Synthesis, Screening and Cocrystallization of Adenosine Based Inhibitors with Methyltransferases, ErmC' and KsgA

Matthew Baker
Virginia Commonwealth University

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Synthesis, Screening and Cocrystallization of Adenosine Based Inhibitors with Methyltransferases, ErmC' and KsgA

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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Richmond, Virginia
November 17th, 2011.
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<tr>
<td>AcN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>bd</td>
<td>broad doublet NMR peak</td>
</tr>
<tr>
<td>bs</td>
<td>broad singlet NMR peak</td>
</tr>
<tr>
<td>CDCl$_3$</td>
<td>chloroform</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>d</td>
<td>doublet NMR peak</td>
</tr>
<tr>
<td>dd</td>
<td>doublet of doublets NMR peaks</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Erm</td>
<td>erythromycin resistance methylase</td>
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<tr>
<td>Et$_3$N</td>
<td>triethylamine</td>
</tr>
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<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HRMS</td>
<td>high-resolution mass spectra</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>HTS</td>
<td>high throughput screening</td>
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<tr>
<td>IPA</td>
<td>isopropyl alcohol</td>
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<tr>
<td>K₂CO₃</td>
<td>potassium carbonate</td>
</tr>
<tr>
<td>K</td>
<td>degrees kelvin</td>
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<tr>
<td>Ksg</td>
<td>kasugamycin</td>
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<tr>
<td>Li₂SO₄</td>
<td>lithium sulfate</td>
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<tr>
<td>m</td>
<td>multiplet NMR peak</td>
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<tr>
<td>MeOH</td>
<td>methanol</td>
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<tr>
<td>MgOAc</td>
<td>magnesium acetate</td>
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<tr>
<td>MLS₈</td>
<td>macrolides, lincosamides, and streptogramin B</td>
</tr>
<tr>
<td>Ms</td>
<td>mesyl</td>
</tr>
<tr>
<td>Ms-Cl</td>
<td>methanesulfonyl chloride</td>
</tr>
<tr>
<td>MTase(s)</td>
<td>methyltransferase(s)</td>
</tr>
<tr>
<td>N₂</td>
<td>nitrogen gas</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>sodium bicarbonate</td>
</tr>
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<td>NH₄Cl</td>
<td>ammonium chloride</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NPE</td>
<td>non-proximity effect</td>
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<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>Rᶠ</td>
<td>retention factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
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<td>------------------------------------------------</td>
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<tr>
<td>RMT</td>
<td>RNA methyltransferase</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
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<tr>
<td>rxn</td>
<td>reaction</td>
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<tr>
<td>SAH</td>
<td>S-adenosyl homocysteine</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosyl methionine</td>
</tr>
<tr>
<td>SPA</td>
<td>scintillation proximity assay</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>thin-layer chromatography</td>
</tr>
<tr>
<td>TMS</td>
<td>tetramethylsilane</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>Ts</td>
<td>tosyl</td>
</tr>
<tr>
<td>TsOH</td>
<td>p-toluenesulfonic acid monohydrate</td>
</tr>
<tr>
<td>YSi</td>
<td>yttrium silicate</td>
</tr>
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Abstract

SYNTHESIS, SCREENING AND COCRYSTALLIZATION OF ADENOSINE BASED INHIBITORS WITH METHYLTRANSFERASES, ERMC' AND KSGA

By Matthew Ryan Baker, Ph.D.

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2011

Major Director: Dr. Jason P. Rife
Associate Professor, Department of Physiology & Biophysics

Antibiotic resistance threatens to throw mankind back into an era when infectious disease was the predominant cause of death. In an effort to mitigate this danger, we targeted Ermc’ and KsgA. Both methylate N6-adenosine of ribosomal RNA, though each serve different roles in their bacterial host. Ermc’ dimethylates A2058 on 23S rRNA, conferring resistance to MLS\textsubscript{B} antibiotics (macrolides, lincosamides, streptogramin B). KsgA aids in ribosome assembly, binding inactive 30S until dimethylating A1518/A1519 of 16S rRNA. Like most methyltransferases, Ermc’ and KsgA use cofactor S-adenosylmethionine (SAM) as their methyl source, which binds adjacent to their specific adenosine substrate. Ermc’ inhibitors could restore MLS\textsubscript{B} antibiotics against infections with this resistance mechanism. KsgA inhibitors could form novel antibiotics that stall 30S assembly.
Previous studies reported a potent ErmC’ inhibitor, \( N^6\)-cyclopentyl adenosine (1), binding to the substrate pocket with cyclopentyl bridging into the SAM pocket. We expanded this study by synthesizing 1 and 22 other \( N^6\)-substituted analogs to explore more favorable interactions within the SAM pocket. When these compounds (1mM) were screened against ErmC’ and KsgA, some showed greater inhibition than 1. Two of these inhibitors that were crystallized with ErmC’, \( N^6\)-8-octylamine adenosine (2.60Å) and \( N^6\)-phenethyl adenosine (2.40Å), unexpectedly docked into the SAM pocket with their 5’-C pointing towards the substrate pocket.

New compounds were made to exploit this orientation by adding substituents off the 5’-C to probe the substrate pocket. Through a five step synthesis, the 5’-OH of 1 was substituted with an amine linked to benzyl (30), phenethyl (31), propylphenyl (32) or butylphenyl (33). When 30-33 were screened using 20\( \mu \)M SAM, ErmC’ showed greater inhibition (relative to 1), while KsgA showed virtually none. However, when ErmC’ was tested using 0.5\( \mu \)M SAM, inhibition from 30-33 was nearly unchanged, whereas 1 became significantly more potent than 30-33, suggesting 30-33 were not binding to the SAM pocket. Preliminary data showed that raising 23S concentrations lowered inhibition from 32-33, while inhibition from 1, 30 and 31 was nearly unchanged, suggesting that at least 32-33 bound within the substrate pocket.
CHAPTER 1: Introduction

Antibiotic resistance

Since the discovery of penicillin in 1928 by Alexander Fleming et al., mankind has greatly benefited from the use of antibiotics. Prior to this period, infectious diseases like tuberculosis, typhoid, pneumonia and cholera were the predominant causes of death (contributed to 33% of the deaths in 1900). Yet, thanks largely to the introduction of antibiotics, as well as vaccination programs and improved sanitation during the mid-20th century, many of these diseases were near eradication by century’s end (<5% of deaths were from infectious diseases in 1996). Antibiotics have also enabled dramatic rises in our food production through their use in animal husbandry and sterilization techniques. Also, the many advances in surgery that we enjoy today would otherwise be unthinkable if not for antibiotics. Unfortunately, these popular drugs have been overused and misused, spawning new forms of resistant pathogens that have mushroomed throughout the globe. Antibiotic resistance has become so alarming in recent years that unless solutions are soon found, we risk being thrown back into this pre-antibiotic era.

While the prolific use and improper dosing of antibiotics have been contributing factors, the other reason resistance has spread so quickly stems from the fact that many of these drugs were derived from the natural products of soil-dwelling bacteria or fungi. Most notable is the genus of bacteria Streptomyces, from which most of our antibiotics originate (ex.; streptomycin,
tetracycline, and vancomycin) and the phylum of fungi Ascomycota, which has been the source of β-lactams like penicillin and cephalosporin. These organisms long ago evolved the means of releasing antimicrobials into their surroundings to gain an advantage over other microbes competing for the same food source. Microorganisms that were engaged in this biochemical warfare had also long ago evolved the means for self-defense. Genes responsible for these protective mechanisms are often found in plasmids, and are easily exchanged between a variety of different microbes. As a result, many of the bacterial pathogens that were once treatable by antibiotics soon acquired these resistance genes, which had been present in other microorganisms long before clinical antibiotics were ever used.

There are many ways that a microorganism can develop tolerance to an antibiotic. Those mechanisms of resistance that are acquired by pathogens (i.e., via gene transfer) have been categorized into three main groups. One mechanism is by modifying the antibiotic. For example, β-lactamases (ex: cephalosporinase and penicillinase) will hydrolyze the amide ring of β-lactams, rendering them ineffective against the bacterium. A second mechanism is by modifying the binding site of the antibiotic. For example, some rRNA methyltransferases like ErmC and RmtA methylate key bases on the ribosome to block the binding of macrolides and aminoglycosides, respectively. A third commonly acquired mechanism is to reduce the concentration of antibiotics through efflux pumps (ex: tetracycline efflux & TolC multi-drug efflux). It is also possible for an organism to use these mechanisms or other means (ex: reduced permeability & alternate metabolic pathway) as an intrinsic source of antibiotic resistance (i.e., encoded on chromosomal DNA). The term, “superbug”, refers to a microbe that has gained multiple copies of these genes, allowing for resistance to multiple antibiotics.
Resolving this threat of antibiotic resistance is by no means a simple task, since there are many kinds of antibiotics for which there are many types of resistance. One approach historically used to address this problem has been to develop novel antibiotics. Though the discovery rate of new antibiotics rose steadily from the 1940’s to a peak in the 1970’s, this has since dwindled to few new antibiotics reaching the market by 2000. An alternative approach would be to restore the effectiveness of existing antibiotics by discovering ways to block the mechanisms of resistance that are currently the most threatening. Some success with this approach was achieved through β-lactamase inhibitors (ex. clavulanic acid, tazobactum and sulbactam), which when used as an adjunct therapy with penicillins (ex: amoxicillin), restored the antibiotic’s potency against pathogens that acquired this mechanism of resistance. However, bacterial strains producing β-lactamases that are resistant to such inhibitors have subsequently emerged. In this dissertation work, both of these approaches for combating antibiotic resistance were attempted by targeting two different, but structurally similar, rRNA methyltransferases: ErmC’ and KsgA. Inhibitors to ErmC’ were investigated with the ultimate goal of restoring the effectiveness of the large class of MLSB antibiotics (macrolides, lincosamides, and streptogramin B) when treating infections with this particular form of resistance. Additionally, inhibitors of KsgA were investigated as a potential source for a novel class of antibiotics that act by stalling ribosomal assembly.
Methyltransferases

The large and diverse category of methyltransferases (MTases) occur in an equally large number of biological systems. By facilitating the addition of methyl groups to specific substrates, these enzymes effectively change the biochemical properties of a wide variety of different compounds. Examples of substrates that are modified by MTases include: amino acids, DNA, and RNA. Of particular interest to medicine are MTases that epigenetically silence genes through the methylation of DNA. Another well known example are bacterial MTases, which methylate DNA for self-protection against the restriction enzymes that cleave foreign or viral DNA. MTases are usually very substrate-specific, but nearly all must also bind the cofactor S-adenosyl methionine (SAM) for their methyl source. Examples of alternative methyl donors are betaine and N\textsuperscript{5}-methyl-tetrahydrofolate, which are used by betaine-homocysteine methyltransferase and methionine synthase (respectively) to convert homocysteine to methionine, and N-Methyl-L-glutamate synthase, which transfers the methyl from methylamine to glutamate.

ErmC’ and KsgA belong to the predominant class of MTases, which use SAM as their methyl source. Not surprisingly, SAM-dependent MTases share many of the same structural characteristics. Figure 1 shows a schematic of the core fold that is typical of SAM-dependent MTases. This characteristic pattern of alternating β-strands and α-helices closely resembles the Rossman fold seen in other nucleotide-binding proteins. Also common to all SAM-dependent MTase are two closely adjoining pockets, one of which accepts the cofactor SAM and the other of which binds the substrate to be methylated. This arrangement puts SAM in a position that allows for the methyl group to be directly transferred to a nucleophile on the substrate. Residues
that create the SAM-binding site are highly conserved among these MTases, while residues involved with substrate binding are more specific to the particular MTase substrate. The conservation of the cofactor-binding region has been a challenge for medicinal chemists targeting a specific SAM-dependent MTase. Inhibitors that act solely on the SAM-binding site are liable to cause toxicity by knocking out any of the other SAM-dependent MTases vital in human physiology. Therefore, the substrate-binding site has generally been the target for developing selective MTase inhibitors.\textsuperscript{15}

Figure 1. Core fold typical of SAM-dependent MTases. Alternating $\beta$-strands (1-7) and $\alpha$-helices (A, B, D, E & Z) are shown as red arrows and yellow cylinders, respectively. The gray cylinder (C) represents a less conserved region of SAM-dependent MTases. Chemical structures are of cofactor $S$-adenosyl methionine (SAM) and the by-product of methylation, $S$-adenosyl homocysteine (SAH). Information was adapted from the work of Martin \textit{et al.}\textsuperscript{15}
ErmC’

*MLS*<sub>B</sub> antibiotics

*MLS*<sub>B</sub> antibiotics shown in Table 1 represent a large and important class of drugs used to treat both gram-positive and gram-negative infections, and have come under threat from ErmC’-induced resistance. The first macrolide, erythromycin (a natural product of *Saccharopolyspora erythraea*), was introduced in 1952 and offered one of a few alternatives at that time to penicillin. However, resistance to erythromycin was soon found in clinical isolates from the US, Europe and Japan within one year of its being introduced. Later, semi-synthetic derivatives of erythromycin, such as roxithromycin (introduced in 1984), clarithromycin and azithromycin (both licensed in 1991), and dirithromycin (licensed in 1995) had improved absorption and acid-stability with fewer side-effects, but still shared cross-resistance with their parent compound.\(^{16,17}\)

In response to the prevailing resistance to traditional macrolides, efforts were focused on a new sub-class of semi-synthetic derivatives of erythromycin known as Ketolides. These include telithromycin (approved 2001 in EU, 2004 in US), cethromycin (currently in FDA review) and solithromycin (now in phase II), which have been shown to be active against many of the macrolide-resistant strains.\(^{18}\) However, isolates of telithromycin-resistant bacteria have since been found in Taiwan, despite the fact that this drug had not yet been made available in Taiwan.\(^{19}\)
Table 1. Examples of MLS$_B$-antibiotics. Structures were rendered in ChemDraw.

<table>
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<tr>
<td>Erythromycin A: $R^1=H$, $R^2=O$</td>
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<td>Clarithromycin: $R^1=CH_3$, $R^2=O$</td>
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<tr>
<td>Roxithromycin: $R^1=H$, $R^2=\text{N}<em>2O</em>{\text{O}}\text{O}_{\text{O}}\text{OCH}_3$</td>
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<td>Azithromycin</td>
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<td>Dirithromycin (prodrug, enteric coated)</td>
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<td>Erythromyclamine (active product of acid hydrolysis)</td>
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<table>
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<th>Ketolides (subclass of macrolides)</th>
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<tbody>
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<th>Lincosamides</th>
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<td>Clindamycin, $R=\text{Cl}$</td>
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Lincomycin (a natural product from *Streptomyces lincolnensis*) was introduced in 1963 as the first of two antibiotics in the class of lincosamides, yet was surpassed a few years later by its more effective, semi-synthetic derivative, clindamycin. This small class of compounds broadened the spectrum of MLS$_B$ antibiotics by also being able to treat fungus and protozoans (ex. malaria). Unfortunately, lincosamides have shared a similar fate of tolerance in several pathogens that were already resistant to existing macrolides, as well as eliciting resistance in newly indicated pathogens (in some cases of malaria).\textsuperscript{20}
The last group of MLS$_B$ antibiotics is streptogramin B. Streptogramins are a class of natural or semi-synthetic derivatives from certain *Streptomyces*. These bacteria simultaneously release two different types of streptogramins (A and B types), which are structurally distinct and act by separate mechanisms, yet have a powerful synergistic effect when dosed together. Streptogramin B utilizes the same mechanism of action as macrolides and lincosamides, and has similarly become susceptible to many of the same forms of resistance.²¹ [Note: Streptogramin A binds near the B type and also interferes with tRNA binding (has a bacteriostatic effect by itself), but more importantly the binding of the A-type induces a conformational change that greatly improves the binding affinity of streptogramin B.]²²

Although structurally diverse, MLS$_B$-antibiotics act through a common mechanism of inhibiting protein synthesis by binding to the large ribosomal subunit of bacteria (i.e., 50S). More specifically, these compounds bind at overlapping sites near the peptidyl transferase center on the 23S rRNA portion of 50S, thereby displacing the peptidyl-tRNA during elongation.¹⁶, ¹⁹, ²³, ²⁴

The most widespread and medically concerning route of resistance to MLS$_B$-antibiotics has been the modification of this binding site via Erm (Erythromycin resistance methylase),²⁵ although other mechanisms may also be used by some MLS$_B$-antibiotic resistant pathogens (ex. efflux pumps and drug inactivation).²⁶

*Erm family of methyltransferases*

The Erm family includes over 60 different methyltransferases that have been found in a variety of bacterial species.¹⁶ All of these enzymes prevent MLS$_B$-antibiotics from binding to the ribosome by adding one or two methyl groups to the N$_6$-position of a specific adenosine (A2058
in *Escherichia coli* or A2085 in *Bacillus subtilis*), which is located in domain V of 23S rRNA (Figure 2).\textsuperscript{25} Research into the Erm family has largely focused on a couple of members in particular. ErmE, from the erythromycin-producing bacteria *Saccharopolyspora erythraea*, had been used in determining the recognition elements of 23S.\textsuperscript{27, 28} Another member that is of great medical interest is ErmC, from *Staphylococcus aureus*, whose gene has been found in many of the clinical isolates of MLS\textsubscript{B}-antibiotic resistant infections.\textsuperscript{29} A more stable and soluble substitute for ErmC that is typically used for experimental work is ErmC’ (from *B. subtilis*), which varies from ErmC by only 4 amino acids.\textsuperscript{25} ErmC’ has also been used to produced the only published X-ray crystal structures for this family of MTases, although one structure of ErmAM has been solved by solution NMR.\textsuperscript{30}
Figure 2. Secondary structure of 23S rRNA substrate from *E. coli*. Magnified in the green box is the Erm methylation site on domain V (the red arrow indicates the target adenosine to be methylated, and highlighted nucleotides show key elements for recognition by Erm).

**Binding interactions of ErmC’**

For the reasons mentioned above, ErmC’ was chosen as the main subject for the inhibition and crystallographic studies presented herein. This member of the Erm family is a monomer of 244 amino acids (28.9 kDa), and acts by dimethylating its rRNA substrate using two equivalents of SAM in a random bi-bi mechanism (Figure 3). The preferred and natural substrate of ErmC’ is 23S rRNA from *B. subtilis*, but ErmC’ will also methylate synthetic RNA...
oligos as small as 32 nucleotides. ErmC’ has been found to also methylate the 23S rRNA from *E. coli*, although with about half of the same efficiency *in vitro.* [Note: The primary structures of 23S from *B. subtilis* and *E. coli* are 77% homologous.]  

![Diagram of ErmC’ dimethylating the target adenosine on 23S rRNA.](image)

Figure 3. Reaction scheme of ErmC’ dimethylating the target adenosine on 23S rRNA.

Since one objective of our research was to find ways to block this reaction facilitated by ErmC’, previous crystal structures were studied to see how this enzyme might interact with the SAM cofactor and RNA substrate. As of yet, five crystal structures of ErmC’ have been deposited into the RCSB Protein Data Bank (PDB). Two of these exist in their apo form (2ERC at 3.03 Å, and 1QAM at 2.20 Å), while the other three structures show ErmC’ with ligands in the SAM-binding pocket (SAM in 1QAO at 3.03 Å, SAH in 1QAN at 2.40 Å, and sinefungin in 1QAQ at 2.80 Å). S-Adenosyl homocysteine (SAH) is a product of SAM-dependent methylation, and sinefungin is a natural product from *Streptomyces incarnatus* or *S. griseolus*. Both of these structurally related compounds are often used experimentally as non-specific
methyltransferase inhibitors. Figure 4 shows these three co-structures of ErmC’ superimposed on one another to illustrate the similar binding modes of SAM and the two inhibitors. In each case, the ligand binds into the SAM pocket with the adenosine moiety oriented in a similar manner. In fact, this orientation closely aligns with the adenine moiety of SAM in complex with other MTases, and is likely conserved in all SAM-dependent methyltransferases.\textsuperscript{36, 38, 39}

Figure 4. Superimposed co-structures of ErmC’ bound with SAM (green), SAH (blue) and sinefungin (red).\textsuperscript{36} Chemical structures of each ligand are shown on the right side.
A closer look at the SAM-binding pocket in co-structure 1QAO reveals how ErmC’ interacts with its cofactor (Figure 5). The purine ring of SAM is held in place largely through hydrophobic interactions with surrounding residues (Ile-60, Ile-85, Leu-86 and Ile-106). In addition, hydrogen bonds are donated from the N^6 of SAM to the carboxyl on Asp-84, and the N^1 on SAM accepts a hydrogen bond from the backbone amide of Ile-85. The ribose portion of SAM makes a critical contact with ErmC’ by donating a pair of hydrogen bonds from its 2’-OH and 3’-OH to the carboxylate of Glu-59 [Note: This interaction will become very relevant when evaluating activity of second-generation inhibitors 26-29 in Chapter 5]. The amino group on the methionine portion of SAM makes one H-bond with the backbone carbonyl of Gly-38. Two more H-bonds are accepted at the carboxyl end of SAM from the amines of Asn-101 and backbone of Ile-13. The positive-charged sulfur on SAM forms ionic bonds with two carbonyl groups on the backbones of Asn-11 and Asn-101.36 While this co-structure has been very useful in explaining how ErmC’ binds to SAM, it still leaves unanswered questions about the interaction with its RNA substrate.
Despite the attempts of several groups, there are currently no structures of ErmC’ complexed with 23S or any other substrates bound to the adenosine pocket. There is, however, a structure of a related methyltransferase, M.TaqI, which is complexed with its nucleic acid substrate (PDB 1G38). M.TaqI (from *Thermus aquaticus*) mono-methylates DNA at the N6-position of adenine as part of the type II restriction-modification system that recognizes a 5’-TCGA-3’ sequence. While having a slightly different function, the catalytic domain of M.TaqI is very similar to those in ErmC’ and KsgA, and thus, has been a useful comparison tool for our research. Below is a sequence alignment for M.TaqI, ErmC’ and KsgA (Figure 6 ).
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Figure 6. Structure-based sequence alignment of ErmC' (PDB 1QAO),\textsuperscript{36} KsgA (PDB 1QYR)\textsuperscript{43} and M.TaqI (PDB 1G38).\textsuperscript{41} This task was performed on a T-Coffee web server, version 8.99.\textsuperscript{44}

For the X-ray model of PDB 1G38, M.TaqI was co-crystallized with a 10-base pair duplex of DNA and a non-reactive SAM analogue, 5'-[2-(amino)ethylthio]-5'-deoxyadenosine (AETA). This structure shows AETA positioned in the SAM pocket in a similar fashion as the endogenous cofactor (compared with co-structure of M.TaqI + SAM, PDB 2ADM).\textsuperscript{45} More importantly, this co-structure also shows the target adenine flipped out of the DNA-helix and binding into the substrate pocket of M.TaqI. The target-adenine is held in close proximity to the would-be methyl donor through face-to-face $\pi$-stacking with Tyr-108, and edge-to-face $\pi$-
stacking with Phe-196. [Note: These residues are analogous to Tyr-104 and Phe-163 in ErmC’, respectively.] Additionally, H-bonds from the backbone amide of Tyr-108 and the side-chain amide of Asn-105 (analogous to Asn-101 in ErmC’) are donated to N\textsuperscript{7} and N\textsuperscript{1} on the target adenine, respectively. Furthermore, the N\textsuperscript{6} of adenine donates H-bonds to the carbonyl oxygens on Asn-105 and backbone of Pro-106 (Ile-102 in ErmC’). These interactions made by M.TaqI with its adenine substrate were extrapolated to make the predicted model of ErmC’ binding to its adenosine substrate (Figure 7).

Figure 7. Predicted binding mode of the target-adenine into the substrate pocket of ErmC’. Phe-163 and Tyr-104 of ErmC’ are predicted to form \(\pi-\pi\) stacking with the putative adenine substrate. Dashed yellow lines show H-bonds of this nucleotide with key residues of ErmC’. In gray are the adenine substrate and “flipped” Tyr-108 from M.TaqI (PDB 1G38)\textsuperscript{41} that were modeled into ErmC’ with cofactor (PDB 1QAO)\textsuperscript{36}. Catalytic regions aligned in PyMOL with a root mean square (RMS) = 0.709 (70 to 70 atoms), aligning residues 11-13, 59-61, 63, 84, 98, 101-105 and 163 of 1QAO with analogous residues of 21-23, 71-73, 75, 89, 91, 102, 105-109 and 196 of 1G38.
Catalytic residues of ErmC’ and M.TaqI closely overlap in PyMol, and the adenine substrate of M.TaqI fits into the putative adenosine-binding pocket of ErmC’ to form nearly identical interactions. One important distinction, however, is the rotation of the highly conserved tyrosine (Tyr-104 in ErmC’, Tyr-108 in M.TaqI). In all published structures of ErmC’, the Tyr-104 points away from this pocket as shown in Figure 7. It has been speculated that the binding of RNA substrate to ErmC’ involves this tyrosine flipping inwards to make favorable π-π stacking with the target adenosine,\textsuperscript{41, 46} which may also aid in catalysis\textsuperscript{42} (discussed below in Predicted Mechanism for ErmC’ and KsgA Catalysis).

Another difference between these two MTases is an isoleucine in ErmC’ (Ile-102) rather than a proline at this same location in M.TaqI (Pro-106). The reason for this discrepancy in an otherwise highly conserved region likely reflects the strict mono-methylating activity of M.TaqI.\textsuperscript{36} The C\textsubscript{γ} and C\textsubscript{β} of Pro-106 would limit the space needed for an N\textsuperscript{6}-monomethyl adenine to bind in the substrate pocket of M.TaqI, whereas Ile-102 in ErmC’ is far less confining and may provide favorable hydrophobic interactions with the N\textsuperscript{6} methyl when a monomethylated substrate binds.\textsuperscript{36}

Many of the interactions of ErmC’ with SAM or the putative adenosine substrate will be considered when designing inhibitors. This knowledge will also be valuable when evaluating the activity of inhibitors \textit{in vitro}, as well as providing information used to interpret new co-structures presented in this dissertation.
Also in the crosshairs of this inhibition study was a paralog of ErmC’, the rRNA methyltransferase KsgA (from *E. coli*). This enzyme belongs to a family that is uniquely conserved throughout the three domains of life. In bacteria, members of this family are named KsgA, whereas, in archaeal and eukaryotic organisms, these orthologs are called Dim1. ErmC’ most likely originated from the same ancestor in this family and, not surprisingly, shares a similar mechanism of action. With only a couple of exceptions, the KsgA/Dim1 family dimethylates the N\(^6\) atom of two adjacent adenosine bases (A1518 and A1519 in *E. coli*) on helix 45 of the small rRNA (16S rRNA in bacteria, Figure 8). The actions of KsgA/Dim1 are centrally involved with the ribosome assembly process.
Figure 8. Secondary structure of 16S rRNA from *Escherichia coli*\(^\text{31}\) KsgA methylation sites on helix 45 are magnified in the green box (the red arrows indicate the target adenosine bases to be methylated, and the highlighted nucleotides are predicted to make interactions with KsgA\(^\text{50}\) (Personal communication, Drs. Jason Rife and Nenad Ban).
KsgA from E. coli

KsgA was first noticed when a strain of E. coli became resistant to the aminoglycoside kasugamycin (ksg) as the result of a mutation on the ksgA gene. Later it was found that KsgA modifies a pair of nucleotides at the end of a hairpin loop on helix 45, which is near or indirectly associated with the binding area of kasugamycin on helixes 44, 28 and 25 (Figure 8). This antibiotic is believed to block protein synthesis by displacing the initiator tRNA from the P-site of the ribosome. Paradoxically, it is the absence of KsgA (or rather, its methylation function) that causes resistance to kasugamycin. When bases A1518/1519 are unmethylated, the hairpin loop on helix 45 is thought to assume a slightly different conformation that is enough to disrupt the adjacent binding sites of this antibiotic. The lack of KsgA in this strain of kasugamycin resistant (ksgR) E. coli, however, comes with a consequence of reduced growth rate, translation errors, and sensitivity to stress. While such a mutation has proven to be advantageous under the pressures of kasugamycin, this form of resistance has not been observed in the wild. [Note: ksgR E. coli arose from mutational experiments with nitrosoguanidine.]

In vivo, nucleotides A1518/1519 are not methylated by KsgA until 16S rRNA has been modified and assembled into a nearly complete, yet translationally inactive, small ribosomal subunit. The assembly of 16S into translationally active 30S involves incorporating a total of 21 ribosomal proteins (S1-S21), which is aided by and in concert with dozens of ribosome-associated enzymes and biogenesis factors. These include: chaperones (ex. DnaK) and GTPases (ex. Era) that speed up other binding and folding events; RNases trimming off the leading and tailing ends of pre-16S rRNA; isomerization of a uridine to a pseudouridine (U516 to pU516 by RsuA); and MTases (ex. KsgA and RsmB to RsmF) that modify up to 10 rRNA bases. At
some point near the final stages of this maturation (maybe just prior to the addition of the essential S21), KsgA completes its methylation. Afterwards, 30S forms a conformation that can bind 50S and begin translation. Fortunately, for the sake of our experiments, certain salt conditions will trap 30S into a translationally inactive conformation that is a suitable substrate for KsgA methylation \textit{in vitro}. Such conditions typically consist of low divalent and intermediate monovalent ion concentrations (ex. 4 mM Mg$^{++}$ and 40 mM NH$_3^+$).

It has been proposed that KsgA’s role in ribosome biogenesis goes far beyond just methylating rRNA and that it also functions as a biogenesis factor or gatekeeper during the final stages of 30S assembly. While methylated A1518/1519 do offer a slight improvement of reading accuracy during ribosome translation \textit{(in vivo)}, unmethylated 30S will still join with 50S to form functional ribosomes, albeit at about half of the normal activity \textit{(in vitro)}. Therefore, if KsgA’s methylation is not essential (at least in \textit{E. coli}) despite its ubiquitous conservation, it may have a secondary and possibly more important role than just adding four methyl groups to 16S rRNA. Evidence for this claim can be found in recent experiments which showed that when the \textit{ksgA} gene was deleted from a strain of \textit{E. coli} (Δ\textit{ksgA}), cell growth was significantly hindered by cold temperatures (20-25 °C) compared with wild-type \textit{E. coli}. It was also observed that Δ\textit{ksgA} cells had a greater ratio of subunits (30S and 50S) to fully formed ribosomes (70S) compared with wild type. Cold-sensitivity and the buildup of ribosomal subunits are also phenotypes in bacteria that have defective or deleted biogenesis factors, such as GTPases Era and YieQ. Therefore, the binding of KsgA to 30S may similarly aid with subsequent or concurrent maturation steps that are separate from its catalytic function.
This same study also had very interesting results using a catalytically inactive mutant of KsgA (E66A), inserted into a vector for inducible expression. When Glu-66 (analogous to Glu-59 in ErmC’) is mutated to an alanine, SAM binding is blocked, but does not otherwise prevent KsgA from binding to the 30S substrate. Expression of E66A KsgA in either ΔksgA or wild-type E. coli showed significant inhibition of cell growth. Furthermore, cells expressing E66A had ratios of subunits:ribosomes that were shifted to nearly all 30S and 50S. It was concluded from this study that KsgA binding may also act as a checkpoint during ribosome assembly by recognizing the proper conformation of the nearly complete 30S. Methylation by KsgA then triggers the release of 30S just before being allowed to join with 50S. The last maturation steps that make 30S translationally active may happen while still bound to KsgA, or occur just following this release. Irrespective of this order, the nearly complete 30S particle would be sequestered by KsgA until methylation is completed.63

The greater implication of Connolly et al.’s work is that KsgA is a viable target for developing a novel class of antibiotics.63 Inhibitors that block the methylation by KsgA (but otherwise don’t block 30S from binding) could stall ribosomal assembly through the buildup of 30S-KsgA complexes. Such an inhibitor could act as a biostatic antibiotic when given alone, or it could be combined with other antibiotics that might have a synergistic effect on an otherwise weakened cell. While many current antibiotics directly target the ribosome, KsgA inhibitors could be the first in a class of antibiotic that indirectly target the ribosome during biogenesis.

