using Topomer CoMFA and HQSAR for the training set

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Table 1.11. A comparison of experimental and predicted PIC\textsubscript{50} values using Topomer CoMFA and HQSAR for the test set

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1.12.8 General chemical methods:

All chemical reagents and solvents used were reagent grade or purified using standard methods. TLC analyses were conducted on silica gel plates (Sorbent Silica G UV254). Column chromatography was carried out on flash silica gel (Sorbent 230–400 mesh). NMR spectra were recorded at $^1$H (400 MHz) and $^{13}$C (100 MHz) on a Bruker instrument. Coupling constants ($J$) and chemical shifts ($\delta$) are given in hertz and ppm respectively, using TMS ($^1$H NMR) and solvents ($^{13}$C NMR) as internal standards.
General synthetic procedure for 14a-d: A previously published\textsuperscript{63b} procedure was followed. Briefly, to a solution of ethanol (50 mL) and appropriate aldehyde (RCHO, 5 mmol) was added ethyl cyanoacetate (0.5 mL, 5 mmol), thiourea (0.38 g, 5 mmol) and piperidine (1.0 mL, 10 mmol). The mixture was heated under reflux for overnight and then cooled to room temperature. The precipitate was dissolved in 0.5 M NaOH (20 mL) and washed with ethyl acetate (15 ml × 3). Then the aqueous solution was acidified to pH ~ 2 by slow addition of 1 M HCl. This caused the product to precipitate, which was then filtered using vacuum filtration. Yield: 60-65%.

General synthetic procedure for 15a-b and 15e-p: To a solution of 6-((1, 1'-biphenyl)-4-yl)-4-oxo-2-thioxo-1, 2, 3, 4-tetrahydropyrimidine-5-carbonitrile derivatives (1.37 mmol) in CH\textsubscript{3}CN (8 ml), K\textsubscript{2}CO\textsubscript{3} (6.85 mmol) was added and the resulting mixture was stirred for 10-15 min. To this was added the appropriate (bromomethyl)benzene derivatives (1.23 mmol) and the reaction was stirred at room temperature for 16-18 h. Upon completion, the reaction mixture was cooled to ambient temperature and the solvent removed \textit{in vacuo}. The dried residue was washed by H\textsubscript{2}O (pH = 9-10, 20 mL × 2) and brine (15 ml × 2) followed by product extraction in ethyl acetate (20 ml). The solvent was evaporated \textit{in vacuo} to obtain crude product, which was purified using silica gel column chromatography. Yields: 65-80%.

4-((1,1'-Biphenyl)-4-yl)-2-((4-azidobenzyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (15a):
Yield: 72%; \textsuperscript{1}H NMR (DMSO-\textit{d}_6) \(\delta\) 8.07 (d, \(J = 8.0\) Hz, 2H), 7.90 (d, \(J = 8.0\) Hz, 2H), 7.79 (d, \(J = 8.0\)Hz, 2H), 7.49 (m, 5H), 7.09 (d, \(J = 8.0\) Hz, 2H), 4.56 (s, 2H); \textsuperscript{13}C NMR (DMSO-\textit{d}_6) \(\delta\) 170.2, 167.1, 166.0, 161.6, 143.8, 139.3, 139.1, 134.5, 133.9, 131.1, 129.9, 129.5, 128.8, 127.4, 119.7, 116.4, 93.4, 34.1. HRMS (ESI-TOF): Calc’d for C\textsubscript{24}H\textsubscript{17}N\textsubscript{6}OS [MH\textsuperscript{+}]: 437.1179; found: 437.1183.

4-((1, 1'-Biphenyl)-4-yl)-2-((4-aminobenzyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (15b):
Yield: 65%; \textsuperscript{1}H NMR (DMSO-\textit{d}_6) \(\delta\) 7.62 (d, \(J = 8.0\) Hz, 2H), 7.54 (d, \(J = 8.0\) Hz, 2H), 7.48-7.52 (m, 5H), 6.94 (d, \(J = 8.0\) Hz, 2H), 6.59 (d, \(J = 8.0\) Hz, 2H); \textsuperscript{13}C NMR (DMSO-\textit{d}_6) \(\delta\) 166.0, 163.4, 160.2, 149.7, 139.8, 135.9, 130.0, 129.9, 129.1, 129.0, 128.2, 128.1, 127.0, 114.9, 112.6, 93.4, 36.9. HRMS-ESI (+): Calc. for C\textsubscript{24}H\textsubscript{18}N\textsubscript{4}OS [MH\textsuperscript{+}]: 411.1280; found: 411.1276.
(4-((6-Cyano-5-oxo-4, 5-dihydro-[1, 1′:4′, 1″-terphenyl]-3yl) thio) methyl) phenyl) boronic acid (15c): Yield 72%; $^1$H NMR (DMSO-$d_6$): $\delta$ 8.05 (t, $J = 8.0$ Hz, 3H), 7.89 (d, $J = 8.0$ Hz, 2H), 7.79 (s, $J = 8.0$ Hz, 2H), 7.74 (d, $J = 8.0$ Hz, 2H), 7.52 (t, $J = 8.0$ Hz, 2H), 7.45 (d, $J = 8.0$ Hz, 2H), 7.40 (d, $J = 8.0$ Hz, 2H), 4.56 (s, 2H); $^{13}$C NMR (DMSO-$d_6$): $\delta$ 167.1, 166.2, 163.2, 143.7, 139.3, 138.7, 134.8, 134.7, 130.0, 129.6, 128.8, 128.5, 127.4, 127.3, 116.6, 93.2, 34.7. HRMS (ESI-TOF): Calc’d for C$_{24}$H$_{18}$BN$_3$NaO$_3$S [$MNa^+$]: 462.1054; found: 462.1055.

4-((6-Cyano-5-oxo-4, 5-dihydro-[1, 1′, 4′, 1″-terphenyl]-3-yl) thiomethyl) benzoic acid (15d):

Yield 62%; $^1$H NMR (DMSO-$d_6$): $\delta$ 8.03 (d, $J = 8.0$ Hz, 2H), 7.91 (t, $J = 8.0$ Hz, 4H), 7.79 (d, $J = 8.0$ Hz, 2H), 7.54 (m, 4H), 7.44 (t, $J = 8.0$ Hz, 1H), 4.63 (s, 2H); $^{13}$C NMR (DMSO-$d_6$): $\delta$ 167.4, 167.1, 165.9, 161.8, 143.7, 142.4, 139.3, 134.5, 130.3, 129.9, 129.9, 129.6, 129.5, 128.7, 127.4, 127.2, 116.4, 93.4, 34.2. HRMS (ESI-TOF): Calc’d for C$_{25}$H$_{16}$N$_3$O$_3$S [$MNa^+$]: 438.0918; found: 438.0914.

Methyl 4-(((4-([1, 1′-biphenyl]-4-yl)-5-cyano-6-oxo-1,6-dihydropyrimidin-2 ylthio)methyl) benzoate (15e): Yield 83%; $^1$H NMR (DMSO-$d_6$) $\delta$ 7.98 (d, $J = 8.0$ Hz, 2H), 7.90 (d, $J = 8.0$H, 2H), 7.83 (d, $J = 8.0$ Hz, 2H), 7.76 (d, $J = 8.0$ Hz, 2H), 7.58 (d, $J = 8.0$ Hz, 2H), 7.51 (t, $J = 8.0$ Hz, 2H), 4.57 (s, 2H), 7.42 (t, $J = 4.0$ Hz, 1H), 4.55 (s, 2H), 3.84 (s, 3H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 167.0, 166.4, 143.9, 143.1, 139.5, 135.4, 132.0, 129.9, 129.7, 129.7, 129.6, 129.5, 128.9, 128.6, 127.3, 127.0, 117.8, 92.0, 52.5, 34.0. HRMS (ESI-TOF): Calc’d for C$_{26}$H$_{19}$N$_3$O$_3$S [$MNa^+$]: 476.1039; found: 476.1039.

4-((1, 1′-Biphenyl]-4-yl)-2-(methylthio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (15f): Yield 68%

$^1$H NMR (DMSO-$d_6$): $\delta$ 7.92 (d, $J = 8.0$ Hz, 2H), 7.86 (d, $J = 8.0$ Hz, 2H), 7.74 (d, $J = 8.0$ Hz, 2H), 7.40 (t, $J = 8.0$ Hz, 2H), 7.23 (t, $J = 8.0$ Hz, 1H), 4.05 (s, 3H), 2.64 (s, 3H); $^{13}$C NMR (DMSO-$d_6$): $\delta$ 173.2, 170.1, 166.4, 139.8, 135.4, 129.6, 127.3, 127.1, 114.3, 85.4, 53.5, 14.5. HRMS (ESI-TOF): Calc’d for C$_{19}$H$_{16}$N$_2$OS [$MNa^+$]: 334.1014; found: 334.1019.

4-((1, 1′-Biphenyl]-4-yl)-6-oxo-2-(prop-2-yn-1-ylthio)-1, 6-dihydropyrimidine-5-carbonitrile (15g):

Yield 72%; $^1$H NMR (DMSO-$d_6$): $\delta$ 8.09 (d, $J = 8.0$ Hz, 2H), 7.86 (d, $J = 8.0$ Hz, 2H), 7.78 (d, $J = 8.0$ Hz, 2H), 7.521 (t, $J = 8.0$ Hz, 2H), 7.44 (t, $J = 8.0$ Hz, 1H), 4.06 (s, 2H), 3.23 (s, 1H); $^{13}$C NMR (DMSO-$d_6$):
δ 166.9, 143.4, 139.4, 135.1, 129.8, 129.5, 128.6, 127.3, 127.1, 74.2, 19.3. HRMS (ESI-TOF): Calc’d for C_{20}H_{13}N_{2}OS [MH^+]: 344.0852; found: 344.0845.

4-[[1, 1'-Biphenyl]-4-yl]-2-((2-(4-azidophenyl)-2-oxoethyl)thio)-6-oxo-1,6 dihydropyrimidine-5-carbonitrile (15j): Yield 74%; \(^1^H\) NMR (DMSO-\(d_6\)) \(δ\): 8.07 (d, \(J = 8.0\) Hz, 2H), 7.74 (d, \(J = 8.0\) Hz, 2H), 7.68 (d, \(J = 8.0\) Hz, 2H), 7.60 (d, \(J = 8.0\) Hz, 2H), 7.50 (t, \(J = 8.0\) Hz, 2H), 7.42 (t, \(J = 8.0\) Hz, 1H), 7.27 (d, \(J = 8.0\) Hz, 2H), 4.84 (s, 2H); \(^{13}^C\) NMR (DMSO-\(d_6\)) \(δ\): 192.3, 166.9, 145.1, 143.2, 139.3, 133.0, 130.9, 129.5, 128.7, 127.2, 126.7, 119.7, 115.0. HRMS (ESI-TOF): Calc’d for C_{23}H_{13}N_{2}O_{2}S [MH^+]: 465.1128; found: 465.1207.

Ethyl 4-((((1, 1'-biphenyl)-4-yl)-5-cyano-6-oxo-1,6-dihydropyrimidin-2-thiol)methyl)benzoate (15i): Yield 82%; \(^1^H\) NMR (DMSO-\(d_6\)) \(δ\): 7.96 (d, \(J = 8.0\) Hz, 2H), 7.85 (m, \(J = 8.0\)Hz, 4H), 7.74 (d, \(J = 8.0\) Hz, 2H), 7.55 (d, \(J = 8.0\) Hz, 2H), 7.47 (t, \(J = 8.0\) Hz, 4H), 7.39 (t, \(J = 8.0\) Hz, 1H), 4.57 (s, 2H), 4.24 (q, 2H), 1.25 (t, \(J = 8.0\) Hz, 3H); \(^{13}^C\) NMR (DMSO-\(d_6\)) \(δ\): 167.2, 166.5, 165.9, 143.6, 143.1, 139.3, 134.8, 129.8, 129.7, 129.7, 129.5, 128.7, 127.3, 127.1, 116.8, 93.4, 61.1, 34.2, 14.5. HRMS (ESI-TOF): Calc’d for C_{22}H_{21}N_{3}NaO_{3}S [MNa^+]: 490.1196; found: 490.1204.

4-[[1, 1'-Biphenyl]-4-yl]-2-((3-azidobenzyl(thio)-6-oxo-1,6 dihydropyrimidin-2-thiol)methyl)benzoate (15j): Yield 80%; \(^1^H\) NMR (DMSO-\(d_6\)) \(δ\): 8.06 (d, \(J = 8.0\) Hz, 2H), 7.89 (d, \(J = 8.0\) Hz, 2H), 7.78 (d, \(J = 8.0\) Hz, 2H), 7.52 (t, \(J = 8.0\) Hz, 2H), 7.44 (t, \(J = 8.0\) Hz, 1H), 7.38 (t, \(J = 8.0\) Hz, 1H), 7.27 (d, \(J = 8.0\) Hz, 1H), 7.22 (s, 1H), 7.02 (m, \(J = 4.0\) Hz, 1H), 4.57 (s, 2H); \(^{13}^C\) NMR (DMSO-\(d_6\)) \(δ\): 167.1, 166.0, 143.8, 139.9, 139.6, 139.3, 130.6, 129.9, 129.5, 128.8, 127.4, 127.2, 126.3, 120.0, 118.7, 116.4, 93.4, 34.1. HRMS (ESI-TOF): Calc’d for C_{23}H_{21}N_{3}OS [MH^+]: 437.1179; found: 437.1183.

Tert-butyl (4'-2-(benzylthio)-5-cyano-6-oxo-1, 6-dihydropyrimidin-4-yl)-[1, 1'-biphenyl]-3-yl)carbamate (15k): Yield 75%; \(^1^H\) NMR (DMSO-\(d_6\)) \(δ\): 9.49 (s, 1H), 8.10 (d, \(J = 8.0\) Hz, 2H), 7.46 (d, \(J = 8.0\) Hz, 2H), 7.39 (s, 1H), 7.00 (s, 1H), 4.62 (s, 2H), 3.87-3.83 (m, 9H), 3.59 (s, 3H); \(^{13}^C\) NMR (DMSO-\(d_6\)) \(δ\): 167.8, 167.3, 166.4, 151.5, 148.3, 144.8, 143.1, 135.4, 134.2, 129.8, 129.1, 128.8, 122.1, 166.7, 114.1, 113.2, 93.1, 56.3, 56.2, 52.6, 52.1, 34.2. HRMS (ESI-TOF): Calc’d for C_{29}H_{26}N_{4}O_{3}S [MH^+]: 509.1647; found: 509.1644.
2-(Benzylthio)-4-(3'-hydroxy-[1,1'-biphenyl]-4-yl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (15l): Yield 80%; $^1$H NMR (DMSO-d$_6$): $\delta$ 9.75 (br, 1H), 8.00 (d, $J = 8.0$ Hz, 2H), 7.77 (d, $J = 8.0$ Hz, 2H), 7.62 (d, $J = 8.0$ Hz, 2H), 7.43 (d, $J = 8.0$ Hz, 2H), 7.30 (m, 3H), 6.89 (d, $J = 8.0$ Hz, 2H), 4.52 (s, 2H); $^{13}$C NMR (DMSO-d$_6$) $\delta$: 167.0, 166.7, 158.4, 143.6, 140.8, 137.2, 133.6, 130.5, 129.9, 129.8, 129.4, 128.9, 128.5, 127.8, 127.0, 126.1, 118.1, 117.0, 116.3, 115.7, 114.1, 92.3, 34.6. HRMS (ESI-TOF): Calc’d for C$_{24}$H$_{18}$N$_3$O$_2$S [M+H]$: 412.1120$; found: 412.1112.

Methyl 4'-(2-((4-azidobenzyl)thio)-5-cyano-6-oxo-1,6-dihydropyrimidin-4-yl)-4,5-dimethoxy-[1,1'-biphenyl]-2-carboxylate (15m): Yield 81%; $^1$H NMR (DMSO-d$_6$): $\delta$ 7.99 (d, $J = 8.0$ Hz, 2H), 7.47 (d, $J = 8.0$ Hz, 4H), 7.39 (s, 1H), 7.07 (d, $J = 8.0$ Hz, 2H), 6.99 (s, 1H), 4.55 (s, 3H), 3.86 (s, 3H), 3.85 (s, 3H), 3.58 (s, 3H); $^{13}$C NMR (DMSO-d$_6$): $\delta$: 167.7, 167.3, 166.0, 161.7, 151.6, 148.4, 144.9, 139.0, 135.4, 134.0, 133.9, 131.1, 129.1, 128.8, 122.1, 119.6, 116.4, 114.2, 113.3, 93.3, 56.3, 56.2, 52.1, 34.1. HRMS (ESI-TOF): Calc’d for C$_{29}$H$_{21}$N$_6$O$_5$S [MH$^+$]: 553.1300; found: 553.1306.

Methyl 4'-(5-cyano-2-((4-(methoxycarbonyl)benzyl)thio)-6-oxo-1,6-dihydropyrimidin-4-yl)-4,5-dimethoxy-[1,1'-biphenyl]-2-carboxylate (15n): Yield 72%; $^1$H NMR (DMSO-d$_6$): $\delta$ 7.92 (d, $J = 8.0$ Hz, 4H), 7.59 (d, $J = 8.0$ Hz, 2H), 7.46 (d, $J = 8.0$ Hz, 2H), 7.39 (s, 1H), 7.00 (s, 1H), 4.62 (s, 2H), 3.87 (s, 3H), 3.86 (s, 3H), 3.83 (s, 3H), 3.59 (s, 3H); $^{13}$C NMR (DMSO-d$_6$): $\delta$: 167.8, 167.3, 166.4, 151.5, 148.3, 144.8, 143.1, 135.4, 134.2, 129.8, 129.1, 128.8, 122.1, 166.7, 114.1, 113.2, 93.1, 56.3, 56.2, 52.6, 52.1, 34.2. HRMS (ESI-TOF): Calc’d for C$_{30}$H$_{25}$N$_3$NaO$_5$S [MNa$^+$]: 594.1305; found: 594.1324.