Finally, it has been found that knocking out KsgA can also hyper-sensitize E. coli to the antibiotic effects of the aminoglycoside gentamicin.67 This study showed that 30S unmethylated by KsgA was a less suitable substrate for ArmA (a methyltransferase conferring resistance to the
aminoglycosides). Therefore, KsgA inhibitors could also restore the effectiveness of certain aminoglycosides in bacteria that had acquired the ArmA mechanism of resistance.

Likely Binding interactions of KsgA

Designing inhibitors for KsgA requires an understanding of the binding interactions made with its substrates, and much of this can be gleaned from previous crystallographic work. Our group has solved the only crystal structure of KsgA from *E. coli*, which exists in the apo form (PDB entry 1QYR at 2.10 Å).\(^{43}\) While another group has obtained a crystal structure of KsgA from *Aquifex aeolicus* complexed with an RNA duplex and SAH (PDB entry 3FTF),\(^{68}\) some of their methodology and results remain speculative and was not fully informative for our purposes. Fortunately, the similarities of KsgA with ErmC’ or M.TaqI permits the use of these co-structures to help fill in the blanks (i.e., SAM and target adenosine). These three MTases were superimposed together to illustrate how well the catalytic domains align (Figure 9).
To illustrate how the cofactor likely binds into the SAM pocket of KsgA (Figure 10), the co-structure of ErmC’ + SAM (1QAO)\textsuperscript{36} was aligned with the the apo structure of KsgA (1QYR).\textsuperscript{43} The SAM molecule modeled into KsgA forms nearly the same interactions that were observed with ErmC’ in Figure 5. One difference was KsgA had slightly less space around the N⁰-position of SAM (discussed further in Chapter 3).
Figure 10. Predicted binding mode for cofactor in the SAM-pocket of KsgA. Shown in gray is SAM from the co-structure of ErmC’ + SAM (PDB entry 1QAO\textsuperscript{36}), which was modeled into the apo form of KsgA (PDB entry 1QYR\textsuperscript{43}). Catalytic regions aligned in PyMOL with a root mean square (RMS) = 0.734 (95 to 95 atoms), aligning residues 18, 20, 45, 66, 91, 92, 111-120 and 181 of 1QYR, and the analogous residues 11, 13, 38, 59, 84, 85, 99-109 and 163 of 1QAO.

Similarly, the co-structure of M.TaqI + DNA + AETA (PDB 1G38\textsuperscript{41}) was aligned with the apo structure of KsgA to predict how the target-adenosine would likely interact with this substrate’s binding pocket. When the target-adenine from the DNA of M.TaqI (PDB 1G38\textsuperscript{41}), was modeled into the adenosine pocket of KsgA (Figure 11), interactions closely resemble those predicted for ErmC’ (Figure 7), though, this model of KsgA did not show the full residue of Tyr-116 (possibly due to its dynamic nature).
Predicted Mechanism for ErmC’ and KsgA Catalysis

Understanding the orientation of the target-adenosine relative to the cofactor and catalytic residues of ErmC’ or KsgA has allowed us to envision how a methyl group from SAM is transferred to the N⁶-position of adenosine. Previously, Goedecke et al. proposed a mechanism for M.TaqI catalysis based on his crystal structures,⁴¹ which has since been applied to ErmC’ and KsgA to give their predicted mechanism (Figure 12). In short, the target adenosine becomes more labile for methylation after forming H-bonds with the asparagine residue and the adjacent peptide bond, and is then stabilized by π-interactions from a flipped tyrosine.
Figure 12. Proposed mechanism for RNA methylation by ErmC' and KsgA.

The N<sup>6</sup> atom on adenosine normally exists in a more stable co-planar sp<sup>2</sup> hybridization state. This is a result of the lone pair of electrons on N<sup>6</sup> conjugating into the aromatic system of the connected purine ring. When the target adenosine binds to its respective pocket, hydrogens from the N<sup>6</sup> atom should be pulled towards the carboxamide oxygens of both asparagine (Asn-101 in ErmC’, Asn-113 in KsgA) and the backbone of the neighboring amino acid (Ile-102 in ErmC’, Leu-114 in KsgA), both of which lie slightly adjacent to the plane of the putative purine ring. This distortion should push the N<sup>6</sup> atom into a more reactive sp<sup>3</sup> hybridization as electrons are conjugated out of the purine ring and return to the lone orbital of N<sup>6</sup>. As a result, the lone pair of electrons of N<sup>6</sup> would point directly towards the methyl group on SAM, which lies on the opposite side of the plane from Asn-101/113 and Ile-102/Leu-114. The relative position of the methyl group would also be an ideal geometry for the nucleophilic N<sup>6</sup> atom to perform an inline attack on SAM. The resulting charged intermediate (quaternary N<sup>6</sup><sup>-</sup>-adenosine) could then be stabilized though cation-π interactions with the electron rich tyrosine (Tyr-104 in
ErmC’, Tyr-116 in KsgA). The methylated adenosine should return to the co-planar sp² hybridization after the extra proton on N⁶ is carried off by another nucleophile, such as a hydroxyl ion (Figure 12).

**Research Aim**

There is clearly an urgency to act upon the threat posed by antibiotic resistance. In the past, scientists have responded to this danger by discovering new classes of antibiotics. However, most of these drugs were derived from soil-dwelling bacteria that are inherently prone to resistance. As an alternative, we suggest that the effectiveness of the large class of MLSB antibiotics can be restored by targeting one of the most widespread and threatening mechanisms of resistance, ErmC. In addition, we will look for a potentially new class of antibiotics that stalls ribosome assembly by inhibiting the ubiquitously conserved KsgA.

Conveniently, the similarities between ErmC’ and KsgA justifies using a common library of compounds to test against both of these methyltransferases. After an efficient assay was developed for measuring RNA methylation (Chapter 2), compounds were synthesized for screening against ErmC’ and KsgA in vitro (Chapter 3). Potential inhibitors were used with the adenosine scaffold with the objective of binding to the target-adenosine pockets of ErmC’ and KsgA. Several alkyl, aromatic, or other functionalized substituents were added at the N⁶-position of adenosine in the first-generation of compounds to probe for favorable interactions with the neighboring SAM pocket. Selected inhibitors were then co-crystallized with ErmC’, and the co-structure of two of these crystals were solved and shown to have an unexpected binding mode.
The revelations of how two of these inhibitors bind to the SAM pocket of ErmC’ redirected our focus to a second-generation of compounds (Chapter 4). Here, we added phenyl groups with varying linker lengths to the 5’-position of our lead compound with the intent of bridging from the SAM pocket into the target-adenosine pocket. Initial screening showed that adding this second substituent provided a marked improvement in the inhibition of ErmC’ but not of KsgA. However, when SAM concentration was lowered in IC$_{50}$ studies with ErmC’, we saw an apparent loss of inhibition for some of the second-generation compounds relative to the first-generation lead compound (Chapter 5). These surprising results and other preliminary data led us to conclude that some of these second-generation compounds bind outside of the SAM pocket, possibly in the target-adenosine pocket.

While many questions remain unanswered and new questions created, this report should provide useful information about a new screening technique, structural data for two new ligands complexed with ErmC’ and synthesis procedures for several previously unreported compounds.
CHAPTER 2: Scintillation Proximity Assay for Measurement of RNA methylation

Background

As part of our study to investigate potential inhibitors for ErmC’ and KsgA, we needed an assay that would allow us to efficiently screen libraries of compounds for activity. At the time this method was developed, our research was also focused on another methyltransferase, RmtA. This protein is a member of the ArmA/RmtA family of rRNA methyltransferases, which confer resistance to aminoglycoside antibiotics. RmtA and related members monomethylate the N7-position of G1405 (E. coli numbering) on 16S rRNA in the 30S ribosomal particle. Like ErmC’ and KsgA, RmtA also uses the S-adenosyl-methionine (SAM) cofactor as the methyl donor. While the work done with RmtA was very instrumental in the development of this screening assay, RmtA was not included in subsequent inhibitions studies and is limited to this chapter.

In vitro characterization of RNA methyltransferases usually requires 3H-methyl-SAM (radiolabeled on the methyl group) to be reacted with the RNA substrate. The resulting radiolabeled RNA product can then be measured to determine the extent of methylation (i.e., how many 3H-methyl groups are added to substrate). This is commonly used for kinetically characterizing an enzyme and detecting inhibition for high throughput screening (HTS).

Typically, these assays also require radiolabeled RNA product to be separated from the
unreacted \(^3\)H-methyl-SAM before methylation activity can be properly measured. This separation is usually accomplished by filter binding\(^73\) or with a spin column.\(^74\) In either case, positively charged surfaces on the media allow RNA to adhere through their negatively charged phosphate groups and allow residual SAM to elute. While these methods are commonly used, they suffer from a variety of shortcomings, including empirical determination of wash conditions, variation in deposition efficiencies, large volumes of radioactive waste and the inability to scale up to a large number of parallel measurements as would be used in HTS. One way to circumvent these deficiencies has been to use fluorescence-based assays that measure the \(S\)-(\(5'\)-adenosyl)-L-homocysteine (SAH) product rather than the methylated RNA. This type of assay has the advantages of being adaptable for HTS and not requiring radiolabeled reagents, but it does not directly measure the methylated product and makes the assumption that RNA methylation will equal SAH production. One version of this assay uses SAH antibodies with conjugate tracers to measure activity,\(^75\) but high cross-reactivity of the anti-SAH antibody with SAM limits its use to low concentrations of SAM (i.e., 3 \(\mu\)M or less). Other indirect assays using coupled enzymes to convert SAH to detectable products\(^76\)\(^-\)\(^78\) are potentially ambiguous, since test compounds (e.g., inhibitor screens) could inhibit these enzymes but not the methyltransferase.

We have developed a scintillation proximity assay (SPA) for measuring RNA methylation using commercially available yttrium silicate (YSi) scintillant beads that capture DNA or RNA by non-specific electrostatic binding. In our application, radiolabeled methylated RNA product bound to the bead activates the scintillant whose emission can be measured and quantitated. The intrinsic physical separation of the radiolabeled methylated RNA product from radiolabeled
SAM reactant permits the accurate direct quantitation of methylated RNA product without the need for a separation step. This method should be generally applicable to assay RNA-methyltransferases and their products and adaptable for HTS.

**Optimization of SPA Assay Conditions**

*Bead concentration affects the signal to noise ratio*

To optimize the YSi bead concentration, we tested signal and background reactions with different amounts of YSi beads (Figure 13). We found that for our assay conditions, 1 mg of YSi beads gave the highest signal:noise ratio with a signal greater than 6000 cpm. These experiments also suggest that the apparent failure to reach saturation of the beads with bound, methylated 30S ribosomal particles is due to the non-proximity effect (NPE) as bead concentration increases (Figure 13). The NPE arises from excitation of beads by radiolabeled substrate that is within the path length of the isotope’s β-particle (1.5 µm for 3H; GE Health Sciences), but is either free in solution or bound to other nearby beads. It is recommended that bead concentrations be optimized for specific applications.
Figure 13. Optimization of assay conditions. (A) Dilution of samples of $^3$H-methyl-SAM with water: (●) beads mixed with 50 µL of sample (buffer R, 20 µM SAM 780 cpm/pmol) before adding water; (■) beads mixed with 50 µL of sample (buffer R, 20 µM SAM 780 cpm/pmol, 10 pmol 30S) before adding water; (◇) beads mixed with 50 µL of sample (buffer R, 20 µM SAM 780 cpm/pmol) followed by addition of 3 mL of water; (□) beads mixed with 50 µL of sample (buffer R, 20 µM SAM 780 cpm/pmol, 10 pmol 30S) after adding 3 mL of water. Data shown were from single samples.

Minimizing background from $^3$H-methyl-SAM

$^3$H-methyl-SAM alone in reaction buffer produced surprisingly high counts (Figure 14.) that would raise background levels in the assay. Addition of cold 30S (10 pmol) ribosomal particles to the incubation of $^3$H-methyl-SAM with YSi beads lowered the counts, particularly for samples with smaller amounts of YSi beads, indicating that SAM and 30S ribosomal particles compete for sites on the YSi beads.

A significant diminution of background counts from $^3$H-methyl-SAM binding to the beads was achieved by dilution of the sample with 3 mL of water (Figure 14). This indicated that the major contributor of $^3$H-methyl-SAM to the background counts is through the NPE and not through binding to the beads. Dilution of the reaction solution with water to a volume of ~250 µl
(the well volume of a typical 96 well plate) in addition to the volume of the YSi beads almost entirely eliminated this background. Attempts to lower background counts by dilution with solutes (adenosine, unlabeled SAM, spermine, HCl or MeOH) showed that only unlabeled SAM lowered background without lowering signal (data not shown). We conclude that background contributions from the non-specific binding of $^3$H-methyl-SAM to YSi beads is intrinsically optimized because the higher affinity 30S ribosomal particles in the sample and the cold SAM used in the quenching step reduce bound $^3$H-methyl-SAM to an inconsequential level.

**Figure 14.** Optimizing bead concentration. (●) beads mixed with quenching SAM and 50 µL of signal reaction (10 pmol RmtA, 10 pmol 30S, buffer R, 20 µM SAM 780 cpm/pmol, 37 ºC for 1 hour) and diluted to 250 µL with water; (▲) beads mixed with quenching SAM and 50 µL of background reactions (10 pmol 30S, buffer R, 20 µM SAM 780 cpm/pmol, 37 ºC for 1 hour) and diluted to 250 µL with water. Assays were done in triplicate, plotting the average with error bars of +/- 1 standard deviation.
Sample centrifugation increases the signal to noise ratio

We found that centrifuging samples just prior to scintillation counting improved the signal:noise significantly (> 5:1). Counts from signal reactions more than doubled, while those of background reactions increased only slightly (Figure 15). This phenomenon may be due to the NPE, arising in this case from more tightly packed radiolabeled 30S particle-bound beads exciting proximal YSi beads within the 1.5 μm path length of the tritium β-particles. Background counts did not increase significantly on centrifugation, consistent with the low level of bound radiolabeled substrate. More importantly, centrifugation of the beads after product binding also reduced variability among replicate samples, possibly by detaching beads bound to the walls of the vial and packing them more uniformly. A centrifugation step can be incorporated into HTS experiments by centrifuging 96-well plates in a special rotor as described in a similar SPA HTS assay.47

Figure 15. Centrifuging samples from time courses of RmτA + wild-type 30S. (□) signal reactions before centrifugation; (■) signal reactions after centrifugation; (Δ) background reactions before centrifugation; (▲) background reactions after centrifugation. Assays were done in triplicate, plotting the average with error bars of +/- 1 standard deviation.
Binding of methylated 30S ribosomal particles to YSi beads is instantaneous

Incubation time for YSi beads with product mix was optimized to ensure maximum capture of 30S ribosomal particles. A large batch (70 pmol) of 30S rRNA particles was methylated by RmtA for 1 hour and separated into individual 10 pmol portions to test the effect of different incubation times (0, 20, 40, 60, 80, 100 and 120 min). YSi beads (density = 4.1 g/cm$^3$) quickly settle in solution, so beads were thoroughly mixed with a pipette on addition of the reaction sample (time 0) and then mixed by inversion every 20 min for the specified time. The signal from time 0 was approximately the same as the other incubation times (data not shown), indicating that 30S binding occurs almost instantaneously and is complete with the first mixing. For convenience, all succeeding samples were incubated for 40 min (i.e., mixed by pipette initially and again at 20 and 40 min before centrifugation).

With these optimized conditions, we attained minimum count levels of $\sim$3000 cpm and signal:noise ratio of $\sim$ 5:1, which are the recommended benchmarks for this bead-based assay (GE Health Sciences). While these conditions are useful as a guide to implementing the assay, optimization should be done for applications to different assay systems.

SPA vs. Filter-Binding Method in Measurement of Time Courses and Extent of Methylation

Our standard filter-binding assay for measurement of methyltransferase activity is based on an earlier method$^{79}$ in which reaction solutions are pipetted onto cationic filter discs, washed twice with $\sim$200 mL of 5% TCA and rinsed with $\sim$3 mL ethanol. After drying for an hour, filter discs are placed in 3 mL of scintillation fluid for counting.
We compared this method of measuring RNA methylation with our SPA method in parallel time course experiments. Using our standard optimized reaction conditions, we ran time courses of reaction for RmtA and KsgA, including separate background measurements (Figure 16A). Identical time course experiments were also done using ErmC’ (Figure 16B). For each time point, double the normal amount was removed from the reaction, quenched and divided so that equal amounts were measured by either the SPA method or the traditional filter binding technique. Both sets of experiments with RmtA and KsgA showed the expected exponential curve with an early linear phase extending up to about 5 min and an extended asymptotic plateau (Figure 16A) while, the time course for ErmC’ showed a linear phase extending up to about 32 min (Figure 16B). The more gradual rate of methylation by ErmC’ could be attributed to using 23S from *E. coli* rather than the preferred substrate of 23S from *B. subtilis*. Although the SPA produced a higher background than the filter binding method, signals from the SPA were greater than for filter binding after backgrounds were subtracted. The consistent values of SPA background indicate that the high background does not compromise the results. The SPA background increased slightly over time in a linear fashion. This could be due to degradation of $^3$H-methyl SAM to a radiolabeled product with higher affinity for SPA beads; or substrate being methylated by contaminant MTases; or non-enzymatic reactions that transfer $^3$H-methyl to RNA or other products capable of binding to SPA beads.
Figure 16. Parallel time-course assays measured with SPA and filter binding methods. (a) Maroon are RmtA + wild-type 30S; orange are KsgA and ksgR 30S. (b) Black are ErmC’ + 23S (from *E. coli*). Solid symbols indicate signal reactions (10 pmol 30S or 23S, 10 pmol enzyme, 20 μM SAM 780 cpm/pmol); empty symbols indicate background reactions (10 pmol 30S or 23S, 20 μM SAM 780 cpm/pmol). Triangle symbols are measurements by YSi SPA beads; square (a) or diamond (b) symbols are the corresponding filter binding measurements. Assays were done in triplicate, plotting the average with error bars of +/- 1 Std Dev.
The data from these parallel time courses allowed us to calculate the amount of \(^{3}\text{H}\)-methyl transferred to RNA from the SPA measurements. In the filter binding method, the extent of methylation can be easily determined from knowledge of the amount of RNA substrate in the assay and the measurable specific activity of the \(^{3}\text{H}\)-methyl-SAM used. However, in the SPA format, the amount of \(^{3}\text{H}\)-methyl-SAM used cannot be directly measured with a specific-activity test, because the different binding affinities of \(^{3}\text{H}\)-methyl SAM and \(^{3}\text{H}\)-methylated RNA to the SPA beads will yield a different response for the same amount of \(^{3}\text{H}\)-methyl group.

This problem is circumvented by plotting the SPA and filter binding responses from the same samples and obtaining the linear equation that relates them (Figure 17). SPA measurements can then be converted into “filter binding equivalent” values, from which RNA methylation is quantified (Figure 18) using the specific activity of \(^{3}\text{H}\)-methyl-SAM. The number of methyl groups incorporated into RNA by KsgA or RmtA determined from the SPA assay is consistent with full methylation of the 10 pmol of 30S substrate by two of the enzymes: 10 pmol of methyl groups for RmtA and 40 pmol for KsgA. As for ErmC’, around half of the expected 20 pmol of methyl groups were calculated to be incorporated into 23S, probably because this RNA substrate had been extracted from \textit{E. coli} rather than the preferred source of \textit{B. subtilis}.

Performing a parallel filter binding assay may be needed only when quantifying methyl group incorporation. In this case, once a correlation of SPA and filter binding measurements is established for a particular enzyme reaction, subsequent SPA measurements could be quantified by use of the previously obtained SPA to filter binding conversion factor and the measured specific activity of the \(^{3}\text{H}\)-methyl-SAM.
Figure 17. Correlation of SPA and filter binding measurements. Net counts from time points in Figure 16 are plotted with SPA counts along the X-axis and filter binding counts along the Y-axis. (a): Maroon are RmtA + wild-type 30S; orange are KsgA and ksg\textsuperscript{R} + 30S. (b): Black are ErmC' + 23S (from \textit{E. coli}). Combined replicates were used to fit the least squares trend line.
Figure 18. Extent of methylation. Time courses measured by SPA in Figure 18 are expressed in number of methyl groups incorporated into RNA. Formulas derived from Figure 17 were used to convert SPA measurements into “filter binding equivalent” values for quantitation of methyl group incorporation. (a): Maroon are RmtA + wild-type 30S; orange are KsgA + ksgR 30S. (b): Blue are ErmC’ + 23S (from *E. coli*).
SPA Analysis of Extracted $^3$H-methyl RNA

The experiments described above measure methylation of 30S subunit particles that bind intact to YSi beads. We also tested the SPA assay format on methylated RNA extracted with phenol to remove proteins. Phenol-extracted, methylated 16S rRNA from RmtA or KsgA catalyzed reactions was tested for binding to YSi beads in the SPA assay. Signals for both reaction products increased linearly with amount of extracted $^3$H-methyl-RNA up to 10 pmol (Figure 19). The counts measured for 10 pmol of extracted RNA were higher than those for the same amount of 30S particles, suggesting that free RNA has a higher affinity for YSi beads than RNA bound to protein in the 30S subunit, or that the NPE is larger for bound RNA in the absence of protein. The background for the extracted RNA measurements was essentially nil, since all $^3$H-methyl SAM is washed away in the extraction process.

![Figure 19](image)

**Figure 19.** Free $^3$H-methyl-RNA measured with YSi SPA beads. RNA extracted from 30S subunits following a 2 hr reaction with RmtA + wild-type 30S (maroon) or KsgA + ksg$^R$ 30S (orange), and incubated with different amounts of YSi beads. RNA concentration was determined by measuring the OD$_{260}$ following extraction. 10 pmol of RNA extracted from reactions without enzyme were used as a negative control. Data are for single samples.
SPA Assay for Methylation of Free RNA Substrate

To verify that this SPA procedure can measure methylation of an RNA substrate alone, parallel time course experiments were performed as above using the ErmC’ enzyme and phenol-extracted 23S rRNA from 50S ribosomal particles. The kinetics of this reaction differ from those for KsgA and RmtA, but with 10 pmol of *E. coli* 23S rRNA substrate we measured ErmC’-catalyzed incorporation of ~10 pmol of methyl group (Figure 18), in close agreement with the results of Denoya and Dubnau 81 for this reaction. Furthermore, the correlation of SPA and filter binding values for the ErmC’-catalyzed reaction is closely similar to that obtained for the KsgA and RmtA assays.

Use of a similar YSi SPA bead to monitor methylation of tRNA has been reported 82 for considerably different assay conditions, suggesting that this method can be applied widely to both methylated RNA and RNA-protein complexes.

Dependence on Enzyme Concentration

Based on the time course, we took early samples (8 min for RmtA, 3.5 min for KsgA) of reactions carried out with varying amounts of RmtA or KsgA (Figure 20) to determine the dependence on enzyme concentration. Under the reaction conditions used, the activity measured from the SPA assay is linear for enzyme concentrations up to at least 200 nM (10 pmol enzyme in the reaction), validating the use of this assay for determining enzyme concentrations.
**Figure 20.** Dependence on enzyme concentration. 10 pmol 30S ribosomal particles were incubated with RmtA (maroon) for 8 min and 10 pmol ksg\(^R\) 30S ribosomal particles were incubated with KsgA (orange) for 3.5 min. Samples were done in triplicate and the average plotted with error bars of +/- 1 standard deviation.

**Inhibitor Assays**

We tested the SPA assay for RmtA inhibition by the known methyltransferase inhibitors sinefungin and SAH (Figure 21A). Both compounds inhibited RmtA in a concentration-dependent manner with calculated IC\(_{50}\) values of 19.95 ± 0.09 µM for sinefungin and 9.33 ± 0.07 µM for SAH. We also tested the effect of DMSO in the inhibition reactions, since this solvent is often used in HTS assays to dissolve test compounds (Figure 21B). RmtA in the presence of DMSO appeared to be fully active up to 15% (V/V), but the response from SPA beads dropped off at ≥ 20% DMSO. Whether the drop in signal was from the effect of DMSO on enzyme activity or on the binding of methylated RNA product to the SPA beads was not determined.
**Figure 21.** Inhibitors of methylation. For each concentration of inhibitor, inhibitor/DMSO was added to 30 pmol RmtA + 30 pmol wild-type 30S ribosomal particles + 20 µM SAM 780 cpm/pmol for an 8 min reaction. One third of the reaction volume was added to each of the 3 vials of SPA beads and the average plotted with error bars of +/- 1 standard deviation. (a) reactions with sinefungin (●) and with SAH (○). (b) reactions with DMSO alone.
CONCLUSIONS

Yttrium silicate nucleic acid binding SPA beads are shown here to be a useful method for measuring methylation of rRNA and almost certainly for other RNA and RNA-protein complexes. This assay method gave signal:noise ratios > 5:1 under our optimized conditions and can be used to measure methylation of RNA alone or in a 30S ribosomal particle complex with protein. While optimization of conditions for a specific assay and its products is advisable, all assays of RNA methylation will involve the common step of non-specific binding of the RNA product to the YSi beads. Therefore the conditions described here are likely to be a good starting point for any further optimization. Through use of a standard reference curve relating SPA counts to counts from the traditional filter binding assay, the SPA assay can give reproducible and accurate measures of the stoichiometry of methyl group incorporation. While the creation of this standard reference curve adds a step to the assay when methylation stoichiometry is to be determined, this would not be necessary for routine assays of relative activities or HTS applications, and may otherwise need only be done once. This SPA method can be used in time course assays for kinetic measurements, for quantitation of methyltransferases and for assay of inhibitors in DMSO concentrations up to 15% by volume. It is easily adapted to HTS processes and for manual procedures offers the advantages over the filter binding assay of less time, materials, radioactive waste and lower overall cost.

MATERIALS AND METHODS

Protein expression and purification

KsgA was expressed and purified as described.73 RmtA and ErmC’ were expressed from
a cloned, synthetic gene inserted into pET15b plasmids, rmtA in Rosetta 2 (DE3) cells (Novagen) and ermC’ in BL21 (codon+ DE3-RIL) cells (Stratagene). All three enzymes were purified on a HiTrap Ni-chelating column (Amersham) to > 95% homogeneity on SDS gel.

30S purification

Wild type and ksgR strains of E. coli were provided by Dr. Heather O’Farrell (our lab) and 30S subunits were prepared according to a previously described method. Concentration of 30S was determined by multiplying OD$_{260}$ by 6 pmol/mL.

30S Particle and 23S rRNA Methylation Reaction

A standard 50 µL reaction contained 10 pmol enzyme (RmtA, KsgA or ErmC’), 10 pmol 30S (wild type for RmtA, unmethylated ksgR for KsgA) or 23S (phenol extracted from E. coli 50S for ErmC’), 0.02 mM $^3$H-methyl-SAM (780 cpmp/pmol) (Perkin Elmer) and reaction buffer incubated at 37 °C for a specified time. Prior to mixing the reactants, 30S particles were heated at 42 °C for 5 min to anneal subunits into a homogenous conformation (for reactions with RmtA and KsgA). Reactants were mixed and the reaction initiated by addition of SAM. Background reactions were performed in the same way without enzyme. In reactions with RmtA and for its background measurement, reaction buffer (buffer R) consisted of 40 mM Tris, pH 7.2, 40 mM NH$_4$Cl, 8 mM MgOAc, and 1 mM DTT. Reaction buffer for KsgA and ErmC’ reactions and for its background measurement was 40 mM Tris at pH 7.4, 40 mM NH$_4$Cl, 4 mM MgOAc, 6 mM 2-mercaptoetanol.

At the end of the reaction time, the 50 µL reaction volume was removed and added to
clear 1.5 mL vials containing 180 µL deionized water, 10 µL (100 mg/mL) YSi binding beads (cat. RPNQ0013, GE Life Sciences) and 10 µL 100 mM unlabeled SAM (Sigma) and mixed thoroughly by pipette. Vials were then incubated in the dark for 40 min with mixing by inversion at 20 and 40 min and then centrifuged at 13,000 rpm for 3 min, placed in the mouth of a 15 mL scintillation vial, and counted in a Packard 1500 Tri-Carb Liquid Scintillation Analyzer. For experiments to optimize bead concentration (Figure 13) and water dilution (Figure 14), sample/bead mixtures were pipetted into the bottom of the scintillation vial and allowed to settle for an additional 20 min in the dark without mixing before being counted. Time course assays of samples before and after centrifugation (Figure 15) were done in one vial in volumes sufficient for 8 samples. Samples of 50 µL were taken at each of the 8 designated time points and measured by the standard SPA method with the additional step of counting samples just prior to centrifugation. Signal and background reactions were measured in triplicate at 1, 2, 4, 8, 16, 32, 64 and 128 min.

Parallel time course assays (Figure 16) were done as above except the reaction volume was sufficient for 16 samples for signal reactions and 6 samples for background reactions. Samples of 100 µL were removed at each time point, quenched with 20 µL 100 mM unlabeled SAM, and divided into two 60 µL aliquots for determination by the SPA and filter binding methods. Signal reactions were measured at 1, 2, 4, 8, 16, 32, 64 and 128 min, while background reactions were measured at 1, 8, and 128 min, all in triplicate. Excel was used to obtain a linear fit of data in Figures 17 and 21 and background data in Figure 16, and a logarithmic fit of signal data in Figures 16 and 18.

Inhibition studies were done using RmtA and wild type 30S in a triple volume reaction.
Inhibitor or solvent was added at a specified concentration prior to the addition of SAM to initiate the reaction. Reactions were stopped after 8 min and dispensed in equal volumes into 3 vials with YSi beads. Sinefungin (Sigma) was dissolved in deionized H$_2$O, SAH (Sigma) was dissolved in DMSO (American Bioanalytical). Data in Figures 21A and B were fitted with a sigmoidal dose-response curve provided in Sigma Plot 8.0; IC$_{50}$ values were calculated as described.$^{83}$

**Extraction of 16S and 23S rRNA from 30S and 50S Particles.**

Methylated 16S rRNA after reaction with KsgA or RmtA was extracted with buffer-saturated phenol (Invitrogen) and precipitated with ethanol before dilution and counting. 23S rRNA was similarly extracted from 50S particles with phenol prior to methylation by incubation with ErmC’ (this method is further detailed in Chapter 3).

**ACKNOWLEDGEMENTS**

We thank Dr. Heather C. O’Farrell (VCU, Physiology & Biophysics) for providing purified KsgA and wild type and ksg$^R$ strains of *E. coli*, Dr. Aiye Liang (VCU, Medicinal Chemistry) for her help with Sigma Plot, and Dr. Gordona Maravic Vlahovicke (University of Zagreb, Biochemistry & Molecular Biology) for providing us with a plasmid containing the ErmC’ gene. This work was funded from grants from the Commonwealth Health Research Board (JPR and HTW) and the NIH (JPR, GM66900).
CHAPTER 3: First-Generation Compounds

Background

In order for ErmC’ or KsgA inhibitors to be considered for drug development, compounds should demonstrate both potency and selectivity. The former might be easy if the latter were disregarded. While many potent MTase inhibitors have been discovered, few have been developed into drugs. Catechol-\(O\)-methyltransferase (COMT) inhibitors like entacapone or tolcapone are rare exceptions.\(^{84}\) The biggest hurdle has been selectively blocking the intended MTase without also affecting any of the many vital MTases in humans, which could lead to toxicity.Traditionally, this obstacle has been approached by aiming for the more unique substrate-binding pocket of MTases rather than the more common SAM-binding pocket. In the cases of our targets, ErmC’ and KsgA, this is particularly difficult since both pockets for the substrate and cofactor are binding molecules that contain the adenosine moiety. There appeared to be a breakthrough for this challenge when Pfizer presented work on selective ErmC inhibitors, which allegedly did not compete for the SAM binding site.\(^{85}\) This was followed up by a molecular modeling study from Feder \textit{et al.} that showed one of these inhibitors (compound 1) binding to the adenosine-pocket of ErmC’ and not the SAM-binding pocket (Figure 22).\(^{40}\) The conclusions made from these two studies formed bases for much of our original research plan.
In the first report,\textsuperscript{85} Clancy \textit{et al.} performed high throughput screening of synthetic compounds on ErmC (from \textit{S. aureus}), using COMT (from rat livers) and \textit{EcoRI} methylase (from \textit{E. coli}) to test for selectivity. Of the 160,000 compounds screened, seven of the inhibitors were selective towards ErmC, and were also structurally unrelated. One compound that had our interest (1) produced IC\textsubscript{50} values of 63 µM (ErmC), 390 µM (COMT) and >1 mM (\textit{EcoRI} MTase). For comparison, the positive control (sinefungin), gave IC\textsubscript{50} values of 5 µM (ErmC), 2 µM (COMT) and 80 µM (\textit{EcoRI} MTase). It was also reported that raising the concentration of SAM did not affect IC\textsubscript{50} values, suggesting that 1 was not acting through the SAM-binding pocket, although supporting data for this conclusion was not shown.\textsuperscript{85}
In the second report, Feder et al. screened *in silico* all compounds in the Maybridge collection using three different models of ErmC’s adenosine-binding pocket: 1) an “M.TaqI-like” model, where Tyr-104 was rotated to match the rotamer of Tyr-108 in M.TaqI. (PDB entry 1G38), 2) from the co-structure with SAM bound (1QAO), and 3) from the structure without SAM. Those compounds that docked into the adenosine pocket had the greatest affinity in model 1, supporting the idea that Tyr-104 “flips-out” during binding of the target substrate (affinity scoring done by Suflex software). Of the top 200 scoring compounds, 77 were visually selected for MIC studies with an ErmC’-expressing clone of *E. coli*. Seventeen of these compounds were shown to restore the potency of erythromycin *in vivo*. When these 17 were tested against ErmC’ *in vitro*, 8 compounds had IC$_{50}$ values between 180 to 500 µM. Compound 1 was also tested by this group for comparison, and was found to be more potent than the other test compounds, with an IC$_{50}$ = 80 µM. [Note: IC$_{50}$ assays used 0.13 µM SAM, 0.2 µM ErmC’, 1 µM synthetic RNA of 32 nucleotides.]

More interestingly, when 1 was docked into the “M.TaqI-like” model of ErmC’ (Figure 22), the adenosine moiety of this inhibitor oriented against the “flipped” tyrosine in a similar manner as the putative target-adenosine (Figure 7, Chapter 1). Furthermore, the cyclopentyl substituent on the N$_6$ atom could be seen branching into the SAM pocket, reaching about as far as the sulfur atom of the modeled SAM ligand (Figure 22). Feder et al. predicted that this substituent improved potency by extending it into the SAM pocket to make favorable interactions. [Note: Feder et al. briefly stated 1 was competitive with SAM when tested between 0.05 - 0.5 µM (SAM), contrary to their docking model and the claim by Clancy et al.]
**Rationale**

The assumption was made that the lead compound (1) bound to the target-adenosine pocket to form many of the same interactions as the putative substrate, namely \( \pi \)-stacking with Tyr-104 and Phe-163, and H-bonding with Asn-101 and Ile-102. It was reasoned that if the cyclopentyl substituent branched into the SAM-binding pocket of ErmC’, then other substituents may also be added to this position in order to explore more favorable interactions. We anticipated that 1 (or related analogs) might also inhibit KsgA because of the similarities with ErmC’. Therefore, a small library of N\(^6\)-substituted adenosine analogs was designed for a preliminary survey of potential interactions in ErmC’ and KsgA. Any analog that showed particular promise as a good inhibitor could later be expanded upon in subsequent libraries to further improve these interactions. Such a strategy would be similar to the Topliss scheme often used to optimize inhibitors during drug design.\(^{86}\)

For the first library, 23 substituents were selected from a general array of chemical groups that could be placed in the N\(^6\)-position of adenosine. These included aliphatic single chains ranging from one to eight carbons (as on 2-7, see Table 2), which were designed to probe for hydrophobic interactions at various lengths. Two of these chains had a terminal amine (as on 6 & 7) able to form ionic bonds with acidic residues. Also selected were small branched alkyl groups (as on 8 & 9), which could probe for steric hindrance near the N\(^6\) region of the adenosine analog. Several aromatic rings were also picked to explore \( \pi \)-stacking, hydrophobic or other interactions. For example, the benzyl groups on 10 - 12 were attached by linkers varying from 0 - 2 carbon bonds, which might also tell us about a preferential linker length. Three aromatic rings with para-substitutions (as on 13-15) could explore additional steric and electronic effects.
Another two substituents were heterocyclic (as on 16 & 17), which could form H-bonds. Non-aromatic rings were also selected (as on 1, 18-21), which could explore hydrophobic interactions with slightly more flexibility than the aromatic substituents, but with more spatial constraints than the aliphatic chains. Lastly, two short, unsaturated chains (as on 22 & 23) could test for π-interactions close to the N⁶-position.

We hypothesized that these new adenosine analogs would bind to the substrate pockets of ErmC’ or KsgA with substituents branching into the adjacent SAM pocket, similarly to how 1 was proposed to dock into the ErmC’ model.⁴⁰ Compounds 1-23 should offer us an initial assessment about preferred binding interactions when tested against ErmC’ and KsgA in vitro. The lead compound (1) will be used as a reference inhibitor, and a benchmark we aim to surpass.

**Synthesis**

Compounds 1, 3 - 23 were made by following the general reaction shown atop Table 2. [Note: Compound 2 (N⁶-methyladenosine) was also part of the original synthesis plan, but had been previously purchased from Sigma.] Those substituents that we chose to add at the N⁶-position of adenosine were purchased in the form of a primary amine attached to the desired substituent. This substituted amine was then reacted with 6-chloropurine riboside under the presence of a base, triethylamine (Et₃N), and heat (60-110 °C). [Note: K₂CO₃ was initially used as the base, but gave poor yields.] In this reaction, the primary amine performs a nucleophilic attack on the C⁶ of 6-chloropurine riboside, thereby replacing the Cl⁶ with the desired N⁶-substituent. Structures of these products and their respective yields are summarized below (Table 2). Further details of each reaction can be found at the end of the experimental section.
Table 2. Synthesis of first-generation compounds.

<table>
<thead>
<tr>
<th>product</th>
<th>-R</th>
<th>(name)</th>
<th>yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>cyclopentyl</td>
<td>82%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>methyl</td>
<td>(Sigma)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>ethyl</td>
<td>75%</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>n-butyl</td>
<td>8%</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>octyl</td>
<td>22%</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>ethylamine</td>
<td>23%</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>octylamine</td>
<td>64%</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>isopropyl</td>
<td>73%</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>tert-butyl</td>
<td>84%</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>phenyl</td>
<td>44%</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>benzyl</td>
<td>54%</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>phenethyl</td>
<td>67%</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>4-methylbenzyl</td>
<td>62%</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>4-chlorobenzyl</td>
<td>72%</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>4-methoxybenzyl</td>
<td>60%</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>4-methylpyridine</td>
<td>97%, crude</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>tetrazole</td>
<td>34%, crude</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>cyclohexyl</td>
<td>31%</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>tetrahydropyran</td>
<td>41%</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>piperidine</td>
<td>75%</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>methylecyclopropyl</td>
<td>82%</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>propargyl</td>
<td>44%</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>allyl</td>
<td>42%</td>
<td></td>
</tr>
</tbody>
</table>

Structures of the N^6-substituents and yields for each compound are shown above.
Following each reaction, product was purified by normal phase or reverse phase chromatography, and analyzed by proton ($^1$H) and carbon ($^{13}$C) NMR. In addition, two-dimensional NMR experiments, Correlation Spectroscopy (COSY) and Heteronuclear Single Quantum Coherence (HSQC) were performed on each product to help in the assignment of proton and carbon peaks. COSY is typically used to show which protons are within two to three bond lengths of a particular proton. Similarly, HSQC can indicate which protons are directly attached to a particular carbon. The resulting spectra from these NMR experiments corroborate the formation of the desired products 1, 3 - 23 (see appendix A). The assignment of NMR peaks ($^1$H and $^{13}$C) to their respective atoms are listed for each product in the experimental section. [Note: Compound 4 was the one exception, which did not have enough material to produce a quality $^{13}$C NMR spectra, though other NMR data supported the formation of this product.] Some of these products were also analyzed by mass spectrometry, which are reported in the experimental section.

**Inhibition Assays**

First-generation compounds were tested *in vitro* for inhibition against ErmC’ and KsgA using methods similar to those described in Chapter 2. In short, compounds (1-23) and positive control (sinefungin) were tested at 1 mM in 10% DMSO with 20 µM SAM, incubated at 37 °C for 32 min (ErmC’, Figures 23) or 5 min (KsgA, Figures 24), and activity measured by SPA.
Figure 23. Results of screening first-generation compounds with ErmC’.  a) Methylation activity was measured by SPA.  b) Percent inhibition was calculated by relating activity from test-compound with DMSO control, after subtracting the background.  The blue bars show the average of three replicate reactions, and error bars of +/- 1 std dev.
Figure 24. Results of screening first-generation compounds with KsgA. 

- Methylation activity was measured by SPA.
- Percent inhibition was calculated by relating activity from test-compound with DMSO control, after subtracting the background. The blue bars show the average of three replicate reactions, and error bars of +/- 1 std dev.

N6-substituent on adenosine analog
To our delight, the results from ErmC’ reactions showed that at least one of our compounds (7, and maybe 18) was more inhibitory than the lead compound 1, and a few others (4, 12 and 19) were not far behind in activity (Figure 23). Likewise, we were pleased to see that for the first time 1 had also inhibited KsgA (albeit, to a lesser extent than with ErmC’), and that a few other analogs were as inhibitory (7 and 18) or more inhibitory (5) towards KsgA than was 1 (Figure 24). Compound 6 was uniquely inhibitive to KsgA in our tests, while inhibitors 4, 12 and 19 were active only against ErmC’. Overall, the inhibitors from the first-generation compounds showed slightly more of an effect against ErmC’ than with KsgA. While these results may suggest partial selectivity towards ErmC’, some inhibitors (1, 7 and 18) were still active against both MTases.

It was difficult to decipher a governing trend of inhibition for the different substituents we had added. Generally, groups that were more aliphatic and less spatially constrained seemed to be preferred. The octylamine substituent (as on 7) was particularly inhibitory for both ErmC’ and KsgA, though its shorter equivalent, ethylamine (as on 6) was not inhibitory for either MTases. The only aromatic substituent that showed inhibition greater than 15% was the phenethyl (as on 12), which only affected ErmC’. In order to better understand the binding interactions of first-generation compounds, we attempted to crystallize ErmC’ and KsgA complexed with some of these inhibitors.

**Co-crystal Structures of ErmC’ and Selected Inhibitors**

*Crystallization*

A few of the more potent inhibitors (1, 7, 12, and 21) were selected for co-crystallization with either ErmC’ or KsgA. In short, protein was expressed, purified, and concentrated (8-13...
mg/mL), then inhibitor (at 1-20x protein conc.) was added immediately before crystallization. ErmC’ was crystallized by hanging drop vapor diffusion, equilibrated against a range of reservoir buffers (0.1 M Tris pH 8, 50-300 mM Li₂SO₄, 20-35% PEG 3350). ErmC’ usually formed large and diffraction-quality crystals (Figure 25) after just 1-2 days, although the protein was often lost during the preceding dialysis or concentration steps (as discussed in experimental section). Of those crystals that did grow to a reasonable size and quality, two were successfully diffracted by X-rays (co-crystals of ErmC’ with 7 or 12), while co-crystals of ErmC’ with 1 or 21 were lost during the mounting procedures. The apo form of ErmC’ was also crystallized under these conditions, but was not used for soaking in any of the first-generation inhibitors as was originally intended.

Figure 25. Photos of ErmC’ co-crystals complexed with inhibitors 7 (left) and 12 (right). Dimensions of crystals were approximated at 520 x 260 x 90 µm and 230 x 140 x 60 µm, respectively.

KsgA was similarly crystallized with inhibitor 7, using a reservoir buffer of 1.4 M MgSO₄ and 0.1 M 1 MES pH 6.5. However, this crystal did not diffract to a resolution greater than 10 Å. Other attempts with KsgA produced crystals that were too small for analysis. Interestingly, KsgA seemed to only crystallize when inhibitor was present, as attempts to crystallize the apo form were not successful. This may suggest that the bound ligand pushes
KsgA into a more stable or rigid conformation than its apo form, allowing for a more orderly aggregation of protein during crystallization. The work done on co-crystallizing KsgA was rather limited. Instead, efforts were focused more on crystallizing ErmC’ with inhibitors. Nevertheless, there is certainly potential for successfully co-crystallizing KsgA if these experiments were revisited in the future.

Structure Refinement

The structures of ErmC’ complexed with either $N^\circ$-8-octylamine-adenosine (7) or $N^\circ$-phenethyl-adenosine (12) were solved by X-ray diffraction to resolutions of 2.60 Å and 2.40 Å, respectively. Initial phasing of the diffraction data used molecular replacement from the apo form of ErmC’ (1QAM) to generate electron density maps. Both of our structures, like 1QAM, were solved from crystals with the space group P4$_3$2$_1$2. After refinement, the models that were created showed reasonable R and $R_{free}$ values of 23.7% and 28.3% for ErmC’ complexed with 7, and 23.8% and 26.9% for ErmC’ complexed with 12, respectively (see Table 3). Both models consisted of residues 9 - 244 out of the total 244 amino acids in ErmC’. Residues 1 - 8 were omitted owing to the lack of electron density at the N-terminus.
Table 3. Refinement statistics of ErmC’ co-crystallized with inhibitors 7 and 12

<table>
<thead>
<tr>
<th></th>
<th>8-amino octyl (7)</th>
<th>Phenethyl (12)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Data Collection</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Space group</td>
<td>P4₃2₁2 (No. 96)</td>
<td>P4₃2₁2 (No. 96)</td>
</tr>
<tr>
<td>Unit-Cell parameters</td>
<td>a = 81.34 Å, b = 81.34 Å, c = 122.07 Å, β = 90.00°</td>
<td>a = 81.62 Å, b = 81.62 Å, c = 122.68 Å, β = 90.00°</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>24.78 - 2.60 (2.69 - 2.60)</td>
<td>33.37 - 2.40 (2.49 - 2.40)</td>
</tr>
<tr>
<td>Total Number of Reflections</td>
<td>59,438</td>
<td>159,517</td>
</tr>
<tr>
<td>Number of Unique Reflections</td>
<td>13,112</td>
<td>16,788</td>
</tr>
<tr>
<td>Average redundancy</td>
<td>4.53 (4.58)</td>
<td>9.50 (9.23)</td>
</tr>
<tr>
<td>Completeness (%)d</td>
<td>99.6 (99.8)</td>
<td>99.8 (100.0)</td>
</tr>
<tr>
<td>R&lt;sub&gt;merge&lt;/sub&gt;</td>
<td>0.049 (0.319)</td>
<td>0.047 (0.384)</td>
</tr>
<tr>
<td>Reduced ChiSquared</td>
<td>0.84 (0.94)</td>
<td>0.90 (1.04)</td>
</tr>
<tr>
<td>I/σ(I)d</td>
<td>17.6 (4.3)</td>
<td>25.9 (5.7)</td>
</tr>
<tr>
<td>Reduced ChiSquared</td>
<td>0.84 (0.94)</td>
<td>0.90 (1.04)</td>
</tr>
<tr>
<td><strong>Refinement</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>2.60 - 24.00 (2.600 - 2.667)</td>
<td>2.40 - 33.00 (2.400 - 2.462)</td>
</tr>
<tr>
<td>Reflections: working/free</td>
<td>11,799/1,311</td>
<td>14,954/1,661</td>
</tr>
<tr>
<td>Protein Atoms</td>
<td>1978</td>
<td>1978</td>
</tr>
<tr>
<td>Ligand Atoms</td>
<td>28</td>
<td>27</td>
</tr>
<tr>
<td>Water Atoms</td>
<td>36</td>
<td>0</td>
</tr>
<tr>
<td>Residues not Modeled</td>
<td>1 - 9</td>
<td>1 - 9</td>
</tr>
<tr>
<td>R&lt;sub&gt;work&lt;/sub&gt;</td>
<td>0.237 (0.302)</td>
<td>0.238 (0.318)</td>
</tr>
<tr>
<td>R&lt;sub&gt;free&lt;/sub&gt;</td>
<td>0.283 (0.346)</td>
<td>0.269 (0.340)</td>
</tr>
<tr>
<td>R&lt;sub&gt;overall&lt;/sub&gt;</td>
<td>0.242</td>
<td>0.241</td>
</tr>
<tr>
<td>Mean B value (Å²)</td>
<td>56.857</td>
<td>38.523</td>
</tr>
<tr>
<td>Estimated Maximum Coordinate Error (Å)</td>
<td>0.247</td>
<td>0.177</td>
</tr>
<tr>
<td>RMSD Bond Lengths (Å)</td>
<td>0.009</td>
<td>0.010</td>
</tr>
<tr>
<td>RMSD Bond Angles</td>
<td>1.190°</td>
<td>1.147°</td>
</tr>
</tbody>
</table>

Note: Values in () are for the last resolution shell.
Unexpected binding mode

Surprisingly, these refined crystal structures of ErmC’ revealed that both 7 and 12 bound to the SAM pocket rather than the intended adenosine pocket. In fact, ligands in these new co-structures aligned nearly identically with the cofactor from the previous co-structure of ErmC’ + SAM (1QAO).\textsuperscript{36} Figure 26 shows how well the adenosine moiety of these three ligands overlap when co-structures are superimposed. This was in stark contrast to the binding mode predicted by Feder \textit{et al.} for the similar analog 1.\textsuperscript{40}

Figure 26. Superimposed structures of ErmC’ complexed with their respective ligand: SAM (red) from PDB no. 1QAO,\textsuperscript{36} compound 7 (blue), compound 12 (green).
A closer look at the SAM-binding pocket of our co-structures shows that inhibitors 7 and 12 form many of the same interactions with ErmC’ as seen with the cofactor in the previously solved structure of ErmC + SAM (1QAO). With our ligands, however, an additional hydrogen bond is made through the 5’-OH to the peptide-backbone of Asn-101 (Figure 27). This figure also shows the electron density around the N6-substituents to be sparse, suggesting that these groups remained dynamic during binding. We also observed how these inhibitors oriented with the 5’-carbon pointed towards the adenosine pocket. This unexpected binding mode would later be exploited to make a second library of test compounds (discussed in Chapters 4 & 5).

Discussion

As mentioned before, the trend among first-generation inhibitors appeared to be that aliphatic substituents that were less spatially constrained made better inhibitors. For ErmC’, those substituents on compounds that showed an inhibition greater than 15% were n-butyl (4 at
24.8%), 8-octylamine (7 at 51.1%), cyclopentyl (1 at 32.2%), cyclohexyl (18 at 32.2%),
tetrahydropyran (19 at 20.3%), and phenethyl (12 at 18.5%) (Table 4). Now that we have co-
structures for two of these inhibitors in complex with ErmC’, we can better explain why certain
substituents may have been preferred over other groups, and why KsgA was overall less affected
by these inhibitors than was ErmC’.
Table 4. Summary of Inhibition for ErmC’ and KsgA with 1 mM of first-generation compounds.

<table>
<thead>
<tr>
<th>#</th>
<th>Test Compound</th>
<th>ErmC' Inhibition</th>
<th>KsgA Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chemical Name</td>
<td>Average  SD</td>
<td>Average  SD</td>
</tr>
<tr>
<td>1</td>
<td>N&lt;sup&gt;6&lt;/sup&gt;-cyclopentyl-adenosine</td>
<td>32.2% 7.4</td>
<td>16.2% 9.4</td>
</tr>
<tr>
<td>2</td>
<td>N&lt;sup&gt;6&lt;/sup&gt;-methyl-adenosine</td>
<td>1.5% 10.2</td>
<td>5.9% 14.3</td>
</tr>
<tr>
<td>3</td>
<td>N&lt;sup&gt;6&lt;/sup&gt;-ethyl-adenosine</td>
<td>7.1% 6.6</td>
<td>7.0% 10.0</td>
</tr>
<tr>
<td>4</td>
<td>N&lt;sup&gt;6&lt;/sup&gt;-n-butyl-adenosine</td>
<td>24.8% 10.4</td>
<td>5.5% 12.2</td>
</tr>
<tr>
<td>5</td>
<td>N&lt;sup&gt;6&lt;/sup&gt;-octyl-adenosine</td>
<td>10.7% 11.0</td>
<td>38.9% 8.6</td>
</tr>
<tr>
<td>6</td>
<td>N&lt;sup&gt;6&lt;/sup&gt;-ethylamine-adenosine</td>
<td>-8.5% 6.7</td>
<td>-4.1% 3.3</td>
</tr>
<tr>
<td>7</td>
<td>N&lt;sup&gt;6&lt;/sup&gt;-octylamine-adenosine</td>
<td>51.1% 10.7</td>
<td>25.5% 14.0</td>
</tr>
<tr>
<td>8</td>
<td>N&lt;sup&gt;6&lt;/sup&gt;-isopropyl-adenosine</td>
<td>13.1% 12.5</td>
<td>6.1% 13.9</td>
</tr>
<tr>
<td>9</td>
<td>N&lt;sup&gt;6&lt;/sup&gt;-t-butyl-adenosine</td>
<td>-6.7% 16.2</td>
<td>7.3% 16.8</td>
</tr>
<tr>
<td>10</td>
<td>N&lt;sup&gt;6&lt;/sup&gt;-phenyl-adenosine</td>
<td>-0.3% 12.7</td>
<td>3.4% 6.6</td>
</tr>
<tr>
<td>11</td>
<td>N&lt;sup&gt;6&lt;/sup&gt;-benzyl-adenosine</td>
<td>-1.5% 8.8</td>
<td>12.8% 17.8</td>
</tr>
<tr>
<td>12</td>
<td>N&lt;sup&gt;6&lt;/sup&gt;-phenethyl-adenosine</td>
<td>18.5% 10.3</td>
<td>-1.1% 5.8</td>
</tr>
<tr>
<td>13</td>
<td>N&lt;sup&gt;6&lt;/sup&gt;-4-methylbenzyl-adenosine</td>
<td>2.8% 0.2</td>
<td>6.5% 14.6</td>
</tr>
<tr>
<td>14</td>
<td>N&lt;sup&gt;6&lt;/sup&gt;-4-chlorobenzyl-adenosine</td>
<td>10.9% 2.0</td>
<td>6.5% 3.6</td>
</tr>
<tr>
<td>15</td>
<td>N&lt;sup&gt;6&lt;/sup&gt;-4-methoxybenzyl-adenosine</td>
<td>3.3% 3.0</td>
<td>1.9% 18.2</td>
</tr>
<tr>
<td>16</td>
<td>N&lt;sup&gt;6&lt;/sup&gt;-4-pyridinemethyl-adenosine</td>
<td>-0.7% 12.7</td>
<td>2.4% 11.4</td>
</tr>
<tr>
<td>17</td>
<td>N&lt;sup&gt;6&lt;/sup&gt;-tetrazole-adenosine</td>
<td>-10.6% 3.9</td>
<td>4.6% 9.5</td>
</tr>
<tr>
<td>18</td>
<td>N&lt;sup&gt;6&lt;/sup&gt;-cyclohexyl-adenosine</td>
<td>32.2% 11.9</td>
<td>19.2% 23.1</td>
</tr>
<tr>
<td>19</td>
<td>N&lt;sup&gt;6&lt;/sup&gt;-tetrahydropyran-adenosine</td>
<td>20.3% 11.5</td>
<td>7.4% 15.5</td>
</tr>
<tr>
<td>20</td>
<td>N&lt;sup&gt;6&lt;/sup&gt;-piperidine-adenosine</td>
<td>-10.9% 14.5</td>
<td>8.1% 20.9</td>
</tr>
<tr>
<td>21</td>
<td>N&lt;sup&gt;6&lt;/sup&gt;-methylcyclopropyl-adenosine</td>
<td>10.2% 9.6</td>
<td>-2.1% 10.7</td>
</tr>
<tr>
<td>22</td>
<td>N&lt;sup&gt;6&lt;/sup&gt;-propargyl-adenosine</td>
<td>-1.3% 12.7</td>
<td>2.4% 9.7</td>
</tr>
<tr>
<td>23</td>
<td>N&lt;sup&gt;6&lt;/sup&gt;-allyl-adenosine</td>
<td>-4.2% 7.6</td>
<td>-1.5% 8.7</td>
</tr>
<tr>
<td>C+</td>
<td>sinefungin</td>
<td>97.7% 2.1</td>
<td>97.2% 0.3</td>
</tr>
</tbody>
</table>
Using our co-structure of ErmC’ + 12 as an example (Figure 28), we see that there is little space immediately in front of the N^6 atom of this ligand bound into the SAM pocket. The phenyl group on 12 branches off the N^6-position by three bond lengths, which is just long enough to clear the narrowing at the distal end of the SAM pocket. In contrast, the phenyl substituents on 10 and 11, which did not cause inhibition, are linked to the N^6 atom by only one and two bond lengths, respectively. The discrepancy of how 10 and 11 did not cause inhibition, while the similar compound 12 did inhibit ErmC’, may be explained by the spatial constraints associated with the shorter linkers of 10 and 11.

Figure 28. Spatial constraints of the SAM pocket for ErmC’ around the N^6-substituent of 12. Distances between residues are shown as yellow dotted lines and were measured in Å using MacPyMOL. The red dotted lines represent polar contacts between 12 and residues of ErmC’.
In contrast, compounds 1, 18 and 19 showed inhibition with ErmC’ despite having only one bond length between their cyclic substituent and the N6 atom. This could be the result of these substituents adopting conformations that are more easily accommodated within this narrowing of the SAM pocket than an aromatic ring with the same linker-length (as on 10 & 17). For all of these first-generation inhibitors, the N6-substituents could have formed hydrophobic interactions with the surrounding residues of ErmC’ (i.e., Ile-60, Ile-84, Leu-86, or Ile-106).

In regard to KsgA, those substituents on compounds that showed an inhibition greater than 15% were octyl (5 at 38.9%), 8-octylamine (7 at 25.5%), cyclopentyl (1 at 16.2%), and cyclohexyl (18 at 19.2%). For the sake of this discussion, it will be assumed that these inhibitors bound to the SAM pocket of KsgA in the classical mode seen with adenosine analogs complexed with other MTases. In that case, substituents branching off the N6-position likely benefited from hydrophobic interactions with nearby residues of Leu-67, Leu-122, Ile-118, or Ile-114 (Figure 29), similar to what has been proposed for ErmC’.

Those inhibitors that were active against both ErmC’ and KsgA, were still less inhibitory towards KsgA. This could be the result of KsgA having an even narrower opening in the SAM pocket than that of ErmC’. The distances between three atoms that triangulate around the N6-position of 12 were measured from the SAM pocket of ErmC’ and compared with the analogous atoms on KsgA. It was found that this opening at the distal end of the SAM pocket was about 1.5 Å narrower in KsgA. For ErmC’, these measurements were as follows: Ile-106 Cγ2 was 7.7 Å to Asp-84 Oδ2, which was 4.5 Å to Ile-60 Cδ1, which was 9.1 Å to Ile-106 Cγ2 (Figure 28). The analogous positions in KsgA were measured as follows: Ile-118 Cγ2 was 5.9 Å to Asp-91 Oδ1, which was 4.8 Å to Leu-67 Cδ1, which was 7.6 Å to Ile-118 Cγ2 (Figure 29).
Figure 29. Spatial constraints of KsgA around the supposed N6-substituent of first-generation inhibitors. Distances between residues are shown as yellow dotted lines and were measured in Å using MacPyMOL and the apo structure of KsgA (1QYR).

\textit{Trans-cis} isomerization of X-Proline in co-structure of ErmC’ with inhibitor 8.

The model for ErmC’ in complex with 7 originally showed two conformations for Pro-103, one a \textit{cis} isomer and the other a \textit{trans} isomer (Figure 30). In all reported ErmC’ structures (including ErmC’ + 12), this peptide bond has a \textit{trans} configuration. In the trans configuration, the C\(\alpha\) of Pro-103 is trans to the C\(\alpha\) of Ile-102. [Note: amides have partial double-bond character due to resonance between the lone pair of electrons from nitrogen and the carbonyl group. Loss of this double bond character can lead to rotation about the –C – N- bond resulting in isomerization.] However, at the very end of this project our model (ErmC’ + 7) was refined again to reveal that the peptide bond of Ile-102 - Pro-103 is entirely in the \textit{cis} isomer. This atypical configuration shows the side chain of Ile-102 pointing into the adenosine-binding
pocket of ErmC’. Such a rotation might preclude binding of the target adenosine, and may explain why 7 was such a good inhibitor in our tests.