4-(((4-(1,1'-Biphenyl)-4-yl)-5-cyano-6-oxo-1,6-dihydropyrimidin-2-yl)thio)methyl)-N-3-(2-(2-(3-aminopropoxy)ethoxy)ethoxy)propyl)benzamide (15o): Yield 68%; $^1$H NMR (MeOD): $\delta$ 8.02 (d, $J = 8.0$ Hz, 2H), 7.75 (m, $J = 8.0$ Hz, 4H), 7.66 (d, $J = 8.0$ Hz, 4H), 7.46 (t, $J = 8.0$ Hz, 2H), 7.39 (t, $J = 8.0$ Hz, 1H), 4.55 (s, 2H), 3.58 (m, 12H), 3.46 (t, $J = 8.0$ Hz, 4H), 3.07 (t, $J = 8.0$ Hz, 2H), 1.99 (m, 4H); $^{13}$C NMR (MeOD): $\delta$: 168.0, 167.9, 167.46, 143.7, 141.0, 139.3, 134.2, 132.9, 128.9, 128.6, 128.3, 127.5, 126.8, 126.4, 126.3, 116.2, 91.3, 69.7, 69.3, 69.2, 68.6, 68.5, 38.4, 37.0, 33.8, 28.6, 26.3. HRMS (ESI-TOF): Calc’d for C$_{35}$H$_{40}$N$_3$O$_5$S [MH$^+$]: 642.2745; found: 642.2742.
**Tert-butyl(1-(4-(((4-(1'-biphenyl)-4-yl)-5-cyano-6-oxo-1,6-dihydropyrimidin-2-yl)thio)methyl)phenyl)-1-oxo-6,9,12-trioxa-2-azapentadecan-15-yl)carbamate (15p):**

1H NMR (DMSO-d$_6$): δ 8.06 (d, J = 8.0 Hz, 2H), 7.89 (d, J = 8.0 Hz, 2H), 7.79 (d, J = 8.0 Hz, 2H), 7.48 (m, 6H), 7.09 (d, J = 8.0 Hz, 2H), 4.55 (s, 2H), 2.09 (s, 1H); 13C NMR (DMSO-d$_6$): δ 173.8, 173.4, 166.6, 162.0, 143.1, 139.5, 138.3, 135.4, 130.9, 129.6, 129.5, 127.3, 127.1, 119.6, 115.2, 83.5, 34.5. HRMS (ESI-TOF): Calc’d for C$_{25}$H$_{19}$N$_3$OS [MH$^+$]: 450.1137; found: 450.1132.

**General synthetic procedure for 15c-d:**

To a solution of 6-((1', 1'-biphenyl)-4-yl)-4-oxo-2-thioxo-1, 2, 3, 4-tetrahydropyrimidine-5-carbonitrile derivatives (0.32 mmol) in THF: H$_2$O (3:1, 4 ml) was added NaOH (0.35 mmol) and the resulting solution was stirred for 10-15 min. To this mixture, appropriate (bromomethyl) benzene derivatives (0.29 mmol) were added and the reaction was stirred at room temperature for 8 h. On completion, the reaction mixture was cooled to ambient temperature and the solvent removed in vacuo. The dried residue was washed with acidified water (pH = 4-5, 20 mL × 2), brine (15 ml × 2) and the product was extracted in ethyl acetate (20 ml). The solvent was evaporated under vacuum to obtain crude residue, which was purified using silica gel column chromatography. Yields: 75-80%

(4-(((6-Cyano-5-oxo-4, 5-dihydro-[1, 1':4', 1''-terphenyl]-3yl)thio)methyl)phenyl)boronic acid (15c): Yield 72%; 1H NMR (DMSO-d$_6$): δ 8.05 (t, J = 8.0 Hz, 3H), 7.89 (d, J = 8.0 Hz, 2H), 7.79 (s, J = 8.0 Hz, 2H), 7.59 (d, J = 8.0 Hz, 2H), 7.54 (d, J = 8.0 Hz, 2H), 7.46 (s, 2H); 13C NMR (DMSO-d$_6$): δ 167.1, 166.2, 163.2, 143.7, 139.3, 138.7, 134.8, 134.7, 130.0, 129.6, 128.8, 128.5, 127.4, 127.3, 116.6, 93.2, 34.7. HRMS (ESI-TOF): Calc’d for C$_{24}$H$_{18}$BN$_3$NaO$_3$ [MNa$^+$]: 462.1054; found: 462.1055.

4-(((6-Cyano-5-oxo-4, 5-dihydro-[1, 1', 4', 1''-terphenyl]-3-yl)thio)methyl)benzoic acid (15d).

Yield 62%; 1H NMR (DMSO-d$_6$): δ 8.03 (d, J = 8.0 Hz, 2H), 7.91 (t, J = 8.0 Hz, 4H), 7.79 (d, J = 8.0 Hz, 2H), 7.54 (m, 4H), 7.44 (t, J = 8.0 Hz, 1H), 4.63 (s, 2H); 13C NMR (DMSO-d$_6$): δ: 167.4, 167.1, 165.9, 161.8, 143.7, 142.4, 139.3, 134.5, 130.3, 129.9, 129.6, 129.5, 128.7, 127.4, 127.2, 116.4, 93.4, 34.2

HRMS (ESI-TOF): Calc’d for C$_{25}$H$_{16}$N$_3$O$_3$S [MH$^+$]: 438.0918; found: 438.0914.
General synthetic procedure for 17a-j: To a solution of 3 (0.109 mmol) in 2 ml of CH$_3$CN: DMF (3:1) was added R-NH$_2$/R-OH (0.164 mmol) and the reaction was allowed to stir at room temperature overnight. Alternatively the reaction was monitored by TLC and on disappearance of the starting material the reaction solvent was dried in vacuo. The dried residue product was dissolved in ethyl acetate (20 ml) and washed with H$_2$O (5 ml x 3) and brine (5 ml x 3). The solvent was evaporated under vacuum to obtain crude residue, which was purified using silica gel column chromatography. Yields: 65-85%

4-[[1,1'-Biphenyl]-4-yl]-2-((4-azidobenzylthio)-6-(hydroxyamino)pyrimidine-5-carbonitrile (17a):

Yield 65%; $^1$H NMR (DMSO-d$_6$): $\delta$ 8.06 (d, $J$ = 8.0 Hz, 2H), 7.89 (d, $J$ = 8.0 Hz, 2H), 7.79 (d, $J$ = 8.0 Hz, 2H), 7.48 (m, 6H), 7.09 (d, $J$ = 8.0 Hz, 2H), 4.55 (s, 2H), 2.09 (s, 1H). $^{13}$C NMR (DMSO-d$_6$): $\delta$ 173.8, 173.4, 166.6, 162.0, 143.1, 139.5, 138.3, 135.4, 130.9, 129.5, 127.3, 127.1, 119.6, 116.6, 93.2, 34.1. HRMS (ESI-TOF): Calc’d for C$_{23}$H$_{17}$N$_3$OS [MH$^+$] 450.1137; found: 450.1132.

4-[[1,1'-Biphenyl]-4-yl]-2-(benzylthio)-6-((2-hydroxyethyl)amino)pyrimidine-5-carbonitrile (17b):

Yield 62%; $^1$H NMR (DMSO-d$_6$): $\delta$ 7.94 (t, $J$ = 8.0 Hz, 3H), 7.85 (d, $J$ = 8.0 Hz, 2H), 7.76 (d, $J$ = 8.0 Hz, 2H), 7.51 (t, $J$ = 8.0 Hz, 2H), 7.44 (d, $J$ = 8.0 Hz, 2H), 7.33 (t, $J$ = 8.0 Hz, 2H), 7.26 (d, $J$ = 8.0 Hz, 1H), 4.83 (s, 1H), 4.44 (s, 2H), 3.57 (s, 4H); $^{13}$C NMR (DMSO-d$_6$): $\delta$ 173.2, 166.6, 161.5, 142.8, 139.1, 137.9, 134.9, 129.3, 129.2, 128.9, 128.6, 128.3, 127.2, 127.0, 126.8, 116.4, 83.4, 59.1, 43.5, 34.4. HRMS (ESI-TOF): Calc’d for C$_{23}$H$_{23}$N$_3$OS [MH$^+$]:439.1587; found: 439.1588.

4-[[1,1'-Biphenyl]-4-yl]-2-(benzylthio)-6-methoxypyrimidine-5-carbonitrile (17c): Yield 88%;

$^1$H NMR (CDCl$_3$): $\delta$ 8.13 (d, $J$ = 8.0 Hz, 2H), 7.74 (d, $J$ = 8.0 Hz, 2H), 7.65 (d, $J$ = 8.0 Hz, 2H), 7.46 (m, 6H), 7.31 (m, 3H), 4.51 (s, 2H), 4.13 (s, 3H); $^{13}$C NMR (CDCl$_3$): $\delta$ 174.7, 170.0, 168.0, 144.6, 139.9, 136.7, 133.8, 129.5, 128.9, 128.6, 128.1, 127.5, 127.3, 127.2, 114.9, 87.8, 55.5, 35.8. HRMS (ESI-TOF): Calc’d for C$_{23}$H$_{20}$N$_3$OS [MH$^+$]: 410.1322; found: 410.1343.

4-[[1,1'-Biphenyl]-4-yl]-2-((4-azidobenzyl)thio)-6-(methylamino)pyrimidine-5-carbonitrile (17d):

Yield 88%; $^1$H NMR (DMSO-d$_6$): $\delta$ 7.94 (d, $J$ = 8.0 Hz, 2H), 7.85 (d, $J$ = 8.0 Hz, 2H), 7.74 (d, $J$ = 8.0 Hz, 2H), 7.50 (q, $J$ = 8.0 Hz, 4H), 7.43 (d, $J$ = 8.0 Hz, 1H), 7.08 (d, $J$ = 8.0 Hz, 2H), 4.45 (s, 2H), 2.95 (s, 3H). $^{13}$C NMR (DMSO-d$_6$): $\delta$ 173.4, 166.6, 162.0, 143.1, 139.5, 138.6, 135.4, 130.9, 129.6, 129.5, 127.3,
127.1, 119.6, 116.7, 83.7, 34.2, 28.7. HRMS (ESI-TOF): Calc’d for C_{25}H_{29}N_{5}S [MH^+]: 450.1495; found: 450.1491.

**Tert-butyl(3-(2-(2-(3-((6-[[1,1']-biphenyl]-4-yl)-2-((4-azidobenzyl)thio)-5-cyanopyrimidin-4-yl)amino)propoxy)ethoxy)ethoxy)propyl)carbamate (17e):** Yield 75%; \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 8.05 (d, \(J = 8.0\) Hz, 2H), 7.72 (d, \(J = 8.0\) Hz, 2H), 7.64 (d, \(J = 8.0\) Hz, 2H), 7.463 (m, 5H), 6.97 (d, \(J = 8.0\) Hz, 2H), 5.05 (br, 1H), 4.42 (s, 2H), 3.51-3.74 (m, 14H), 3.32 (d, 2H), 1.94 (t, \(J = 8.0\) Hz, 2H), 1.76 (m, 2H), 1.44 (s, 10H); \(^{13}\)C NMR (CDCl\(_3\)): \(\delta\) 173.9, 166.2, 161.7, 156.0, 143.98, 140.0, 138.9, 134.8, 134.4, 130.3, 129.1, 128.93, 127.9, 127.2, 127.2, 119.1, 116.7, 83.6, 70.7, 70.6, 70.4, 70.2, 70.2, 69.5, 40.7, 38.5, 34.8, 29.6, 28.5, 28.4. HRMS (ESI-TOF): Calc’d for C_{39}H_{47}N_{5}O_{5}S [MH^+]: 739.3385; found: 739.3367.

**Tert-butyl(3-(2-(2-(3-((6-[[1,1']-biphenyl]-4-yl)-2-(benzylthio)-5-cyanopyrimidin-4-yl)amino)propoxy)ethoxy)ethoxy)propyl)carbamate (17f):** Yield 75%; \(^1\)H NMR (DMSO-d\(_6\)): \(\delta\) 8.08 (t, 1H), 7.94 (d, \(J = 8.0\) Hz, 2H), 7.82 (d, \(J = 8.0\) Hz, 2H), 7.74 (d, \(J = 8.0\) Hz, 2H), 7.45 (m, 5H), 7.32 (t, \(J = 8.0\) Hz, 2H), 7.26 (t, \(J = 6.0\) Hz, 1H), 4.44 (s, 2H), 3.44 (m, 16H), 2.97 (d, \(J = 8.0\) Hz, 2H), 1.82 (t, \(J = 6.0\) Hz, 2H), 1.59 (t, \(J = 8.0\) Hz, 2H). \(^{13}\)C NMR (DMSO-d\(_6\)): \(\delta\) 174.0, 167.8, 166.3, 161.5, 143.5, 139.6, 137.6, 134.7, 132.1, 130.9, 129.0, 128.6, 128.6, 128.2, 127.7, 126.8, 126.7, 126.5, 116.1, 83.1, 79.4, 70.0, 70.0, 69.7, 69.3, 68.9, 39.3, 38.65, 34.9, 31.4, 29.4, 28.5. HRMS (ESI-TOF): Calc’d for C_{39}H_{41}N_{5}O_{5}S [MH^+]: 698.3371; found: 698.3381.

**4-((1,1'-Biphenyl)-4-yl)-6-((3-(2-(2-(3-aminopropoxy)ethoxy)ethoxy)propyl)amino)-2-(benzylthio)-1,6-dihydropyrimidine-5-carbonitrile (17g):** Yield 82%; \(^1\)H NMR (MeOD): \(\delta\) 7.90 (d, \(J = 8.0\) Hz, 2H), 7.63 (d, \(J = 8.0\) Hz, 2H), 7.58 (d, \(J = 8.0\) Hz, 2H), 7.38 (t, \(J = 8.0\) Hz, 4H), 7.30 (d, \(J = 8.0\) Hz, 1H), 7.26 (t, \(J = 8.0\) Hz, 2H), 7.21 (d, 1H), 4.38 (s, 2H), 3.53 (m, 15H), 2.70 (br, 2H), 1.81 (t, \(J = 8.0\) Hz, 2H), 1.67 (t, \(J = 8.0\) Hz, 2H). \(^{13}\)C NMR (MeOD): \(\delta\) 174.0, 167.8, 166.3, 161.5, 143.5, 139.6, 137.6, 134.1, 132.1, 130.9, 129.0, 128.6, 128.6, 128.2, 127.7, 126.8, 126.7, 126.5, 116.1, 83.1, 70.0, 70.0, 69.7, 69.3, 68.9, 39.3, 38.6, 34.9, 31.4, 29.4, 28.6. HRMS (ESI-TOF): Calc’d for C_{34}H_{40}N_{5}O_{5}S [MH^+]: 598.2846; found: 598.2866.
4-[[1,1'-Biphenyl]-4-yl]-2-([4-azidobenzyl]thio)-6-([2-hydroxyethyl]amino)-1,6-dihydropyrimidine-5-carbonitrile (17h): Yield 65%; 1H NMR (DMSO-d6): δ: 7.94 (d, J = 8.0 Hz, 2H), 7.85 (d, J = 8.0 Hz, 2H), 7.76 (d, J = 8.0 Hz, 2H), 7.50 (q, J = 8.0 Hz, 4H), 7.42 (t, J = 8.0 Hz, 1H), 7.08 (d, J = 8.0 Hz, 2H), 4.83 (s, 1H), 4.43 (s, 2H), 3.56 (s, 4H); 13C NMR (DMSO-d6): δ: 173.4, 166.9, 161.8, 143.1, 139.5, 138.6, 135.3, 135.2, 130.9, 129.6, 128.6, 127.3, 127.0, 119.6, 116.7, 83.8, 59.4, 43.9, 34.2. HRMS (ESI-TOF): Calc’d for C29H22OS [MH+]: 480.1601; found: 480.1606.

4-[[1,1'-Biphenyl]-4-yl]-2-([4-azidobenzyl]thio)-6-hydrazinyl-1,6-dihydropyrimidine-5-carbonitrile (17i): Yield 60%; 1H NMR (DMSO-d6): δ 7.91 (d, J = 8.0 Hz, 2H), 7.84 (d, J = 8.0 Hz, 2H), 7.77 (t, J = 8.0 Hz, 2H), 7.52 (m, J = 8.0 Hz, 4H), 7.50 (d, J = 8.0 Hz, 1H), 7.06 (d, J = 8.0 Hz, 2H), 4.43 (s, 2H), 1.97 (d, J = 4.0 Hz, 2H); 13C NMR (DMSO-d6): δ 174.0, 167.8, 166.3, 161.5, 143.5, 139.6, 137.6, 134.7, 132.1, 130.9, 129.0, 128.6, 128.6, 128.2, 127.7, 126.8, 126.7, 126.5, 116.1, 83.1, 70.1, 70.0, 69.7, 69.3, 68.9, 39.3, 38.6, 34.9, 31.4, 29.4, 28.6. HRMS-ESI (+): Calc. for C29H20N8S [M-H]: 451.1453; found: 451.1444.

4-[[1,1'-Biphenyl]-4-yl]-2-([4-azidobenzyl]thio)-6-(prop-2-yn-1-ylamino) pyrimidine-5-carbonitrile (17j): Yield 72%; 1H NMR (DMSO-d6): 7.95 (d, J = 8.0 Hz, 2H), 7.85 (d, J = 8.0 Hz, 2H), 7.76 (d, J = 8.0 Hz, 2H), 7.50 (m, J = 8.0 Hz, 4H), 7.43 (t, J = 8.0 Hz, 1H), 7.07 (d, J = 8.0 Hz, 2H), 4.48 (s, 2H), 4.22 (d, J = 4.0 Hz, 2H), 3.20 (s, 1H); 13C NMR (DMSO-d6): δ 173.5, 167.1, 161.4, 143.2, 139.4, 138.6, 135.4, 135.0, 130.9, 129.7, 129.5, 128.6, 127.3, 127.20, 119.6, 116.4, 84.1, 81.2, 73.6, 34.2, 30.9. HRMS (ESI-TOF): Calc’d for C27H20N7S [MH+]: 474.1495; found: 474.1490.

**General synthetic procedure for 18a-g:** A solution of 4-[[1, 1'-biphenyl]-4-yl]-2-([4-azidobenzyl]thio)-6-oxo-1, 6-dihydropyrimidine-5-carbonitrile 2 (30 mg, 0.068 mmol) was prepared in 0.3 ml of DMF in a 4 ml glass vial. To this solution were added appropriate alkynes (0.103 mmol) and the resulting mixture was dissolved by stirring. To this mixture was added 0.015 ml of 1M CuSO4·5H2O in H2O and sodium ascorbate (6.81 mg, 0.0344 mmol). The reaction is left to stir overnight at room temperature. On completion, the reaction solvent is evaporated under vacuum and the dried residue in dissolved in ethyl acetate. The resulting solution is washed with acidified water (pH = 4-5, 10 ml × 3) and brine (5 ml × 2). The sol-
vent was evaporated under vacuum to obtain crude residue, which was purified using silica gel column chromatography. Yields: 65-80%

4-[[1,1'-Biphenyl]-4-yl]-6-oxo-2-((4-(4-phenyl-1H-1,2,3-triazol-1-yl)benzyl)thio)-1, 6-dihydro pyrimidine-5-carbonitrile (18a): Yield 85%; $^1$H NMR (DMSO-$d_6$): $\delta$ 9.25 (s, 1H), 8.07 (d, $J = 8.0$ Hz, 2H), 7.91 (m, 6H), 7.79 (d, $J = 8.0$ Hz, 2H), 7.70 (d, $J = 8.0$ Hz, 2H), 7.50 (m, 4H), 7.43 (m, 2H), 4.65 (s, 2H); $^{13}$C NMR (DMSO-$d_6$): $\delta$ 167.1, 166.4, 147.7, 143.7, 139.3, 138.1, 136.2, 134.7, 131.1, 130.9, 130.6, 129.8, 129.5, 129.4, 128.7, 128.7, 127.8, 127.4, 127.2, 125.8, 120.5, 119.7, 116.6, 93.2, 36.2. HRMS (ESI-TOF): Calc’d for C$_{33}$H$_{23}$N$_6$OS [MH$^+$]: 539.1649; found: 539.1656.