Figure 30. Alternate conformations of ErmC’ in complex with 7. Residues Ile-102 and Pro-103 were initially found to be in both the atypical cis configuration (shown in hot-pink) as well as the typical trans configuration (shown in green). Later refinements revealed said residues to be all in the cis configuration.

While our research into this phenomenon is just in its infancy, the implications of this trans to cis isomerization may actually be quite profound. From this early observation, it appears inhibitor 7 may be catalyzing the conversion of the Ile102 - Pro103 peptide bond from a trans isomer to the cis isomer. Normally, the interconversion of an X-Pro in native proteins is limited due to a high energy barrier (~20 kcal/mol). However, it has been found that certain proteins (ex. peptidylprolyl isomerases) or strong acids can facilitate a similar cis to trans isomerization of X-Pro. This probably occurs through a mechanism where the partial double-
bond character of the peptide bond is reversed by protonation of the amide, thus allowing the two Cα’s to rotate about the adjoining C-N bond to form a more energetically favored arrangement overall.\textsuperscript{88} It is conceivable that the protonated terminal amine on 7 could also disrupt the partial double-bond character between Ile102 - Pro103 and catalyze a trans to cis isomerization in ErmC’. Further experimentation will be needed to confirm this.

Acknowledgements

We would like to thank Dr. Keith C. Ellis of Virginia Commonwealth University (VCU) for his contributions made with designing the library of test compounds 1-23 and the reaction scheme used to synthesize compounds 1, 3-23. We also thank Dr. Neil Scarsdale of VCU for refining and building molecular models of ErmC’ co-structures. Additionally we thank Dr. Faik N. Musayev of VCU for suggesting conditions used to crystallize ErmC’ and KsgA.

Experimental Section

Buffers

The following two buffers were used for pelleting 70S particles.

Buffer I: 50 mM Tris pH 7.4, 10 mM MgOAc, 0.5 mM EDTA, 6 mM DTT, and 100 mM NH₄Cl.

Buffer II: Same as Buffer I except for the addition of 1.1 M sucrose, and NH₄Cl was at 1 M.

ErmC’ Lysis Buffer. The following was used to resuspend cells prior to breaking and loading onto Ni-NTA column: 50 mM NaPO₄ (pH 8), 300 mM NaCl, 5 mM imidazole, 10% glycerol, 1
mM phenylmethylsulfonyl fluoride (PMSF) dissolved in EtOH (17.4 mg/mL), 2 mM \( \beta \)-mercaptoethanol (BME), and 0.2% triton X-100.

\textit{ErmC’ Washes and Elution Buffers}. These were the same as ErmC’ Lysis Buffer except no triton X-100, and increasing concentrations of imidazole (5, 15, 30, to 75 mM) were used for each washing step (15-20 mL each). Protein was eluted from the Ni-NTA column with buffer containing 175 mM imidazole.

\textit{ErmC’ Storage Buffer}. Purified protein for activity assays or crystallization was kept in 50 mM Tris pH of 7.4, 100 mM NaCl, 10% glycerol, and 2 mM dithiothreitol (DTT). ErmC’ elution (175 mM imidazole) was first dialyzed against storage buffers containing 100 mM imidazole, then the same with 50 mM imidazole, and finally storage buffer with no imidazole.

\textit{Buffer K}. The following was used for enzymatic reaction with KsgA and ErmC’: 40 mM Tris at pH 7.4, 40 mM \( \text{NH}_4 \text{Cl} \), 4 mM \( \text{MgOAc} \), and 6 mM 2-mercaptoethanol.

\textbf{Protein expression and purification.}

\textit{ErmC’}. The ermC’ gene was inserted into the pET15b plasmid to have a 6x-polyhistidine tag at the C-terminus. This plasmid was then transfected into \textit{E. coli} BL21 (codon+ DE3-RIL) cells (Stratagene). Cells were cultured in 1 L of LB media at 37 \(^\circ\)C to an OD\(_{600}\) of 0.6, at which time expression of protein was induced with 1 mM IPTG and incubated at 25 \(^\circ\)C for 5 more hours. Cells were grown in the presence of Ampicillin (50 \( \mu \text{g/mL} \)) and Chloramphenicol (30 \( \mu \text{g/mL} \)) to
preserve the plasmid and cell line, respectively. Cells were harvested by centrifugation and divided into two equal halves for storage at -20 °C. Cell pellet (equal to 500 mL of growth) was resuspended in ~25 mL of ErmC’ Lysis Buffer, passed twice through Emulsiflex cell breaker (Avestin) at ~15,000 psi, and centrifuged twice (20 min at 11,000 rpm, then 45 min at 15,000 rpm), discarding cell debris after each time. Lysate was passed through a column with 5 mL HiTrap Ni-chelating column (Amersham), then rinsed with ErmC’ Washing Buffers (5, 10, 30 and 75 mM imidazole; 20 mL of each) and eluted with 20 mL of same buffer with 175 mM imidazole. Purity of collected fractions was later checked by gel-electrophoresis and found to be greater than 95%. Fractions 2 - 12 (~1 mL each) were usually selected as the most pure, and dialyzed in ErmC’ Storage Buffers with gradually decreasing concentrations of imidazole, then concentrated using Centricon YM-10 tubes (Millipore).

*Expression and purification of KsgA. “see Chapter 2”*

**Preparation of 23S**

*Bacillus subtilis* (BD170) was purchased from ATCC.

One liter of BHI (brain heart infusion) broth was inoculated with an overnight growth of *B. subtilis*, and grown at 34 °C for 4 hours until OD$_{600}$ = 1.0. Then, cells were pelleted, washed with lysis buffer and stored in 2 aliquots at -20 °C. One aliquot of cells was resuspended in 30 mL Buffer I, and lysed at 10,000 psi (French press). Cell debris was pelleted and discarded by centrifugation at 11,000 RPM for 20 min, and again at 14,500 RPM for 60 min. In order to collect the 70S pellets, cell lysate was layered onto a sucrose cushion (Buffer II) in 4 x 40 mL
tubes, and centrifuged at 35,000 RPM for 22 hours. The glassy 70S pellets (from 2 of the 4 tubes) were slowly resuspended in 1 mL of low [Mg] buffer, twice dialyzed in 500 mL for 1 hr in same buffer, before layering onto 10-40% sucrose gradient in 6 x 30 mL tubes, and centrifuged for 17 hours at 19,000 rpm. Ribosomal subunits (50S and 30S) were separated from sucrose gradient with a fraction collector, discarding the 30S portion. After dialyzing the 50S fractions (~37 mL) in low [Mg] buffer (3 x ~420 mL for 1 hr each), subunits were concentrated with 100,000 MWCO spin column until OD$_{260}$ x 44 µg/mL = 2.5 µg/µL (concentrate divided into 6 x 0.5 mL aliquots and stored at -80 °C).

Extraction of 23S rRNA from 50S subunits followed an earlier procedure. In short, 600 µL of phenol:chloroform:isoamyl alcohol (25:24:1) was mixed with each aliquot of 50S (6 x 0.5 mL of 2.5 µg/µL), and centrifuged at 14,000 rpm for 5 min. The top 90% of the aqueous layer was pipetted out and used in the same extraction process twice more. Then, 15 µL of 5 M NaCl were added to each vial of ~400 µL of aqueous portion, followed by 1 mL of ice cold EtOH (100%) and mixed by hand. After vials sat on dry-ice for 30 min, precipitated RNA was spun down at 14,000 RPM for 20 min (in the cold room). Pellets were rinsed with 0.5 mL of EtOH (70%), centrifuged, decanted, and air-dried for ~3 hrs (taped upside down in the ventilation-hood). Dried RNA pellets were each dissolved in 200 µL of H$_2$O for a final concentration of 4.8 µg/µL (OD$_{260}$ x 44 µg/mL). The amount of 23S was determined to be 5 pmol/µL based on the assumption that 96% of the RNA was 23S and 4% was 5S rRNA (23S has 2923 bases, 5S has 122 bases), and that 23S has a MW of 1 x 10$^6$ g/mol.
Inhibition Assays

First-Generation compounds were tested *in vitro* for inhibition against ErmC’ and KsgA using methods similar to those described in Chapter 2. In short, compounds (1-23) and positive control (sinefungin) were tested at a final concentration of 1 mM dissolved in 10% DMSO. Also in this reaction (final vol = 50 µL) was 0.2 µM MTase (ErmC’ or KsgA), 0.2 µM rRNA (23S for ErmC’ or unmethylated ksgR 30S for KsgA) and buffer K. Reactions where initiated with the addition of 20 µM ³H-methyl-SAM (780 cpm/pmol), and incubated at 37 °C for a set duration (32 min for ErmC’, 5 min for KsgA), at which point reactions were quenched by the addition of 10 µL of 100 mM cold SAM. Activity of ErmC’ and KsgA methylation was measured by RNA binding SPA beads (1 mg YSi per reaction) as described in Chapter 2.

For the negative control, the same reaction was performed in 10% DMSO, but without inhibitor. Likewise, background reactions (i.e., “no enzyme”) were also done in 10% DMSO without inhibitor and without MTase present. All reactions were done in triplicate. Figures 23a (ErmC’) and 24a (KsgA) show the resulting activity from these reactions, measured in counts per minute (cpm). To calculate the percent inhibition shown in Figures 23b and 24b, the background signal was first subtracted from all of the other signals. Then, it was determined how much lower (in percentage) each of the test signals were compared to the signal from the negative control (i.e., “no inhibitor”). The following formula simplifies this calculation: % inhibition = [1-(test compound - “no enzyme”)/(“no inhibitor” - “no enzyme”))] x 100% .
**ErmC’ crystallization**

Protein was obtained in the same manner as above then concentrated to 13.1 mg/mL (0.45 mM) and co-crystallized with either 5x or 20x concentration of inhibitor 7 by hanging drop vapor diffusion. Each drop contained 3 µL of protein/inhibitor in storage buffer plus 3 µL of reservoir buffer (0.1 M Tris pH 8, 50-300 mM Li₂SO₄, 20-35% PEG 3350). Crystals were seen after 15 hours and were allowed to grow for ~2 weeks before attempting to diffract by X-rays. The selected crystal was cryoprotected by soaking in reservoir solutions with increasing glycerol concentrations, as follows: 1 min soak in 10% glycerol, then a quick dip in 15% glycerol. With a nylon loop, the crystal was fished from solutions and mounted immediately on the goniometer to be frozen in a stream of N₂ gas (98 K). Note that inhibitor 7 had been inadvertently omitted from cryoprotectant solutions, nonetheless data collected from this crystal was still of good quality and the ligand was present.

On a later occasion, ErmC’ was co-crystallized with compounds 1, 12, or 21. The previous procedure was followed with the exception of ErmC’ concentrated to 9.8 mg/mL and co-crystallized with either 1x, 5x or 10x inhibitor concentration using reservoir buffers of (0.1 M Tris pH 8, 200-300 mM Li₂SO₄, 25-30% PEG 3350). Co-crystals of 1 and 21 were both lost during the mounting procedures. Co-crystals of 12 (from 200 mM Li₂SO₄, 30% PEG 3350 and 1x inhibitor conc.) was successfully mounted for X-ray diffraction from cryoprotectant solution containing ligand.
Synthesis

Chemicals and Solvents. 6-Chloropurine riboside, triethylamine, N6-methyladenosine (2), ethylamine, isopropylamine, tert-butylamine, octylamine, 4-(aminomethyl)pyridine, 5-aminotetrazole, and deuterated dimethoxy sulfide (DMSO-d6) were purchased from Sigma-Aldrich (USA). n-Butylamine, ethylenediamine, 1,8-diaminoctane, cyclopentylamine, cyclohexylamine, aminomethylcyclopropane, aniline, benzylamine, 4-methylbenzylamine, 4-methoxybenzylamine, propargylamine, and allylamine were purchased from Acrös Organics (Belgium). 4-Aminotetrahydropyran was purchased through Maybridge (England). 4-Aminopiperidine, 4-chlorobenzylamine, and 2-phenethylamine were bought from Alfa Aesar (England). Methanol (MeOH), dichloromethane (DCM), acetonitrile (AcN), isopropanol (IPA), tetrahydrofuran (THF), hexanes, and formic acid were purchased through Fisher Scientific (USA).

Chromatography. Thin-layer chromatography (TLC) was performed on silica plates purchased from Analtech (USA). Preparative chromatography was done with RediSep®Rf columns containing silica, C18 or amine-functionalized silica, and a Combiflash®Rf instrument, all purchased through Teledyne Isco, Inc (USA).

Instruments and Analyses. 1H and 13C NMR spectra were obtained using a 400 MHz Bruker spectrometer with Bruker Topspin 2.1 software. Chemical shifts are reported as ppm (δ) using solvent peaks or tetramethylsilane (TMS) as the internal standard. Mass spectra (MS) were performed at the Department of Pharmacology and Toxicology, Virginia Commonwealth University.
General procedure for N⁶ substitution/amination of 6-chloropurine riboside into compounds 1 and 3 through 23. To a suspension of 6-chloropurine riboside (57.3 mg, 0.2 mmol) in MeOH (2.86 mL), 3.6 eq. of triethylamine (0.1 mL, 0.72 mmol) was added. Then one of 21 different alkylamines was added and allowed to reflux at 60 °C for 18 hr to yield compounds 1, 3-23.

N⁶-cyclopentyl-adenosine (1). To a suspension of 6-chloropurine riboside (2.0 g, 7.0 mmol) in 50 mL of dry methanol, 3.6 eq of cyclopentylamine (2.5 mL, 25.3 mmol) and 4.9 eq of dry triethylamine (4.75 mL, 34.1 mmol) were added then stirred at 60 °C for 20 hours. Reaction was concentrated in vacuo and separated by reverse phase chromatography (130 g C18 RediSep Column, 5-100% THF in DI H₂O over 23.4 min with flow rate of 85 mL/min). Product was co-evaporated with hexanes to yield 1.9 g of white powder (5.7 mmol, 82%). Rf 0.37 (MeOH:DCM = 1:9); ¹H NMR (DMSO-d₆) δ 8.34 (s, 1H, 8-H), 8.20 (bs, 1H, 2-H), 7.73 (bd, 1H, N⁶-H, J = 7.52 Hz), 5.90 (d, 1H, 1'-H, J = 6.20 Hz), 5.45-5.38 (m, 2H, 5'-OH + 2'-OH), 5.16 (d, 1H, 3'-OH, J = 4.64), 4.62 (dd, 1H, 2'-H, J = 6.12, 11.24 Hz), 4.55 (bs, 1H, 1''-H, cyclopentyl), 4.19 - 4.13 (m, 1H, 3'-H), 3.98 (dd, 1H, 4'-H, J = 3.4, 6.5 Hz), 3.74 - 3.65 (m, 1H, 5'-Ha), 3.64 - 3.52 (m, 1H, 5'-Hb), 2.04 - 1.88 (m, 2H, 2''-Ha + 5''-Ha, cyclopentyl), 1.79 - 1.67 (m, 2H, 3''-Ha + 4''-Ha, cyclopentyl), 1.67 - 1.47 (m, 4H, 2''-Hb, 5''-Hb, 3''-Hb + 4''-Hb cyclopentyl); ¹³C NMR (DMSO-d₆) δ 154.38 (C-6), 152.24 (C-2), 148.38 (C-4), 139.51 (C-8), 119.56 (C-5), 87.94 (C-1'), 85.87 (C-4'), 73.45 (C-2'), 70.63 (C-3'), 61.66 (C-5'), 51.46 (C-1'', cyclopentyl), 32.07 (C-2''+ C-5'', cyclopentyl), 23.45 (C-3''+ C-4'', cyclopentyl); HRMS m/z 336.1689, expected 336.1672 (M + H)⁺.
\textsuperscript{N}\textsuperscript{6}-\textsuperscript{ethyl}-adenosine (3). To a suspension of 6-chloropurine riboside (57 mg, 0.20 mmol) in 2.8 mL of MeOH, 2 eq of ethylamine (26 µL, 0.4 mmol) and 3.6 eq of dry triethylamine (100 µL, 0.72 mmol) were added and stirred for 18 hr at 60 °C, concentrated in vacuo and purified by normal phase chromatography (4 g silica RediSep Column, 0-15\% MeOH in DCM over 45 cv) to yield 44 mg (0.15 mmol, 75\%) as a white powder. \textit{R}_{f} 0.58 (MeOH:DCM = 1:9); \textsuperscript{1}H NMR (DMSO-\textit{d}_6) \delta 8.34 (s, 1H, 8-H), 8.21 (bs, 1H, 2-H), 7.73 (bs, 1H, \textit{N}\textsuperscript{6}-H), 5.89 (d, 1H, 1’-H, \textit{J} = 6.16 Hz), 5.45-5.37 (m, 2H, 5’-OH & 2’-OH), 5.17 (d, 1H, 3’-OH, \textit{J} = 4.60), 4.62 (dd, 1H, C\textsuperscript{2’}-H, \textit{J} = 6.12, 11.24 Hz), 4.15 (m, 1H, 3’-H), 3.97 (dd, 1H, 4’-H, \textit{J} = 3.4, 6.6), 3.74 - 3.63 (m, 4H, 5’-H\textsubscript{2} & 1’’-H\textsubscript{2}, ethyl), 1.18 (t, 3H, 2’’-H\textsubscript{3}, \textit{J} = 7.12 Hz, ethyl); \textsuperscript{13}C NMR (DMSO-\textit{d}_6) \delta 154.50 (C-6), 152.33 (C-2), 148.21 (C-4), 139.60 (C-8), 119.71 (C-5), 87.92 (C-1’), 85.88 (C-4’), 73.44 (C-2’), 70.63 (C-3’), 61.66 (C-5’), 34.50 (C-1’’, ethyl), 14.78 (C-2’’, ethyl); MS m/z 296.3, expected 296.1 (M + H)\textsuperscript{+}.

\textsuperscript{N}\textsuperscript{6}-\textit{n}-\textsuperscript{butyl}-adenosine (4). To a suspension of 6-chloropurine riboside (57 mg, 0.20 mmol) in 2.8 mL of MeOH, 2 eq of \textit{n}-butylamine (40 µL, 0.4 mmol) and 3.6 eq of dry triethylamine (100 µL, 0.71 mmol) were added then stirred overnight at 60 °C. Reaction was concentrated in vacuo and crystallized in ~2 mL MeOH. This solid was captured on filter connected to a vacuum manifold and rinsed with a small amount of MeOH to yield 5 mg of white powder (0.02 mmol, 8\%). \textit{R}_{f} 0.66 (MeOH:DCM = 3:17); \textsuperscript{1}H NMR (DMSO-\textit{d}_6) \delta 8.33 (s, 1H, 8-H), 8.20 (bs, 1H, 2-H), 7.85 (bs, 1H, \textit{N}\textsuperscript{6}-H), 5.88 (d, 1H, 1’-H, \textit{J} = 6.20 Hz), 5.45-5.36 (m, 2H, 2’-OH, & 5’-OH), 5.16 (d, 1H, 3’-OH, \textit{J} = 4.60 Hz), 4.61 (dd, 1H, 2’-H, \textit{J} = 6.0, 11.2 Hz), 4.17-4.12 (m, 1H, 3’-H), 3.97
(dd, 1H, 4'-H, J= 3.4, 6.6 Hz), 3.72 - 3.64 (m, 1H, 5'-Hₐ), 3.60 - 3.52 (m, 1H, 5'-Hₐ) 3.52 - 3.42 (m, 2H, 1''-H₂, n-butyl), 1.63 - 1.52 (m, 2H, 2''-H₂, n-butyl), 1.40-1.28 (m, 2H, 3''-H₂, n-butyl), 0.90 (t, 3H, 4''-H₃, n-butyl, J= 7.3 Hz).

N⁶-octyl-adenosine (5). To a suspension of 6-chloropurine riboside (57 mg, 0.20 mmol) in 2.8 mL of MeOH, 2 eq of octylamine (66 µL, 0.4 mmol) and 3.6 eq of dry triethylamine (100 µL, 0.71 mmol) were added then stirred overnight at 60 °C. Reaction was concentrated in vacuo and crystallized in ~2 mL MeOH. This solid was captured on filter connected to a vacuum manifold and rinsed with a small amount of MeOH to yield 17 mg (0.04 mmol, 22%) of white powder. Rf 0.60 (MeOH:DCM = 3:17); ¹H NMR (DMSO-d₆) δ 8.33 (s, 1H, 8-H), 8.20 (bs, 1H, 2-H), 7.85 (bs, 1H, N⁶-H), 5.88 (d, 1H, 1'-H, J = 6.20 Hz), 5.45-5.37 (m, 2H, 2'-OH, & 5'-OH), 5.16 (d, 1H, 3'-OH, J = 4.64 Hz), 4.62 (dd, 1H, 2'-H, J = 6.1, 11.3 Hz), 4.18-4.12 (m, 1H, 3'-H), 3.97 (dd, 1H, 4'-H, J= 3.40, 6.56 Hz), 3.73 - 3.63 (m, 1H, 5'-Hₐ), 3.60 - 3.52 (m, 1H, 5'-Hₐ) 3.47 (bs, 2H, 1''-H₂, octyl), 1.59 (p, 2H, 2''-H₂, octyl, J = 7.0 Hz), 1.39-1.52 (m, 10H, 3''-H₂, 4''-H₂, 5''-H₂, 6''-H₂, 7''-H₂, octyl), 0.85 (t, 3H, 8''-H₃, octyl, J= 7.88 Hz); ¹³C NMR (DMSO-d₆) δ 154.67 (C-6), 152.32 (C-2), 148.27 (C-4), 139.55 (C-8), 119.89 (C-5), 87.94 (C-1'), 85.87 (C-4'), 73.43 (C-2'), 70.64 (C-3'), 61.66 (C-5'), 40.1 (C-1'', octyl), 31.19 (C-4'', octyl), 29.00 (C-2'', octyl), 28.73 (C-3'', octyl), 28.64 (C-5'', octyl), 26.37 (C-6'', octyl), 22.03 (C-7'', octyl), 13.89 (C-8'', octyl).

N⁶-ethylamine-adenosine (6). To a suspension of 6-chloropurine riboside (57 mg, 0.20 mmol) in 2.8 mL of MeOH, 2 eq of ethylenediamine (27 µL, 0.4 mmol) and 3.6 eq of dry triethylamine
To a suspension of 6-chloropurine riboside (57 mg, 0.20 mmol) in 10 mL of MeOH, 3 eq of 1,8-Diaminoctane (86.6 mg, 0.6 mmol) and 3.6 eq of dry triethylamine (100 µL, 0.71 mmol) were added and stirred overnight at 60 °C. Reaction was separated by normal phase chromatography (4 g silica RediSep Column, 0-50% MeOH in DCM, over 55 cv) to yield 50.2 mg (0.13 mmol, 64%) as a white powder. Rf 0.15 (MeOH:DCM = 1:3); ¹H NMR (DMSO-d₆) δ 8.34 (s, 1H, 8-H), 8.20 (bs, 1H, 2-H), 8.05 (bs, 2H, N⁶-H₂, octylamine), 7.85 (bs, 1H, N⁶-H), 5.89 (d, 1H, 1’-H, J = 6.20 Hz), 5.46 (bs, 2H, 2’-OH & 5’-OH), 5.23 (bs, 1H, 3’-OH), 4.61 (dd, 1H, 2’-H, J = 5.56, 5.52 Hz), 4.16 (dd, 1H, 3’-H, J = 3.04, 4.80 Hz), 3.97
(dd, 1H, 4’-H, J = 3.44, 6.60 Hz), 3.72 - 3.62 (m, 1H, 5’-H₃), 3.60 - 3.51 (m, 1H, 5’-H₅), 3.46 (bs, 2H, 1”-H₂, octylamine), 2.72 (dd, 2H, 8”-H₂, octylamine, J = 7.48, 7.68 Hz), 1.65 - 1.49 (m, 4H, 2”-H₂ & 7”-H₂, octylamine), 1.27 (bs, 8H, 3”-H₂, 4’’-H₂, 5”-H₂ & 6”-H₂, octylamine).¹³C NMR (DMSO-d₆) δ 154.65 (C-6), 152.32 (C-2), 148.33 (C-4), 139.52 (C-8), 119.68 (C-5), 87.87 (C-1’), 85.85 (C-4’), 73.50 (C-2’), 70.62 (C-3’), 61.62 (C-5’), 39.91 (C-1”’, octylamine), 38.94 (C-8”, octylamine), 28.97 (C-2”’, octylamine), 28.54 (C-3”’, octylamine), 28.45 (C-4”’, octylamine), 26.86 (C-7”’, octylamine), 26.23 (C-5”’, octylamine), 25.78 (C-6”’, octylamine).

N⁶-isopropyl-adenosine (8). To a suspension of 6-chloropurine riboside (57 mg, 0.20 mmol) in 2.8 mL of MeOH, 2 eq of isopropylamine (33 µL, 0.4 mmol) and 3.6 eq of dry triethylamine (100 µL, 0.72 mmol) were added and stirred for 18 hr at 60 °C, concentrated in vacuo and purified by normal phase chromatography (4 g silica RediSep Column, 0-20% MeOH in DCM over 35 cv) to yield 45 mg (0.15 mmol, 73%) as a white powder. Rₚ 0.67 (MeOH:DCM = 1:9); ¹H NMR (DMSO-d₆) δ 8.34 (s, 1H, 8-H), 8.21 (bs, 1H, 2-H), 7.73 (bs, 1H, N⁶-H), 5.89 (d, 1H, 1’-H, J = 6.20 Hz), 5.45-5.40 (m, 2H, 5’-OH & 2’-OH), 5.17 (d, 1H, 3’-OH, J = 4.64), 4.62 (dd, 1H, 2’-H, J = 6.08, 11.2 Hz), 4.46 (bs, 1H, 1’’-H, i-propyl), 4.18-4.13 (m, 1H, 3’’-H), 3.97 (dd, 1H, 4’-H, J= 3.4, 6.6), 3.73 - 3.64 (m, 1H, 5’-H₃), 3.61 - 3.51(m, 1H, 5’-H₅),1.18 (d, 6H, 2”-H₃ & 3”-H₃, J = 7.12 Hz, i-propyl). ¹³C NMR (DMSO-d₆) δ 153.96 (C-6), 152.30 (C-2), 148.34 (C-4), 139.53 (C-8), 119.58 (C-5), 87.91 (C-1’), 85.88 (C-4’), 73.43 (C-2’), 70.63 (C-3’), 61.66 (C-5’), 41.34 (C-1”’, i-propyl), 28.83 (C-2”’ + C-3”’, i-propyl); MS m/z 310.3, expected 310.2 (M + H)⁺.
N6-ter-butyl-adenosine (9). To a suspension of 6-chloropurine riboside (57 mg, 0.20 mmol) in 10 mL of IPA, 10 eq of tert-butylamine (504 µL, 2.0 mmol) and 7.2 eq of dry triethylamine (200 µL, 1.43 mmol) were added then stirred in a sealed tube at 110 °C for 48 hours. Reaction was separated by reverse phase chromatography (4.3 g C18 RediSep Column, 0-30% AcN in DI H2O with 0.1% formic acid, over 70 cv). Product dried in vacuo to yield 54.3 mg of white powder (0.17 mmol, 84%). Rf 0.43 (MeOH:DCM = 1:9); 1H NMR (DMSO-d6) δ 8.35 (s, 1H, 8-H), 8.24 (s, 1H, 2-H), 6.81 (s, 1H, N6-H), 5.88 (d, 1H, 1’-H, J = 6.12 Hz), 5.81 - 4.91 (bs, 3H, 2’-OH, 3’-OH & 5’-OH), 4.62 (dd, 1H, 2’-H, J = 5.2, 5.8 Hz), 4.16 (dd, 1H, 3’-H, J = 4.8, 3.1 Hz), 3.97 (dd, 1H, 4’-H, J = 3.48, 6.72 Hz), 3.72 - 3.64 (m, 1H, 5’-Hα), 3.60 - 3.51 (m, 1H, 5’-Hβ), 1.52 (s, 9H, 2’’-H3, 3’’-H3 & 4’’-H3, t-butyl); 13C NMR (DMSO-d6) δ 154.52 (C-6), 151.67 (C-2), 148.12 (C-4), 139.56 (C-8), 120.20 (C-5), 87.96 (C-1’), 85.83 (C-4’), 73.43 (C-2’), 70.58 (C-3’), 61.62 (C-5’), 51.58 (C-1’’, t-butyl), 28.83 (C-2’’, C-3’’+ C-4’’, t-butyl).

N6-phenyl-adenosine (10). To a suspension of 6-chloropurine riboside (57 mg, 0.20 mmol) in 10 mL of MeOH, 10 eq of aniline (182 µL, 2.0 mmol) and 7.2 eq of dry triethylamine (200 µL, 1.43 mmol) were added and stirred at 60 °C for 24 hours. Reaction was separated first by normal phase chromatography (4 g silica RediSep Column, 0-20% MeOH in DCM, over 55 cv) and later by reverse phase chromatography (4.3 g C18 RediSep Column, 0-30% AcN in DI H2O with 0.1% formic acid, over 70 cv) to yield 30.3 mg (0.09 mmol, 44%) as a white powder. Rf 0.63 (MeOH:DCM = 1:9); 1H NMR (DMSO-d6) δ 9.93 (s, 1H, N6-H), 8.55 (s, 1H, 8-H), 8.41 (s, 1H, 2-H), 7.95 (dd, 2H, 2’’-H + 6’’-H, phenyl, J = 8.50, 0.85 Hz), 7.37 -7.30 (m, 2H, 3’’-H + 5’’-H, phenyl), 7.05 (t, 1H, 4’’-H, phenyl, J = 7.35 Hz), 5.97 (d, 1H, 1’-H, J = 6.04 Hz), 5.50 (d, 1H,
2'-OH, $J = 6.18$ Hz), 5.29 (dd, 1H, 5'-OH, $J = 6.28, 11.31$ Hz), 5.23 (d, 1H, 3'-OH, $J = 4.68$ Hz), 4.65 (dd, 1H, 2'-H, $J = 5.8, 11.1$ Hz), 4.19 (dd, 1H, 3'-H, $J = 4.5, 7.9$ Hz), 4.03 - 3.97 (m, 1H, 4'-H), 3.76 - 3.66 (m, 1H, 5'-Ha), 3.64 - 3.53 (m, 1H, 5'-Hb). 13C NMR (DMSO-d6) $\delta$ 152.14 (C-6), 151.89 (C-2), 149.33 (C-4), 140.64 (C-8), 139.50 (C-1”, phenyl), 128.35 (C-3” + C-5”, phenyl), 122.69 (C-4”, phenyl), 120.88 (C-2” + C-6”, phenyl), 120.33 (C-5), 87.83 (C-1’), 85.82 (C-4’), 73.61 (C-2’), 70.52 (C-3’), 61.52 (C-5’).