4-[[1,1'-Biphenyl]-4-yl]-2-((4-(4-(4-aminophenyl)-1H-1,2,3-triazol-1-yl)benzyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (18b): Yield 75%; $^1$H NMR (DMSO-$d_6$): $\delta$: 8.90 (s, 1H), 7.89 (d, $J = 8.0$ Hz, 2H), 7.84 (d, $J = 8.0$ Hz, 2H), 7.75 (m, $J = 8.0$ Hz, 5H), 7.64 (d, $J = 8.0$ Hz, 2H), 7.57 (t, $J = 8.0$ Hz, 2H), 7.49 (t, $J = 8.0$ Hz, 2H), 7.39 (t, $J = 8.0$ Hz, 1H), 6.63 (t, $J = 8.0$ Hz, 2H), 4.39 (s, 2H). $^{13}$C NMR (DMSO-$d_6$): $\delta$ 174.0, 167.8, 166.3, 161.5, 143.5, 139.6, 137.6, 134.7, 132.1, 130.9, 129.0, 128.7, 128.6, 128.2, 127.7, 126.8, 126.7, 126.5, 115.7, 116.1, 93.5, 36.1. HRMS (ESI-TOF): Calc’d for C$_{32}$H$_{24}$N$_7$OS [MH$^+$]: 554.1758; found: 554.1759.

1-((4-(4-[[1,1'-Biphenyl]-4-yl]-5-cyano-6-oxo-1,6-dihydropyrimidin-2yl)thio)methyl)phenyl)-1H-1,2,3-triazole-4-carboxylic acid (18c): Yield 80%; $^1$H NMR (DMSO-$d_6$): $\delta$ 9.38 (s, 1H), 8.07 (d, $J = 8.0$ Hz, 2H), 7.93 (m, 4H), 7.80 (d, $J = 8.0$ Hz, 2H), 7.68 (d, 2H), 7.53 (t, $J = 6.0$ Hz, 2H), 7.44 (d, $J = 8.0$ Hz, 1H), 4.65 (s, 2H); $^{13}$C NMR (DMSO-$d_6$): $\delta$ 171.4, 169.2, 166.5, 161.4, 143.2, 139.4, 138.6, 135.4, 135.0, 130.9, 129.7, 129.5, 128.6, 127.3, 127.2, 119.6, 131.5, 93.4, 36.4. HRMS (ESI-TOF): Calc’d for C$_{27}$H$_{16}$N$_6$O$_3$S [MH$^+$]: 507.1234; found: 507.1246.

(1-(((4-((4-[[1,1'-Biphenyl]-4-yl]-5-cyano-6-oxo-1,6-dihydropyrimidin-2yl)thio)methyl)phenyl)-1H-1,2,3-triazol-4-yl)methyl acetate (18d): Yield 92%; $^1$H NMR (DMSO-$d_6$): $\delta$ 8.83 (s, 1H), 8.05 (d, $J = 8.0$ Hz, 2H), 7.88 (t, $J = 8.0$ Hz, 4H), 7.79 (d, $J = 8.0$ Hz, 2H), 7.70 (s, 1H), 7.52 (t, $J = 8.0$ Hz, 2H), 7.43 (d, $J = 8.0$ Hz, 1H), 5.20 (s, 2H), 4.68 (s, 2H), 2.05 (s, 3H); $^{13}$C NMR (DMSO-$d_6$): $\delta$ 170.6, 142.5, 139.6,
135.9, 131.1, 129.5, 129.2, 128.4, 127.3, 127.1, 123.5, 120.8, 75.2, 57.3, 56.4, 52.0, 21.0, 18.9. HRMS (ESI-TOF): Calc’d for C_{29}H_{23}N_{6}O_{5}S [MH^+]: 535.1547; found: 535.1552.

3-(1-(4-(((4-(1,1'-Biphenyl)-4-yl)-5-cyano-6-oxo-1,6-dihydropyrimidin-2-yl)(thio)methyl)phenyl)-1H-1,2,3-triazol-4-yl)propanoic acid (18e): Yield 66%; \(^1\)H NMR (DMSO-\(d_6\)): \(\delta\) 12.21 (s, 1H), 8.54 (s, 1H), 8.05 (d, \(J = 8.0\) Hz, 2H), 7.89 (d, \(J = 8.0\) Hz, 2H), 7.80 (q, \(J = 8.0\) Hz, 4H), 7.64 (d, \(J = 12.0\) Hz, 2H), 7.51 (t, \(J = 8.0\) Hz, 2H), 7.41 (d, 1H), 4.62 (s, 2H), 2.91 (t, \(J = 8.0\) Hz, 2H), 2.64 (t, \(J = 4.0\) Hz, 2H); \(^{13}\)C NMR (DMSO-\(d_6\)): \(\delta\) 173.9, 167.3, 166.0, 161.7, 147.4, 143.7, 139.3, 137.6, 136.3, 134.5, 130.9, 129.9, 129.5, 128.8, 127.4, 127.2, 120.7, 120.3, 116.4, 93.4, 34.0, 33.3, 21.1. HRMS (ESI-TOF): Calc’d for C_{29}H_{23}N_{6}O_{5}S [MH^+]: 535.1547; found: 535.1559.

2-(1-(4-(((4-(1,1'-biphenyl)-4-yl)-5-cyano-6-oxo-1,6-dihydropyrimidin-2-yl)(thio)methyl)phenyl)-1H-1,2,3-triazol-4-yl)acetic acid (18f): Yield 65%; \(^1\)H NMR (DMSO-\(d_6\)): \(\delta\) 8.12 (d, \(J = 8.0\) Hz, 2H), 7.83 (d, \(J = 8.0\) Hz, 4H), 7.74 (d, \(J = 8.0\) Hz, 2H), 7.69 (d, \(J = 8.0\) Hz, 2H), 7.50 (t, \(J = 8.0\) Hz, 2H), 7.42 (t, \(J = 8.0\) Hz, 2H), 4.70 (s, 2H), 3.01 (s, 1H), 2.87 (s, 1H); \(^{13}\)C NMR (DMSO-\(d_6\)): \(\delta\) 173.9, 167.3, 166.0, 161.7, 147.4, 143.7, 139.3, 137.6, 136.3, 134.5, 130.9, 129.9, 129.5, 128.8, 127.4, 127.2, 120.7, 120.3, 116.4, 93.4, 34.0, 33.3, 21.1. HRMS (ESI-TOF): Calc’d for C_{29}H_{19}N_{5}O_{5}S [MH^+]: 519.1239; found: 519.1250.

4-((1,1'-Biphenyl)-4-yl)-2-((4-(4-(hydroxymethyl)-1H-1,2,3-triazol-1-yl)benzyl)(thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (18g): Yield 70%; \(^1\)H NMR (DMSO-\(d_6\)): \(\delta\) 8.64 (s, 1H), 7.96 (d, \(J = 8.0\) Hz, 2H), 7.835 (q, 4H), 7.76 (d, \(J = 8.0\) Hz, 2H), 7.63 (d, 2H), 7.50 (d, \(J = 8.0\) Hz, 2H), 7.41 (t, \(J = 8.0\) Hz, 1H), 4.59 (s, 2H), 4.50 (s, 2H); \(^{13}\)C NMR (DMSO-\(d_6\)): \(\delta\) 170.4, 166.2, 160.5, 144.2, 140.2, 139.6, 136.0, 134.7, 129.8, 129.2, 128.5, 127.3, 127.0, 121.4, 120.4, 114.5, 93.6, 55.4, 33.8. HRMS (ESI-TOF): Calc’d for C_{27}H_{19}N_{5}O_{4}S [MH^+]: 491.1296; found: 491.1292.

**General synthetic procedure for 19a-c:** To a solution of 2a (0.109 mmol) in 2 ml of CH_{3}CN: DMF (3:1) was added R-X (0.164 mmol) and the reaction was monitored by TLC. Upon disappearance of the starting material, the reaction solvent is dried in *vacuo*, followed by washing with H_{2}O (5 ml × 3) and brine (5 ml × 3). The product is extracted in ethyl acetate (10 ml) and the solvent was evaporated under
vacuum to obtain crude residue, which was purified using silica gel column chromatography. Yields: 70-80%

4-([1,1'-Biphenyl]-4-yl)-2-((4-azidobenzyl)thio)-1-methyl-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (19a): Yield 25%; 1H NMR (DMSO-d6): δ 8.03 (d, J = 8.0 Hz, 2H), 7.95 (d, J = 8.0 Hz, 2H), 7.86 (d, J = 8.0 Hz, 2H), 7.61 (m, 5H), 7.18 (d, J = 8.0 Hz, 2H), 4.54 (s, 2H), 3.05 (s, 3H); 13C NMR (DMSO-d6): δ 166.8, 164.9, 160.3, 143.9, 139.3, 134.3, 133.1, 131.3, 129.9, 129.58, 128.8, 127.4, 127.3, 119.7, 116.4, 92.2, 36.0, 31.3. HRMS (ESI-TOF): Calc’d for C25H19N6NaOS [MNa]+ 451.1341; found: 473.1150.

4-([1,1'-Biphenyl]-4-yl)-2-((4-azidobenzyl)thio)-6-methoxypyrindine-5-carbonitrile (19b): Yield 45%; 1H NMR (DMSO-d6): δ 8.09 (d, J = 8.0 Hz, 2H), 7.90 (d, J = 8.0 Hz, 2H), 7.79 (d, J = 8.0 Hz, 2H), 7.50 (m, 6H), 7.10 (d, J = 8.0 Hz, 2H), 4.66 (s, 2H), 3.48 (s, 3H); 13C NMR (DMSO-d6): δ 167.4, 165.1, 161.5, 159.3, 155.3, 131.3, 130.3, 128.9, 126.7, 124.7, 120.3, 117.4, 115.4, 114.2, 92.2, 55.3, 35.3. HRMS (ESI-TOF): Calc’d for C25H19N6NaOS [MNa]+ 473.1160; found: 473.1161.

4-([1,1'-Biphenyl]-4-yl)-6-((4-azidobenzyl)oxy)-2-((4-azidobenzyl)thio)pyrimidine-5-carbonitrile (19c): Yield 72%; 1H NMR (DMSO-d6): δ 8.12 (d, J = 8.0 Hz, 2H), 7.90 (d, J = 8.0 Hz, 2H), 7.78 (d, J = 8.0 Hz, 2H), 7.44 (m, 8H), 7.08 (m, 4H), 5.26 (s, 2H), 4.62 (s, 2H); 13C NMR (DMSO-d6): δ 166.5, 160.6, 144.0, 139.4, 139.3, 139.2, 134.1, 132.8, 131.3, 131.2, 130.9, 130.4, 130.0, 129.5, 129.5, 128.8, 127.4, 127.3, 119.8, 119.7, 119.6, 116.3, 92.9, 47.7, 36.3. HRMS (ESI-TOF): Calc’d for C19H22N6O2S [MH]+ 567.1663; found: 568.1689.

General synthetic procedure for 20a-c: Same as used for 14a. Yields: 70-75%

4-(4-(benzylolxy)phenyl)-2-mercapto-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (20a). Yield 65%; 1H NMR (DMSO-d6): δ: 7.65 (d, J = 8.0 Hz, 2H), 7.38 (m, 5H), 7.19 (t, J = 8.0 Hz, 2H), 5.22 (s, 2H); 13C NMR (DMSO-d6): δ: 176.6, 167.9, 161.8, 160.8, 159.1, 157.9, 136.9, 133.2, 131.7, 130.6, 128.9, 128.3, 121.6, 118.1, 116.1, 115.5, 90.2, 69.9. HRMS (ESI-TOF): Calc’d for C19H17N6OS [MH]: 334.0650; found: 334.0644.

2-mercapto-6-oxo-4-(4-phenoxyphenyl)-1,6-dihydropyrimidine-5-carbonitrile (20b): Yield 65%
$^1$H NMR (DMSO-$d_6$): $\delta$ 7.73 (d, $J = 8.0$ Hz, 2H), 7.46 (t, 3H), 7.25 (d, $J = 8.0$ Hz, 1H), 7.11 (t, $J = 8.0$ Hz, 4H), 1.62 (s, 1H); $^{13}$C NMR (DMSO-$d_6$): $\delta$ 177.8, 161.7, 160.5, 159.7, 155.5, 131.5, 130.8, 125.5, 125.1, 120.3, 117.6, 116.1, 93.5. HRMS (ESI-TOF): Calc’d for C$_{25}$H$_{18}$N$_6$O$_2$S [MH$^+$]: 320.0494; found: 320.0500.

2-Mercapto-6-oxo-4-(4-phenylethynyl)phenyl)-1,6-dihydropyrimidine-5-carbonitrile (20c): Yield 72%; $^1$H NMR (CDCl$_3$): $\delta$ 10.03 (s, 1H), 7.87 (d, $J = 8.0$ Hz, 2H), 7.69 (d, $J = 8.0$ Hz, 2H), 7.57 (t, $J = 8.0$ Hz, 2H), 7.39 (t, $J = 8.0$ Hz, 2H); $^{13}$C NMR (CDCl$_3$): $\delta$ 171.4, 135.4, 132.1, 131.8, 129.6, 129.0, 128.5, 122.5, 93.4, 88.5. HRMS (ESI-TOF): Calc’d for C$_{19}$H$_{10}$N$_3$OS [MH$^+$]: 328.0545; found: 328.0541.

**General synthetic procedure for 21a-o**: Same as used for 15a. Yields: 65-70%

2-((4-Azidobenzyl)thio)-4-(4-(benzylxoy)phenyl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (21a): Yield 68%; $^1$H NMR (CDCl$_3$): $\delta$ 7.99 (d, $J = 8.0$ Hz, 2H), 7.40 (m, 7H), 7.19 (d, $J = 8.0$ Hz, 2H), 7.05 (d, $J = 8.0$ Hz, 2H), 5.21 (s, 2H), 4.52 (s, 2H); $^{13}$C NMR (CDCl$_3$): $\delta$ 166.7, 165.6, 162.0, 161.7, 139.0, 136.9, 134.0, 131.2, 131.1, 128.9, 128.5, 128.3, 127.9, 119.6, 116.8, 115.3, 91.9, 70.0, 34.0. HRMS (ESI-TOF): Calc’d for C$_{25}$H$_{18}$N$_6$O$_2$S [MH$^+$]: 465.1139; found: 465.1127.

Methyl4-((4-(4-(benzylxoy)phenyl)-5-cyano-6-oxo-1,6-dihydropyrimidin-2-yl)thio)methyl)benzoate (21b): Yield 75%; $^1$H NMR (DMSO-$d_6$): $\delta$ 7.90 (m, $J = 8.0$ Hz, 4H), 7.55 (d, $J = 8.0$ Hz, 2H), 7.47 (d, $J = 8.0$ Hz, 2H), 7.40 (t, $J = 8.0$ Hz, 2H), 7.34 (d, 1H), 7.16 (d, $J = 8.0$ Hz, 2H), 5.20 (s, 2H), 4.56 (s, 2H), 3.82 (s, 3H); $^{13}$CNMR (DMSO-$d_6$): $\delta$ 166.7, 166.4, 166.1, 161.5, 143.2, 136.9, 131.1, 129.7, 129.7, 129.0, 128.9, 128.4, 128.2, 128.1, 117.2, 115.2, 114.3, 91.6, 69.9, 52.5, 34.1. HRMS (ESI-TOF): Calc’d for C$_{27}$H$_{21}$N$_3$O$_4$S [MH$^+$]: 482.1180; found: 482.1165.

4-(4-(Benzyloxy)phenyl)-2-((4-methoxybenzyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (21c): Yield 75%; $^1$H NMR (CDCl$_3$): $\delta$ 8.03 (d, $J = 8.0$ Hz, 2H), 7.48 (d, $J = 8.0$ Hz, 2H), 7.37 (m, 5H), 7.21 (d, $J = 8.0$ Hz, 2H), 6.87 (d, $J = 8.0$ Hz, 2H), 5.22 (s, 2H), 4.49 (s, 2H), 3.71 (s, 3H); $^{13}$C NMR (CDCl$_3$): $\delta$ 166.7, 165.5, 161.8, 161.6, 159.3, 136.9, 131.3, 130.7, 128.9, 128.5, 128.41, 128.3, 127.9, 116.7, 115.3, 114.4, 91.9, 69.9, 55.5, 34.2. HRMS (ESI-TOF): Calc’d for C$_{26}$H$_{20}$N$_3$O$_3$S [MH$^+$]: 454.1225; found: 454.1227.
4-(4-(Benzyloxy)phenyl)-2-((4-bromobenzyl)(thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (21d): Yield 70%; \(^1\)H NMR (DMSO-\(d_6\)): \(\delta\) 7.97 (d, \(J = 8.0 \text{ Hz}, 2\)H), 7.50 (t, \(J = 8.0 \text{ Hz}, 4\)H), 7.39 (m, 5H), 7.20 (d, \(J = 8.0 \text{ Hz}, 2\)H), 5.22 (s, 2H), 4.51 (s, 2H); \(^{13}\)C NMR (DMSO-\(d_6\)): \(\delta\) 171.9, 166.9, 165.4, 161.7, 137.0, 136.8, 131.8, 131.6, 131.2, 128.9, 128.5, 128.3, 121.0, 116.8, 115.3, 91.8, 70.0, 33.8. HRMS (ESI-TOF): Calc’d for C\(_{23}\)H\(_{17}\)BrN\(_3\)O\(_2\)S [MH]: 503.3904; found: 504.0203.

4-(4-(Benzyloxy)phenyl)-2-((2-bromobenzyl)(thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (21e): Yield 67%; \(^1\)H NMR (DMSO-\(d_6\)): \(\delta\) 7.97 (d, \(J = 8.0 \text{ Hz}, 2\)H), 7.65 (d, \(J = 8.0 \text{ Hz}, 1\)H), 7.55 (d, \(J = 8.0 \text{ Hz}, 1\)H), 7.47 (d, \(J = 8.0 \text{ Hz}, 2\)H), 7.26 (m, 7H), 5.21 (s, 2H), 4.61 (s, 2H); \(^{13}\)C NMR (DMSO-\(d_6\)): \(\delta\) 166.9, 165.4, 162.2, 161.7, 136.8, 135.7, 133.2, 131.9, 131.3, 130.4, 129.0, 128.5, 127.7, 124.5, 116.8, 115.3, 91.9, 69.9, 35.4. HRMS (ESI-TOF): Calc’d for C\(_{23}\)H\(_{17}\)BrN\(_3\)O\(_2\)S [MH]: 503.3904; found: 502.0288.

4-(4-(Benzyloxy)phenyl)-2-((cyclohexylmethyl)(thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (21f): Yield 68%; \(^1\)H NMR ((CD\(_3\))\(_2\)CO): \(\delta\) 8.09 (d, \(J = 8.0 \text{ Hz}, 2\)H), 7.54 (d, \(J = 8.0 \text{ Hz}, 2\)H), 7.36 (m, 3H), 7.20 (d, \(J = 8.0 \text{ Hz}, 2\)H), 5.27 (s, 2H), 3.27 (d, 2H), 1.26 (m, 10H); \(^{13}\)C NMR (DMSO-\(d_6\)): \(\delta\) 171.7, 166.4, 161.7, 136.9, 130.8, 128.4, 128.3, 127.9, 127.7, 116.2, 114.6, 69.85, 45.5, 37.7, 37.3, 32.2, 28.3, 26.0, 25.7. HRMS (ESI-TOF): Calc’d for C\(_{27}\)H\(_{21}\)N\(_3\)O\(_2\)S [MH]: 431.1667; found: 430.1605.

4-(4-(Benzyloxy)phenyl)-2-((3, 5-dimethoxybenzyl)thio)-6-oxo-1,6 dihydropyrimidine-5-carbonitrile (21g): Yield 68%; \(^1\)H NMR (DMSO-\(d_6\)): \(\delta\) 8.00 (d, \(J = 8.0 \text{ Hz}, 2\)H), 7.33 (m, 5H), 7.17 (d, \(J = 8.0 \text{ Hz}, 2\)H), 6.56 (s, 2H), 6.34 (s, 1H), 5.20 (s, 2H), 4.43 (s, 2H). \(^{13}\)C NMR (DMSO-\(d_6\)): \(\delta\) 166.7, 165.6, 161.9, 161.7, 160.8, 139.5, 136.9, 131.3, 128.9, 128.5, 128.2, 127.9, 116.84, 115.3, 115.0, 107.4, 99.9, 91.8, 69.9, 55.4, 34.7, 29.7. HRMS (ESI-TOF): Calc’d for C\(_{27}\)H\(_{16}\)N\(_3\)O\(_2\)S [MH]: 484.1331; found: 484.1343.

4-(4-(Benzyloxy)phenyl)-2-((3,5-dimethylbenzyl)thio)-6-oxo1,6 dihydropyrimidine-5-carbonitrile (21h): Yield 72%; \(^1\)H NMR (DMSO-\(d_6\)): \(\delta\) 8.00 (d, \(J = 8.0 \text{ Hz}, 2\)H), 7.34 (m, 5H), 7.20 (d, \(J = 8.0 \text{ Hz}, 2\)H), 7.01 (s, 2H), 6.88 (s, 1H), 5.23 (s, 2H), 4.43 (s, 2H), 2.18 (s, 6H); \(^{13}\)C NMR (DMSO-\(d_6\)): \(\delta\) 171.5, 170.3, 166.5, 142.7, 141.7, 141.3, 136.0, 134.0, 133.7, 133.2, 133.0, 132.7, 132.0, 121.5, 120.0, 96.9, 74.7, 39.4, 25.9. HRMS (ESI-TOF): Calc’d for C\(_{27}\)H\(_{21}\)N\(_3\)O\(_2\)S [MH]: 452.1433; found: 452.1435.
2-((4-Azidobenzyl)(thio)-6-oxo-4-(4-phenoxypyphenyl)-1,6-dihydropyrimidine-5-carbonitrile (21i): Yield 65%; \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 8.10 (d, \(J = 8.0\) Hz, 2H), 7.40 (m, 5H), 7.19 (d, \(J = 8.0\) Hz, 2H), 7.11 (m, 4H), 6.98 (d, 2H), 4.54 (s, 2H); \(^1^3\)C NMR (CDCl\(_3\)): \(\delta\) 167.4, 164.2, 163.1, 161.7, 155.2, 139.9, 131.5, 131.3, 130.5, 128.7, 124.8, 120.3, 119.4, 117.4, 115.1, 92.6, 35.0. HRMS (ESI-TOF): Calc’d for C\(_{24}\)H\(_{16}\)N\(_6\)O\(_3\)S [MH\(^+\)]: 451.0983; found: 451.0977.

Methyl4-(((5-cyano-6-oxo-4-(4-phenoxypyphenyl)-1,6-dihydropyrimidin-2-yl)(thio)methyl)benzoate (21j): Yield 62%; \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 7.91(t, \(J = 8.0\) Hz, 4H), 7.38 (m, 4H), 7.20 (t, \(J = 8.0\) Hz, 2H), 7.07 (d, 2H), 6.97 (d, 2H), 4.43 (s, 2H), 4.43 (s, 2H), 3.87 (s, 3H); \(^1^3\)C NMR (CDCl\(_3\)): \(\delta\) 169.7, 167.1, 166.5, 161.2, 155.3, 140.9, 131.1, 130.0, 129.9, 129.5, 129.07, 128.9, 124.6, 120.2, 117.3, 91.8, 52.1, 35.0. HRMS (ESI-TOF): Calc’d for C\(_{25}\)H\(_{19}\)N\(_3\)O\(_4\)S [MH\(^+\)]: 470.1169; found: 470.1165.

2-((4-Methoxybenzyl)(thio)-6-oxo-4-(4-phenoxypyphenyl)-1,6-dihydropyrimidine-5 carboxanitrile (21k): Yield 65%; \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 8.10 (d, \(J = 8.0\) Hz, 2H), 7.42 (t, \(J = 8.0\) Hz, 2H), 7.29 (d, \(J = 8.0\) Hz, 2H), 7.22 (t, \(J = 8.0\) Hz, 1H), 7.09 (m, 4H), 6.84 (d, 2H), 4.51 (s, 2H), 3.79 (s, 3H); \(^1^3\)C NMR (CDCl\(_3\)): \(\delta\) 167.4, 165.1, 161.5, 159.3, 155.3, 131.3, 130.3, 130.1, 128.9, 126.7, 124.7, 120.3, 117.4, 115.4, 114.2, 92.2, 55.3, 35.3. HRMS (ESI-TOF): Calc’d for C\(_{25}\)H\(_{19}\)N\(_3\)O\(_4\)S [MH\(^+\)]: 440.1074; found: 440.1070.

2-((4-Azidobenzyl)(thio)-6-oxo-4-(4-(phenylethynyl)phenyl)-1, 6-dihydropyrimidine-5 carbonitrile (21l): Yield 78%; \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 7.96 (d, \(J = 8.0\) Hz, 2H), 7.73 (d, \(J = 8.0\) Hz, 2H), 7.58 (t, 2H), 7.43 (m, 5H), 7.05 (d, \(J = 8.0\) Hz, 2H), 4.48 (s, 2H); \(^1^3\)C NMR (CDCl\(_3\)): \(\delta\) 167.0, 166.7, 141.5, 138.9, 135.8, 134.2, 132.0, 131.9, 131.1, 129.7, 129.5, 129.3, 125.8, 122.2, 119.6, 116.7, 92.3, 89.1, 34.1. HRMS (ESI-TOF): Calc’d for C\(_{24}\)H\(_{16}\)N\(_6\)O\(_3\)S [MH\(^+\)]: 460.1106; found: 461.1185.

Methyl-4-(((5-cyano-6-oxo-4-(4-(phenylethynyl)phenyl)-1,6-dihydropyrimidin-2-yl)(thio)methyl)benzoate (21m): Yield 73%; \(^1\)H NMR (DMSO-d\(_6\)): \(\delta\) 7.91 (t, \(J = 8.0\) Hz, 4H), 7.72 (d, \(J = 8.0\) Hz, 2H), 7.59 (m, 4H), 7.45 (t, 3H), 4.58 (s, 2H), 3.81 (s, 3H); \(^1^3\)C NMR (DMSO-d\(_6\)): \(\delta\) 174.9, 166.7, 166.4, 143.0, 135.5, 132.0, 131.8, 129.7, 129.5, 129.3, 125.9, 122.2, 116.4, 92.4, 89.0, 52.6, 34.2. HRMS (ESI-TOF): Calc’d for C\(_{25}\)H\(_{18}\)N\(_3\)O\(_3\)S [MH\(^+\)]: 476.1069; found: 476.1057.
2-((4-Methoxybenzyl)thio)-6-oxo-4-(4-(phenylethynyl)phenyl)-1,6-dihydropyrimidine-5-carbonitrile (21n): Yield 71%; $^1$H NMR (DMSO-$d_6$): δ 7.91 (t, J = 8.0 Hz, 4H), 7.72 (d, J = 8.0 Hz, 2H), 7.59 (m, 4H), 7.45 (t, 3H), 4.58 (s, 2H), 3.81 (s, 3H); $^{13}$C NMR (DMSO-$d_6$): δ 166.6, 166.5, 161.6, 159.1, 135.6, 132.0, 131.9, 130.8, 129.7, 129.5, 129.3, 128.4, 126.0, 122.2, 116.3, 114.4, 93.6, 92.3, 89.1, 55.5, 34.4. HRMS (ESI-TOF): Calc'd for C$_{23}$H$_{18}$N$_5$O$_2$S [MH$^+$]: 448.1120; found: 448.1118.

2-((3,5-Dimethoxybenzyl)thio)-6-oxo-4-(4-(phenylethynyl)phenyl)-1,6-dihydropyrimidine-5-carbonitrile (21o): Yield 74%; $^1$H NMR (DMSO-$d_6$): δ 8.02 (d, J = 8.0 Hz, 2H), 7.73 (d, J = 8.0 Hz, 2H), 7.60 (s, 2H), 7.45 (s, 3H), 6.57 (s, 2H), 6.36 (s, 1H), 4.49 (s, 2H), 3.72 (s, 6H); $^{13}$C NMR (DMSO-$d_6$): δ 167.0, 160.8, 139.4, 135.6, 132.0, 131.9, 129.7, 129.5, 129.3, 125.9, 122.2, 116.6, 107.5, 99.8, 92.4, 89.0, 55.6, 55.5, 34.8. HRMS (ESI-TOF): Calc’d for C$_{24}$H$_{16}$N$_6$O$_2$S [MH$^+$]: 478.1225; found: 478.1243

General synthetic procedure for 24:

Compound 24 was synthesized in two steps. For biotin-NHS activation, 200 mg (0.819 mmol) of biotin was dissolved in DMF, followed by addition of 95 mg (0.819 mmol) NHS and 219 mg (1 mmol) of EDCI. The reaction was monitored using TLC, upon completion the DMF evaporated in vacuo and the resulting semi-solid mixture is dissolved in cold water, causing precipitates which are filtered to obtain 23, which was used for next step without purification. In a 10 ml glass vial, 34.2 mg (0.098 mmol) of 23 was dissolved in 5 ml anhydrous DMF and to this was added 63 mg (0.098 mmol) of 15o and 0.02 ml of TEA (0.196 mmol). The reaction is allowed to stir at room temperature and monitored using TLC. Upon completion, DMF is evaporated in vacuo and the resulting semi-solid mixture is dissolved in ethyl acetate (10 ml) and washed with cold water (3 ml × 3) and brine (3 ml × 2). The resulting mixture is purified using silica gel chromatography and the product was eluted using ethyl acetate: hexane (2:1).

General synthetic procedure for 25 and 26: In a 4 ml vial, 2 ml suspension of NHS-activated beads (in acetone, Pierce Prod # 20259) was taken and washed with DMF (2 ml × 5) (HPLC-grade). To it was added 26 mg 15o (0.040 mmol) or 25 mg (0.041 mmol) of 17g, and 0.008 ml of TEA (0.081 mmol). The mixture was sealed and shaken at room temperature for 24 h. Then, the reaction mixture is washed
with DMF (4 ml × 5) to get rid of the side products and unbound residues. Then, the beads were washed in PBS buffer (pH = 7.38) and stored at 4°C till use.

4-(((4-((1,1'-Biphenyl)-4-yl)-5-cyano-6-oxo-1,6-dihydropyrimidin-2-yl)thio)methyl)-N-(15-oxo-19-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-4,7,10-trioxa-14 azanonadecyl) benzamide (24): Yield 75%; $^1$H NMR (CDCl$_3$): δ: 8.02 (d, $J = 8.0$ Hz, 2H), 7.71 (d, $J = 8.0$ Hz, 2H), 7.63 (d, $J = 8.0$ Hz, 2H), 7.41 (m, 5H), 6.96 (d, $J = 8.0$ Hz, 2H), 6.82 (t, $J = 8.0$ Hz, 1H), 6.69 (t, $J = 8.0$ Hz, 1H), 6.58 (br, 1H), 5.88 (br, 1H), 4.52 (t, $J = 8.0$ Hz, 1H), 4.41 (s, 2H), 4.37 (t, $J = 8.0$ Hz, 1H), 3.64 (m, 11H), 3.58 (t, $J = 8.0$ Hz, 2H), 3.53 (t, $J = 8.0$ Hz, 2H), 3.31 (d, $J = 8.0$ Hz, 2H), 2.89 (m, 1H), 2.83 (m, 1H), 2.70 (m, 1H), 2.17 (t, $J = 8.0$ Hz, 2H), 1.92 (t, $J = 8.0$ Hz, 2H), 1.75 (t, $J = 8.0$ Hz, 2H), 1.66 (m, 3H), 1.41 (t, $J = 4.0$ Hz, 2H); $^{13}$C NMR (CDCl$_3$): δ 173.9, 173.2, 166.4, 164.1, 161.7, 143.9, 139.9, 138.9, 134.8, 134.3, 130.3, 129.2, 129.1, 128.9, 128.0, 127.2, 127.1, 119.1, 116.8, 83.5, 77.4, 77.1, 76.8, 69.8, 61.7, 60.2, 55.7, 40.5, 37.6, 36.0, 34.8, 28.9, 28.5, 28.3, 28.1, 25.7. HRMS (ESI-TOF): Calc’d for C$_{45}$H$_{35}$N$_7$O$_7$S$_2$ [MH$^+$]: 868.3526; found: 868.3549.
2 DISCOVERY OF 1, 2, 4-TRIAZOLE PYRIMIDINE ANALOGS AS NOVEL ANTIMICROBIAL AGENTS TARGETING SEC A

Abstract. The following chapter reports the discovery, structure optimization and structure-activity relationship study of 1, 2, 4-triazole containing pyrimidine as novel, highly potent antimicrobial agents. A series of triazole-pyrimidine analogs were synthesized and evaluated, a number of inhibitors have been found to inhibit microbial growth at high nanomolar concentrations.

2.1 Introduction

In earlier sections, we have discussed the need for developing novel methods for targeting multidrug resistance against existing antibiotics. Our continuous efforts in this direction involve screening, design, syntheses and structure-optimization to develop novel structural classes of small molecule SecA inhibitors having antimicrobial properties. We have discovered another class of 1, 2, 4-triazole containing pyrimidine compounds using similar approach. The hit (27, Scheme 2.1) obtained was evaluated for SecA and bacterial growth inhibition and the results obtained (IC$_{50}$ = 30 µM; MIC = 3.4 µM) showed 27 to be a good starting point for structure optimization.

2.1.1 Rationale

The structural attributes of 27 make it symmetrical, hydrophobic and poorly water-soluble (logS = -5.29). The initial medicinal chemistry intuition was to simplify the structure into half so as to enhance drug-likeness by reducing the MW and hydrophobicity. As shown in Scheme 2.1, the monomer compound (28) obtained by halving the hit (27) showed improvement in enzyme inhibition (IC$_{50}$ = 12 µM), increased calculated aqueous solubility (logS = -0.92) and reduced MW. In order to modify the lead structure, two different strategies were adopted; the first was to explore the functional group modifications on the molecule while preserving the core scaffold. The second strategy involved changing the substitution pattern on the pyrimidine ring, in order to change the relative positions of rings B and C with respect to A, while keeping the functional groups intact. We used density functional theory calculations to understand
the underlying changes brought upon by various structural modifications such as orbital interaction energies, bond angles, bond distances and atomic charges. Our analyses provided useful information and valuable insights for future design work.

2.2 Results and Discussion

2.2.1 Chemistry

The key intermediate 32 was synthesized in two steps as shown in Scheme 2.2. First, an amidation of commercially available benzoyl chlorides (29a-b) with hydrazinecarboamides (30a-b) was done to synthesize compound 31, followed by condensation using 5% NaOH under reflux conditions. For intermediate series 34, different thiols or amines were reacted with 2, 4, 6 trichloro pyrimidine under cold conditions to obtain 2-substituted pyrimidines (34a-q) as major product (Scheme 2.2). Series 36 (Scheme 2.3) was obtained by reacting 32a with commercially available 35a-d and series 37 (Scheme 2.4) was
obtained by reacting 32b-c with 34a-q, under basic conditions in acetone at ambient temperature. Compound 37b was further used to synthesize series 38 (Scheme 2.4) by replacement of –Cl using different amines at room temperature. Table 3.1 summarizes the different R2 and R3 groups in series 34, 37 and 38. In series 40 the N-4 position on the triazole was modified by reaction with various alkyl/aryl halides with 34b in the presence of triethylamine in DMF (Scheme 2.5, Table 2.2). Synthesis of compound 42 was done in two steps, first by reacting 32b with Iodomethane at 0°C to obtain 41, followed by reaction with 4,6-dichloro-2-(methylthio)pyrimidine in acetone under basic conditions (Scheme 2.6). Finally, compounds 43-a-b were synthesized by reacting 32a-b with 2, 4, 6-trichloropyrimidine in acetone under basic conditions (Scheme 2.7).

Scheme 2.2. Synthesis of series 31, 32 and 34
Reagents and conditions: a) Hydrazinecarbothioamide, 30a or Hydrazinecarboximidamide, 30b, THF, 0°C ~ rt, overnight; b) 5% NaOH, reflux, 5 h, 65-87% over two steps; c) THF, -5°C-rt, 2-8 h, 70-85%
Scheme 2.3. Synthesis of series 36
Reagents and conditions: a) K$_2$CO$_3$, acetone, rt, 2~3 h, 65%-80%

Scheme 2.4. Synthesis of series 37 and 38
Reagents and conditions: a) 34a-q, K$_2$CO$_3$, acetone, rt, 2~3 h, 70%-75%; b) R$_2$-NH$_2$, acetone/DMF, rt, 5-12 h, 65%-75%
Table 2.1. R₂ and R₃ groups for series 34, 37 and 38

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Scheme 2.5. Synthesis of series 40

Reagents and conditions: a) TEA, DMF, rt-40°C, 2-8 h, 70-85%

Table 2.2. Various R₄ groups in series 40

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Scheme 2.6. Synthesis of compound 42

Reagents and conditions: a) Iodomethane, 0°C, 5 h; b) TEA, 4,6-dichloro-2-(methylthio)pyrimidine, acetone, rt, 10 h, 85%

Scheme 2.7. Synthesis of series 43

Reagents and conditions: a) 2, 4, 6-trichloropyrimidine, K$_2$CO$_3$, acetone, 0-4°C, 10 h, 60%

2.2.2 Biological Evaluation

2.2.2.1 Enzyme and bacterial growth inhibition assays

The enzyme inhibition studies were done using *B. subtilis*168 using the literature colorimetric mala-chite green ATPase assay to measure the release of free inorganic phosphate.\textsuperscript{63b} The growth inhibition activities of the compounds were assessed against a leaky mutant NR698 of *E. coli* by determining the minimum inhibitory concentrations (MIC).
2.3 Results and Discussion

Our optimization efforts started with the modifications of –CF$_3$ groups on the phenyl ring. CF$_3$ is a well-known functional group used in several drugs due to its unique electronic/steric properties and desolvation effects. We replaced this sterically hindered, strongly electron-withdrawing –CF$_3$ group with smaller, electron-releasing methyl groups. Scheme 2.3 (36a-d) shows the methyl group (R$_1$) bearing analogs that were synthesized with modification also done to R$_2$ and R$_3$ positions on pyrimidine. In comparison to lead 28 (IC$_{50}$ = 6 µM; MIC$_{50}$ = 35 µM), all four show decreased inhibition potency (Scheme 2.1, Table 2.3). From this series, 36d with R$_2$ = –SMe and R$_3$ = -Cl is the most potent –CH$_3$ containing compound (IC$_{50}$ = 15 µM; MIC$_{50}$ = 32 µM). This point towards an important role played by –CF$_3$ for enzyme inhibition, and thus in later modifications –CF$_3$ was preserved.