**N6-benzyl-adenosine (11).** To a suspension of 6-chloropurine riboside (57 mg, 0.20 mmol) in 10 mL of MeOH, 3 eq of benzylamine (66 µL, 0.6 mmol) and 3.6 eq of dry triethylamine (100 µL, 0.71 mmol) were added and stirred overnight at 60 °C. Reaction was separated first by normal phase chromatography (4 g silica RediSep Column, 0-20% MeOH in DCM, over 55 cv) and again by reverse phase chromatography (4.3 g C18 RediSep Column, 0-30% AcN in DI H2O with 0.1% formic acid, over 70 cv) to yield 38.6 mg (0.11 mmol, 54%) as a white powder. Rf 0.72 (MeOH:DCM = 1:9); 1H NMR (DMSO-d6) $\delta$ 8.44 (bs, 1H, N6-H), 8.38 (s, 1H, 8-H), 8.21 (bs, 1H, 2-H), 7.37 - 7.25 (m, 4H, 3’’-H, 4’’-H, 6’’-H + 7’’-H, benzyl), 7.24 -7.17 (m, 1H, 5’’-H, benzyl), 5.90 (d, 1H, 1’-H, $J = 6.16$ Hz), 5.40 (bs, 3H, 2’-OH, 3’-OH + 5’-OH), 4.72 (bs, 2H, 1’’-H2, benzyl), 4.63 (t, 1H, 2’-H, $J = 5.5$ Hz), 4.16 (dd, 1H, 3’-H, $J = 3.1, 4.8$ Hz), 4.01 - 3.95 (m, 1H, 4’-H), 3.68 (dd, 1H, 5’-Hb, $J = 3.6, 12.1$ Hz), 3.56 (dd, 1H, 5’-Hb, $J = 3.6, 12.1$ Hz). 13C NMR (DMSO-d6) $\delta$ 154.51 (C-6), 152.30 (C-2), 148.01 (C-4), 139.98 (C-2””, benzyl), 139.87 (C-8), 128.16 (C-4” + C-6””, benzyl), 127.07 (C-3” + C-7””, benzyl), 126.57 (C-5””, benzyl), 119.73 (C-5), 87.92 (C-1’), 85.86 (C-4’), 73.47 (C-2’), 70.61 (C-3’), 61.62 (C-5’”), 42.86 (C-1’”, benzyl).
**N<sup>6</sup>-phenethyl-adenosine (12).** To a suspension of 6-chloropurine riboside (57 mg, 0.20 mmol) in 10 mL of MeOH, 3 eq of 2-phenethylamine (76 µL, 0.6 mmol) and 3.6 eq of dry triethylamine (100 µL, 0.71 mmol) were added and stirred overnight at 60 °C. Reaction was separated by normal phase chromatography (4 g silica RediSep Column, 0-20% MeOH in DCM, over 55 cv) to yield 50.1 mg of product as a white powder (0.13 mmol, 67%). R<sub>f</sub> 0.79 (MeOH:DCM = 1:9); ¹H NMR (DMSO-<i>d</i><sub>6</sub>) δ 8.36 (bs, 1H, 8-H), 8.25 (bs, 1H, 2-H), 7.92 (bs, 1H, N<sup>6</sup>-H), 7.34 - 7.23 (m, 4H, 4''-H, 5''-H, 7''-H + 8''-H), 7.22 - 7.16 (m, 1H, 6''-H, phenethyl), 5.90 (d, 1H, 1'-H, <i>J</i> = 6.12 Hz), 5.46 - 5.37 (m, 2H, 2'-OH + 5'-OH), 5.18 (d, 1H, 3'-OH), 4.62 (dd, 1H, 2'-H, <i>J</i> = 6.12, 11.25 Hz), 4.19 - 4.13 (m, 1H, 3'-H), 3.98 (dd, 1H, 4'-H, <i>J</i> = 3.4, 6.4 Hz), 3.80 - 3.64 (m, 3H, 1''-H<sub>2</sub>, phenethyl, & 5''-H<sub>6</sub>), 3.62 - 3.57 (m, 1H, 5''-H<sub>6</sub>), 2.93 (dd, 2H, 2''-H<sub>2</sub>, phenethyl, <i>J</i> = 7.83, 15.00 Hz). ¹³C NMR (DMSO-<i>d</i><sub>6</sub>) δ 154.53 (C-6), 152.37 (C-2), 148.26 (C-4), 139.71 (C-8), 139.48 (C-3''', phenethyl), 128.64 (C-5''' + C-7''', phenethyl), 128.28 (C-4''' + C-8''', phenethyl), 126.01 (C-6''', phenethyl), 119.75 (C-5'), 87.90 (C-1'), 85.87 (C-4'), 73.46 (C-2'), 70.62 (C-3''), 61.64 (C-5'), 41.25 (C-1''', phenethyl), 34.95 (C-2''', phenethyl).

**N<sup>6</sup>-4-methylbenzyl-adenosine (13).** To a suspension of 6-chloropurine riboside (57 mg, 0.20 mmol) in 10 mL of MeOH, 3 eq of 4-methylbenzylamine (76 µL, 0.6 mmol) and 3.6 eq of dry triethylamine (100 µL, 0.71 mmol) were added and stirred overnight at 60 °C. Reaction was separated first by normal phase chromatography (4 g silica RediSep Column, 0-20% MeOH in DCM, over 55 cv) and again by reverse phase chromatography (4.3 g C18 RediSep Column, 0-30% AcN in DI H<sub>2</sub>O with 0.1% formic acid, over 70 cv) to yield 46.4 mg of product as a white granular powder (0.12 mmol, 62%). R<sub>f</sub> 0.59 (MeOH:DCM = 1:9); ¹H NMR (DMSO-<i>d</i><sub>6</sub>) δ 8.39
(bs, 1H, N⁶-H), 8.37 (s, 1H, 8-H), 8.20 (bs, 1H, 2-H), 7.23 (d, 2H, 3''-H + 7''-H, methylbenzyl, \( J = 7.97 \) Hz), 7.09 (d, 2H, 4''-H + 6''-H, methylbenzyl, \( J = 7.84 \) Hz), 5.90 (d, 1H, 1'-H, \( J = 6.16 \) Hz), 5.43 (d, 1H, 2'-OH, \( J = 6.24 \) Hz), 5.38 (dd, 1H, 5'-OH, \( J = 4.64, 6.28 \) Hz), 5.17 (d, 1H, 3'-OH, \( J = 4.60 \) Hz), 4.67 (bs, 2H, 1''-H₂, methylbenzyl), 4.63 (dd, 1H, 2'-H, \( J = 6.0, 11.3 \) Hz), 4.19 - 4.13 (m, 1H, 3'-H), 3.98 (dd, 1H, 4'-H, \( J = 3.44, 6.64 \) Hz), 3.74 - 3.64 (m, 1H, 5'-Hₐ), 3.61 - 3.51 (m, 1H, 5'-Hₐ). ¹³C NMR (DMSO-\( d_6 \)) \( \delta \) 154.49 (C-6), 152.29 (C-2), 148.43 (C-4), 139.82 (C-8), 136.91 (C-2'', methylbenzyl), 135.57 (C-5'', methylbenzyl), 128.70 (C-4'' + C-6'', methylbenzyl), 127.08 (C-3'' + C-7'', methylbenzyl), 119.72 (C-5), 87.92 (C-1'), 85.87 (C-4'), 73.45 (C-2'), 70.62 (C-3'), 61.63 (C-5'), 42.60 (C-1'', methylbenzyl).

\( N^6-4 \)-chlorobenzyl-adenosine (14). To a suspension of 6-chloropurine riboside (57 mg, 0.20 mmol) in 10 mL of MeOH, 3 eq of 4-chlorobenzylamine (73 µL, 0.6 mmol) and 3.6 eq of dry triethylamine (100 µL, 0.71 mmol) were added and stirred overnight at 60 °C. Reaction was separated first by normal phase chromatography (4 g silica RediSep Column, 0-20% MeOH in DCM, over 55 cv) and again by reverse phase chromatography (4.3 g C18 RediSep Column, 0-30% AcN in DI H₂O with 0.1% formic acid, over 70 cv) to yield 56.7 mg (0.14 mmol, 72%) as light amber crystals. Rₚ 0.60 (MeOH:DCM = 1:9); ¹H NMR (DMSO-\( d_6 \)) \( \delta \) 8.48 (bs, 1H, N⁶-H), 8.40 (s, 1H, 8-H), 8.21 (bs, 1H, 2-H), 7.35 (s, 4H, 3''-H, 4''-H, 6''-H + 7''-H, chlorobenzyl), 5.91 (d, 1H, 1'-H, \( J = 6.12 \) Hz), 5.40 (bs, 3H, 2'-OH, 3'-OH + 5'-OH), 4.70 (bs, 2H, 1''-H₂, chlorobenzyl), 4.62 (dd, 1H, 2'-H, \( J = 5.6, 11.0 \) Hz), 4.17 (dd, 1H, 3'-H, \( J = 3.1, 4.8 \) Hz), 4.00 - 3.94 (m, 1H, 4'-H), 3.68 (dd, 1H, 5'-Hₐ, \( J = 3.7, 12.1 \) Hz), 3.56 (dd, 1H, 5'-Hₐ, \( J = 3.7, 12.1 \) Hz). ¹³C NMR (DMSO-\( d_6 \)) \( \delta \) 154.38 (C-6), 152.29 (C-2), 148.52 (C-4), 139.94 (C-8), 139.03 (C-2'').
chlorobenzyl), 131.12 (C-5'', chlorobenzyl), 128.95 (C-4'' + C-6'', chlorobenzyl), 128.11 (C-3'' + C-7'', chlorobenzyl), 119.73 (C-5), 87.90 (C-1’), 85.85 (C-4’), 73.50 (C-2’), 70.60 (C-3’), 61.60 (C-5’), 42.30 (C-1’’, chlorobenzyl).

\textbf{\textit{N}}^6-\textit{4-methoxybenzyl-adenosine (15).} To a suspension of 6-chloropurine riboside (57 mg, 0.20 mmol) in 10 mL of MeOH, 3 eq of 4-methoxybenzylamine (78 µL, 0.6 mmol) and 3.6 eq of dry triethylamine (100 µL, 0.71 mmol) were added and stirred overnight at 60 °C. Reaction was separated by normal phase chromatography (4 g silica RediSep Column, 0-20% MeOH in DCM, over 55 cv) to yield 46.3 mg of product as a white powder (0.12 mmol, 60%). \textit{R}^f 0.64 (MeOH:DCM = 1:9); ^1\text{H} NMR (DMSO-\textit{d}$_6$) \(\delta\) 8.37 (bs, 2H, 8-H + N\textit{6}-H), 8.21 (bs, 1H, 2-H), 7.28 (d, 2H, 3''-H + 7''-H, methoxybenzyl, \(J\) = 8.65 Hz), 6.88 - 6.83 (m, 2H, 4''-H + 6''-H, methoxybenzyl), 5.90 (d, 1H, 1’-H, \(J\) = 6.12 Hz), 5.44 (d, 1H, 2’-OH, \(J\) = 4.24 Hz), 5.39 (bs, 1H, 5’-OH), 5.18 (bs, 1H, 3’-OH), 4.63 (bs, 3H, 2’-H + 1’’-H$_2$, methylbenzyl), 4.16 (bs, 1H, 3’-H), 3.97 (dd, 1H, 4’-H, \(J\) = 3.44, 6.64 Hz), 3.71 (s, 3H, 8’’-H$_3$, methoxy), 3.68 - 3.64 (m, 1H, 5’-H$_3$), 3.60 - 3.52 (m, 1H, 5’-H$_3$). \textit{\textit{13}}C NMR (DMSO-\textit{d}$_6$) \(\delta\) 158.09 (C-5’’, methoxybenzyl), 154.43 (C-6), 152.29 (C-2), 148.41 (C-4), 139.80 (C-8), 131.91 (C-2’’, methoxybenzyl), 128.47 (C-3’’ + C-7’’, methoxybenzyl), 119.75 (C-5), 113.59 (C-4’’ + C-6’’, methoxybenzyl), 87.92 (C-1’), 85.86 (C-4’), 73.45 (C-2’), 70.61 (C-3’), 61.63 (C-5’), 54.98 (C-8’’, methoxy), 42.29 (C-1’’, methoxybenzyl).

\textbf{\textit{N}}^6-\textit{4-pyridinemethyl-adenosine (16).} To a suspension of 6-chloropurine riboside (57 mg, 0.20 mmol) in 10 mL of MeOH, 3 eq of 4-(aminomethyl)pyridine (77 µL, 0.6 mmol) and 3.6 eq of dry triethylamine (100 µL, 0.71 mmol) were added and stirred overnight at 60 °C. Reaction was separated by normal phase chromatography (4 g silica RediSep Column, 0-20% MeOH in DCM, over 55 cv) to yield 46.3 mg of product as a white powder (0.12 mmol, 60%). \textit{R}^f 0.64 (MeOH:DCM = 1:9); ^1\text{H} NMR (DMSO-\textit{d}$_6$) \(\delta\) 8.37 (bs, 2H, 8-H + N\textit{6}-H), 8.21 (bs, 1H, 2-H), 7.28 (d, 2H, 3''-H + 7''-H, methoxybenzyl, \(J\) = 8.65 Hz), 6.88 - 6.83 (m, 2H, 4''-H + 6''-H, methoxybenzyl), 5.90 (d, 1H, 1’-H, \(J\) = 6.12 Hz), 5.44 (d, 1H, 2’-OH, \(J\) = 4.24 Hz), 5.39 (bs, 1H, 5’-OH), 5.18 (bs, 1H, 3’-OH), 4.63 (bs, 3H, 2’-H + 1’’-H$_2$, methylbenzyl), 4.16 (bs, 1H, 3’-H), 3.97 (dd, 1H, 4’-H, \(J\) = 3.44, 6.64 Hz), 3.71 (s, 3H, 8’’-H$_3$, methoxy), 3.68 - 3.64 (m, 1H, 5’-H$_3$), 3.60 - 3.52 (m, 1H, 5’-H$_3$). \textit{\textit{13}}C NMR (DMSO-\textit{d}$_6$) \(\delta\) 158.09 (C-5’’, methoxybenzyl), 154.43 (C-6), 152.29 (C-2), 148.41 (C-4), 139.80 (C-8), 131.91 (C-2’’, methoxybenzyl), 128.47 (C-3’’ + C-7’’, methoxybenzyl), 119.75 (C-5), 113.59 (C-4’’ + C-6’’, methoxybenzyl), 87.92 (C-1’), 85.86 (C-4’), 73.45 (C-2’), 70.61 (C-3’), 61.63 (C-5’), 54.98 (C-8’’, methoxy), 42.29 (C-1’’, methoxybenzyl).
triethylamine (100 µL, 0.71 mmol) were added and stirred overnight at 60 ºC. Reaction was separated by normal phase chromatography (4 g silica RediSep Column, 0-60% MeOH in DCM, over 120 cv) to yield 69.2 mg of product as an orange powder (0.19 mmol, 97%, crude, ~20% of weight may be 4-pyridinemethanamine based on NMR). Rf 0.81 (MeOH:DCM = 1:5); 1H NMR (DMSO-d6) δ 8.53 (bs, 1H, N6-H), 8.47 (dd, 2H, 4''-H + 6''-H, pyridinemethyl, J = 1.60, 4.44 Hz), 8.35 (s, 1H, 8-H), 8.20 (s, 1H, 2-H), 7.30 (d, 2H, 3''-H + 7''-H, pyridinemethyl, J = 5.80 Hz), 5.91 (d, 1H, 1'-H, J = 6.12 Hz), 5.34 (bs, 3H, 2'-OH, 3'-OH + 5'-OH), 4.73 (bs, 2H, 1''-H, pyridinemethyl), 4.62 (dd, 1H, 2'-H, J = 5.5, 10.8 Hz), 4.16 (dd, 1H, 3'-H, J = 3.1, 4.8 Hz), 4.00 - 3.95 (m, 1H, 4'-H), 3.68 (dd, 1H, 5'-Ha, J = 3.68, 12.13 Hz), 3.56 (dd, 1H, 5'-Hb, J = 3.68, 12.09 Hz); 13C NMR (DMSO-d6) δ 154.48 (C-6), 152.27 (C-2), 149.41 (C-4’’ + C-6’’, pyridinemethyl), 149.02 (C-4), 140.08 (C-8), 122.00 (C-3’’ + C-7’’, pyridinemethyl), 119.89 (C-5), 87.87 (C-1’), 85.84 (C-4’), 73.50 (C-2’), 70.58 (C-3’), 61.58 (C-5’), 41.94 (C-1’’, pyridinemethyl); MS m/z 359.034, expected 359.1468 (M + H)+.

N6-tetrazole-adenosine (17). To a suspension of 6-chloropurine riboside (57 mg, 0.20 mmol) in 10 mL of MeOH, 9 eq of 5-aminotetrazole (151 mg, 1.8 mmol) and 7.2 eq of dry triethylamine (200 µL, 1.42 mmol) were combined in a sealed tube and stirred for 72 hours at 80 ºC. Reaction was separated by normal phase chromatography (4 g silica RediSep Column, 0-50% MeOH in DCM, over 55 cv) to yield 23 mg of product as an orange powder (0.07 mmol, 34%, crude, ~10% of weight may be Et3N based on NMR). Rf 0.11 (MeOH:DCM = 1:4); 1H NMR (DMSO-d6) δ 8.58 (s, 1H, 8-H), 8.42 (s, 1H, 2-H), 6.47 (bs, 1H, N6-H), 5.98 (d, 1H, 1’-H, J = 5.88 Hz), 5.70 - 4.85 (bs, 3H, 2’-OH, 3’-OH & 5’-OH), 4.63 (dd, 1H, 2’-H, J = 5.56, 11.84 Hz), 4.19 (dd,
$^{1}$H, 3'-H, $J = 3.52, 4.80$ Hz), 4.00 - 3.95 (m, 1H, 4'-H), 3.71 (dd, 1H, 5'-H$_a$, $J = 3.84, 12.04$ Hz), 3.56 (dd, 1H, 5'-H$_b$, $J = 3.84, 12.04$ Hz). $^{13}$C NMR (DMSO-$d_6$) $\delta$ 153.72 (C-6), 151.67 (C-2), 151.27 (C-1”), tetrazole) 150.02 (C-4), 141.31 (C-8), 120.23 (C-5), 87.78 (C-1’), 85.76 (C-4'), 73.67 (C-2’), 70.42 (C-3’), 61.42 (C-5’).

$N^6$-cyclohexyl-adenosine (18). To a suspension of 6-chloropurine riboside (57 mg, 0.20 mmol) in 5 mL of MeOH, 3 eq of cyclohexylamine (69 µL, 0.6 mmol) and 3 eq of dry triethylamine (84 µL, 0.6 mmol) were added then stirred overnight at 60 °C. Based on TLC, a substantial amount of product was formed but did not separate adequately from the small amount of starting material even after two attempts of normal phase chromatography (4 g silica RediSep Column, 0-20% & 0-10% MeOH in DCM). Separation was improved slightly using amine-functionalized silica (4.7 g Amine RediSep Column, 0-10% DCM in MeOH over 60 column volumes) to yield 22 mg (0.06 mmol, 31%) as clear glassy solid. Rf 0.67 (MeOH:DCM = 1:9); $^1$H NMR (DMSO-$d_6$) $\delta$ 8.34 (s, 1H, 8-H), 8.19 (bs, 1H, 2-H), 7.85 (bd, 1H, N$^6$-H, $J = 7.88$ Hz), 5.88 (d, 1H, 1'-H, $J = 6.20$ Hz), 5.50 - 5.35 (m, 2H, 2’-OH & 5’-OH), 5.16 (d, 1H, 3'-OH, $J = 4.64$ Hz), 4.61 (dd, 1H, 2’-H, $J = 6.04, 11.28$ Hz), 4.15 (t, 1H, 3'-H, $J = 3.16, 4.72, 7.76$ Hz), 4.09? (m, 1H, 1’-H, cyclohexyl), 3.97 (dd, 1H, 4’-H, $J= 3.4, 6.5$ Hz), 3.72 - 3.64 (m, 1H, 5'-H$_a$), 3.60 - 3.51 (m, 1H, 5’-H$_b$), 1.88 (bd, 2H, 2’'-H$_{\text{equatorial}}$ + 6’’-H$_{\text{equatorial}}$, cyclohexyl, $J = 7.96$ Hz), 1.75 (bd, 2H, 3’’-H$_{\text{equatorial}}$ + 5’’-H$_{\text{equatorial}}$, cyclohexyl, $J = 12.28$ Hz), 1.62 (bd, 1H, 4’’-H$_{\text{equatorial}}$, cyclohexyl, $J = 12.72$ Hz), 1.52 - 1.22 (m, 4H, 2''-H$_{\text{axial}}$, 3''-H$_{\text{axial}}$, 5''-H$_{\text{axial}}$ & 6’’-H$_{\text{axial}}$, cyclohexyl), 1.21 - 1.03 (m, 1H, 4’’-H$_{\text{axial}}$, cyclohexyl).$^{13}$C NMR (DMSO-$d_6$) $\delta$ 153.91 (C-6), 152.29 (C-2), 148.31 (C-4), 139.53 (C-8), 119.64 (C-5), 87.96 (C-1’), 85.88 (C-4’), 73.43 (C-2’), 70.64 (C-3’), 61.66 (C-4’).
(C-5’), 48.76 (C-1’’, cyclohexyl), 32.25 (C-2’’ & C-6’’, cyclohexyl), 25.19 (C-4’’’, cyclohexyl),
24.95 (C-3’’ & C-5’’’, cyclohexyl).

\(N^6\)-tetrahydropyran-adenosine (19). To a suspension of 6-chloropurine riboside (57 mg, 0.20
mmol) in 5 mL of MeOH, 3 eq of 4-aminotetrahydropyran (60.7 mg, 0.6 mmol) and 3 eq of dry
triethylamine (84 µL, 0.6 mmol) were added then stirred overnight at 60 °C. Reaction was
separated by normal phase chromatography (4 g silica RediSep Column, 0-10% MeOH in DCM,
over 40 cv) to yield 28.6 mg (0.08 mmol, 41%) as a clear glassy solid. \(R_f\) 0.46 (MeOH:DCM =
1:9); \(^1\)H NMR (DMSO-\(d_6\)) \(\delta\) 8.37 (s, 1H, 8-H), 8.21 (bs, 1H, 2-H), 7.80 (bs, 1H, \(N^6\)-H), 5.89 (d,
1H, 1’-H, \(J = 6.16\) Hz), 5.46 - 5.36 (m, 2H, 2’-OH & 5’-OH), 5.17 (d, 1H, 3’-OH, \(J = 4.64\) Hz),
4.61 (dd, 1H, 2’-H, \(J = 6.1, 11.3\) Hz), 4.34 (bs, 1H, 1’’-H, tetrahydropyran), 4.17 - 4.13 (m, 1H,
3’-H), 3.97 (dd, 1H, 4’-H, \(J = 3.40, 6.60\) Hz), 3.90 (dd, 2H, 3’’-H\(_{equatorial}\) + 4’’-H\(_{equatorial}\),
tetrahydropyran, \(J = 2, 12\) Hz), 3.72 - 3.64 (m, 1H, 5’-H\(_a\)), 3.60 - 3.52 (m, 1H, 5’-H\(_b\)), 3.41 (dt,
2H, 3’’-H\(_{axial}\) + 4’’-H\(_{axial}\), cyclohexyl, \(J = 12, 2\) Hz), 1.81 (bd, 2H, 2’’’-H\(_{equatorial}\) + 5’’’-H
\(_{equatorial}\), tetrahydropyran, \(J = 10.8\) Hz), 1.81 (bd, 2H, 2’’-H\(_{axial}\) + 5’’-H\(_{axial}\), tetrahydropyran, \(J\)
= 9.52 Hz). \(^13\)C NMR (DMSO-\(d_6\)) \(\delta\) 153.90 (C-6), 152.26 (C-2), 148.56 (C-4), 139.69 (C-8),
119.75 (C-5), 87.92 (C-1’), 85.86 (C-4’), 73.47 (C-2’), 70.61 (C-3’), 66.24 (C-3’’ & C-4’’’,
tetrahydropyran), 61.66 (C-5’), 46.17 (C-1’’, tetrahydropyran), 32.40 (C-2’’ & C-5’’’,
tetrahydropyran).

\(N^6\)-piperidine-adenosine (20). To a suspension of 6-chloropurine riboside (57 mg, 0.20 mmol)
in 5 mL of MeOH, 3 eq of 4-Aminopiperidine (64 µL, 0.6 mmol) and 3 eq of dry triethylamine
(84 µL, 0.6 mmol) were added then stirred overnight at 60 ºC. Reaction was separated by normal phase chromatography (4 g silica RediSep Column, 0-25% MeOH in DCM, over 70 cv) to yield 52.3 mg (0.15 mmol, 75%) as light yellow crystals. \( R_f \) 0.08 (MeOH:DCM = 1:9); \(^1\)H NMR (DMSO-\(d_6\)) \( \delta \) 8.44 (s, 1H, 8-H), 8.26 (s, 1H, 2-H), 5.93 (d, 1H, 1'-H, \( J = 5.93 \) Hz), 5.32 (bs, 2H, 3'”-H(equatorial) + 5’”-H(equatorial), piperidine), 4.58 (dd, 1H, 2’-H, \( J = 5.62, 5.34 \) Hz), 4.17 (dd, 1H, 3’-H, \( J = 3.46, 4.83 \) Hz), 3.97 (dd, 1H, 4’-H, \( J = 3.5, 7.0 \) Hz), 3.68 (dd, 1H, 5’-H, \( J = 3.66, 12.07 \) Hz), 3.56 (dd, 1H, 5’-H, \( J = 3.73, 12.07 \) Hz), 3.36 - 3.26 (m, 1H, 1’’-H, cyclohexyl), 3.26 - 3.17 (m, 2H, 3’’-H(axial) + 5’’-H(axial), piperidine), 2.02 (bd, 2H, 2’’-H(equatorial) + 6’’-H(equatorial), piperidine, \( J = 12.47 \) Hz), 1.58 - 1.42 (m, 2H, 2’’-H(axial) + 6’’-H(axial), piperidine). \(^{13}\)C NMR (DMSO-\(d_6\)) \( \delta \) 153.10 (C-6), 151.78 (C-2), 150.32 (C-4), 138.89 (C-8), 119.60 (C-5), 87.75 (C-1’), 85.72 (C-4’), 73.52 (C-2’), 70.44 (C-3’), 61.44 (C-5’), 47.59 (C-1’’, piperidine), 42.96 (C-3” & C-5”’, piperidine), 30.43 (C-2”’ & C-6”’, piperidine); MS m/z 350.932, expected 351.178 (M + H). 

\( N^6 \)-methylene cyclopropyl-adenosine (21). To a suspension of 6-chloropurine riboside (57 mg, 0.20 mmol) in 5 mL of MeOH, 3 eq of aminomethylcyclopropane (52 µL, 0.6 mmol) and 3 eq of dry triethylamine (84 µL, 0.6 mmol) were added then stirred overnight at 60 ºC. Reaction was separated by normal phase chromatography (4 g silica RediSep Column, 0-10% MeOH in DCM, over 40 cv) to yield 52.7 mg (0.16 mmol, 82%) as fluffy crystals/powder. \( R_f \) 0.81 (MeOH:DCM = 3:17); \(^1\)H NMR (DMSO-\(d_6\)) \( \delta \) 8.35 (s, 1H, 8-H), 8.20 (bs, 1H, 2-H), 7.93 (bs, 1H, \( N^6 \)-H), 5.89 (d, 1H, 1’-H, \( J = 6.24 \) Hz), 5.46 - 5.38 (m, 2H, 2’-OH & 5’-OH), 5.17 (d, 1H, 3’-OH, \( J = 4.60 \) Hz), 4.62 (dd, 1H, 2’-H, \( J = 6.1, 11.3 \) Hz), 4.18 - 4.13 (m, 1H, 3’-H), 3.98 (dd, 1H, 4’-H, \( J = 3.5, \)
6.5 Hz), 3.73 - 3.64 (m, 1H, 5’-H_a), 3.61 - 3.52 (m, 1H, 5’-H_b), 3.37 (bs, 2H, 1’’-H_2, methylcyclopropyl), 1.21 - 1.09 (m, 1H, 2’’-H, methylcyclopropyl), 0.45 - 0.38 (m, 2H, 3’’-H_a + 4’’-H_a, methylcyclopropyl), 0.31 - 0.24 (m, 2H, 3’’-H_b + 4’’-H_b, methylcyclopropyl). 13C NMR (DMSO-d6) δ 154.61 (C-6), 152.29 (C-2), 148.31 (C-4), 139.31 (C-8), 119.65 (C-5), 87.94 (C-1’), 85.88 (C-4’), 73.45 (C-2’), 70.63 (C-3’), 61.65 (C-5’), 43.95 (C-1’’), methylcyclopropyl), 11.11 (C-2’’), methylcyclopropyl), 3.21 (C-3’’ & C-4’’, methylcyclopropyl).

N^6-propargyl-adenosine (22). To a suspension of 6-chloropurine riboside (57 mg, 0.20 mmol) in 10 mL of MeOH, 3 eq of propargylamine (38 µL, 0.6 mmol) and 3.6 eq of dry triethylamine (100 µL, 0.71 mmol) were added and stirred overnight at 60 °C. Reaction was separated by normal phase chromatography (4 g silica RediSep Column, 0-15% MeOH in DCM, over 38 cv), a yellowish residue on a dried fraction was rinsed off 3x with ~1 mL MeOH to yield 27 mg (0.09 mmol, 44%) as a spongy white powder. R_f 0.30 (MeOH:DCM = 1:9); 1H NMR (DMSO-d6) δ 8.41 (s, 1H, 8-H), 8.29 (bs, 1H, 2-H), 8.26 (bs, 1H, N^6-H), 5.92 (d, 1H, 1’-H, J = 6.08 Hz), 5.46 (d, 1H, 2’-OH, J = 6.21 Hz), 5.35 (dd, 1H, 5’-OH, J = 6.97, 4.68 Hz), 5.20 (d, 1H, 3’-OH, J = 4.72 Hz), 4.65 - 4.58 (m, 1H, 2’-H), 4.27 (bs, 2H, 1’’-H_2, propargyl), 4.19 - 4.14 (m, 1H, 3’-H), 4.01 - 3.95(m, 1H, 4’-H), 3.74 - 3.65 (m, 1H, 5’-H_a), 3.61 - 3.53 (m, 1H, 5’’-H_b), 3.04 (bs, 1H, 3’’-H, propargyl). 13C NMR (DMSO-d6) δ 153.91 (C-6), 152.19 (C-2), 148.80 (C-4), 140.14 (C-8), 119.83 (C-5), 87.85 (C-1’), 85.81 (C-4’), 81.79 (C-2’’, propargyl), 73.50 (C-2’’), 72.34 (C-3’’, propargyl), 70.55 (C-3’), 61.57 (C-5’), 29.18 (C-1’’, propargyl).
\textbf{N^6-allyl-adenosine (23).} To a suspension of 6-chloropurine riboside (57 mg, 0.20 mmol) in 10 mL of MeOH, 3 eq of allylamine (45 µL, 0.6 mmol) and 3.6 eq of dry triethylamine (100 µL, 0.71 mmol) were added and stirred overnight at 60 °C. Reaction was separated by normal phase chromatography (4 g silica RediSep Column, 0-12\% MeOH in DCM, over 34 cv) to yield 26 mg (0.08 mmol, 42\%) as a white powder. \textit{R_f} 0.40 (MeOH:DCM = 1:9); \textit{^1}H NMR (DMSO-\textit{d}_6) \delta 8.37 (s, 1H, 8-H), 8.21 (s, 1H, 2-H), 8.06 (bs, 1H, N^6-H), 6.02 - 5.92 (m, 1H, 2''-H, allyl), 5.90 (d, 1H, 1'-H, \textit{J} = 6.21 Hz), 5.16 5.45 (d, 1H, 2'-OH, \textit{J} = 6.2 Hz), 5.40 (dd, 1H, 5'-OH, \textit{J} = 4.56, 7.12 Hz), 5.19 (d, 1H, 3'-OH, \textit{J} = 6.2 Hz), 5.18 - 5.11 (m, 1H, 3''-H\textit{cis}, allyl), 5.06 (ddd, 1H, 3''-H\textit{trans}, allyl, \textit{J} = 1.48, 3.20, 10.28 Hz), 4.68 - 4.56 (m, 1H, 2'-H), 4.23 - 4.05 (m, 3H, 3'-H, + 1''-H\textit{cis}, allyl), 4.02 - 3.94 (m, 1H, 4'-H), 3.73 - 3.63 (m, 1H, 5'-H\textit{a}), 3.60 - 3.52 (m, 1H, 5'-H\textit{b}). \textit{^13}C NMR (DMSO-\textit{d}_6) \delta 154.43 (C-6), 152.28 (C-2), 148.35 (C-4), 139.78 (C-8), 135.58 (C-2'', allyl), 119.72 (C-5), 114.97 (C-3'', allyl), 87.88 (C-1’), 85.86 (C-4’), 73.43 (C-2’), 70.62 (C-3’), 61.63 (C-5’), 41.91 (C-1’’, allyl).
CHAPTER 4: Synthesis of Second-Generation Compounds

Rationale

The original goal for synthesizing first-generation compounds was to design inhibitors that targeted the more selective, substrate-binding pocket of ErmC’ or KsgA. We had expected these adenosine analogs (1-23) to orient in a similar manner as the putative target adenosine, and for the N6-substituents to branch into the adjacent SAM-binding pocket. However, X-ray data of ErmC’ in complex with 7 and 12 revealed that the SAM pocket was the preferred binding site for at least these two inhibitors, and that the N6-substituents were directed away from the substrate-binding pocket. Furthermore, these co-crystals of ErmC’ showed the 5’-C of 7 and 12 were pointed directly at the originally targeted substrate pocket of ErmC’ (Figure 31).

Figure 31. First-generation compounds 7 (pink) and 12 (cyan) bound in SAM pocket of ErmC’ (wheat) with 5’-C pointed towards the adenosine pocket. Shown in gray are the target adenine and the “flipped” tyrosine 104, (Tyr-108) in M.TaqI + DNA (PDB 1G38),41 which were superimposed over ErmC’ same as in Figure 7 (Chapter 1).
This unanticipated binding mode presented us with the opportunity to design a new library of compounds which could bridge into the substrate pocket through a second substituent. Our new goal was to add a phenyl substituent to the 5’-carbon on one of the first-generation inhibitors in order to improve potency and/or selectivity. We hypothesized that an aromatic group extending off the 5’-C of an adenosine analog could form π-π stacking with the essential tyrosine (Tyr-104 in ErmC’, or Tyr-116 in KsgA), similar to the interactions that have been predicted for the target adenosine and the “flipped” tyrosine.

To test this principle, we synthesized four derivatives of N6-cyclopentyl adenosine (1). For each of these analogues, the 5’-carbon was attached with an aromatic amine, which replaced the 5’-OH of 1 with a 5’-N. For these second-generation compounds, the linker between the 5’-N and the phenyl ring varied from 1 to 4 carbons (Figure 32). Additionally, the replacement of 5’-OH with a secondary amine could have acted as a bioisostere for the sulfur atom in SAM. We selected 1 to use as the scaffold in this limited series of compounds because this inhibitor: i) had been referenced by other relevant publications; ii) showed inhibition for both KsgA and ErmC’; and iii) did not have a potentially reactive N⁶-substituent (ex: the octylamine on 7).

Figure 32. Chemical structures of second-generation compounds (30-33)
Synthesis

Several different strategies were explored for synthesizing second-generation compounds. The follow section first describes the many synthetic routes that were attempted, but were ultimately unsuccessful at producing the desired final product (Figures 33-39). After a successful reaction scheme was found to yield 31, the remaining second-generation compounds (30, 32 and 33) were synthesized using this same strategy (Table 5).

Mitsunobu Reactions

The simplest and most direct route that we conceived for adding the 5’-substituent was tried first, the Mitsunobu reaction. This reaction is often used to couple an alcohol with another group containing a nucleophilic function (ex. carboxylic acids, phenols, or imides). Typically, reagents such as diisopropyl azodicarboxylate (DIAD) and triphenylphosphine (TPP) are used to form a reactive intermediate (Morrison–Brunn–Huisgen betaine), which acts to both deprotonate the nucleophile and bind the alcohol. The deprotonated nucleophile then substitutes the alcohol resulting in the loss of an H₂O, thereby reducing DIAD to diisopropyl hydrazinedicarboxylate and oxidizing TPP to triphenylphosphine oxide.

For our reaction, we expected the amine on phenethylamine to act as the nucleophilic component and substitute the 5’-OH on 6-chloropurine riboside (Figure 33). If successful, we could have made 31 by later adding the N⁶-cyclopentyl (as reacted in Chapter 3). In the first attempt, 60 mg of starting material (6-chloropurine riboside) was used to yield ~5 mg of a product that resembled 31 by NMR (albeit, at a low purity and poor yield). However, mass analysis of this compound reported 372.140 m/z and not the expected 390.126 m/z (M + H)⁺.
Therefore, we repeated this reaction on a larger scale (1 g of starting material) and purified the product more extensively. However, the NMR spectra from the latter reaction clearly matched the spectra of the similar but distinct product 12 (previously generated in Chapter 3). Furthermore, the mass analysis from the former reaction also indicated that the product was 12 (expected 372.1666 m/z) (M + H)^+. It was concluded that the 6-Cl group on the starting material was more readily substituted than the 5’-OH group during this Mitsunobu reaction.

![Mitsunobu reaction with 6-chloropurine riboside and phenethylamine. This reaction made the N^6-substitution product (12) rather than the desired C5’-substitution. The dotted arrow shows the subsequent reaction that would have been used to obtain the final product 31.](image)

The Mitsunobu reaction was performed again on adenosine, without the reactive 6-Cl group (Figure 34). If successful, we could have later added the N^6-substituent by reductive amination or by converting the N^6 to the more familiar 6-Cl group (based on a previous report). The first attempt used adenosine that had been protected with isopropylidene on the 2’-OH and 3’-OH groups, but no products were generated (top, Figure 34). Similarly, when this reaction was repeated using the unprotected adenosine (plus 1 eq. of HCl), no products were formed (bottom, Figure 34).
Figure 34. Mitsunobu reaction with adenosine and phenethylamine. Neither attempts with the protected adenosine (top) or the unprotected adenosine + 1 eq. of HCl (bottom) yielded any products, as seen by TLC.

As a new strategy, we tried performing substitution reactions on the ribose alone, which could later be joined to the adenine portion via a glycosylation reaction (based on a previous report). After all secondary alcohols of the ribose were protected with isopropylidene, as previously described (1st step, Figure 35), we tried the Mitsunobu reaction to substitute the 5-OH group with phenethylamine (2nd step, Figure 35), but no products were formed.

Figure 35. Mitsunobu reaction with protected ribose and phenethylamine. After protecting the ribose with isopropylidene (left), the Mitsunobu reaction was attempted to add phenethylamine (right), but TLC showed only starting material.

† Product matched NMR from previous work by Gyepes et al.97
It’s unclear why the Mitsunobu reaction failed to substitute the primary alcohol with phenethylamine in any of the aforementioned reactions. It was suggested in the literature that the order in which reagents are added may make the difference in whether this reaction is successful or not.\textsuperscript{98} [Note: For the reactions in Figures 34 and 35, the alcohol (i.e., the 5-OH on ribose or the 5’-OH on adenosine) and phenethylamine were first dissolved in tetrahydrofuran (THF). Then, TPP was added and the reaction vial was cooled to -20 °C before adding DIAD.] Perhaps the reaction would have worked if DIAD and TPP were first mixed, then added to phenethylamine or the alcohol. Nonetheless, the Mitsunobu reaction was abandoned for the remainder of this report, though the product from the first step in Figure 35 (the protected ribose) was carried forward to subsequent reactions with slightly better success.

\textit{Substitution of 5-OH on ribose}

Continuing with our strategy of reacting ribose before the adenine portion is attached, we replaced the 5-OH of ribose with a leaving group, which could then be substituted with phenethylamine (Figure 36). This first reaction successfully added the mesylate group to ribose, following a previous method\textsuperscript{97} (top left, Figure 36). We then attempted to replace the mesylate with phenethylamine (1.5 eq.) in the presence of potassium carbonate (1.25 eq.) via an $S_{\text{n}2}$ reaction (top right, Figure 36). However, no products were formed despite raising the temperature to 80 °C and extending the reaction overnight (monitored by TLC stained with Ninhydrin and Hanessian’s stain). Likewise, when this reaction was repeated using a stronger base (1 eq. of sodium hydride) no products were formed (middle right, Figure 36).
Taking a slightly different route, we added a more labile leaving group, tosylate, to the 5-OH of our protected ribose (bottom left, Figure 36), in accordance with a previously published procedure.\(^9^9\) Next, the tosylated ribose was dissolved into phenethylamine (10 eq.) for a neat reaction at 85 °C (bottom right, Figure 36). After being purified by normal phase chromatography, the desired product was confirmed by NMR.

**Attempts to deprotect ribose**

After substituting the 5-OH of ribose with phenethylamine, the remaining hydroxyls needed to be deprotected before we could attempt to join the adenine portion of **31** by
glycosylation. Our first attempt followed a procedure previously used to deprotect an adenosine analog, which also had an amine substituent at the 5’ position. In a similar manner, our protected 5-phenethylamine ribose (400 mg) was treated with 8.5 mL of 70% trifluoroacetic acid (TFA) for 30 min at RT (1st reaction, Figure 37). Afterwards, a TLC of this reaction reported several products in addition to unreacted starting material. [Note: TLC was stained with Ninhydrin & Hanessian’s stain. The more prominent spot was also the most polar (R_f = 0.25, DCM:MeOH 4:1), and was indicative of the deprotected ribose.] However, after purifying the reaction contents by normal phase chromatography, NMR analysis did not show the desired product or anything resembling a ribose.

![Figure 37](image_url)

Figure 37. Four deprotection reactions attempted on 5-phenethylamine ribose. Only a partially deprotected product was recovered (3rd step).
The second attempt followed a procedure used to deprotect a ribose, which had an ethyl group attached to the 5-O atom. Likewise, we refluxed our protected ribose (389 mg) in 4.2 mL of 40 mM HCl at 100 °C for 2 hours (2nd reaction, Figure 37). However, the TLC of this reaction showed only starting material. It was also noticed that starting material remained in an insoluble layer at the bottom of the reaction vial. Therefore, after recovering this starting material (346 mg) with DCM extractions, we added 2 mL of dioxanes to improve solubility during our next attempt at deprotection (3rd reaction, Figure 37). [Note: This reaction originally had 40 mM HCl, but the pH was ~7, so HCl was raised to 200 mM, and the pH was 1-2. Strangely, at the end of the reaction the pH read ~7.] After extracting products with EtOAc (3 x 15 mL), the TLC of this reaction reported two polar compounds (R\(_f\) = 0.64 & 0.27 with DCM:MeOH at 4:1, or R\(_f\) = 0.23 & 0.12 with DCM:MeOH at 9:1), and only traces of starting material (R\(_f\) = 0.99 with DCM:MeOH at 4:1, or R\(_f\) = 0.95 with DCM:MeOH at 9:1), suggesting that this reaction may have produced both the fully deprotected ribose (as seen by the most polar spot on TLC) and a partially deprotected ribose (indicated by 2nd most polar spot on TLC). However, after separating these compounds by normal phase chromatography, NMR analysis showed only the partially deprotected ribose, which still contained the C1-methoxy group (3rd reaction, Figure 37).

The last attempt at deprotecting our ribose used methanolic HCl (0.33 mL of 3 N, 1.1 eq.) and 2 mL of 50% dioxanes in DI H\(_2\)O (v/v) to dissolve 0.28 g of starting material (4th reaction, Figure 37). [Note: Additional HCl (40 µL of 12.1 M) was added to this reaction to lower the pH from ~7 to a pH of ~ 1.] Unfortunately, NMR analysis showed no products that were indicative of a deprotected ribose after this reaction was separated by normal phase and reverse phase...
chromatography. No further attempts were made to synthesis second-generation compounds using this strategy of reacting ribose independently.

**Addition of azide to adenosine**

As a new strategy for making 31, we sought to replace the 5’-OH of 6-chloropurine riboside with an azide (N₃), which could later be reacted by reductive amination¹⁰² to introduce the 5’-phenethylamine substituent (Figure 38). First, the 2’-OH and 3’-OH groups on 6-chloropurine riboside were protected with isopropylidene, following an earlier method¹⁰³ (1ˢᵗ step, Figure 38). Next, the 5’-OH was replaced with tosylate (Ts) by reacting the purified product with Ts-Cl (1 eq.) in dry pyridine under N₂ (2ⁿᵈ step, Figure 38). NMR analysis confirmed that the 5’-OH was successfully replaced with the tosylate group. [Note: TLC showed product, starting material, and unreacted Ts-Cl, respectively at R_f = 0.51, 0.79 & 0.89 in DCM:MeOH of 19:1. This reaction was then co-evaporated with toluene and washed with 10% Cu(OAc)₂ to remove residual pyridine. Product was extracted with DCM, concentrated *in vacuo*, and purified by normal phase and then reverse phase chromatography.]
We then tried substituting the 5’-tosylate group with an azide group by adding 1.2 eq of NaN₃ and refluxing in sealed tube with an aprotic solvent, dry DMSO (3rd step, Figure 38). After 20 min, no product was seen by TLC, therefore, excess NaN₃ (4.1 eq.) was added and heated 20 more min. However, the TLC again reported no product that was distinguishable from the starting material. Irrespective of the TLC, the reaction was quenched (2x vol. H₂O) and extracted (3x vol. CHCl₃) for NMR analysis. Interestingly, this spectra showed “twinned peaks” from two species resembling adenosine analogues with attached azide(s). Further analysis by mass spectrometry indicated one species was the desired product (reported 352.4 m/z, expected 352.1 m/z), and a second species was the “double-azide” byproduct (3rd step, Figure 38) (reported 359.4 m/z, expected 359.1 m/z)(M+H). Ultimately, this strategy for making 31 was abandoned when we were unable to separate either species by normal phase or reverse phase
chromatography. Regrettably, this reaction may have succeeded if the second addition of NaN₃ was avoided.

*Substitution of 5’-OH on lead compound 1*

We reasoned from previous attempts at substituting the 5’-OH on 6-chloropurine riboside that it may be necessary to first add the N⁶-cyclopentyl (as to make 1) to avoid unwanted substitutions with the 6-Cl group as had occurred with reactions in Figures 33 & 38. Additionally, we saw how the tosylate group at the 5-position of ribose was successfully replaced by phenethylamine when reacted neat (shown in Figure 36). Therefore, our final strategy was to similarly add a leaving group at the 5’-position of 1, then react neat with phenethylamine (or other aromatic amines) to produce the 5’ N-substituents on second-generation compounds.

After protecting the starting material with isopropylidene (top left, Figure 39) as previously done,⁹⁷ the N⁶-cyclopentyl was added (as in Chapter 3) to give compound 24 with an overall yield of 50% (top middle, Figure 39). As an aside, when the order of these last two steps was reversed (i.e., N⁶-cyclopentyl added before isopropylidene), we saw a better overall yield of 66% for 24 (middle left, Figure 39), thus subsequent reactions followed this more productive route.
Figure 39. Leaving groups added to 5’-OH of 1. Tosylation of 24 gave both product and cyclized byproduct (top right). Mesylation of 24 successfully produced 25 (middle right).

The first leaving group we attempted to add to 24 was the tosylate (top right, Figure 39), using the same conditions from the similar tosylation reaction in Figure 36. However, in this reaction NMR analysis indicated a “cyclized byproduct” had formed in addition to the desired 5’-tosylate product (top right, Figure 39). We were unable to separate these two species by reversed phase chromatography. Cyclization of adenosine analogs during similar tosylation reactions has also been reported in earlier literature.\textsuperscript{104-107} [Note: Evidence of our cyclized byproduct came from the NMR spectra, which showed only one of the two purine protons in the aromatic region at 7.14 ppm (likely the C\textsuperscript{2}-H), in addition, the other purine proton (C\textsuperscript{8}-H) was
suspected of being the singlet found in the non-aromatic region at 5.64 ppm, which did not coordinate with a neighboring proton during a COSY NMR experiment.]

The second attempt to add a leaving group to 24 used the less labile mesylate, which was reported to not cause cyclization of the adenosine analog during a similar reaction. Similarly in our reaction, the mesylate was successfully added to the 5'-OH of 24 without cyclizing, to give 25 in a good yield (middle right, Figure 39). This same mesylation reaction was attempted on the unprotected 1 to test if the protecting step could be eliminated from the overall reaction scheme (bottom left, Figure 39). However, the TLC of this reaction reported multiple byproducts, thus validating the need to protect 1 before the mesylation reaction. [Note: 25 appeared unstable, and was thus used in the subsequent reactions immediately after purification.]

2nd Generation Final Products; 5'-Substitution and Deprotection

The addition of a 5’-substituent to our adenosine analog was finally realized by reacting neat the mesylated precursor (25) with the substituted amine (1st step, Table 5). In short, 25 was dissolved into 10 eq. of amine (benzylamine, phenethylamine, propylphenylamine or butylphenylamine) and heated for ~9 hr at 60 ºC to give the protected intermediate 26 - 29, respectively. Generally, the TLC analysis suggested that these reactions had gone to near completion, reporting only trace amounts of starting material (25) with a small amount of byproduct or impurity (possibly a degradant of 25). The lower than expected yields for 26 - 29 were likely the results of purification steps used to remove the excess aromatic amines, which often eluted closely with the desired product during normal phase chromatography.
Lastly, 26 - 29 were deprotected with trifluoroacetic acid (TFA) according to an earlier method\textsuperscript{102} (2\textsuperscript{nd} step, Table 5). [Note: Initially, we attempted to deprotect 27 using TFA anhydride (not TFA) without success. Other attempts with 50\% formic acid\textsuperscript{108} or 0.5 M HCl were also unsuccessful at deprotecting 27.] Further details of reaction conditions and results for products 24 - 33 are provided in the experimental section. Structural identity for compounds 24 - 33 was confirmed by proton and carbon NMR, assisted by COSY and HSQC (see Appendix B). Additionally, high resolution mass spectrometry (HRMS) of 24 - 33 agreed with the expected masses (see experimental section).

Table 5. Addition of 5\textsuperscript{'-}substitution and deprotection of 2nd generation products.

<table>
<thead>
<tr>
<th>R\textsuperscript{2}</th>
<th>(name)</th>
<th>Product</th>
<th>O\textsuperscript{2} &amp; O\textsuperscript{3} yield</th>
<th>Product</th>
<th>O\textsuperscript{2} &amp; O\textsuperscript{3} yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Image]</td>
<td>benzyl</td>
<td>26</td>
<td>protected 30%, crude</td>
<td>30</td>
<td>deprotected 25%</td>
</tr>
<tr>
<td>[Image]</td>
<td>phenethyl</td>
<td>27</td>
<td>protected 31%</td>
<td>31</td>
<td>deprotected 87%</td>
</tr>
<tr>
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<td>propylphenyl</td>
<td>28</td>
<td>protected 36%</td>
<td>32</td>
<td>deprotected 99%</td>
</tr>
<tr>
<td>[Image]</td>
<td>butylphenyl</td>
<td>29</td>
<td>protected 23%</td>
<td>33</td>
<td>deprotected 98%</td>
</tr>
</tbody>
</table>
Conclusion

The goal of adding substituents to both $N^6$ and 5’-C of adenosine was realized through a five-step synthesis to give compounds 30 - 33 (Figure 40), which were previously unreported in the literature. While this reaction scheme used only cyclopentyl as the $N^6$-substituent, our process may still be acceptable with other substituents at this position. However, a substituent such as $N^6$-8-octylamine in 7 may need to be first protected (ex. with BOC) to prevent polymerization or other by-products from forming in the subsequent reaction steps.

Likewise, this process may be suitable for adding a wide variety of other substituted amines to the 5’-position of 25 besides the 4 aromatic amines used on 30-31. One example that we plan to try is to use the primary amine on $N^6$-ethylamine-adenosine (6) to substitute the 5’-tosylate of 25 in order to make a bi-substrate analog containing two adenosine components.

Figure 40. Overall yields for second-generation compounds 30-33.

Overall, we have successfully developed a strategy to generate a library of adenosine-based compounds that can be elaborated at two positions ($N^6$ and 5’). The motivation for generating these compounds was to make ErmC’/KsgA inhibitors that function by targeting both active site pockets. These compounds were then tested for their ability to inhibit the methyltransferase activity of ErmC’ and KsgA, as discussed next in Chapter 5.
Experimental Section

**Chemicals and Solvents.** Triethylamine, p-toluenesulfonic acid monohydrate (TsOH), methanesulfonyl chloride (Ms-Cl), 3-phenyl-1-propylamine, 4-phenylbutylamine, 2,2-dimethoxypropane, trifluoroacetic acid (TFA), and deuterated chloroform (CDCl$_3$) were purchased from Sigma-Aldrich (USA). Benzylation was purchased from Acròs Organics (Belgium). 2-Phenethylamine was bought from Alfa Aesar (England). Methanol (MeOH), dichlromethane (DCM), isopropanol (IPA), hexanes, triethylamine (Et$_3$N), and tetrahydrofuran (THF) were purchased through Fisher Scientific (USA).

**Chromatography.** Thin-layer chromatography (TLC) was performed on silica plates purchased from Analtech (USA). Preparative chromatography was done with RediSep®$R_f$ columns containing silica or C18, and a Combiflash®$R_f$ instrument, all purchased through Teledyne Isco, Inc. (USA).

**Instruments and Analyses.** $^1$H and $^{13}$C NMR spectra were obtained using 400 MHz Bruker spectrometer with a Bruker Topspin 2.1 software. Chemical shifts are reported as ppm (δ) using solvent peaks or tetramethylsilane (TMS) as the internal standard. High-resolution mass spectrometry (HRMS) were outsourced to the Chemistry Department of Virginia Commonwealth University.
Synthesis

2', 3'-O-Isopropylidene-\textit{N}^6-cyclopentyl adenosine (24). To a solution of compound 1 (0.64 g, 1.92 mmol) in 3.3 mL dry acetone, 5 eq. of 2,2-Dimethoxypropane (1.18 mL, 9.63 mmol) and 1.1 eq. of p-toluensulfonic acid monohydrate (0.41 g, 2.16 mmol) were added under N\textsubscript{2} and stirred for 2.5 hours at room temp. Reaction was quenched with saturated solution of NaHCO\textsubscript{3} (3.2 mL) until pH of reaction was 7-8. Neutralized reaction was concentrated in vacuo then separated by normal phase chromatography (12 g Silica RediSep Column, 5:95 MeOH:DCM for 8 min with flow rate of 30 mL/min). Product was further purified by crystallization in hexanes to give 0.64 g of white crystalline foam (1.7 mmol, 88.4%). R\textsubscript{f} 0.48 (MeOH:DCM = 1:19); \textsuperscript{1}H NMR (CDCl\textsubscript{3}) \(\delta\) 8.26 (bs, 1H, 2-H), 7.71 (bs, 1H, 8-H), 6.74 (bs, 1H, 5'-OH), 5.81 (bs, 1H, N\textsubscript{6}-H), 5.77 (d, 1H, 1'-H, \textit{J} = 4.80 Hz), 5.13 (dd, 1H, 2''-H, \textit{J} = 5.55, 5.16 Hz), 5.04 (dd, 1H, 3'-H, \textit{J} = 0.8, 5.8 Hz), 4.52 (bs, 1H, 1''-H, cyclopentyl), 4.47 (d, 1H, 4'-H, \textit{J} = 0.92 Hz), 3.91 (dd, 1H, 5'-H\textsubscript{a}, \textit{J} = 1.4, 12.8 Hz), 3.72 (d, 1H, 5’-H\textsubscript{b}, \textit{J} = 12.61 Hz), 2.13 - 1.99 (m, 2H, 2'''-H\textsubscript{a} + 5'''-H\textsubscript{a}, cyclopentyl), 1.77 - 1.59 (m, 4H, 3''-H\textsubscript{2} + 4''-H\textsubscript{2}, cyclopentyl), 1.58 (s, 3H, 2''''-H\textsubscript{3}, isopropylidene), 1.56 - 1.41 (m, 2H, 2''''-H\textsubscript{b} + 5''''-H\textsubscript{b}, cyclopentyl), 1.31 (s, 3H, 3''''-H\textsubscript{3}, isopropylidene); \textsuperscript{13}C NMR (CDCl\textsubscript{3}) \(\delta\) 154.92 (C-6), 152.68 (C-2), 147.14 (C-4), 139.43 (C-8), 120.44 (C-5), 113.97 (C-1''''', isopropylidene), 94.44 (C-1’), 86.09 (C-4’), 83.04 (C-2’), 81.74 (C-3’), 63.46 (C-5’), 52.36 (C-1”, cyclopentyl), 33.43 (C-2”+ C-5”, cyclopentyl), 27.68 (C-2”’), (C-3”’), isopropylidene), 25.25 (C-3”’’, isopropylidene), 23.70 (C-3”’+ C-4”, cyclopentyl); HRMS m/z 376.1998, expected 376.1985 (M + H).
2', 3'-Isopropylidene-5'-mesyl-N6-cyclopentyl adenosine (25).*

*Note: the following describes the synthesis of the batch used to make 26 and 29.

To a vial of 24 (0.21 g, 0.57 mmol) purged with N₂, dry DCM (6 mL) and 2.5 eq. triethylamine (0.2 mL, 1.44 mmol) were added. In a separate vial purged with N₂, 0.1 mL methanesulfonyl chloride (Ms-Cl) was added to 0.9 mL dry DCM, then 663 µL of this 1:10 diluted solution of Ms-Cl (0.85 mmol, 1.5 eq.) was added to the vial with 24 and stirred at RT for 45 min. The reaction was loaded directly to 40 g silica and separated by normal phase chromatography (12 g Silica RediSep Column, 0 to 15% MeOH in DCM over 16 cv) to yield 255.77 mg of white crystalline solid (0.564 mmol, 98.7%, crude). Rf 0.63 (MeOH:DCM = 1:19); ¹H NMR (CDCl₃) δ 8.29 (bs, 1H, 2-H), 7.75 (s, 1H, 8-H), 6.03 (d, 1H, 1'-H, J = 2.00 Hz), 5.65 (bs, 1H, N₆-H), 5.41 (dd, 1H, 2'-H, J = 2.00, 6.32 Hz), 5.10 (dd, 1H, 3'-H, J = 3.08, 6.28 Hz), 4.62 - 4.48 (m, 1H, 1''-H, cyclopentyl), 4.48 - 4.43 (m, 1H, 4'-H), 4.42 - 4.31 (m, 2H, 5'-H₂), 2.83 (s, 3H, 1''''-H₃, mesylate), 2.15 - 2.02 (m, 2H, 2''-Hₐ + 5''-Hₐ, cyclopentyl), 1.76 - 1.58 (m, 4H, 3''-H₂ + 4''-H₂, cyclopentyl), 1.54 (s, 3H, 2'''-H₃, isopropylidene), 1.53 - 1.41 (m, 2H, 2''-Hₐ + 5''-Hₐ, cyclopentyl), 1.33 (s, 3H, 3'''-H₃, isopropylidene); ¹³C NMR (CDCl₃) δ 154.69 (C-6), 153.41 (C-2), 148.23 (C-4), 139.04 (C-8), 120.40 (C-5), 114.70 (C-1''', isopropylidene), 90.94 (C-1'''), 84.87 (C-4''), 84.06 (C-2''), 81.51 (C-3'''), 68.56 (C-5''), 52.51 (C-1'', cyclopentyl), 37.52 (C-1''''', mesylate), 33.43 (C-2'''+ C-5''', cyclopentyl), 27.08 (C-2''', isopropylidene), 25.30 (C-3'''', isopropylidene), 23.70 (C-3'''+ C-4''', cyclopentyl); HRMS m/z 454.1762, expected 454.1760 (M + H)^+. 

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2', 3'-Isopropylidene-5'-mesyl-\(\text{N}^6\)-cyclopentyl adenosine (25).**

**Note: The following describes the synthesis for a batch used to make 25 and 27.

To a vial of 24 (0.25 g, 0.68 mmol, from 5/10/10) purged with \(\text{N}_2\), dry DCM (8.5 mL) and 2.5 eq. triethylamine (236 µL, 1.69 mmol) were added. In a separate vial purged with \(\text{N}_2\), 78.66 µL Ms-Cl was added to 1 mL dry DCM, then 1079 µL of this diluted Ms-Cl (1.02 mmol, 1.5 eq.) was added to vial with 24 and stirred at RT for 45 min. The reaction was loaded directly to 40 g silica and separated by normal phase chromatography to yield 298.78 mg of white crystalline solid (0.659 mmol, 97.2 %, crude).