Table 2.3. Inhibition results for series 36, 38, 39 and 43

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Table 2.4. Inhibition results for series 37

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Next, the 2 and 6- pyrimidine positions (series 37a-q and 38a-h respectively) were modified with different functional groups as shown in Scheme 3.4 (Table 2.1). The inhibition results for series 37 and 38 are shown in Tables 2.3 and 2.4 respectively. At position 2, various functional groups such as alkyl, cyclic and aromatic were used to study the preferred electronic and steric environment on the pyrimidine ring. The inhibition data shows this position accommodates several different functional groups and yet retains the potency. As shown in Figure 2.1, a variety of functional groups such as -H (37a, 0.8 µM), -SMe (37b, 0.3 µM), -OPh (37i, 0.8 µM) and –SPh (37q, 0.5 µM) showed MIC below 1 µM (Tier-I modi-
fications). Besides, modifications such as pyrimidine (37c), pyrroline (37h), o-trifluoromethyl thiophenyl (37j) and 4-methy thiophenyl (37n) shows MIC values between 1-2 µM (Tier-II modifications).

However, there is another striking trend seen in compounds having –NH linked functional groups at 2-position. In all such compounds (37d, 37e, 37f, 37l and 37m) the antimicrobial activity is nearly abolished with MIC values > 250 µM. Although, 37l (IC\textsubscript{50} = 6.2 µM) and 37m (IC\textsubscript{50} = 5.0 µM) show moderate enzyme inhibition values, but all others show a complete loss of enzyme inhibition in addition to antimicrobial activity. As we further continue to explore this position, so far our results indicate that 2-pyrimidine tolerates a variety of functional groups very well. It is also evident that the -S/O/NH at 2-position has some important role to play for inhibition.

Figure 2.1. Top tier functional group modifications on pyrimidine ring in comparison to 37b

In the hit compound, the 6-position is occupied by a -Cl atom and just like –CF\textsubscript{3}, halogen atoms due to their electronegative properties and small size are of significance in drug design. We analyzed the
importance of the electronegative environment offered by the –Cl group at 4-pyrimidine position. So far, 37b showed the best potency, so further modifications were performed using 37b as the starting point. Various modifications including –N₃, -NHMe, -NHEt (linear), -NHPr, (branched) and –NHCyp (cyclic) were done as seen in Table 2.1. A comparison of -Cl (37b, IC₅₀ = 0.3 µM) with substitutions such as –H (38a, IC₅₀ = 12 µM), -NH(Me)₂ (38c, IC₅₀ > 100 µM), -NHMe (38d, IC₅₀ > 100 µM), –NHCyp (38f, IC₅₀ > 100 µM) show highly decreased (38a) or completely abolished potency. It appears the electronegative environment is preferred to electron releasing at this position. If we compare 37b, 38a to others in this series 38, the trend is, electron-withdrawing group > none > electron-donating group. This observation can be further supported by –N₃ substitution (38b, MIC: 1.5 µM), an electron withdrawing group, although less potent than 37b, still retains potency. Overall, the pyrimidine ring at both 2-and 6-positions is more sensitive to electronic effects than the steric effects.

Compound 39 (Scheme 2.4) was synthesized in order to study the importance of the linker between pyrimidine and triazole, thus -S- linkage was substituted by –NH. Intriguingly, 39 showed a complete elimination of activity (MIC and IC₅₀ > 100 µM) as compared to 37b (IC₅₀ = 0.3 µM) with only a small structural modification (-S, 37b to –NH, 39). Next, we wanted to explore the importance of 1,2,4-triazole moiety and thus we started out with the modification on its 4-NH position (40a-f). Several functional group modifications (Scheme 2.5, Table 2.2) such as linear (-Me, -Et) or branched (i-Pr) alkyl, cyclobutyl and phenyl ring were done as shown. Interestingly, the IC₅₀ values for all the compounds in series 40 were > 250 µM. In effect, all these modifications have masked the ability of triazole’s -NH to H-bond, which seems to have led to a complete loss of activity. It appears that the H-bonding ability of triazole -NH plays an extremely crucial role in making the compounds potent. Compound 42 (Scheme 2.6) also showed a complete loss of potency (IC₅₀ > 100 µM) for both enzymatic and bacterial growth inhibition, pointing towards the significance of both –NH and S-triazole linkage.

As we mentioned, our alternate strategy for designing inhibitors was to change the substitution of 5-(3,5-bis(trifluoromethyl)phenyl)-4H-1,2,4-triazole-3-thiol moiety on the pyrimidine ring to 2-position instead of 4-position. Interestingly, the two molecules synthesized in this series (43a-b) led to a deeper
understanding (Scheme 2.7, Table 2.3) of the structure-activity relationship. For our initial studies, we synthesized analogs with both –CH$_3$ (43a) and -CF$_3$ (43b). Here, 43b (IC$_{50}$ = 0.7 µM, MIC = 8 µM) shows nearly as good potency as 37b (IC$_{50}$ = 0.3 µM, MIC = 6.3 µM). Also, the trend of decreased potency with -CF$_3$ to CH$_3$ substitution is the same as earlier, a decrease in inhibition potency for 43a (IC$_{50}$ = 30 µM, MIC= 70 µM).

Taken collectively, all the inhibition results point towards a) the importance of triazole –NH’s H-bonding ability b) the electron withdrawing groups (-CF$_3$) on phenyl playing crucial roles c) the significance of electronic environment generated by pharmacophores triazole and pyrimidine taken together. To obtain better insights, we turned to computational analysis of these molecules to understand the effect of structural modifications on orbital interaction energies, bond angles, atomic charges and H-bonding abilities.

2.3.1.1 Energy Minimization studies using Density Functional Theory (DFT):

There are numerous precedents in literature indicating the effect of intramolecular H-bonding of inhibitors on molecular structure and properties causing huge variations in potency. Design and syntheses of potent inhibitors containing pseudo-rings$^{90}$ formed by intramolecular H-bonding interactions is well-known. When the donor and acceptors are in close proximity, equilibrium exists between closed conformation (intramolecular H-bonded) forming a temporary ring system and open conformation (intermolecular H-bonded) when exposed to polar solvent.$^{91}$ Such closed-conformations hide the polarity/charges and are more lipophilic thus showing enhanced membrane permeability. Such properties have been explored for the development of lipophilic drugs$^{92}$ for enhanced CNS penetration. Several studies reported the importance of conformational restrictions$^{93}$ brought about by intramolecular H-bonding$^{94}$ and their significant effects on receptor-inhibitor binding.$^{95}$

The inhibition results obtained for 1,2,4-triazole analogs showed trends similar to reported literature, in terms of possibility of a 6-membered H-bonded pseudo ring formation between the triazole and pyrimidene, effect of –CF$_3$ substitution and H-bond donors/acceptors playing crucial role to the potency. There-
fore, we decided to explore the effect of some crucial functional group modifications on interactions energies, bond lengths, bond angles and atomic charges. To begin with, we analyzed our most active compound 37b. As shown in Figure 2.2, in silico energy minimization of open conformation 37b (1) resulted in closed conformation 37b (2), suggesting that the latter is favored. An evidence of H-bonding was obtained from calculating the Lp (N)→σ* (N-H) interaction energies (13.5 kcal/mol) and N---H bond distance (1.964 Å) of 37b (2). Replacing the electron withdrawing –CF3 group with an electron donating –CH3 group 36d (2), (Figure 2.3) resulted in lower interaction energy of Lp (N)→σ* (N-H) in 36d (2), (10.4 kcal/mol) as compared with 37b (2) (13.5 kcal/mol). This reduced the strength of H-bond by ~3 kcal and increased the distance between N---H by 0.042 Å (2.006 Å). Interestingly, experimental results show these –CH3 analogs having remarkably reduced enzyme inhibition compared to the –CF3 substituted compounds. This suggests that the electron withdrawing substituents on the phenyl ring make the triazole ring a good H-bond donor thereby stabilizing structure 37b (2) by forming the H-bond with the triazole.

We also calculated the energies in water using the PCM-SCRF/B3LYP/6-311g (d,p) level, for both 37b (1) and 37b (2), which showed nearly the same energy suggesting that the polar solvent, water, compensates for their stabilization, similar to reports in literature.96, 91 To our understanding, the enhanced lipophilicity of 37b could either cause enhanced cell permeability due to masking of polarity92 or in a relatively non-polar or hydrophobic environment, such as the enzyme binding pocket, structure 37b (2) with H-bonding between triazole and pyrimidine should predominate.94, 95 Besides, Our 6-membered pseudo ring hypothesis is supported by the assay inhibition results. For example, the complete loss of activity seen with modifications on triazole’s –NH (40a-f) and compound 42. In series 40, NH bonding ability was masked using a variety of functional groups, which led to all the compounds in this series showing complete loss of activity. Likewise, the control compound 42, which was specifically designed to study the importance of pyrimidine-triazole proximity, shows a complete loss of activity. This is because there is no possibility of H-bonding between triazole and pyrimidine given the structural attributes
of 42. This also explains the intriguing activity seen in series 43, which instead of having a completely different substitution pattern on the pyrimidine, shows nearly as good activity as 37b.

Figure 2.2. Computed geometry for CF<sub>3</sub>-substituted 37b (2) and its rotamer 37b (1) on B3LYP/6-311g (d,p) level (gas phase). Interaction energies (kcal/mol) of Lp (N)→ σ* (N-H) obtained were from second order perturbation analysis.

Figure 2.3. Computed geometry for CH<sub>3</sub>-substituted 36d (2) and its rotamer 36d (1) on B3LYP/6-311g (d, p) level (gas phase). Interaction energies (kcal/mol) of Lp (N)→ σ* (N-H) obtained were from second order perturbation analysis.
Apart from the electronic nature of the triazole and the pyrimidine ring, the H-bond strength is dependent on the $\angle C_2$-$S_4$-$C_3$ angle. In the present cases, lone pair of electrons in the 3p orbitals of sulfur overlaps with the adjacent triazole or the pyrimidine rings Lp (S)$\rightarrow$ $\sigma^*$ (C2-N1): 28 kcal/mol 37b (2), and Lp (S)$\rightarrow$ $\sigma^*$ (C7-N4): 22 kcal/mol 36d (2) thus preserving the angle at 106°. Using this, we tried to explain the loss of inhibition observed for 39 (MIC$_{50}$ and IC$_{50}$ > 100µM) having an NH linkage instead of S-linkage. In theory, one would imagine that substituting sulfur with the smaller hetero atoms such as oxygen or nitrogen, whose 2p lone pairs can effectively overlap with the pyrimidine ring, should increase the electron density on the pyrimidine ring and in turn increase its ability as a H-bond acceptor.

![Figure 2.4. B3LYP/6-311g (d,p) optimized geometry for NH- and O-analogs. Interaction energies (kcal/mol) of Lp (N$_i$) $\rightarrow$ $\sigma^*$ (N$_5$-H$_6$) & Lp (O$_i$) $\rightarrow$ $\sigma^*$ (N$_5$-H$_6$) obtained were from second order perturbation analysis.](image)

However, a minor change of S-atom from the most active compound 37b (IC$_{50}$ = 0.3 µM, MIC = 6.3 µM) to NH linkage in 39, lead to nearly abolished activity (MIC$_{50}$ and IC$_{50}$ > 100µM). The optimized structure for 39 (Figure 2.4) showed wider $\angle C2$-N3-C4 angle (128°) and longer H-bond (2.110Å) compared to the sulfur analogs. The interaction energy for Lp (N$_i$) $\rightarrow$ $\sigma^*$ (N$_5$-H$_6$) obtained (7.4 kcal/mol) from the second order perturbation analysis was also smaller than the sulfur analogs. Interestingly, the
electron donating ability of NH as shown in structure is making the pyrimidine nitrogen more nucleophilic and better H-bond acceptor (NBO charge on N1: -0.592) than the sulfur analog, 37b (2) (NBO charge on N1: -0.575), but the same phenomenon also increases the \( \angle \text{C2-N3-C4} \) bond angle from 106° to 128° and thus weakens the H-bond. This observation adds further credibility to our hypothesis of a possible 6-membered ring formed between the triazole and pyrimidine, which causes the molecular geometry to become planar. To what we understand so far, this planarity in our inhibitors is crucial for effective enzyme-inhibitor interactions, possibly because the targeted pocket can only accommodate the planar molecule.

2.3.2 Conclusion and future work:

Besides our discovery of novel 1, 2, 4 triazole pyrimidine analogs as antimicrobial agents, we have successfully achieved the design, syntheses and a structure-activity relationship analysis using various computational tools. Having done in-depth analyses of subtle electronic changes leading to large variations in potency of the molecules, we want to further test the validity of our 6-membered pseudo ring model by synthesizing more analogs designed and predicted on the basis of computational analysis. For example, we expect the oxygen analogs (Figure 3.4, right panel) to show intermediate enzyme inhibition activity between 37b and 36d, while the analogs with methylene linkage, due to their bent structure, should be inactive (Figure 2.5). This is based on the N1---H6 bond distance (2.215 Å) and the interaction energy of \( \text{Lp (O\text{,1})} \rightarrow \sigma^* \text{(N5-H6)} \) (4.8 kcal/mol) which shows the weakest H-bond is predicted for the C3-methylene-analogs. However, in theory this bond can be strengthened by compressing \( \angle \text{C2-C3-C4} \) using Thorpe-Ingold or gem-dimethyl effect (Figure 2.5). We also plan to design analogs to make the intramolecular H-bond stronger by testing a variety of different functional groups linking the triazole and pyrimidine moieties.
Figure 2.5. B3LYP/6-311g (d,p) optimized geometry for the methylene analog. \( \angle \) C2-C3-C4 angle compression and subsequent strengthening of H-bond could be achieved by substituting hydrogen on C3 methylene with the bulky substituents (Thorpe-Ingold Effect).

2.4 Acknowledgements

I would like to thank Dr. Jianmei Cui for her contribution to this project. She synthesized compounds \textbf{37a, 37i, 37o, 38a, 36a-d} and \textbf{43a-b}, which have led to a deeper understanding of the structure activity relationship studies. I also thank Dr. Jinshan Jin (P.C. Tai’s lab, Department of Biology, Georgia State University) for the antimicrobial and enzymatic assay work. I also want to thank Dr. Siming Wang and Dr. Lifang Wang for the MS analyses.

2.5 Experimental

2.5.1 Computational methods

All geometries were optimized using the Density Functional Theory (DFT) at B3LYP level with 6-311G (d,p) which performed well for the organic compounds. \textbf{Gaussian 03}, Revision E.01, M. J. Frisch,
Table 2.5. Cartesian coordinates for structures

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**Structure – 36d (1)**

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**Structure – 36d (2)**

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**Total Energy (Water):** -2110.664
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2.5.2 **General chemical methods:**

All chemical reagents and solvents used were reagent grade or purified using standard methods. TLC analyses were conducted on silica gel plates (Sorbent Silica G UV254). Column chromatography was carried out on flash silica gel (Sorbent 230–400 mesh). NMR spectra were recorded at $^1$H (400 MHz) and $^{13}$C (100 MHz) on a Bruker instrument. Coupling constants ($J$) and chemical shifts ($\delta$) are given in hertz and ppm respectively, using TMS ($^1$H NMR) and solvents ($^{13}$C NMR) as internal standards.

**General synthetic procedure for 31a-c:** To a mixture of benzoyl chloride (5mmol, 1 equiv) in anhydrous tetrahydrofuran (25 mL) was slowly added hydrazinecarboamide at 0~5°C. Then, the reaction mixture was brought to ambient temperature and stirred overnight. The reaction was quenched with saturated sodium carbonate solution, and mixture was extracted with ethyl acetate (15 ml × 2) and washed by brine (10 ml × 2). The solvent was evaporated *in vacuo* and the crude product was used directly for next step.

**General synthetic procedure for 32a-c:** Crude product 31a-c (5mmol) was added in 5% sodium hydroxide solution (50 mL) and the mixture was heated to reflux for 5-6 h. Then 2N Hydrochloric acid was added to adjust the pH of the mixture to ~5, and the precipitated white solid was filtered and washed with warm water for (20 ml × 4). The white solid was dried under vacuum at room temperature to give pure 32a-c (65-80% for two steps).

**5-(3, 5-Dimethylphenyl)-1H-1, 2, 4-triazole-3-thiol (32a):** Yield: 65%; $^1$H NMR (DMSO-$d_6$) $\delta$ 13.71 (s, 1H), 13.62 (s, 1H), 7.54 (s, 2H), 7.14 (s, 1H), 2.31 (s, 6H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 167.3, 150.8, 138.7, 132.4, 125.7, 123.8, 21.2. 205.0. ESI-MS (+): Calc. for C$_{10}$H$_{11}$N$_3$S [M+H]$^+$: 206.0; found: 206.2.
5-(3, 5-Bis(trifluoromethyl)phenyl)-4H-1, 2, 4-triazole-3-thiol (32b): Yield: 72%; $^1$H NMR (DMSO-$d_6$): $\delta$ 14.16 (br, 1H), 13.97 (s, 1H), 8.53 (s, 2H), 8.26 (s, 1H); $^{13}$C NMR (DMSO-$d_6$): $\delta$ 168.1, 148.3, 131.8, 131.4, 128.3, 126.5, 124.7, 124.3, 122.0. ESI-MS (+): Calc. for C$_{10}$H$_3$F$_5$S [M+H]$^+$: 314.0; found: 314.4.

5-(3, 5-Bis(trifluoromethyl)phenyl)-4H-1,2,4-triazol-3-amine (32c): Yield: 80%; $^1$H NMR (DMSO-$d_6$): $\delta$ 8.38 (s, 2H), 8.06 (s, 1H), 6.33 (s, 2H); $^{13}$C NMR (DMSO-$d_6$): $\delta$ 158.6, 156.1, 135.0, 131.3, 131.0, 125.5, 125.0, 122.3, 121.9. ESI-MS (+): Calc. for C$_{10}$H$_6$F$_6$ [M+H]$^+$: 297.0; found: 297.0.

**General synthetic procedure for 34a-q:** To a solution of 2, 4, 6 trichloropyrimidine (1.30 mmol) in THF (8 ml), appropriate R-NH$_2$/R-SH/R-OH (1.35 mmol) was slowly added at 0 to -5°C and stirred for 4-5 h. Then, the reaction was stirred at room temperature for 2-3 h and monitored using TLC. Upon completion, THF was removed in vacuo and dried residue was dissolved in ethyl acetate (20 mL), washed with water (15 ml × 2) and brine (15 ml × 2). The solvent was evaporated in vacuo to obtain crude product, which was purified using silica gel column chromatography. Yields: 70-85%

4,6-Dichloro-2-(piperidin-1-yl)pyrimidine (34a): Yield: 72%; $^1$H NMR (CDCl$_3$): $\delta$ 6.46 (s, 1H), 3.79 (d, $J$ = 4.0 Hz, 4H), 1.61-1.67 (m, 6H); $^{13}$C NMR (CDCl$_3$): $\delta$ 163.3, 162.0, 105.4, 55.8, 25.4, 24.6. ESI-MS (+): Calc. for C$_9$H$_7$N$_3$Cl$_2$ [M+H]$^+$: 232.0; found: 232.4.

4,6-Dichloro-N-cyclopentylpyrimidin-2-amine (34b): Yield: 75%; $^1$H NMR (CDCl$_3$): $\delta$ 6.51 (s, 1H), 3.63 (d, $J$ = 4.0 Hz, 2H), 3.33 (d, $J$ = 4.0 Hz, 2H), 2.67 (m, 1H), 2.03 (m, 4H); $^{13}$C NMR (CDCl$_3$): $\delta$ 167.2, 162.4, 110.5, 55.2, 26.5. ESI-MS (+): Calc. for C$_9$H$_{13}$N$_3$Cl$_2$ [M+H]$^+$: 232.0; found: 232.6.

4,6-Dichloro-N-(prop-2-yn-1-yl)pyrimidin-2-amine (34c): Yield: 82%; $^1$H NMR (CDCl$_3$): $\delta$ 7.10 (s, 1H), 4.20 (br, 1H), 4.13 (d, $J$ = 8.0 Hz, 2H), 3.33 (d, $J$ = 4.0 Hz, 2H), 2.55 (s, 1H); $^{13}$C NMR (CDCl$_3$): $\delta$ 163.5, 161.2, 111.5, 80.2, 71.2, 35.8. ESI-MS (-): Calc. for C$_{7}$H$_{13}$N$_3$Cl$_2$ [M-H]$^-$: 199.9; found: 199.8. [M+H]$^+$

4,6-Dichloro-N-cyclobutylpyrimidin-2-amine (34d): Yield: 70%; $^1$H NMR (CDCl$_3$): $\delta$ 6.57 (s, 1H), 5.63 (s, 1H), 4.45 (m, $J$ = 8.0 Hz, 1H), 2.41 (m, 2H), 1.68-1.94 (m, 4H); $^{13}$C NMR (CDCl$_3$): $\delta$ 167.4, 162.3, 111.5, 65.5, 32.5, 15.8. ESI-MS (-): Calc. for C$_{8}$H$_{13}$N$_3$Cl$_2$ [M-H]$^-$: 216.0; found: 216.2.
4-(4,6-Dichloropyrimidin-2-yl)morpholine (34e): Yield: 75%; $^1$H NMR (CDCl₃): $\delta$ 6.54 (s, 1H), 3.79 (t, $J = 12.0$ Hz, 2H), 3.72 (t, $J = 12.0$ Hz, 2H); $^{13}$C NMR (CDCl₃): $\delta$ 161.7, 160.5, 108.2, 66.5, 44.3. ESI-MS (+): Calc. for C₈H₇N₂Cl₂O [M+H]$^+$: 234.0; found: 234.1.

4,6-Dichloro-2-(pyrrolidin-1-yl)pyrimidine (34f): Yield: 68%; $^1$H NMR (CDCl₃): $\delta$ 6.20 (s, 1H), 3.63 (d, $J = 4.0$ Hz, 2H), 3.33(d, $J = 4.0$ Hz, 2H), 2.03 (m, 4H); $^{13}$C NMR (CDCl₃): $\delta$ 167.2, 162.4, 110.5, 55.2, 26.5. ESI-MS (+): Calc. for C₈H₇N₂Cl₂ [M+H]$^+$: 217.0; found: 218.4.

4,6-Dichloro-N-(2-(trifluoromethyl)benzyl)pyrimidin-2-amine (34g): Yield: 75%; $^1$H NMR (CDCl₃): $\delta$ 7.69 (d, $J = 8.0$ Hz, 1H), 7.61 (d, $J = 8.0$ Hz, 1H), 7.53 (t, 1H), 7.41 (t, $J = 8.0$ Hz, 1H), 6.66 (s, 1H), 5.91 (br, 1H), 4.81 (d, $J = 8.0$ Hz, 2H); $^{13}$C NMR (CDCl₃): $\delta$ 163.4, 161.4, 135.0, 132.2, 130.2, 127.7, 126.2, 126.1, 109.6, 42.1. ESI-MS (-): Calc. for C₁₂H₁₄F₃N₂Cl₂ [M-H]$^-$: 320.0; found 320.0.

4,6-Dichloro-2-((4-methoxyphenyl)thio)pyrimidine (34h): Yield: 67%; $^1$H NMR (CDCl₃): $\delta$ 7.48 (d, $J = 8.0$ Hz, 2H), 7.02 (d, $J = 8.0$ Hz, 2H), 6.54 (s, 1H), 3.87 (s, 3H); $^{13}$C NMR (CDCl₃): $\delta$ 173.4, 163.5, 158.4, 125.8, 123.4, 118.5, 112.3, 54.4. ESI-MS (-): Calc. for C₁₁H₈N₂Cl₂S [M-H]$^-$: 285.9; found: 287.0.

4,6-Dichloro-N-(2-methoxybenzyl)pyrimidin-2-amine (34i): Yield: 75%; $^1$H NMR (CDCl₃): $\delta$ 7.22 (d, $J = 8.0$ Hz, 1H), 7.13 (s, 1H), 6.85 (m, 3H), 6.75 (s, 1H), 4.62 (s, 2H), 3.84 (s, 3H); $^{13}$C NMR (CDCl₃): $\delta$ 163.4, 161.2, 155.2, 128.4, 118.6, 113.4, 112.4, 55.3, 45.4. ESI-MS (-): Calc. for C₁₂H₁₁N₂Cl₂O [M-H]$^-$: 282.0; found: 282.4.

4,6-Dichloro-2-(p-tolylthio)pyrimidine (34k): Yield: 70%; $^1$H NMR (CDCl₃): $\delta$ 7.47 (d, $J = 8.0$ Hz, 2H), 7.33 (d, $J = 8.0$ Hz, 2H), 6.58 (s, 1H), 2.45 (s, 1H); $^{13}$C NMR (CDCl₃): $\delta$ 172.4, 163.3, 138.4, 128.4, 128.3, 118.3, 22.3. ESI-MS (-): Calc. for C₁₁H₈N₂Cl₂S [M+H]$^+$: 271.0; found: 271.3.

4,6-Dichloro-2-((1,4,5,6-tetrahydropyrimidin-2-yl)thio)pyrimidine (34l): Yield: 75% $^1$H NMR (CDCl₃): $\delta$ 8.40 (s, 1H), 4.06 (t, 2H), 3.47 (t, 2H), 2.18-2.21 (m, 2H); $^{13}$C NMR (CDCl₃): $\delta$ 164.5, 159.2, 120.4, 46.5, 42.4, 22.7. ESI-MS (-): Calc. for C₈H₉N₂Cl₂S [M+H]$^+$: 262.9; found 263.0.

4-(2, 6-Dichloropyrimidin-4-yl)morpholine (34n): Yield: 68%; $^1$H NMR (CDCl₃): $\delta$ 6.35 (s, 1H), 3.12-3.68 (m, 8H); $^{13}$C NMR (CDCl₃): $\delta$ 163.3, 160.7, 160.4, 99.8, 66.3, 44.3. ESI-MS: 234.4 [M+H]$^+$

ESI-MS (-): Calc. for C₈H₉N₂Cl₂O [M+H]$^-$: 234.0; found: 234.4
2,4-Dichloro-6-(piperidin-1-yl)pyrimidine (34a): Yield: 70%; \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 6.37 (s, 1H), 3.58 (br, 4H), 1.60-1.68 (m, 6H); \(^13\)C NMR (CDCl\(_3\)): \(\delta\) 162.6, 160.1, 159.7, 99.5, 45.6, 25.4, 24.2. ESI-MS (-): Calc. for C\(_9\)H\(_{12}\)N\(_3\)Cl\(_2\) [M+H]\(^+\): 232.0; found: 232.2.

**General synthetic procedure for 36a-d and 37a-q:** To a solution of 32a or 32b-c (1.5 mmol) in acetone (8 ml), were added 35 or 34a-q respectively (1.5 mmol), followed by addition of potassium carbonate (3 mmol) and stirred at room temperature. Then, the reaction was monitored by TLC and upon completion, the solvent removed in vacuo. The dried residue was dissolved in ethyl acetate (20 mL) and washed with water (15 ml । 2) and brine (15 ml । 2). The solvent was evaporated in vacuo to obtain crude product, which was purified using silica gel column chromatography. Yields: 65-80%

4-Chloro-6-((3-(3, 5-dimethylphenyl)-1H-1,2,4-triazol-5-yl)thio)pyrimidine (36a): Yield: 68%; \(^1\)H NMR (DMSO-\(d_6\)): \(\delta\) 15.07 (br, 1H), 8.84 (s, 1H), 7.66 (s, 2H), 7.53 (s, 1H), 7.18 (s, 1H), 2.35 (s, 6H); \(^13\)C NMR (DMSO-\(d_6\)): \(\delta\) 158.7, 157.9, 138.4, 132.1, 129.1, 128.6, 127.7, 126.4, 123.8, 112.8, 20.0. HRMS-ESI (+): Calc. for C\(_{16}\)H\(_{12}\)N\(_6\)ScI [M+H]\(^+\): 426.0625; found: 426.0612.

4-Chloro-6-((3-(3, 5-dimethylphenyl)-1H-1, 2, 4-triazol-5-yl)thio)-2-(phenylthio)pyrimidine (36b): Yield: 75%; \(^1\)H NMR (MeOD): \(\delta\) 7.56 (m, 2H), 7.38 (m, 2H), 7.17 (m, 5H), 2.40 (s, 6H); \(^13\)C NMR (MeOD): \(\delta\) 172.5, 170.7, 159.9, 138.7, 138.6, 143.7, 132.1, 129.1, 128.6, 127.7, 126.4, 123.9, 123.8, 102.8, 20.0. HRMS-ESI (+): Calc. for C\(_{26}\)H\(_{10}\)ClN\(_6\)S\(_2\) [M+H]\(^+\): 462.0625; found: 426.0612.

4-((3-(3, 5-Dimethylphenyl)-1H-1, 2, 4-triazol-5-yl)thio)-2-(methylthio)pyrimidine (36c): Yield: 78%; \(^1\)H NMR (DMSO-\(d_6\)): \(\delta\) 8.78 (d, \(J = 5.2 \text{ Hz}\), 1H), 8.60 (d, \(J = 5.2 \text{ Hz}\), 1H), 7.92 (d, 1H, \(J = 5.2 \text{ Hz}\), 7.62 (m, 2H), 7.11 (s, 1H), 2.61 (s, 3H), 2.34 (s, 6H); \(^13\)C NMR (DMSO-\(d_6\)): \(\delta\) 166.1, 160.5, 157.9, 138.4, 132.4, 124.4, 116.9, 21.3, 14.2, 13.9. HRMS-ESI (+): Calc. for C\(_{15}\)H\(_{13}\)N\(_3\)S\(_2\) [M+H]\(^+\): 330.0847; found: 330.0841.

4-Chloro-6-((3-(3, 5-dimethylphenyl)-1H-1,2,4-triazol-5-yl)thio)-2-(methylthio)pyrimidine (36d): Yield: 80%; \(^1\)H NMR (DMSO-\(d_6\)): \(\delta\) 15.06 (br, 1H), 7.64 (s, 2H), 7.15 (s, 2H), 2.37 (s, 3H), 2.34 (s, 6H); \(^13\)C NMR (DMSO-\(d_6\)): \(\delta\) 172.5, 160.0, 138.8, 132.5, 124.3, 112.7, 60.2, 21.3, 14.0. HRMS-ESI (+): Calc. for C\(_{16}\)H\(_{14}\)N\(_3\)S\(_2\)Cl [M+H]\(^+\): 364.0457; found: 364.0457.
4-((5-(3,5-Bis(trifluoromethyl)phenyl)-4H-1,2,4-triazol-3-yl)thio)-6-chloropyrimidine (37a): Yield: 72%; \( ^1H \) NMR (DMSO-\( d_6 \)): \( \delta \) 15.52 (br, 1H), 8.85 (s, 1H), 8.61 (s, 2H), 8.31 (s, 1H), 7.66 (s, 1H); \( ^{13}C \) NMR (DMSO-\( d_6 \)): \( \delta \) 160.6, 158.8, 132.1, 131.8, 131.5, 131.1, 126.8, 124.8, 124.0, 122.1, 118.3. HRMS-ESI (+): Calc. for C\(_{14}H_8ClF\_6N\_5S [M+H]^+\): 426.0020; found: 426.0027.

4-((5-(3,5-Bis(trifluoromethyl)phenyl)-4H-1,2,4-triazol-3-yl)thio)-6-chloro-2-(methylthio)pyrimidine (37b): Yield: 68%; \( ^1H \) NMR (DMSO-\( d_6 \)): \( \delta \) 15.50 (br, 1H), 8.57 (s, 2H), 8.25 (s, 1H), 7.28 (s, 1H), 2.34 (s, 3H); \( ^{13}C \) NMR (DMSO-\( d_6 \)): \( \delta \) 172.7, 160.1, 131.8, 131.5, 131.1, 126.7, 124.8, 122.1, 113.0, 14.0. HRMS-ESI (+): Calc. for C\(_{14}H_8N_5S\_2F_6Cl [M+H]^+\): 471.9874; found: 471.9856.

4-((5-(3,5-Bis(trifluoromethyl)phenyl)-4H-1,2,4-triazol-3-yl)thio)-6-chloro-2-(piperidin-1-yl)pyrimidine (37c): Yield: 71%; \( ^1H \) NMR (CDCl\(_3\)): \( \delta \) 8.61 (s, 2H), 7.93 (s, 1H), 6.43 (s, 1H), 3.72 (br, 4H), 1.63-1.69 (m, 6H); \( ^{13}C \) NMR (CDCl\(_3\)): \( \delta \) 165.4, 161.1, 160.4, 160.0, 147.7, 132.7, 132.3, 132.2, 132.0, 131.7, 127.2, 126.5, 124.5, 123.0, 121.8, 119.1, 105.2, 45.4, 25.5, 24.3. HRMS-ESI (-): Calc. for C\(_{19}H_{16}ClF_6N_6S [M-H]^+\): 507.0593; found: 507.0596.

4-((5-(3,5-Bis(trifluoromethyl)phenyl)-4H-1,2,4-triazol-3-yl)thio)-6-chloro-N-cyclopentylpyrimidin-2-amine (37d): Yield: 75%; \( ^1H \) NMR (DMSO-\( d_6 \)): \( \delta \) 8.57 (s, 2H), 8.27 (s, 1H), 7.89 (s, 1H), 1.48-1.85 (m, 9H); \( ^{13}C \) NMR (DMSO-\( d_6 \)): \( \delta \) 160.8, 160.6, 160.0, 132.1, 131.8, 131.5, 131.1, 127.5, 126.6, 124.8, 123.8, 122.1, 119.4, 104.6, 53.0, 52.8, 32.3, 31.9, 23.7, 23.4. HRMS-ESI (+): Calc. for C\(_{19}H_{15}ClF_6N_6S [M+H]^+\): 509.750; found: 509.0760.

4-((5-(3,5-Bis(trifluoromethyl)phenyl)-4H-1,2,4-triazol-3-yl)thio)-6-chloro-N-(prop-2-yn-1-yl) pyrimidin-2-amine (37e): Yield: 74%; \( ^1H \) NMR ((CD\(_3\))\(_2\)CO): \( \delta \) 8.64 (s, 2H), 8.07 (s, 1H), 6.70 (s, 1H), 4.15 (d, J = 8.0 Hz, 2H), 2.53 (s, 1H); \( ^{13}C \) NMR (CD\(_3\))\(_2\)CO): \( \delta \) 168.6, 160.8, 158.9, 147.5, 132.4, 132.1, 131.8, 131.4, 127.3, 126.3, 124.6, 122.8, 121.9, 119.2, 106.2, 80.0, 71.3, 70.2, 36.4. HRMS-ESI (-): Calc. for C\(_{17}H_9ClF_6N_6S [M-H]^+\): 477.0121.

4-((5-(3,5-Bis(trifluoromethyl)phenyl)-4H-1,2,4-triazol-3-yl)thio)-6-chloro-N-cyclobutylpyrimidin-2-amine (37f): Yield: 75%; \( ^1H \) NMR (CDCl\(_3\)): \( \delta \) 8.61 (d, J = 8.0 Hz, 2H), 7.93 (s, 1H), 6.66 (s, 1H), 2.55 (br, 2H), 2.00 (br, 4H). HRMS-ESI (-): Calc. for C\(_{18}H_{13}ClF_6N_6S[M-H]^+\): 493.0437; found: 493.0456.
4-((5-(3,5-Bis(trifluoromethyl)phenyl)-1H-1,2,4-triazol-3-yl)thio)-6-chloropyrimidin-2-yl)morpholine (37g): Yield: 70%; $^1$H NMR (CDCl$_3$): δ 8.60 (s, 2H), 7.95 (s, 1H), 6.53 (s, 1H), 3.77 (s, 8H); $^{13}$C NMR (CDCl$_3$): δ 166.1, 161.3, 160.7, 160.2, 147.2, 132.8, 132.4, 132.1, 132.0, 131.8, 127.2, 126.4, 124.4, 123.2, 123.1, 121.7, 119.0, 106.3, 66.4, 44.4. HRMS-ESI (-): Calc. for C$_{18}$H$_{13}$ClF$_6$N$_6$O$_5$S [M-H]$^-$: 509.0386; found: 509.0386.

4-((3-(3,5-Bis(trifluoromethyl)phenyl)-1H-1,2,4-triazol-5-yl)thio)-6-chloro-2-(pyrrolidin-1-yl)pyrimidine (37h): Yield: 72%; $^1$H NMR (CDCl$_3$): δ 8.60 (s, 2H), 7.92 (s, 1H), 6.60 (s, 1H), 3.67-3.75 (s, 4H), 2.06 - 2.17 (m, 4H), 5.43 (s, 2H), 3.78 (s, 6H), 2.24 (s, 3H); $^{13}$C NMR (CDCl$_3$): δ 164.5, 160.8, 159.9, 158.5, 148.8, 132.5, 132.3, 131.9, 126.4, 124.5, 122.9, 121.8, 105.4, 47.4, 47.1, 25.5, 25.2. HRMS-ESI (-): Calc. for C$_{18}$H$_{13}$ClF$_6$N$_6$S [M-H]$^-$: 493.0437; found: 493.0435.