2', 3'-Isopropylidene-5'-mesyl-\(\text{N}^6\)-cyclopentyl adenosine (25).***

***Note: the following describes the synthesis of a batch that was used to make 28.

To a vial of 24 (0.22 g, 0.59 mmol) purged with \(\text{N}_2\), dry DCM (6 mL) and 2.5 eq. triethylamine (0.2 mL, 1.48 mmol) were added. In a separate vial purged with \(\text{N}_2\), 0.1 mL Ms-Cl was added to 0.9 mL dry DCM, then 690 µL of this diluted solution Ms-Cl (0.89 mmol, 1.5 eq.) was added to vial with 24 and stirred at RT for 45 min. The reaction was loaded directly to 40 g silica and separated by normal phase chromatography to yield 270.56 mg of white crystalline solid (0.597 mmol, 100%, crude).

2', 3'-O-Isopropylidene-5'-deoxy-5'-benzylamino-\(\text{N}^6\)-cyclopentyl adenosine (26). To a vial of 25 (144.1 mg, 0.32 mmol), 10 eq. of benzylamine (0.35 mL, 3.2 mmol) was added, dissolving the starting material. This “neat” reaction was carried out under \(\text{N}_2\) for 12 hr at 60 °C then loaded directly onto 4 g silica and separated twice by normal phase chromatography (12 g Silica
RediSep Column, 0 to 100% MeOH in DCM over 30 cv) to yield 43.51 mg (0.09 mmol, 29.5%, crude). Rf 0.32 (MeOH:DCM = 1:19); 1H NMR (CDCl3) δ 8.12 (bs, 1H, 2-H), 7.70 (s, 1H, 8-H), 7.32 - 7.09 (m, 5H, 3''''-H, 4''''-H, 5''''-H, 6''''-H + 7''''-H, benzyl), 5.88 (d, 1H, 1'-H, J = 3.24 Hz), 5.66 (d, 1H, N°-H, J = 4.76 Hz), 5.39 (dd, 1H, 2'-H, J = 3.3, 6.4 Hz), 4.97 (dd, 1H, 3'-H, J = 3.1, 6.4 Hz), 4.61 - 4.43 (m, 1H, 1''-H, cyclopentyl), 4.35 - 4.26 (m, 1H, 4'-H), 3.70 (s, 2H, 1''''-H2, benzyl), 2.88 - 2.73 (m, 2H, 5'-H2), 2.10 - 1.99 (m, 2H, 2''-Hb + 5''-Hb, cyclopentyl), 1.75 - 1.55 (m, 4H, 3'''-H2 + 4''-H2, cyclopentyl), 1.53 (s, 3H, 2'''-H3, isopropylidene), 1.52 - 1.42 (m, 2H, 2''-Hb + 5''-Hb, cyclopentyl), 1.30 (s, 3H, 3''''-H3, isopropylidene); 13C NMR (CDCl3) δ 154.63 (C-6), 153.27 (C-2), 148.41 (C-4), 140.06 (C-2'''', benzyl), 139.03 (C-8), 128.35 (C-3'''', benzyl), 127.97 (C-4'''', benzyl), 126.95 (C-5''''', benzyl), 120.49 (C-5), 114.43 (C-1''', isopropylidene), 91.04 (C-1'), 85.61 (C-4'), 83.36 (C-2'), 82.38 (C-3'), 53.79 (C-1'''', benzyl), 52.43 (C-1'', cyclopentyl), 50.73 (C-5'), 33.43 (C-2'''+ C-5'', cyclopentyl), 27.33 (C-2''', isopropylidene), 25.44 (C-3''', isopropylidene), 23.70 (C-3'''+ C-4'', cyclopentyl); HRMS m/z 465.2617, expected 465.2614 (M + H)+.

2', 3'-O-Isopropylidene-5'-deoxy-5'-phenethylamino- N°-cyclopentyl adenosine (27). To a vial of 25 (298.78 mg, 0.659 mmol), 10 eq. of 2-phenethylamine (0.82 mL, 6.52 mmol) was added, dissolving the starting material. This “neat” reaction was carried out under N2 for 9 hr at 60 °C then loaded directly onto 4 g silica and separated once by normal phase chromatography (12 g Silica RediSep Column, 0-100% MeOH in DCM for over 36 cv) then separated by reverse phase chromatography (13 g C18 RediSep Column, 5-100% THF in DI H2O over 24 cv) to yield 98.39 mg of clear yellowish gel (0.206 mmol, 31.2%). Rf 0.57 (MeOH:DCM = 1:9); 1H NMR
(CDCl$_3$) δ 8.29 (bs, 1H, 2-H), 7.73 (s, 1H, 8-H), 7.19 - 7.13 (m, 2H, 5''''-H + 7''''-H, phenethyl), 7.12 - 7.04 (m, 3H, 4''''-H, 6''''-H + 7''''-H, phenethyl), 5.91 (d, 1H, 1''-H, $J = 3.04$ Hz), 5.63 (bd, 1H, N$_6$-H, $J = 6.68$ Hz), 5.37 (dd, 1H, 2''-H, $J = 3.04$, 6.44 Hz), 4.94 (dd, 1H, 3''-H, $J = 3.2$, 6.4 Hz), 4.54 (bs, 1H, 1'''-H, cyclopentyl), 4.31 - 4.24 (m, 1H, 4''-H), 2.92 - 2.73 (m, 4H, 5'''-H + 1'''-H, phenethyl), 2.73 - 2.63 (m, 2H, 2'''-H$_a$), 2.13 - 2.00 (m, 2H, 2''-H$_a$ + 5''-H$_a$, cyclopentyl), 1.74 - 1.56 (m, 4H, 3'H$_a$ + 4''-H$_a$, cyclopentyl), 1.53 (s, 3H, 2'''-H$_a$, isopropylidene), 1.52 - 1.42 (m, 2H, 2''-H$_b$ + 5''-H$_b$, cyclopentyl), 1.30 (s, 3H, 3'''-H$_a$, isopropylidene); $^{13}$C NMR (CDCl$_3$) δ 154.62 (C-6), 153.29 (C-2), 148.? (C-4), 139.78 (C-3'''), phenethyl), 138.94 (C-8), 128.64 (C-4'''+ C-8'''', phenethyl), 128.38 (C-5'''+ C-7'''', phenethyl), 126.11 (C-6''''', phenethyl), 120.44 (C-5), 114.48 (C-1''', isopropylidene), 90.87 (C-1''), 85.60 (C-4''), 83.53 (C-2''), 82.35 (C-3''), 52.45 (C-1'', cyclopentyl), 51.21 (C-1''''', phenethyl), 51.18 (C-5''), 36.16 (C-2''''', phenethyl), 33.42 (C-2'''+ C-5''', cyclopentyl), 27.28, 25.42 (C-2''''+ C-3''', isopropylidene), 23.70 (C-3'''+ C-4'', cyclopentyl); HRMS m/z 479.2776, expected 479.2771 (M + H)$^+$. 

2', 3'-O-Isopropylidene-5'-deoxy-5'-propylphenylamino-N$_6$-cyclopentyl adenosine (28). To a vial of 25 (270.56 mg, 0.597 mmol), 10 eq. of 3-phenyl-1-propylamine (0.848 mL, 5.967 mmol) was added, dissolving the starting material. This “neat” reaction was carried out under N$_2$ for 12 hr at 60 °C. Reaction contents dissolved in 5 mL DCM were treated with CuSO$_4$ (5 mL, 1% in water) to extract amine, but no amine was seen in aqueous layer by TLC, so the green-colored organic layer was loaded onto 4 g silica and separated once by normal phase chromatography (12 g Silica RediSep Column, 0 to 5% MeOH in DCM over 13 cv) to yield 106 mg (0.215 mmol,
36%). Rf 0.30 (MeOH:DCM = 1:19); ^1^H NMR (CDCl\textsubscript{3}) \(\delta\) 8.26 (bs, 1H, 2-H), 7.74 (bs, 1H, 8-H), 7.23 - 7.14 (m, 2H, 6'''-H + 8'''-H, propylphenyl), 7.13 - 7.02 (m, 3H, 5'''-H, 7''''-H + 9''''-H, propylphenyl), 5.89 (d, 1H, 1'-H, \(J = 3.24\) Hz), 5.66 (bd, 1H, N\textsuperscript{6}-H, \(J = 6.08\) Hz), 5.33 (dd, 1H, 2'-H, \(J = 6.3, 3.3\) Hz), 5.16 - 5.07 (m, 1H, 3'-H), 4.54 (bs, 1H, 1''-H, cyclopentyl), 4.38 - 4.30 (m, 1H, 4'-H), 3.03 - 2.93 (m, 1H, 5'-Ha), 2.92 - 2.83 (m, 1H, 5'-Hb), 2.64 (t, 2H, 1''''-H\textsubscript{2}, \(J = 7.3\) Hz, propylphenyl), 2.56 (t, 2H, 3''''-H\textsubscript{2}, \(J = 7.52\) Hz, propylphenyl), 2.12 - 1.98 (m, 2H, 2''-H\textsubscript{a} + 5''-H\textsubscript{a}, cyclopentyl), 1.88 - 1.74 (m, 2H, 2''''-H\textsubscript{2}), 1.74 - 1.56 (m, 4H, 3'''-H\textsubscript{2} + 4''-H\textsubscript{2}, cyclopentyl), 1.53 (s, 3H, 2''''-H\textsubscript{3}, isopropylidene), 1.52 - 1.41 (m, 2H, 2''-H\textsubscript{b} + 5''-H\textsubscript{b}, cyclopentyl), 1.31 (s, 3H, 3''''-H\textsubscript{3}, isopropylidene); ^13^C NMR (CDCl\textsubscript{3}) \(\delta\) 154.72 (C-6), 153.20 (C-2), 148.23 (C-4), 141.44 (C-4'''), propylphenyl), 139.00 (C-8), 128.39 (C-6''''+ C-8''''', propylphenyl), 128.33 (C-5'''''+ C-9''''', propylphenyl), 125.92 (C-7''''', propylphenyl), 120.65 (C-5), 114.64 (C-1''''', isopropylidene), 91.31 (C-1'), 84.55 (C-4'), 83.35 (C-2'), 82.10 (C-3'), 52.47 (C-1'', cyclopentyl), 50.83 (C-5'), 49.12 (C-1'''', propylphenyl), 33.43 (C-2'''+ C-5''', cyclopentyl), 33.36 (C-3'''', propylphenyl), 30.44 (C-2'''', propylphenyl), 27.27 (C-2''', isopropylidene), 25.42 (C-3''', isopropylidene), 23.70 (C-3''+ C-4'', cyclopentyl); HRMS m/z 493.2942, expected 493.2927 (M + H)^+.

2', 3'-O-Isopropylidene-5'-deoxy-5'-butylphenylamino-N\textsuperscript{6}-cyclopentyl adenosine (29). To a vial of 25 (139 mg, 0.31 mmol), 10 eq. of 4-phenylbutylamine (0.48 mL, 3.1 mmol) was added, dissolving the starting material. This “neat” reaction was carried out under \(N\textsubscript{2}\) for 12 hr at 60 \(^\circ\)C then loaded directly onto 4 g silica and separated twice by normal phase chromotography (12 g Silica RediSep Column, 0-100% MeOH in DCM over 27.2 cv) to yield 36.5 mg (0.07 mmol,
23.5%). Rf 0.39 (MeOH:DCM = 1:19); 1H NMR (CDCl3) δ 8.28 (bs, 1H, 2-H), 7.75 (s, 1H, 8-H), 7.22 - 7.14 (m, 2H, 7'''-H + 9'''-H, butylphenyl), 7.12 - 7.03 (m, 3H, 6'''-H, 8'''-H + 10'''-H, butylphenyl), 5.91 (d, 1H, 1’-H, J = 3.04 Hz), 5.63 (bd, 1H, N6-H, J = 6.92 Hz), 5.40 (dd, 1H, 2’-H, J = 6.44, 3.04 Hz), 4.94 (dd, 1H, 3’-H, J = 6.44, 3.36 Hz), 4.55 (bs, 1H, 1’’’-H, cyclopentyl), 4.31 - 4.23 (m, 1H, 4’-H), 2.86 - 2.73 (m, 2H, 5’-H2), 2.60 - 2.45 (m, 4H, 2’’-H2, 5’’-H2, butylphenyl), 2.12 - 2.00 (m, 2H, 2’’-Ha + 5’’-Ha, cyclopentyl), 1.75 - 1.57 (m, 4H, 3’’-H2 + 4’’-H2, cyclopentyl), 1.57 - 1.36 (m, 2H, 2’’-Hb + 5’’-Hb, cyclopentyl), 0.96 - 0.74 (m, 6H, 2’’’’-H, isopropylidene), 1.31 (s, 3H, 3’’’’-H, isopropylidene); 13C NMR (CDCl3) δ 154.65 (C-6), 153.30 (C-2), 148.50 (C-4), 142.41 (C-5’’’’, butylphenyl), 139.00 (C-8), 128.36 (C-6’’’’ + C-10’’’’’, butylphenyl), 128.25 (C-7’’’’’’ + C-9’’’’’’’, butylphenyl), 120.52 (C-5), 114.47 (C-1’’’’, isopropylidene) 90.91 (C-1’), 85.63 (C-4’), 83.53 (C-2’), 82.38 (C-3’), 52.49 (C-1’’’, cyclopentyl), 51.47 (C-5’), 49.81 (C-1’’’’’, butylphenyl), 35.76 (C-4’’’’’, butylphenyl), 33.45 (C-2’’ + C-5’’, cyclopentyl), 29.56 (C-2’’’’, butylphenyl), 29.06 (C-3’’’’’, butylphenyl), 27.29 (C-2’’’, isopropylidene), 25.45 (C-3’’’, isopropylidene), 23.70 (C-3’’’ + C-4’’, cyclopentyl); HRMS m/z 507.3092, expected 507.3084 (M + H)+.

5’-deoxy-5’-benzylamino-N6-cyclopentyl adenosine (30). To a vial of 41 mg (0.09 mmol) of 26 on ice, an ice cold solution of TFA:H2O 10:1 (317 µL, 3.57 mL/mmol) was added stirred on ice for 1 hr, then warmed to RT over 2 hr. The reaction was neutralized with saturated solution of NaHCO3 (3.5 mL), then extracted with CHCl3 (4x 5 mL). An unknown impurity, which NMR suggests was also present in 25, was crystallized out by dissolving the dried extract in hot hexanes (~20 mL, at 60 °C) then concentrated to ~1 mL forming a white fuzz of fine needle
crystals at room temperature. After centrifuging down the precipitate, the liquid portion was purified further by normal phase chromatography (12 g Silica RediSep Column, 0 to 37% MeOH in DCM over 18 cv) to yield 9.53 mg of a light tan powder (0.02 mmol, 25.4%). Rf 0.38 (MeOH:DCM = 1:9); \(^1\)H NMR (CDCl\(_3\)) 8 7.99 (bs, 1H, 2-H), 7.76 (s, 1H, 8-H), 7.30 - 7.14 (m, 5H, 3'''-H, 4'''-H, 5'''-H, 6'''-H + 7'''-H, benzyl), 5.92 - 5.73 (m, 2H, N6-H + 1’-H), 4.63 - 4.44 (m, 2H, 2’-H + 1’’-H, cyclopentyl) 4.37 (dd, 1H, 3’-H, \(J = 3.72, 5.12\) Hz), 4.31 - 4.22 (m, 1H, 4’-H), 3.85 - 3.72 (m, 2H, 1’’’-H, benzyl), 2.97 - 2.84 (m, 2H, 5’-H), 2.11 - 1.98 (m, 2H, 2”-H\(_{a}\) + 5”-H\(_{a}\), cyclopentyl), 1.76 - 1.54 (m, 4H, 3”’-H + 4”’-H, cyclopentyl), 1.53 - 1.40 (m, 2H, 2”’-H\(_{b}\) + 5”’-H\(_{b}\), cyclopentyl); \(^1\)C NMR (CDCl\(_3\)) 8 154.62 (C-6), 152.71 (C-2), 147.83 (C-4), 138.32 (C-8 + C-2’’, benzyl), 128.60 (C-4”’+ C-6”’), benzyl), 128.28 (C-3”’+ C-7”’), benzyl), 127.52 (C-5”’’, benzyl), 120.23 (C-5), 90.40 (C-1’), 84.64 (C-4’), 74.94 (C-2’), 72.13 (C-3’), 53.74 (C-1”’), benzyl), 52.41 (C-1”’, cyclopentyl), 50.23 (C-5’), 33.40 (C-2”’+ C-5”’, cyclopentyl), 27.08, 23.70 (C-3”’+ C-4”’, cyclopentyl); HRMS m/z 425.2300, expected 425.2301 (M + H).)

5’-deoxy-5’-phenethylamino-N6-cyclopentyl adenosine (31). To a vial of 59 mg (0.12 mmol) of 27 on ice, an ice cold solution of TFA:H\(_2\)O 10:1 (400 \(\mu\)L, 3.57 mL/mmol) was added stirred on ice for 1 hr, then warmed to RT over 2 hr. Reaction was neutralized with saturated solution of NaHCO\(_3\) (3.5 mL), then extracted with CHCl\(_3\) (4x 5 mL). The organic layer was concentrated in vacuo then purified further by normal phase chromatography (4 g Silica RediSep Column, 0 to 20% MeOH in DCM over 80 cv). An impurity, which NMR suggests was “grease”, was precipitated out as a white solid/film when dried product was dissolved in MeOH. After centrifuging down the greasy precipitate at 0 °C, the MeOH supernatant was concentrated in
vacuo and co-evaporated with hexanes to yield 43 mg of a light pink powder (0.10 mmol, 87%).

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R_f 0.37 (\text{MeOH:DCM} = 1:9); \quad ^1H \text{ NMR (CDCl}_3\text{)} \delta 8.08 (bs, 1H, 2-H), 7.75 (s, 1H, 8-H), 7.14 - 7.08 (m, 2H, 5''''-H + 7''''-H, phenethyl), 7.07 - 6.98 (m, 3H, 4''''-H, 6''''-H + 8''''-H, phenethyl), 6.34 - 5.60 (m, 4H, 2'-OH, 3'-OH, N^6-H + 1'-H), 4.61 - 4.37 (m, 3H, 2'-H, 3'-H + 1''-H), 4.31 (bd, 1H, 4'-H, J = 3.88 Hz), 3.28 - 3.17 (m, 1H, 5'-H_a), 3.14 - 3.06 (m, 1H, 5'-H_b), 3.05 - 2.94 (m, 2H, 1''''-H_2, phenethyl), 2.88 - 2.73 (m, 2H, 2''''-H_2, phenethyl), 2.10 - 1.94 (m, 2H, 2''''-H_a + 5''''-H_a, cyclopentyl), 1.74 - 1.52 (m, 4H, 3''''-H_2 + 4''''-H_2, cyclopentyl), 1.52 - 1.37 (m, 2H, 2''''-H_b + 5''''-H_b, cyclopentyl); ^13C \text{ NMR (CDCl}_3\text{)} \delta 154.69 (C-6), 152.56 (C-2), 147.37 (C-4), 138.86 (C-8), 137.13 (C-3''''), phenethyl), 128.65 (C-5'''' + C-7''''), phenethyl), 128.53 (C-4'''' + C-8''''), phenethyl), 126.81 (C-6''''), phenethyl), 120.95 (C-5), 90.67 (C-1'), 81.96 (C-4'), 74.69 (C-2'), 71.22 (C-3'), 52.42 (C-1'', cyclopentyl), 50.22 (C-1'''), phenethyl), 49.27 (C-5'), 33.62 (C-2''''), phenethyl), 33.33 (C-2'''' + C-5'', cyclopentyl), 23.69 (C-3'''' + C-4'', cyclopentyl); HRMS m/z 439.2498, expected 439.2458 (M + H)^+.

5'-deoxy-5'-propylphenylamino-N^6-cyclopentyl adenosine (32). To a vial of 104.87 mg (0.21 mmol) of 28 on ice, an ice cold solution of TFA:H_2O 10:1 (760 µL, 3.57 mL/mmol) was added stirred on ice for 1 hr, then warmed to RT over 2 hr. Reaction was neutralized with saturated solution of NaHCO_3 (8.5 mL), then extracted with CHCl_3 (4x 10 mL). The organic layer was concentrated in vacuo and co-evaporated with hexanes to yield 95.3 mg of a light amber powder (0.21 mmol, 99%). \( R_f 0.38 (\text{MeOH:DCM} = 1:9); \quad ^1H \text{ NMR (CDCl}_3\text{)} \delta 8.13 (bs, 1H, 2-H), 7.76 (bs, 1H, 8-H), 7.25 - 7.15 (m, 2H, 6'''' + 8''''-H, propylphenyl), 7.13 - 7.07 (m, 1H, 7''''-H, propylphenyl), 7.06 - 7.0 (m, 2H, 5'''' + 9''''-H, propylphenyl), 6.04 - 5.32 (m, 4H, N^6-H, 1'-H,
2'-OH + 3'-OH), 4.74 - 4.36 (m, 3H, 1''-H, cyclopentyl, 3'-H + 2'-H), 4.25 (bs, 1H, 4''-H), 3.17 (d, 1H, 5''-Ha, J = 10.24 Hz), 2.96 (d, 1H, 5'-Hb, J = 10.64 Hz), 2.74 (t, 2H, 1'''-H2, J = 7.4 Hz, propylphenyl), 2.56 (t, 2H, 3'''-H2, J = 7.4 Hz, propylphenyl), 2.12 - 1.96 (m, 2H, 2''-Ha + 5'''-Hb, cyclopentyl), 1.93 - 1.76 (m, 2H, 2'''-H, propylphenyl), 1.75 - 1.55 (m, 4H, 3''-H2 + 4''-H2, cyclopentyl), 1.54 - 1.39 (m, 2H, 2''-Hb + 5''-Hb, cyclopentyl); 13C NMR (CDCl3) δ 154.79 (C-6), 152.56 (C-2), 147.61 (C-4), 140.51 (C-4''', propylphenyl), 138.74 (C-8), 128.55 (C-6'''+ C-8''', propylphenyl), 128.25 (C-5'''+ C-9''', propylphenyl), 126.23 (C-7''', propylphenyl), 120.58 (C-5), 90.89 (C-1'), 82.51 (C-4'), 74.97 (C-2'), 70.99 (C-3'), 52.42 (C-1'', cyclopentyl), 49.67 (C-5'), 48.83 (C-1''', propylphenyl), 33.37 (C-2'''+ C-5''', cyclopentyl), 33.01 (C-3'''', propylphenyl), 29.16 (C-2'''', propylphenyl), 23.71 (C-3'''+ C-4'', cyclopentyl); HRMS m/z 453.2608, expected 453.2614 (M + H)^+.

5'-deoxy-5'-butylphenyllamino-N6-cyclopentyl adenosine (33). To a vial of 31 mg (0.06 mmol) of 29 on ice, an ice cold solution of TFA:H2O 10:1 (217 µL, 3.57 mL/mmol) was added stirred on ice for 1 hr, then warmed to RT over 2 hr. Reaction was neutralized with saturated solution of NaHCO3 (3.5 mL), then extracted with CHCl3 (4x 5 mL). An impurity, which NMR suggests was “grease”, precipitated out as a white solid/film when dried product was dissolved in MeOH. After centrifuging down the greasy precipitate at 0 °C, the MeOH supernatant was concentrated in vacuo and co-evaporated with hexanes to yield 33 mg of a light orangish brown powder (0.07 mmol, 98%). Rf 0.26 (MeOH:DCM = 1:9); 1H NMR (CDCl3) δ 8.10 (bs, 1H, 2-H), 7.76 (s, 1H, 8-H), 7.21 - 7.14 (m, 2H, 7''''-H + 9''''-H, butylphenyl), 7.11 - 7.05 (m, 1H, 8''''-H, butylphenyl), 7.05 - 7.00 (m, 2H, 6''''-H + 10''''-H, butylphenyl), 618 (bs, 2H, 2'-OH + 3'-OH), 5.90 (bd, 1H,
N^6-H, J = 3.04 Hz), 5.83 (d, 1H, 1'-H, J = 3.88 Hz), 4.61 - 4.53 (m, 1H, 3'-H), 4.53 - 4.44 (m, 1H, 1''-H, cyclopentyl), 4.44 - 4.37 (m, 1H, 2'-H), 4.28 (d, 1H, 4'-H, J = 3.76 Hz), 3.36 (dd, 1H, 5'-'H_a, J = 3.9, 13.18 Hz), 3.06 (dd, 1H, 5'-H_b, J = 3.00, 13.00 Hz), 2.92 - 2.73 (m, 2H, 1'''-H_b, butylphenyl), 2.56 - 2.45 (m, 2H, 4'''-H_2, butylphenyl), 2.08 - 1.98 (m, 2H, 2''-H_a + 5''-H_a, cyclopentyl), 1.75 - 1.51 (m, 8H, 3''-H_2 + 4''-H_2, cyclopentyl, 2'''-H_2 + 3'''-H_2, butylphenyl), 1.51 - 1.39 (m, 2H, 2'''-H_b + 5'''-H_b, cyclopentyl); ^13C NMR (CDCl_3) δ 154.84 (C-6), 152.49 (C-2), 147.27 (C-4), 141.25 (C-5'''), butylphenyl), 139.03 (C-8), 128.43 (C-7'''+ C-9''', butylphenyl), 128.25 (C-6'''+ C-10''', butylphenyl), 126.02 (C-8'''', butylphenyl), 120.75 (C-5), 91.17 (C-1'), 81.19 (C-4'), 74.88 (C-2'), 70.46 (C-3'), 52.41 (C-1'', cyclopentyl), 48.89 (C-1''', phenethyl), 48.81 (C-5'), 35.17 (C-4''', butylphenyl), 33.31 (C-2'''+ C-5''', cyclopentyl), 28.37 (C-3''', butylphenyl), 26.28 (C-2'''', butylphenyl), 23.71 (C-3'''+ C-4''', cyclopentyl); HRMS m/z 467.2772, expected 467.2771 (M + H)^+.
CHAPTER 5: Inhibition Assays for Second-Generation Compounds

After synthesizing the second library of potential inhibitors (Figure 41), these compounds were prepared for \textit{in vitro} screening against ErmC’ and KsgA by dissolving them into DMSO to final concentrations of 1 mM and 10% DMSO. In addition to second-generation compounds (30-33), the protected intermediates (24-29) were also included. The lead compound (1), which provided the scaffold for these new compounds, was used as a reference to gauge how the 5’-substitution on 30-33 affected inhibition. Similarly, sinefungin was used as a positive control.

Figure 41. Chemical structures of second-generation compounds (30-33), protected intermediates (24-29) and controls (sinefungin & reference analog 1) tested at 1 mM against ErmC’ & KsgA.
Screening ErmC’ and KsgA against second-generation compounds at 1 mM

Compounds shown in Figure 41 were tested for inhibition using the same enzymatic reactions to screen first-generation compounds in Chapter 3. The one difference, however, was the use of a filter binding assay to measure methyltransferase activity rather than the previously used method of SPA. [Note: Initial attempts to screen second-generation compounds used SPA, but unexplainably produced excessive background counts, so this method was temporarily suspended.] Results from the preliminary screening with ErmC’ (Figure 42) showed second-generation compounds (30-33) were significantly more inhibitory than 1, suggesting that the addition of the phenyl substituent at the 5’-position improved binding interactions as hypothesized. Surprisingly, compounds 26-29, which had their 2’-OH and 3’-OH protected with isopropylidene, also showed significant inhibition against ErmC’. This was unexpected since all ErmC’ co-structures show that the 2’-OH and 3’-OH on adenosine-based ligands formed hydrogen bonds with Glu-59 (as in Figure 5, Chapter 1). The protecting group on 26-29 should preclude this interaction with ErmC’, and should even cause steric hindrance for ligands binding to the SAM pocket in the classical mode. Therefore, these early data indicated that 26-29 were not binding in a like manner to first-generation compounds.

The activity of KsgA was similarly tested in the presence of compounds 1, 24-33 and sinefungin at 1 mM (Figure 43). Unlike ErmC’, KsgA showed virtually no inhibition from second-generation compounds (30-33). One of the protected intermediates (28), however, did significantly inhibit KsgA. Although the reasons for this outlier are uncertain, 28 may have acted through a mechanism similar to that of 26-29 inhibiting ErmC’ (i.e., 28 may have bound outside the SAM site of KsgA as well).
Figure 42. Screening second-generation compounds for ErmC’ inhibition. Reaction were completed in 50 µL with 1 mM test-compound dissolved in 10% DMSO, 0.2 µM ErmC’, 0.2 µM 23S (from B. subtilis), buffer K, 20 µM SAM (780 cpm/pmol), and incubated at 37 °C for 32 min. Activity was measured by filter binding assay (a), which was used to calculate the percent inhibition (b). Reactions were done in triplicate, and the average was graphed with error bars of +/- 1 Std Dev.
Figure 43. Screening second-generation compounds for KsgA inhibition. Reactions were in 50 μL with 1 mM test-compound dissolved in 10% DMSO, 0.2 μM KsgA, 0.2 μM 30S (from ksg<sup>R</sup> E. coli), buffer K, 20 μM SAM (780 cpm/pmol), 37 °C for 5 min. Activity was measured by filter binding assay (a), which was used to calculate the percent inhibition (b). Reactions were done in triplicate, and the average was graphed with error bars of +/- 1 Std Dev.
The results of screening ErmC’ and KsgA are summarized in Table 6. Despite the structural similarities between these two MTases, experiments with 30-33 indicated that the addition of 5’-phenyl substituents to the scaffold of 1 improved selectivity towards ErmC’ over KsgA. While the lack of KsgA inhibition by 30-33 may have been a setback for our goal of developing a novel class of antibiotics that target ribosomal assembly, this was seen as a breakthrough for our other goal of developing selective ErmC’ inhibitors to restore the effectiveness of MLSB antibiotics. Therefore, the remainder of experiments in this dissertation focused on ErmC’, while KsgA awaits future study.

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<th>KsgA Inhibition</th>
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Co-crystallization of Second-Generation Compounds on ErmC’

After observing an improvement in potency and selectivity with 30-33 towards ErmC’ (relative to 1), we were eager to investigate the binding interactions of these new inhibitors. The following questions were asked regarding the effects of second-generation compounds on ErmC’: (i) are these ligands binding to the SAM site in the classical mode for adenosine analogs; (ii) is the 5’-substituent bridging into the target adenosine pocket; (iii) does this phenyl substituent form π-stacking with a flipped-out Tyr-104; and (iv) does the length of the linker on the 5’-substituent influence these potential interactions?