4-((3-(5-Bis(trifluoromethyl)phenyl)-1H-1,2,4-triazol-5-yl)thio)-6-chloro-2-phenoxypyrimidine (37i): Yield: 75%; $^1$H NMR (DMSO-$d_6$): δ 15.52 (br, 1H), 8.56 (m, 3H), 7.49 (m, 7H); $^{13}$C NMR (DMSO-$d_6$): δ 170.4, 163.5, 161.7, 158.8, 152.2, 151.9, 131.8, 131.4, 130.4, 129.7, 126.8, 125.7, 124.8, 124.1, 122.0, 121.7, 112.7, 107.6, 103.0. HRMS-ESI (+): Calc. for C$_{20}$H$_{10}$ClF$_6$N$_5$OS [M+H]$^+$: 518.0277; found: 518.0289.

4-((3-(3,5-Bis(trifluoromethyl)phenyl)-1H-1,2,4-triazol-5-yl)thio)-6-chloro-2-((4methoxyphenyl)thio) pyrimidine (37k): Yield: 70%; $^1$H NMR ((CD$_3)_2$CO): δ 8.61 (s, 2H), 8.23 (s, 1H), 7.41 (t, J = 8.0 Hz, 2H), 6.76 (d, J = 8.0 Hz, 2H), 6.23 (s, 1H), 3.55 (s, 3H); $^{13}$C NMR ((CD$_3)_2$CO): δ 177.1, 161.5, 159.0, 137.1, 132.2, 131.9, 131.5, 126.1, 124.7, 123.5, 122.0, 116.5, 115.6, 110.5, 54.8. HRMS-ESI (-): Calc. for C$_{21}$H$_{15}$ClF$_6$N$_5$OS$_{22}$ [M-H]$^-$: 561.9998; found: 561.9986

4-((3-(3,5-Bis(trifluoromethyl)phenyl)-1H-1,2,4-triazol-5-yl)thio)-6-chloro-N-(2-methoxybenzyl) pyrimidin-2-amine (37l): Yield: 68%; $^1$H NMR (CDCl$_3$): δ 8.55 (s, 2H), 7.92 (s, 1H), 7.30 (t, J = 8.0 Hz, 3H), 6.93 (d, 2H), 6.69 (s, 1H), 4.55 (d, J = 4.0 Hz, 2H), 3.80 (s, 3H); $^{13}$C NMR (CDCl$_3$): δ 160.8, 159.9, 159.4, 132.6, 132.3, 132.3, 131.9, 131.6, 126.4, 124.5, 122.9, 121.8, 114.5, 113.8, 107.5, 55.3, 45.4. HRMS-ESI (-): Calc. for C$_{22}$H$_{15}$ClF$_6$N$_6$O$_5$S [M-H]$^-$: 559.0543; found: 559.0540
4-((3-(3,5-Bis(trifluoromethyl)phenyl)-1H,1,2,4-triazol-5-ylthio)-6-chloro-N-cyclohexylpyrimidin-2-amine (37m): Yield: 70%; $^1$H NMR (CDCl$_3$): δ 8.56 (s, 2H), 7.93 (s, 1H), 6.44 (s, 1H), 4.19 (s, 1H), 3.83 (s, 1H), 1.36-2.06 (m, 10H); $^{13}$C NMR (CDCl$_3$): δ 160.8, 160.6, 160.0, 132.1, 131.8, 131.5, 131.1, 127.5, 124.8, 122.1, 119.4, 53.0, 33.9, 23.7, 23.4. HRMS-ESI (-): Calc. for C$_{20}$H$_{17}$ClF$_6$N$_6$S [M-H]: 521.0750; found: 521.0745.

4-((3-(3,5-Bis(trifluoromethyl)phenyl)-1H,1,2,4-triazol-5-ylthio)-6-chloro-2-(p-tolylthio)pyrimidine (37n): Yield: 74%; $^1$H NMR ((CD$_3$)$_2$CO): δ 8.57 (s, 2H), 7.94 (s, 1H), 7.46 (d, J = 8.0 Hz, 2H), 7.30 (d, J = 8.0 Hz, 2H), 6.5 (s, 1H), 2.39 (s, 3H); $^{13}$C NMR ((CD$_3$)$_2$CO): δ 176.3, 158.7, 141.0, 135.6, 132.0, 131.6, 130.9, 127.4, 126.8, 124.8, 124.5, 122.5, 122.1, 110.7, 20.5. HRMS-ESI (-): Calc. for C$_{21}$H$_{12}$ClF$_6$N$_5$S$_2$ [M-H]: 546.0049; found: 546.0047.

4-((3-(3,5-Bis(trifluoromethyl)phenyl)-1H,1,2,4-triazol-5-ylthio)-6-chloro-2-(pyrimidin-2-ylthio)pyrimidine (37o): Yield: 70%; $^1$H NMR (CDCl$_3$): δ 8.67 (d, J = 4.0 Hz, 2H), 8.59 (s, 2H), 8.30 (s, 1H), 7.93 (s, 1H), 7.28 (s, 1H); $^{13}$C NMR (CDCl$_3$): δ 172.0, 169.7, 167.0, 160.2, 159.1, 158.0, 146.5, 132.7, 132.4, 132.0, 132.0, 131.7, 127.1, 126.5, 126.4, 124.4, 123.1, 121.7, 119.5, 119.0, 116.2. HRMS-ESI (-): Calc. for C$_{18}$H$_8$ClF$_6$N$_7$S$_2$ [M-H]: 533.9797; found: 533.9797.

4-((3-(3,5-Bis(trifluoromethyl)phenyl)-1H,1,2,4-triazol-5-ylthio)-6-chloro-2-(phenylthio)pyrimidine (37p): Yield: 75%; $^1$H NMR (DMSO-d$_6$) δ 15.34 (br, 1H), 8.58 (s, 2H), 8.29 (s, 1H), 7.44 (m, 3H), 7.20 (m, 2H), 7.11 (m, 1H); $^{13}$C NMR (DMSO-d$_6$): δ 171.8, 160.1, 135.1, 132.1, 131.8, 131.4, 129.7, 127.7, 126.8, 124.8, 124.0, 122.1, 114.1. HRMS-ESI (+): Calc. for C$_{28}$H$_{10}$ClF$_6$N$_7$S$_2$ [M+H]$^+$: 534.0049; found: 534.0062.
General synthetic procedure for 38a-h and 39: To a solution of 32b (1.5 mmol) in acetone/DMF (10 ml), was slowly added R-NH₂ (1.5 mmol) at room temperature and stirred. The reaction was monitored using TLC and on completion the solvent was removed in vacuo. The dried residue was dissolved in ethyl acetate (15-20 mL), washed with water (15 ml × 2) and brine (10 ml × 2). The solvent was evaporated in vacuo to obtain crude product, which was purified using silica gel column chromatography. Yields: 65-75%

4-((3-(3, 5-Bis (trifluoromethyl)phenyl)-1H-1,2,4-triazol-5-yl)thio)-2-(methylthio)pyrimidine (38a): Yield: 75%; ¹H NMR (CDCl₃): δ 13.34 (br, 1H), 8.60 (s, 2H), 8.39 (d, J = 5.6 Hz, 1H), 7.93 (s, 1H), 7.01 (d, J = 5.6 Hz, 1H), 2.57 (s, 3H); ¹³C NMR (CDCl₃): δ 173.3, 164.7, 160.1, 156.2, 132.3, 132.2, 126.4, 124.5, 123.0, 121.8, 114.0, 14.2. HRMS-ESI (+): Calc. for C₁₅H₁₀F₆N₅S₂ [M+H]⁺: 438.0282; found: 438.0280.

4-Azido-6-((3-(3,5-bis(trifluoromethyl)phenyl)-1H-1,2,4-triazol-5-yl)thio)-2-(methylthio)pyrimidine (38b): Yield: 72%; ¹H NMR (CDCl₃): δ 8.60 (s, 2H), 7.93 (s, 1H), 6.53 (s, 1H), 2.65 (s, 3H); ¹³C NMR (CDCl₃): δ 174.3, 165.0, 161.6, 159.9, 147.7, 132.7, 132.3, 131.9, 131.6, 126.4, 122.8, 102.5, 14.2. HRMS-ESI (-): Calc. for C₁₅H₁₀F₆N₈S₂ [M-H]: 477.0139; found: 477.0139.

6-((3-(3, 5-Bis (trifluoromethyl) phenyl)-1H-1, 2, 4-triazol-5-yl)thio)-N-methyl-2-(methylthio)pyrimidin-4-amine (38c): Yield: 74%; ¹H NMR (CDCl₃): δ 8.61 (s, 2H), 7.91 (s, 1H), 6.10 (s, 1H), 3.00 (s, 3H), 2.59 (s, 3H); ¹³C NMR (CDCl₃): δ 171.5, 162.4, 159.4, 148.1, 132.6, 132.3, 132.2, 131.9, 131.6, 127.2, 126.1, 126.0, 124.5, 122.7, 122.6, 122.5, 121.8, 119.1, 26.2, 12.4. HRMS-ESI (+): Calc. for C₁₆H₁₂F₆N₄S₂ [M+H⁺]: 467.0547; found: 467.542.

6-((3-(3, 5-Bis (trifluoromethyl) phenyl)-1H-1, 2, 4-triazol-5-yl)thio)-N,N-dimethyl-2-(methylthio)pyrimidin-4-amine (38d): Yield: 68%; ¹H NMR (CDCl₃): δ 8.60 (s, 2H), 7.90 (s, 1H), 6.15 (s, 1H), 3.13 (br, 6H), 2.57 (s, 3H); ¹³C NMR (CDCl₃): δ 171.1, 160.8, 16.5, 159.5, 149.7, 132.8, 132.2, 131.8, 126.8, 126.3, 122.6, 122.5, 94.7, 37.1, 14.1, 13.8. HRMS-ESI (+): Calc. for C₁₇H₁₄F₆N₆S₂ [M+H⁺]: 481.0704; found: 481.0700.
6-((3-(3, 5-Bis (trifluoromethyl) phenyl)-1H-1, 2, 4-triazol-5-yl) thio)-N-isopropyl-2-(methylthio)pyrimidin-4-amine (38e): Yield: 75%; \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 8.60 (s, 2H), 7.14 (s, 1H), 6.06 (s, 1H), 2.57 (s, 3H), 1.28 (t, 6H, 12 Hz); \(^{13}\)C NMR (CDCl\(_3\)): \(\delta\) 160.2, 159.6, 149.4, 132.8, 132.5, 132.2, 131.9, 131.5, 127.3, 124.6, 121.9, 119.1, 43.3, 22.6, 14.1. HRMS-ESI (+): Calc. for C\(_{18}\)H\(_{16}\)F\(_6\)N\(_6\)S\(_2\) [M+H]\(^+\): 495.0860; found: 495.0874.

6-((3-(3, 5-Bis (trifluoromethyl) phenyl)-1H-1, 2, 4-triazol-5-yl) thio)-N-cyclopropyl-2-(methylthio)pyrimidin-4-amine (38f): Yield: 72%; \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 8.61 (s, 2H), 7.91 (s, 1H), 6.43 (s, 1H), 5.56 (s, 1H), 2.54 (s, 3H), 0.90 (t, \(J = 6.0\) Hz, 4H). \(^13\)C NMR (CDCl\(_3\)): \(\delta\) 171.7, 162.7, 159.7, 149.3, 132.8, 132.6, 132.2, 131.9, 131.6, 124.6, 121.9, 119.19, 95.2, 23.3, 14.0, 7.6. HRMS-ESI (-): Calc. for C\(_{18}\)H\(_{16}\)F\(_6\)N\(_6\)S\(_2\) [M-H]-: 491.0547; found: 491.0553.

4-((3-(3,5-Bis(trifluoromethyl)phenyl)-1H-1,2,4-triazol-5-yl)thio)-2-(methylthio)-6-(piperidin-1-yl)pyrimidine (38g): Yield: 77%; \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 8.60 (s, 2H), 7.90 (s, 1H), 6.23 (s, 1H), 3.65 (br, 3H), 2.57 (s, 3H), 1.65-1.73 (m, 6H); \(^{13}\)C NMR (CDCl\(_3\)): \(\delta\) 171.4, 160.9, 159.7, 159.5, 149.8, 132.8, 132.5, 132.2, 131.8, 131.5, 127.3, 126.4, 126.3, 124.6, 122.6, 122.6, 122.5, 121.9, 119.2, 113.1, 94.9, 45.3, 25.5, 24.4, 14.2. HRMS-ESI (-): Calc. for C\(_{20}\)H\(_{18}\)F\(_6\)N\(_6\)S\(_2\)- [M-H]-: 519.0860; found: 519.0853.

4-((6-(3-(3, 5-Bis(trifluoromethyl)phenyl)-1H-1,2,4-triazol-5-yl)thio)-2-(methylthio)pyrimidin-4-yl)morpholine (38h): Yield: 72%; \(^1\)H NMR (DMSO-d\(_6\)) \(\delta\) 8.59 (m, 2H), 8.22 (m, 1H), 7.91 (m, 2H), 7.70 (m, 1H), 2.59 (s, 1H), 2.61 (s, 3H), 2.34 (s, 6H). HRMS-ESI (-): Calc. for C\(_{19}\)H\(_{10}\)ClF\(_6\)N\(_6\)OS\(_2\)-: 521.0653; found: 521.0656 [M-H]-

N-(3-(3, 5-Bis (trifluoromethyl) phenyl)-1H-1,2,4-triazol-5-yl)-6-chloro-2-(methylthio)pyrimidin-4-amine (39a): Yield: 71%; \(^1\)H NMR (DMSO-d\(_6\)) \(\delta\) 8.51 (m, 2H), 8.22 (m, 1H), 7.91 (m, 2H), 7.70 (m, 1H), 2.59 (s, 1H), 2.61 (s, 3H), 2.34 (s, 6H). HRMS-ESI (+): Calc. for C\(_{15}\)H\(_{16}\)ClF\(_6\)N\(_6\): 455.0280; found: 455.0275 [M+H]+
**General synthetic procedure for 40a-k:** To a solution of 37b (1.30 mmol) in DMF (8 ml), was added triethylamine (2mmol), followed by addition of appropriate alkyl halides, RX (1.35 mmol) and stirred at 40-50°C and monitored using TLC. Upon completion, the reaction mixture was cooled to ambient temperature and the DMF removed in vacuo. The dried residue was dissolved in ethyl acetate (20 mL) and washed with water (15 ml x 2) and brine (10 ml x 2). The solvent was evaporated in vacuo to obtain crude product, which was purified using silica gel column chromatography. Yields: 70-80%

4-((3-(3,5-Bis(trifluoromethyl)phenyl)-1-methyl-1H-1,2,4-triazol-5-yl)thio)-6-chloro-2(methylthio)pyrimidine (40a): Yield: 85%; 1H NMR (CDCl3): δ 8.60 (s, 2H), 7.93 (s, 1H), 6.91(s, 1H), 4.04 (s, 3H), 2.31 (s, 3H); 13C NMR (CDCl3): δ 174.1, 167.0, 160.6, 160.4, 144.2, 132.4, 132.3, 132.0, 126.2, 124.5, 123.0, 123.0, 121.8, 112.3, 36.9, 14.1. HRMS-ESI (+): Calc. for C16H16ClF6N2S2 [M+H]+: 486.0049; found: 486.0045.

4-((3-(3,5-Bis(trifluoromethyl)phenyl)-1-ethyl-1H-1, 2, 4-triazol-5-yl)thio)-6-chloro-2-(methylthio)pyrimidine (40b): Yield: 82%; 1H NMR (CDCl3): δ 8.62 (s, 2H), 7.93 (s, 1H), 6.88 (s, 1H), 4.36 (q, 2H, J = 8.0 Hz), 2.32 (s, 3H), 1.57 (t, J = 8.0 Hz, 3H); 13C NMR (CDCl3): δ 174.0, 167.4, 160.6, 143.2, 132.4, 132.3, 132.0, 126.3, 122.9, 122.9, 112.2, 45.3, 15.2, 14.1. HRMS-ESI (+): Calc. for C17H12ClF6N2S2 [M+H]+: 500.0205; found: 500.0197.

4-((3-(3,5-Bis(trifluoromethyl)phenyl)-1-isopropyl-1H-1,2,4-triazol-5-yl)thio)-6-chloro-2(methylthio)pyrimidine (40c): Yield: 78%; 1H NMR (CDCl3): δ 8.63 (s, 2H), 7.93 (s, 1H), 6.86 (s, 1H), 4.88 (m, J = 8.0 Hz, 1H), 2.34 (s, 3H), 1.57 (d, J = 4.0 Hz, 6H); 13C NMR (CDCl3): δ 174.04, 167.68, 160.64, 142.30, 132.70, 132.32, 131.98, 131.65, 126.28, 124.59, 122.87, 112.24, 112.20, 52.40, 29.70, 22.89, 14.12. HRMS-ESI (+): Calc. for C19H14ClF6N2S2 [M+H]+: 514.0362; found: 514.0358.

4-((3-(3,5-Bis(trifluoromethyl)phenyl)-1-(cyclopropylmethyl)-1H-1,2,4-triazol-5-yl)thio)-6-chloro-2-(methylthio)pyrimidine (40d): Yield: 72%; 1H NMR (CDCl3): δ 8.62 (s, 2H), 7.94 (s, 1H), 6.86 (s, 1H), 4.18 (d, J = 8.0 Hz, 2H), 2.33 (s, 3H), 0.66 (d, 2H), 0.46 (d, 2H); 13C NMR (CDCl3): δ 171.5, 162.4, 159.4, 148.1, 132.6, 132, 132.2, 131.9, 131.6, 127.2, 126.0, 126.0, 124.5, 122.6, 122.6, 121.8, 119.1, 26.2, 12.4. HRMS-ESI (+): Calc. for C19H14ClF6N2S2 [M+H]+: 526.0362; found: 526.0358.
4-((3-(5-Bis(trifluoromethyl)phenyl)-1-(3,5-dimethoxybenzyl)-1H-1,2,4-triazol-5-yl)thio)-6-chloro-2-(methylthio)pyrimidine (40e). Yield: 77%; $^1$H NMR (CDCl$_3$): δ 8.64 (s, 2H), 7.95 (s, 1H), 6.56 (s, 1H), 6.40 (s, 2H), 6.32 (s, 1H), 5.43 (s, 2H), 3.78 (s, 6H), 2.24 (s, 3H); $^{13}$C NMR (CDCl$_3$): δ 173.6, 167.1, 161.0, 160.2, 143.9, 135.6, 132.7, 132.4, 132.0, 131.7, 126.35, 124.5, 123.1, 121.8, 112.1, 106.9, 106.5, 104.9, 99.5, 55.3, 54.2, 14.0. HRMS-ESI (+): Calc. for C$_{24}$H$_{18}$ClF$_6$N$_5$O$_2$S$_2$ [M+H]$^+$: 622.0573; found: 622.0571.

4-((1-(4-Azidobenzyl)-3-(3,5-bis(trifluoromethyl)phenyl)-1H-1,2,4-triazol-5-yl)thio)-6-chloro-2-(methylthio)pyrimidine (40f). Yield: 83%; $^1$H NMR (CDCl$_3$): δ 8.62 (s, 2H), 7.94 (s, 1H), 7.31 (d, J = 8.0 Hz, 2H), 6.95 (d, J = 8.0 Hz, 2H), 6.63 (s, 1H), 6.32 (s, 1H), 2.27 (s, 3H); $^{13}$C NMR (CDCl$_3$): δ 173.8, 167.0, 160.7, 160.5, 143.88, 140.7, 132.4, 132.2, 132.0, 130.3, 129.8, 126.3, 123.1, 123.1, 119.3, 112.3, 53.4, 14.1. HRMS-ESI (+): Calc. for C$_{22}$H$_{13}$ClF$_6$N$_5$S$_2$ [M+H]$^+$: 603.0376; found: 603.0362.

**General synthetic procedure for 42:** To a solution of 32a (1.30 mmol) in CH$_3$CN (8 ml), was added methyl Iodide (1.8mmol) at 0 to -5°C and stirred for 4-5 h. Then, the reaction was stirred at room temperature for 2-3 h and monitored by TLC. Upon completion, the solvent was removed in vacuo. The dried residue thus obtained was dissolved in acetone (10 ml) and added triethylamine (2 mmol) followed by 4,6-dichloro-2-(methylthio)pyrimidine (1.4 mmol) and stirred at room temperature. Upon completion, the reaction solvent was evaporated in vacuo and residue dissolved in ethyl acetate (20 mL) and washed with water (15 ml × 2) and brine (10 ml × 2). The solvent was evaporated in vacuo to obtain crude product, which was purified using silica gel column chromatography. Yields: 85%

**General synthetic procedure for 43a-b:** To a solution of 2,4,6 trichloropyrimidine (1.5 mmol) in acetone (8 ml) at 0-4°C was added K$_2$CO$_3$ (2mmol), followed by slow addition of 32a-b. Then, the reaction was monitored by TLC and upon completion; the reaction solvent was removed in vacuo. The dried residue thus obtained was dissolved in ethyl acetate (20 mL) and washed with water (15 ml × 2) and brine (10 ml × 2). The solvent was evaporated in vacuo to obtain crude product, which was purified using silica gel column chromatography. Yields: 60%.
4-(3-(3,5-Bis(trifluoromethyl)phenyl)-5-(methylthio)-1H-1,2,4-triazol-1-yl)-6-chloro -2(methylthio) pyrimidine (42): Yield: 85%; $^1$H NMR (CDCl$_3$): δ 8.64 (s, 2H), 7.97 (s, 1H), 7.66 (s, 1H), 2.83 (s, 3H), 2.74 (s, 3H); $^{13}$C NMR (CDCl$_3$): δ 174.1, 162.2, 160.4, 159.4, 156.5, 132.3, 132.0, 131.8, 126.8, 123.5, 104.3, 16.4, 14.8. HRMS-ESI (+): Calc. for $C_{16}H_{10}$ClF$_6$N$_5$S$_2$ [M+H]$^+$: 486.0049; found: 486.0042.

2.6 References


29. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from the National Institutes of Health (National Center for Research Resources grant 2P41RR001081, National Institute of General Medical Sciences grant 9P41GM103311).


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APPENDICES

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

Data for 4-[(1, 1'-biphenyl]-4-yl)-2-((4-azidobenzyl) thio)-6-oxo-1, 6-dihydropyrimidine-5-carbonitrile (15a).
Data for (4-(((6-cyano-5-oxo-4, 5-dihydro-[1, 1':4',1''-terphenyl]-3yl) thio) methyl) phenyl) boronic acid (15c).
Data for 4-((6-cyano-5-oxo-4,5-dihydro-[1,1':4',1''-terphenyl]-3-yl)thio)methyl)benzoic acid (15d).
Data for Methyl 4-((4-((1, 1'-biphenyl)-4-yl)-5-cyano-6-oxo-1,6-dihydropyrimidin-2-yl)thio)methyl)benzoate (15e).
Data for 4-((1, 1'-biphenyl)-4-yl)-6-oxo-2-(prop-2-yn-1-ylthio)-1,6-dihydropyrimidine-5-carbonitrile (15g).
Data for 4-((1, 1'-biphenyl)-4-yl)-2-((2-(4-azidophenyl)-2-oxoethyl)thio)-6-oxo-1,6 dihydropyrimidine-5-carbonitrile (15h).
Data for Ethyl 4-(((4-([1,1'-biphenyl]-4-yl)-5-cyano-6-oxo-1,6-dihydropyrimidin-2-yl)thio)methyl)benzoate (15i).
Data for 4-([1,1'-biphenyl]-4-yl)-2-((3-azidobenzyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (15j).
Data for 2-(benzylthio)-4-(3'-hydroxy-[1,1'-biphenyl]-4-yl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (15l).
Data for methyl 4'-(2-((4-azidobenzyl)thio)-5-cyano-6-oxo-1,6-dihydropyrimidin-4-yl)-4,5-dimethoxy-[1,1'-biphenyl]-2-carboxylate (15m).
Data for Methyl 4’-(5-cyano-2-((4-(methoxycarbonyl)benzyl)thio)-6-oxo-1,6 dihydropyrimidin-4-y1)-4,5-dimethoxy-[1,1’-biphenyl]-2-carboxylate (15n).
Data for 4-((4-((1,1'-biphenyl)-4-yl)-5-cyano-6-oxo-1,6-dihydropyrimidin-2-yl)thio)methyl)-N-(3-(2-(2-(3-aminopropoxy)ethoxy)ethoxy)propyl)benzamide (15o).
Data for Tert-butyl (1-(4-(((4-((1,1'-biphenyl)-4-yl)-5-cyano-6-oxo-1,6-dihydropyrimidin-2-yl)(thio)methyl)phenyl)-1-oxo-6,9,12-trioxa-2-azapentadecan-15-yl)carbamate (15p).
Data for 4-([1,1'-biphenyl]-4-yl)-2-((4-azidobenzyl)thio)-6-(hydroxyamino)pyrimidine-5-carbonitrile (17a).
Data for 4-((1, 1'-biphenyl)-4-yl)-2-(benzylthio)-6-((2-hydroxyethyl)amino)pyrimidine-5-carbonitrile (17b).
Data for 4-((1,1'-biphenyl)-4-yl)-2-(benzylthio)-6-methoxypyrimidine-5-carbonitrile (17c).
Data for 4-([1,1'-biphenyl]-4-yl)-2-((4-azidobenzyl)thio)-6-(methylamino)pyrimidine-5-carbonitrile (17d).
Data for Tert-butyl (3-(2-(2-(3-((6-((1,1'-biphenyl)-4-yl)-2-((4-azidobenzyl)thio)-5 cyanopyrimidin-4-yl) amino)propoxy)ethoxy)ethoxy)propyl)carbamate (17e).
Data for Tert-butyl (3-(2-(2-(3-(6-((1,1'-biphenyl)-4-yl)-2-(benzylthio)-5-cyanopyrimidin-4-yl)amino)propoxy)ethoxy)ethoxy)propyl)carbamate (17f).
Data for 4-([1,1’-biphenyl]-4-yl)-6-((3-(2-(3aminopropoxy)ethoxy)ethoxy)propyl)amino)-2-(benzylthio)-1,6-dihydropyrimidine-5-carbonitrile (17g)
Data for 4-[[1,1'-biphenyl]-4-yl]-2-((4-azidobenzyl)thio)-6-((2-hydroxyethyl)amino)-1,6-
  dihydropyrimidine-5-carbonitrile (17h).
Data for 4-((1,1'-biphenyl)-4-yl)-2-((4-azidobenzyl)thio)-6-hydrazinyl-1,6 dihydropyrimidine-5-carbonitrile (17i).
Data for 4-([1,1'-biphenyl]-4-yl)-2-((4-azidobenzyl)thio)-6-(prop-2-yn-1-ylamino) pyrimidine-5-carbonitrile (17j).
Data for 4-([1,1'-biphenyl]-4-yl)-6-oxo-2-((4-(4-phenyl-1H-1,2,3-triazol-1-yl)benzyl)thio)-1,6-dihydropyrimidine-5-carbonitrile (18a).
Data for 4-([(1,1'-biphenyl]-4-yl)-2-((4-(4-aminophenyl)-1H-1,2,3-triazol-1-yl)benzyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (18b).
Data for 1-(4-(((4-([1,1'-biphenyl]-4-yl)-5-cyano-6-oxo-1,6-dihydropyrimidin 2yl)thio methyl)phenyl)-1H-1,2,3-triazole-4-carboxylic acid (18c).

Data for (1-(4-(((4-([1,1'-biphenyl]-4-yl)-5-cyano-6-oxo-1,6-dihydropyrimidin-2-yl)thio)methyl)phenyl)-1H-1,2,3-triazole-4-yl)methyl acetate (18d).
100% MeOH + 0.1% HCOOH

Chemical Formula: C_{20}H_{18}N_4O_3
Exact Mass: 554.1474
[M+H]^+ : 555.1592

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Current Date Parameters
Sample: 2.137
Instrument: 1
Scan: 1

F1 - Acquisition Parameters
Date: 02/06/2012
Time: 15:42:07
Reference: 06-Feb-2012

P1 - Processing parameters
SI: 32
SP: 400.1450 MHz

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Data for 3-(1-(4-((4-((1,1'-biphenyl)-4-yl)-5-cyano-6-oxo-1,6-dihydropyrimidin-2-yl)(thio)methyl)phenyl)-1H-1,2,3-triazol-4-yl)propanoic acid (18e).
Data for 2-(1-(4-((4-((1,1'-biphenyl)-4-yl)-5-cyano-6-oxo-1,6-dihydropyrimidin-2-yl)thio)methyl)phenyl)-1H-1,2,3-triazol-4-yl)acetic acid (18f).
Data for 4-((1,1'-biphenyl)-4-yl)-2-((4-(4-(hydroxymethyl)-1H-1,2,3-triazol-1-yl)benzyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (18g).
Data for 4-([1,1’-biphenyl]-4-yl)-2-((4-azidobenzyl)thio)-6-methoxypyrimidine-5-carbonitrile (19b).
Data for 4-([1,1'-biphenyl]-4-yl)-6-((4-azidobenzyl)oxy)-2-((4-azidobenzyl)thio)pyrimidine-5-carbonitrile (19c).
Data for 2-((4-azidobenzyl)thio)-4-(4-(benzyloxy)phenyl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (21a).
Data for Methyl 4-((4-(4-(benzyloxy)phenyl)-5-cyano-6-oxo-1,6-dihydropyrimidin-2-yl)thio)methyl)benzoate (21b).
Data for 4-(4-(benzyloxy)phenyl)-2-((4-methoxybenzyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (21c).
Data for 4-(4-(benzyloxy)phenyl)-2-((4-bromobenzyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (21d).
Data for 4-(4-(benzyl oxy)phenyl)-2-((2-bromobenzyl)thio)-6-oxo-1,6-dihydropyrimidin e-5-carbonitrile (21e).
Data for 4-(4-(benzyloxy)phenyl)-2-((cyclohexylmethyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (21f).
Data for 4-(4-(benzyloxy) phenyl)-2-((3, 5-dimethoxybenzyl) thio)-6-oxo-1,6 dihydropyrimidine-5-carbonitrile (21g).
Data for 4-(4-(benzyloxy) phenyl)-2-((3,5-dimethylbenzyl)thio)-6oxo1,6dihydropyrimidine-5-carbonitrile (21h).
Data for 2-((4-azidobenzyl)thio)-6-oxo-4-(4-phenoxyphenyl)-1,6-dihydropyrimidine-5-carbonitrile (21i).
Data for Methyl 4-(((5-cyano-6-oxo-4-(4-phenoxyphenyl)-1,6-dihydropyrimidin-2-yl)thio)methyl)benzoate (21j).
Data for 2-((4-methoxybenzyl)thio)-6-oxo-4-(4-phenoxyphenyl)-1,6-dihydropyrimidine-5-carbonitrile (21k).
Data for 2-((4-azidobenzyl)thio)-6-oxo-4-(4-(phenylethynyl)phenyl)-1,6-dihydropyrimidine-5-carbonitrile (21l).
Data for methyl 4-(((5-cyano-6-oxo-4-(phenylethynyl)phenyl)-1,6-dihydropyrimidin-2-y1)(thio)methyl)benzoate (21m).
Data for 2-((4-methoxybenzyl)thio)-6-oxo-4-(4-(phenylethynyl)phenyl)-1,6 dihydropyrimidine-5-carbonitrile (21n).
Data for 2-((3,5-dimethoxybenzyl)thio)-6-oxo-4-(4-(phenylethynyl)phenyl)-1,6-dihydropyrimidine-5-carbonitrile (21o).
Data for 4-(((4-([1,1']-biphenyl)-4-yl)-5-cyano-6-oxo-1,6-dihydropyrimidin-2-yl)thio)methyl)-N-(15-oxo-19-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-4,7,10-trioxa-14-azanonadecyl)benzamide (24).
Appendix A. HRMS, $^1$H and $^{13}$C Spectra of compounds in chapter 2:

Data for 4-((5-(3,5-bis(trifluoromethyl)phenyl)-4H-1,2,4-triazol-3-yl)thio)-6-chloro-2-(methylthio) pyrimidine (37b)
Data for 4-((5-(3, 5-bis(trifluoromethyl)phenyl)-4H-1,2,4-triazol-3-yl)thio)-2-chloro-6-(piperidin-1-yl)pyrimidine (37c).
Data for 4-((3-(3,5-bis(trifluoromethyl)phenyl)-1H-1,2,4-triazol-5-yl)thio)-6-chloro-N cyclopen-
tylylpyrimidin-2-amine (37d).
Data for 4-((3-(3, 5-bis(trifluoromethyl)phenyl)-1H-1,2,4-triazol-5-ylthio)-6-chloro-N-(prop-2-yn-1-yl)pyrimidin-2-amine (37e).
Data for 4-((5-(3,5-bis(trifluoromethyl)phenyl)-4H-1,2,4-triazol-3-yl)thio)-6-chloro-N-cyclobutylpyrimidin-2-amine (37f).
Data for 4-(4-((5-(3,5-bis(trifluoromethyl)phenyl)-4H-1,2,4-triazol-3-yl)thio)-6-chloropyrimidin-2-yl)morpholine (37g).
Data for 4-((3-(3,5-bis(trifluoromethyl)phenyl)-1H-1,2,4-triazol-5-yl)thio)-6-chloro-2-(pyrrolidin-1-yl)pyrimidine (37h).
Data for 4-((5-(3,5-bis(trifluoromethyl)phenyl)-4H-1,2,4-triazol-3-yl)thio)-6-chloro-N-(2 (trifluoromethyl)benzyl)pyrimidin-2-amine (37j).
Data for 4-((3-(3,5-bis(trifluoromethyl)phenyl)-1H-1,2,4-triazol-5-yl)thio)-6-chloro-2-((4-methoxyphenyl)thio)pyrimidine (37k).
Data for 4-((3,5-bis(trifluoromethyl)phenyl)-1H-1,2,4-triazol-5-yl)thio)-6-chloro-N-(2-methoxybenzyl)pyrimidin-2-amine (37l).
Data for 4-((3-(3,5-bis(trifluoromethyl)phenyl)-1H-1,2,4-triazol-5-yl)thio)-6-chloro-N-cyclohexylpyrimidin-2-amine (37m).
Data for 4-((5-(3,5-bis(trifluoromethyl)phenyl)-4H-1,2,4-triazol-3-yl)thio)-6-chloro-2-(p-tolythio)pyrimidine (37n).
Data for 4-((3-(3,5-bis(trifluoromethyl)phenyl)-1H-1,2,4-triazol-5-yl)thio)-6-chloro-2-(pyrimidin-2-ylthio)pyrimidine (37o).
Data for 4-((5-(3,5-bis(trifluoromethyl)phenyl)-4H-1,2,4-triazol-3-yl)thio)-6-chloro-2-((1,4,5,6-tetrahydropyrimidin-2-yl)thio)pyrimidine (37p).

Data for 4-azido-6-((5-(3,5-bis(trifluoromethyl)phenyl)-4H-1,2,4-triazol-3-yl)thio)-2-(methylthio)pyrimidine (38b).
Data for 6-((5-(3,5-bis(trifluoromethyl)phenyl)-1,2,4-triazol-3-yl)thio)-N,N-dimethyl-2-(methylthio)pyrimidin-4-amine (38c).
Data for 6-((3-(3, 5-bis (trifluoromethyl) phenyl)-1H-1, 2, 4-triazol-5-yl) thio)-N-methyl-2- (methylthio) pyrimidin-4-amine (38d).
Data for 6-((3-(3, 5-bis (trifluoromethyl) phenyl)-1H-1, 2, 4-triazol-5-yl) thio)-N-isopropyl-2-(methylthio) pyrimidin-4-amine (38e).
Data for 6-((5-(3,5-bis(trifluoromethyl)phenyl)-4H-1,2,4-triazol-3-yl)thio)-N-cyclopropyl-2-(methylthio)pyrimidin-4-amine (38f).
Data for 4-((3,5-bis(trifluoromethyl)phenyl)-1H-1,2,4-triazol-5-yl)thio)-2-(methylthio)-6-(piperidin-1-yl)pyrimidine (38g).
Data for 4-((6-((3,5-bis(trifluoromethyl)phenyl)-1H-1,2,4-triazol-5-ylthio)-2-((methylthio)pyrimidin-4-yl)morpholine (38h).

Chemical Formula: C_{19}H_{19}F_{3}N_{8}O_{3}S_{2}

Exact Mass: 522.0731

[M-H] : 521.0653
Data for 4-((3-(3, 5-bis(trifluoromethyl)phenyl)-1-methyl-1H-1,2,4-triazol-5-yl)thio)-6-chloro-2-(methylthio)pyrimidine (40a).
Data for 4-((5-(3,5-bis(trifluoromethyl)phenyl)-4-ethyl-4H-1,2,4-triazol-3-yl)thio)-6-chloro-2-(methylthio)pyrimidine (40b).
Data for 4-((5-(3,5-bis(trifluoromethyl)phenyl)-4-isopropyl-4H-1,2,4-triazol-3-yl)thio)-6-chloro-2-(methylthio)pyrimidine (40c).
Data for 4-((5-(3,5-bis(trifluoromethyl)phenyl)-4-(cyclopropylmethyl)-4H-1,2,4-triazol-3-yl)thio)-6-chloro-2-(methylthio)pyrimidine (40d).
Data for 4-((3-(3,5-bis(trifluoromethyl)phenyl)1-(3,5-dimethoxybenzyl)-1H-1,2,4-triazol-5-yl)thio)-6-chloro-2-(methylthio)pyrimidine (40e).
Data for 4-((1-(4-azidobenzyl))-3-(3,5-bis(trifluoromethyl)phenyl)-1H-1,2,4-triazol-5-yl)thio)-6-chloro-2-(methylthio)pyrimidine (40f).

diluted in 100%MeOH+0.1%HCOOH

ARPAN_A5-41-N3_BWANG-ACCU_08-31-2012_ESI-POS02 30 (0.316) AM (Cen,2, 80.00, Ar,50000.0, 14:10:35 31-Aug-2012
Data for 4-(3-(3,5-bis(trifluoromethyl)phenyl)-5-(methylthio)-1H-1,2,4-triazol-1-yl)-6-chloro-2-(methylthio)pyrimidine (42).