We attempted to answer these questions by X-ray crystallography on ErmC’ complexed with one or more of the second-generation compounds. Using the same conditions and techniques to co-crystallize ErmC’ with 7 and 12 (described in Chapter 3), dozens of quality crystals were formed over several crystallization attempts with ErmC’ in the presence of 26-33. While in nearly all cases crystals were formed, diffraction data could only be collected from a few of these crystals, of which none showed a ligand bound to ErmC’. Most of these crystals were lost as a result of happenstance (ex. instrument failures, challenges in looping and mounting crystals to the instrument). Even so, the successful diffraction of ErmC’ crystallized with 30, showed only the apo form of ErmC’. The same result was observed when X-ray data was collected on an apo crystal of ErmC’ that had been previously soaked with 31. The absence of second-generation ligands from these crystals would later be seen as possible evidence for a binding mechanism that was unlike the binding mode observed for first-generation compounds 7 and 12 in complex with ErmC’.
IC$_{50}$ Studies

Although the previous co-crystallization attempts did not provide a clear picture of the binding mode for second-generation compounds, additional enzymatic experimentation could offer us some insight into how these inhibitors were acting (or not acting) on ErmC’. Extensive inhibition assays were performed with ErmC’ to calculate IC$_{50}$ values (the concentration of a test compound that results in 50% inhibition) for compounds 1 and 30-33. Here, the objective was to more precisely compare the inhibitory effects of second-generation compounds with that of our original lead inhibitor (1).

Effects of DMSO on ErmC’

Before starting the IC$_{50}$ studies, we looked more closely at the effects of DMSO on ErmC’ to determine an appropriate amount of this solvent for introducing test-compounds into IC$_{50}$ reactions. The results of testing ErmC’ with different volumes of DMSO showed that 10% DMSO, which had typically been the amount used, contributed to a 26.6% loss of activity relative to the same experiments without DMSO (Figure 44). Therefore, it was decided that subsequent inhibition assays should have 5% DMSO, which resulted in only a 4.4% loss of ErmC’ activity (Figure 44). In addition, we decided that reactions used in the IC$_{50}$ studies would have an end-point time of 8 min instead of 32 min used previously.
First attempt of IC<sub>50</sub> studies using 20 µM SAM

Initially, IC<sub>50</sub> reactions followed the same conditions described in Figure 42, except inhibitor concentrations were varied (1 µM to 1 mM in 5% DMSO) and reactions were quenched at 8 min. However, when ErmC’ activity was plotted against inhibitor concentration (Figure 45), data did not produce a well defined lower plateau (i.e., the maximum inhibitory effect was not seen). Therefore, we sought to alter these conditions in order to potentiate a greater effect from these test-compounds. Traditionally, our reactions used SAM at an excess of 100x the ErmC’ and 23S concentrations (i.e., 20 µM SAM). This may not have been appropriate in IC<sub>50</sub> assays for inhibitors that were expected to compete for the SAM-binding site. Instead, we decided that the SAM concentration should be lowered to near or below its K<sub>M</sub>, previously reported at 3 µM.\textsuperscript{36}
Figure 45. Initial IC$_{50}$ assays for ErmC’ with 30-33, sinefungin and SAH, using 20 µM SAM. Compounds were tested at 0, 1, 5, 10, 25, 50, 100, 250, 500, and 1000 µM. Reactions were completed as described in Figure 42, except reactions had 5% DMSO and were quenched at 8 min. Reactions were done in triplicate, measuring their activity by filter binding assays, and graphing averages with error bars of +/- 1 SEM.

**Optimizing SAM concentration for IC$_{50}$ studies**

Since an objective of this study was to compare IC$_{50}$ values of 1 and second-generation compounds, we sought to peg our SAM concentration to the same level that was used previously by Clancy *et al* to report an IC$_{50}$ value of 63 µM for 1.$^{85}$ Unfortunately, the SAM concentration used in this study was not clearly defined. To estimate this amount, we tested ErmC’ activity with [1] fixed at 63 µM, and varied the SAM concentration until we saw 50% inhibition (Figure 46). Based on this experiment, we concluded that 0.5 µM SAM would be an appropriate concentration for repeating IC$_{50}$ experiments. Note: In order to increase the signal measured, we raised the ratio of Hot:Cold SAM to 1:18 (5% $^3$H-SAM) from the typical 1:97 (1% $^3$H-SAM).
Figure 46. The effects of SAM concentration on ErmC’ inhibition with 1 fixed at 63 µM. Reactions were completed as before except 5% DMSO was used and reactions were quenched at 120 min (as described by Clancy et al.\textsuperscript{85}) [SAM] was tested at 0.05, 0.1, 0.5 and 1.0 µM (5\%³H-SAM), and activity was measured by filter binding assay.

These same optimization tests (Figure 46) also demonstrated that 1 acted competitively with SAM. Although we had already suspected 1 was binding to the SAM pocket (based on crystallographic data of ErmC’ bound to similar adenosine analogs), this experiment provided supporting enzymatic evidence for such a binding mode. These results contradicted the earlier reports by Clancy \textit{et al.}, which claimed that changing the SAM concentration did not affect ErmC’ inhibition by 1\textsuperscript{85}, as well as Feder \textit{et al.’s in silico} model, which showed 1 docking into the adenosine pocket of ErmC’\textsuperscript{40} (previously discussed in Chapter 3, Figure 22).
New time course assay with 0.5 µM SAM

Before repeating IC\textsubscript{50} experiments with this new concentration of SAM, a time-course assay of ErmC’ (Figure 47) was completed using 0.5 µM SAM (5% \textsuperscript{3}H-SAM). Under these conditions, the 8-minute time-point still appeared to be within the linear region of ErmC’ activity (i.e., maximum velocity), and was thus retained as the selected endpoint for new IC\textsubscript{50} assays. However, we decided to again raise the proportion of \textsuperscript{3}H-SAM used in subsequent IC\textsubscript{50} reactions to 10% of the relative SAM concentration in order to further improve the measurable signal.

It should also be noted that while performing these time-course experiments we were able to resolve the earlier dilemma of SPA beads producing excessive background counts. This problem appeared to be corrected by using a newer batch of \textsuperscript{3}H-SAM and/or by lowering the absolute amount of \textsuperscript{3}H-SAM in each reaction. In either case, SPA was returned to as the more convenient assay for measuring ErmC’ activity.

![Graph](image)

Figure 47. Time course assay of ErmC’ with 0.5 µM SAM. Reaction were performed as before except 0.5 µM SAM (5% \textsuperscript{3}H-SAM) was used and the volume was scaled up to 0.5 mL. Activity was measured by SPA at the time points of 1, 15, 30, 60, 120, 180 and 240 min. Assays were done in triplicate, plotting the average with error bars of +/- 1 Std Dev.
**IC₅₀ assays repeated at 0.5 µM SAM**

We repeated IC₅₀ experiments with the SAM concentration of 0.5 µM (10% ³H-SAM) to be in agreement with the previous report by Clancy et al. Under these new conditions we expected ErmC’ to demonstrate a greater sensitivity to inhibitors that bind to the SAM site, thereby allowing IC₅₀ values to be more accurately measured. In addition to test-compounds 1 and 30-33, three known inhibitors of ErmC’ (sinefungin, SAH 32 and adenosine 109) were also tested as positive controls. After subtracting background counts, ErmC’ activity was plotted against the concentrations of each of these 8 inhibitors (Figure 48).

![Figure 48. ErmC’ activity vs. inhibitor concentration. Reactions were done as before except 5% DMSO and 0.5 µM SAM (10% ³H-SAM) were used, and reactions were quenched at 8 min. Reactions were done in triplicate, measuring their activity by SPA, and graphing averages with error bars of +/- 1 SEM. Legend lists compounds in order of least inhibitory (top) to most inhibitory (bottom).](image-url)
These new experiments showed that lowering the SAM concentration produced a greater inhibitory effect by 1, which was expected from a compound shown to compete for the SAM-binding site. However, when 30-33 were also tested at this lower SAM concentration, there was little or no increase of ErmC’ inhibition. In fact, second-generation compounds, which had previously shown greater ErmC’ inhibition than 1 during tests with 20 µM SAM (Figure 42), were now less inhibitive than their parent compound 1 in these new tests using 0.5 µM SAM (Figure 48). To illustrate the different effects from these inhibitors at high and low SAM concentrations, Figure 49 was created by extrapolating the percent inhibitions from tests with 1 mM sinefungin, 1 and 30-33 in Figure 48 (0.5 µM SAM), and plotted next to the percent inhibitions by these compounds in similar tests using 20 µM SAM from Figure 42.

Figure 49. Comparing ErmC’ inhibition from tests at two different concentrations of SAM. Yellow bars are results of tests with 1 mM sinefungin, 1 and 30-33 from Figure 48 (0.5 µM SAM), and blue bars are results of the similar tests from Figure 42 (20 µM SAM).
These surprising results suggest at least two of our second-generation compounds (32 and 33) were not acting at the SAM-pocket as the parent compound (1) had done, but were possibly binding to an alternate site. This claim may be less obvious for 30 and 31, since their activity did increase slightly at the lower SAM concentration, nonetheless, the inhibition from 1 had still increased to a greater extent than for any of the second-generation compounds (Figure 49).

Ultimately, these experiments at 0.5 µM SAM (Figure 48) were used to calculate IC₅₀ values for these eight inhibitors (Table 7). The two compounds that had IC₅₀ values previously reported by Clancy et al. (sinefungin at 5.0 µM, and 1 at 63 µM)⁸⁵ were in close agreement with our calculated values of 6.251 and 60.79 µM, respectively. While we had initially expected IC₅₀ values for second-generation compounds to be much lower than that of our lead inhibitor (based on screening results from Figure 44), these values calculated for 30-33 (146.0, 92.35, 825.3 and 455.7 µM, respectively) were actually higher than that of 1. In fact, even the IC₅₀ value for adenosine (31.80 µM) was lower than those of 30-33, and for that matter, 1 as well.

| Table 7. ErmC’ IC₅₀ values for 1, 30-33, sinefungin, SAH and adenosine, with 0.5 µM SAM. |
|---------------------------------|-------------------------------|-------------------|---|-----------------|
|                                | IC₅₀ (µM)                    | 95% Confidence Intervals | R² | # of points Analyzed |
| Sinefungin                     | 6.251 (5.0)†                 | 5.265 to 7.423        | 0.9881 | 24 |
| SAH                            | 16.35                        | 14.23 to 18.78        | 0.9927 | 24 |
| Adenosine                      | 31.80                        | 27.58 to 36.67        | 0.9910 | 24 |
| Cyclopentyl (1)                | 60.79 (63)†                 | 40.85 to 90.48        | 0.9289 | 24 |
| Benzyl (30)                    | 146.0                        | 117.1 to 182.0        | 0.9747 | 21 |
| Phenethyl (31)                 | 92.35                        | 57.69 to 147.8        | 0.8879 | 21 |
| Propylphenyl (32)              | 825.3                        | 635.8 to 1071         | 0.9134 | 21 |
| Butylphenyl (33)               | 455.7                        | 326.8 to 635.5        | 0.9053 | 21 |

Values calculated from data in Figure 48 using Prism 5.0a software with a nonlinear regression (curve fit) of log(inhibitor) vs. response(background subtracted) and the bottom constrained to 0.
† IC₅₀ values previously published by Clancy et al.⁸
Discussion

Proposed binding mode of second-generation compounds

We had originally asked several questions regarding the interactions of second-generation compounds with ErmC’ based on our assumption that these compounds would bind in the likely SAM pocket, however, the outcome of the previous experiments has since suggested otherwise. This presented a new question - if 30-33 were not binding to the SAM pocket, then how were these compounds acting to inhibit ErmC’?

One mechanism currently being investigated is that of second-generation compounds binding within the substrate pocket of ErmC’. A model illustrating how these inhibitors might bind to the substrate pocket is depicted below (Figure 50).

![Figure 50](image). Proposed binding mechanism of second-generation inhibitors into the target adenosine pocket of ErmC’. Represented by 30, the adenosine moiety would orient like the putative substrate, and the 5’-phenyl substituent would be sandwiched between the hydrophobic cleft of α-helices D & E, and the protonated 5’-N+ would form an ionic bond with Glu-128.
We propose that the adenosine moiety of second-generation inhibitors orients within ErmC’ in a similar fashion as the putative adenosine substrate. As a result, the 5’-C of our ligand would be pointed between the α-helices D and E, where the phenyl substituent could lie favorably within this hydrophobic cleft. Further stability could be achieved through an ionic bond between the protonated 5’-N+ of our inhibitor and the Glu-128 on ErmC’ (Figure 50).

This orientation would also offer an explanation for why protected intermediates 26-29 unexpectedly inhibited ErmC’ (described in Figure 42). By binding to the target adenosine site in this manner, the 2’-O and 3’-O of inhibitors 26-29 would be pointed away from the binding pocket and into the surrounding solvent, thus precluding the potential interference from an isopropylidene that is attached at these positions. Additionally, this binding mechanism in Figure 50 may explain why attempts to crystallize ErmC’ with second-generation compounds failed to show a bound ligand, despite the successes of co-crystallizing ErmC’ with 7 and 12 (Chapter 3). Crystallizing ErmC’ may cause motifs around the target adenosine site (in particular, α-helices D and E) to pack so tightly that an inhibitor at this binding site is displaced. [Note: On-going experiments with molecular dynamic simulations of ErmC’ show that the α-helices D and E are very dynamic in solution, supporting the idea that crystallized ErmC’ could impede binding of second-generation compounds into the target adenosine site (unpublished results, John Hackett, VCU).]

This proposed binding mode was recently tested using increasing concentrations of 23S to see if this affected a change in ErmC’ inhibition by second-generation compounds (Figure 51). Results from this preliminary competition assay showed that raising the concentration of 23S caused a drop of inhibition from 32 and 33, while inhibition by 1, 30 and 31 remained constant.
or increased slightly (Figure 51). Although these tests will need to be investigated further, these early data have provided partial support for the idea that at least 32 and 33 are binding within the target adenosine pocket.

![Graph](image)

Figure 51. Competition assays with increasing concentrations of 23S. Compounds were added at a final concentration of 1 mM to reaction like those described in Figure 42, except 23S was varied (3.5, 10, 20 and 85). Activity was measured by filter binding assay after 32 min.

**Future work**

One future objective will be to elucidate the interactions of second-generation compounds with ErmC’. This might simply be accomplished by further attempts at co-crystallization, since only two of our crystals were previously tried by X-ray diffraction. We will
also try new crystallization conditions that include SAM (at 20 µM, for example) in case cofactor facilitates the binding of 30-33 into the adenosine pocket of ErmC’. [Note: Allosteric binding sites for SAM have been observed in certain DNA methyltransferases,111-115 but have never been investigated with ErmC’.] Alternatively, NMR spectroscopy of ErmC’ in solution with one of the second-generation compounds could provide knowledge about the binding site of these inhibitors. One obstacle to this approach will be the limited solubility of ErmC’ in the absence of glycerol, though this may be ameliorated by adding a minimal amount of DMSO.

If the proposed binding mode for 30-33 is confirmed, it would signify that we have come full circle from our original goal of docking adenosine analogs into the substrate pocket with N6-substituents probing for interactions within the SAM pocket of ErmC’. Our model (Figure 50) shows the N6-cyclopentyl of 30 branching into the SAM pocket in agreement with the in silico docking of 1 performed by Feder et al.40 (Figure 22). Therefore, a third library of test-compounds may be synthesized that uses the scaffold from one of our second-generation compounds and replaces the N6-cyclopentyl with other exploratory groups. For example, we could borrow from the same library of substituents placed on the N6-position of first-generation compounds (1-23), since these groups were originally picked for the purpose of probing the SAM pocket.

Conclusions

In response to the growing threat of antibiotic resistance, two closely related methyltransferases were targeted - ErmC’ to restore MLSB antibiotics, and KsgA to form a novel class of antibiotics that stalls ribosomal assembly. One of the biggest challenges we faced with
designing inhibitors suitable for drug development was to selectively block ErmC’ or KsgA without also affecting methyltransferases important in human physiology. The common approach to this challenge has been to aim for the more specific substrate binding pocket of the intended methyltransferase rather than the more ubiquitous SAM binding pocket. Success with this strategy appeared to have been realized when Clancy et al. presented N⁶-cyclopentyl adenosine (1) as a potent and selective ErmC’ inhibitor that was not competitive with SAM binding. This was followed up by the study from Feder et al., which showed 1 docking into the substrate pocket of ErmC’ and the N⁶-cyclopentyl substituent branching into the neighboring SAM pocket, \textit{in silico}.

Based largely on these findings, we synthesized the first library of compounds 1-23, which included N⁶-cyclopentyl adenosine (as the lead inhibitor and as a benchmark) along with 22 other analogues that varied by the substitution on the N⁶-position. Our objective was for these new adenosine analogs to similarly dock into the substrate pocket of ErmC’ (as the target-adenosine putatively orients) and to use the substituents in this library to probe for more favorable interactions within the adjacent SAM pocket. Additionally, these compounds would be tested for inhibition against KsgA, a paralog of ErmC’.

After developing a scintillation proximity assay (SPA) that efficiently measured rRNA methylation using commercially available yttrium silicate (YSi) scintillant beads, compounds 1-23 were screened at 1 mM (with 20 µM SAM). For ErmC’, several of our compounds were either more potent (7) or nearly as potent (4, 12, 18 & 19) as the lead compound 1. We also saw for the first time that 1 was inhibitory towards KsgA, and that other compounds (5, 7 & 18) were more potent. However, when two of these inhibitors (7 & 12) were co-crystallized with ErmC’
we saw that these compounds docked into the SAM pocket with an orientation similar to other adenosine-based ligands (SAM, SAH and sinefungin) and the 5’-C of 7 and 12 was pointed at the substrate pocket. A similar binding mode was also indicated for 1 after we saw that raising SAM concentrations resulted in a loss of inhibition (contrary to the previous report by Clancy et al.).

Therefore, the second library of compounds was designed to exploit this orientation by adding substituents off the 5’-C of our lead inhibitor. Through a five step synthesis, the 5’-OH of 1 was replaced with an amine linked to either a benzyl, phenethyl, propylphenyl or butylphenyl to give 30 - 33, respectively. Our new objective was for second-generation compounds to dock into the SAM pocket of ErmC’ or KsgA, and for the 5’-phenyl substituent to probe for potential interactions within the neighboring substrate pocket.

Initially we saw these second-generation compounds to be more inhibitory than the lead compound against ErmC’, but they were not active against KsgA, suggesting that the addition of the 5’-phenyl substituent had improved potency and selectivity towards ErmC’. However, during IC₅₀ assays with ErmC’ the SAM concentration was lowered from 20 µM to 0.5 µM, and we saw that the inhibition by 30 - 33 was nearly unchanged. Inhibition by 1 was as expected higher at these lower [SAM] conditions, but was now unexpectedly more potent than 30 - 33. These results and other preliminary data lead us to believe that second-generation inhibitors were not acting primarily at the SAM pocket (as 1, 7 & 12 had done), but were possibly binding within the substrate pocket of ErmC’ as was originally intended for first-generation compounds.

Clearly much further research is needed before inhibitors of ErmC’ or KsgA may be developed into appropriate therapies for antibiotic resistant infections. The screening,
synthesizing and co-crystallization work that have been presented herein should provide a substantial basis for any future work in this area.

**Experimental Section**

**Inhibition Assays**

*Screening 1, 24-33 and sinefungin at 1 mM* (Figures 42 and 43)

Test compounds were added by 5 µL from 10 mM stock dissolved in DMSO to a typical enzyme reaction with either ErmC’ or KsgA, as described in Chapter 3 except filter binding assays were used to measure activity. In short, reactions were completed in 50 µL containing buffer K, 1 mM test-compound, 10% DMSO, 0.2 µM of rRNA substrate (23S from *B. subtilis* for ErmC’ or 30S from ksgR *E. coli* for KsgA), 0.2 µM of enzyme (ErmC’ or KsgA), 20 µM SAM (780 cpm/pmol) (Hot:Cold SAM at 1:97), incubated at 37 °C for 32 min for ErmC’ or 5 min for KsgA, and quenched with 10 µL of 100 mM cold SAM. Each reaction was done in triplicate, and the average was graphed with error bars of +/- 1 Std Dev.

*Testing the effects of DMSO concentrations on ErmC’ activity* (Figure 44)

Varying amounts of DMSO (0, 2.5, 5, 7.5, 10, 15, 20 or 25 µL) were incorporated into typical ErmC’ reactions, as described above except the incubation time was 8 min and no test compound was added with DMSO. These reactions were done in triplicate, measuring their activity by filter binding assay and graphing the averages with error bars of +/- 1 Std Dev using excel software. Background counts were not considered for these reactions.
**First attempt at IC<sub>50</sub> assays using 20 µM SAM** (Figure 45)

Test compounds (30-33, sinefungin & SAH) were added (2.5 µL of 20x stock dissolved in DMSO) to final concentrations of 0, 1, 5, 10, 25, 50, 100, 250, 500, and 1000 µM in typical ErmC’ reactions as described for Figure 42, except reactions had 5% DMSO and were quenched at 8 min. Reactions were done in triplicate, except for SAH (only two replicas completed for 1 µM) and 32 (only two replicas completed for 1, 5 and 10 µM). Methylation activity was measured by filter binding assay and averages were graphed with error bars of +/- 1 SEM using Prism 5.0a software and nonlinear regression (curve fit) of log(inhibitor) vs. response with the bottom constrained to 0. Background counts were not subtracted from signals.

**Testing the effects of SAM concentration on ErmC’ inhibition with I fixed at 63 µM** (Figure 46)

Compound I was added at a final concentration of 63 µM to typical ErmC’ reactions, except the SAM concentration was varied (0.05, 0.1, 0.5 and 1.0 µM), the ratio of Hot:Cold SAM was raised to 1:18 (5% <sup>3</sup>H-SAM), reactions had 5% DMSO and were quenched at 120 min (reaction time same as reactions performed by Clancy et al.<sup>85</sup>). Activity was measured by filter binding assay.

**Time course assay of ErmC’ with 0.5 µM SAM** (Figure 47)

Reactions were performed as before except 0.5 µM SAM (5% <sup>3</sup>H-SAM) was used and the volume was scaled up to 0.5 mL. Activity was measured by SPA at the time points of 1, 15, 30, 60, 120, 180 and 240 min. Assays were done in triplicate, plotting the average with error bars of +/- 1 Std Dev.
IC<sub>50</sub> assays repeated using 0.5 µM SAM (Figure 48 and Table 7)

Compounds (1, 30-33, sinefungin, SAH and adenosine) were tested between 0.1 µM and 1.0 mM in typical ErmC’ reactions (as described for Figure 42), except reactions had 5% DMSO, were quenched at 8 min and activity was measured by SPA. Reactions were done in triplicate, the background counts from reactions without enzyme were subtracted from signals and averages were graphed with error bars of +/- 1 SEM using Prism 5.0a software with nonlinear regression (curve fit) of log(inhibitor) vs. response(background subtracted) and the bottom constrained to 0. Concentrations of test-compounds were determined by UV absorbance using extinction coefficients of 16.68 mM<sup>-1</sup>cm<sup>-1</sup> for 1, 30-33 (measuring the OD<sub>271</sub>) and 15.5 mM<sup>-1</sup>cm<sup>-1</sup> for sinefungin, SAH and adenosine (measuring the OD<sub>260</sub>).

Testing the effects of 23S concentrations on inhibition of ErmC’ by 1 and 30-33 (Figure 51)

Compounds (1 and 30-33) were tested at 1 mM in the same ErmC’ reaction used for Figure 42, except the concentration of 23S was varied (3.5, 10, 20 & 85 pmol) and each reaction was performed once.
References
References


Appendix A
$^1$H NMR of $N^6$-cyclopentyl-adenosine (1)
$^{13}$C NMR of $N^6$-cyclopentyl-adenosine (1)
COSY NMR of N°-cyclopentyl-adenosine (1)
HSQC NMR of $N^6$-cyclopentyl-adenosine (1)
$^1$H NMR of N$^\text{6}$-ethyl-adenosine (3)
$^{13}$C NMR of $N^6$-ethyl-adenosine (3)
COSY NMR of N^6-ethyl-adenosine (3)
HSQC NMR of $N^\beta$-ethyl-adenosine (3)
$^1$H NMR of $N^6$-n-butyl-adenosine (4)
COSY NMR of $N^6$-n-butyl-adenosine (4)
HSQC NMR of N<sup>6</sup>-<i>n</i>-butyl-adenosine (4)
$^1$H NMR of $N^6$-octyl-adenosine (5)
$^{13}$C NMR of N$^6$-octyl-adenosine (5)
COSY NMR of $N^6$-octyl-adenosine (5)
HSQC NMR of N⁶-octyl-adenosine (5)
$^1$H NMR of $N^\epsilon$-ethylamine-adenosine (6)
$^{13}$C NMR of $N^6$-ethylamine-adenosine (6)
COSY NMR of $N^6$-ethylamine-adenosine (6)
HSQC NMR of $N^6$-ethyamine-adenosine (6)
\(^1\)H NMR of \(N^6\)-octylamine-adenosine (7)
$^{13}$C NMR of $N^6$-octylamine-adenosine (7)
COSY NMR of $N^6$-octylamine-adenosine (7)
HSQC NMR of $N^6$-octylamine-adenosine (7)
$^1$H NMR of $N^6$-isopropyl-adenosine (8)
$^{13}$C NMR of $N^6$-isopropyl-adenosine (8)
COSY NMR of N6-isopropyl-adenosine (8)
HSQC NMR of $N^\alpha$-isopropyl-adenosine (8)
$^1$H NMR of $N^6$-t-butyl-adenosine (9)
$^{13}$C NMR of $N^6$-t-butyl-adenosine (9)
COSY NMR of $N^6$-t-butyl-adenosine (9)
HSQC NMR of $N^6$-t-butyl-adenosine (9)
$^1$H NMR of $N^6$-phenyl-adenosine (10)
$^{13}$C NMR of $N^6$-phenyl-adenosine (10)
COSY NMR of N\textsuperscript{6}-phenyl-adenosine (10)
HSQC NMR of $N^6$-phenyl-adenosine (10)
$^1$H NMR of $N^6$-benzyl-adenosine (11)
$^{13}$C NMR of $N^6$-benzyl-adenosine (11)
COSY NMR of N^6-benzyl-adenosine (11)
HSQC NMR of $N^6$-benzyl-adenosine (11)
$^1$H NMR of $N^6$-phenethyl-adenosine (12)
$^{13}$C NMR of $N^6$-phenethyl-adenosine (12)
COSY NMR of $N^6$-phenethyl-adenosine (12)
HSQC NMR of N$_6$-phenethyl-adenosine (12)
$^1$H NMR of $N^6$-4-methylbenzyl-adenosine (13)
$^{13}$C NMR of $N^6$-4-methylbenzyl-adenosine (13)
COSY NMR of \( N^6 \)-4-methylbenzyl-adenosine (13)
HSQC NMR of $N^6$-4-methylbenzyl-adenosine (13)
$^1$H NMR of $N^6$-4-chlorobenzyl-adenosine (14)
$^{13}$C NMR of $N^6$-4-chlorobenzyl-adenosine (14)
COSY NMR of $\text{N}^6$-4-chlorobenzyl-adenosine (14)
HSQC NMR of N\textsuperscript{6}-4-chlorobenzyl-adenosine (14)
$^1$H NMR of $N^6$-4-methoxybenzyl-adenosine (15)
$^{13}$C NMR of $N^6$-4-methoxybenzyl-adenosine (15)
COSY NMR of N⁶-4-methoxybenzyl-adenosine (15)
HSQC NMR of $N^6$-4-methoxybenzyl-adenosine (15)
$^1$H NMR of N$^6$-4-pyridinemethyl-adenosine (16)
$^{13}$C NMR of $N^6$-4-pyridinemethyl-adenosine (16)
COSY NMR of $N^6$-4-pyridinemethyl-adenosine (16)
HSQC NMR of N6-4-pyridinemethyl-adenosine (16)
$^1$H NMR of $N^6$-tetrazole-adenosine (17)
$^{13}$C NMR of $N^6$-tetrazole-adenosine (17)
COSY NMR of $N^6$-tetrazole-adenosine (17)
HSQC NMR of $N^6$-tetrazole-adenosine (17)
$^1$H NMR of $N^6$-cyclohexyl-adenosine (18)
$^{13}$C NMR of $N^6$-cyclohexyl-adenosine (18)
COSY NMR of $N^6$-cyclohexyl-adenosine (18)
HSQC NMR of $N^6$-cyclohexyl-adenosine (18)
$^1$H NMR of $N^6$-tetrahydropyran-adenosine (19)
$^{13}$C NMR of $N^6$-tetrahydropyran-adenosine (19)
COSY NMR of $\text{N}^6$-tetrahydropyran-adenosine (19)
HSQC NMR of $N^\circ$-tetrahydropyran-adenosine (19)
$^1$H NMR of N$^6$-piperidine-adenosine (20)
$^{13}$C NMR of $N^6$-piperidine-adenosine (20)
COSY NMR of $N^6$-piperidine-adenosine (20)
HSQC NMR of N\(^6\)-piperidine-adenosine (20)
$^1$H NMR of $N^6$-methylcyclopropyl-adenosine (21)
$^{13}$C NMR of $N^6$-methylcyclopropyl-adenosine (21)
COSY NMR of $N^6$-methylcyclopropyl-adenosine (21)
HSQC NMR of 6'-methylcyclopropyl-adenosine (21)
$^1$H NMR of $N^6$-propargyl-adenosine (22)
$^{13}$C NMR of $N^6$-propargyl-adenosine (22)
COSY NMR of $N^6$-propargyl-adenosine (22)
HSQC NMR of N⁶-propargyl-adenosine (22)
$^1$H NMR of $N^6$-allyl-adenosine (23)
$^{13}$C NMR of $N^6$-allyl-adenosine (23)
COSY NMR of $N^6$-allyl-adenosine (23)
HSQC NMR of $N^6$-allyl-adenosine (23)
Appendix B
\(^1\)H NMR of 2', 3'-O-Isopropylidene-\(N^6\)-cyclopentyl adenosine (24)
$^{13}$C NMR of 2', 3'-O-Isopropylidene-$N^6$-cyclopentyl adenosine (24)
COSY NMR of 2', 3'-O-Isopropylidene-\textit{N}^6\textit{-cyclopentyl adenosine (24)}
HSQC NMR of 2', 3'-O-Isopropylidene-N^6-cyclopentyl adenosine (24)
$^1$H NMR of 2', 3'-Isopropylidene-5'-mesyl-$N^6$-cyclopentyl adenosine (25)
$^{13}$C NMR of 2', 3'-Isopropylidene-5'-mesyl-$\text{N}^6$-cyclopentyl adenosine (25)
COSY NMR of 2', 3'-Isopropylidene-5'-mesyl-\(N^6\)-cyclopentyl adenosine (25)
HSQC NMR of 2', 3'-Isopropylidene-5'-mesyl-N^6-cyclopentyl adenosine (25)
$^1$H NMR of 2', 3'-O-Isopropylidene-5'-deoxy-5'-benzylamino-N$_6$-cyclopentyl adenosine (26)
$^{13}$C NMR of 2', 3'-O-Isopropylidene-5'-deoxy-5'-benzylamino-$N^6$-cyclopentyl adenosine (26)
COSY NMR of 2', 3'-O-Isopropylidene-5'-deoxy-5'-benzylamino-N6-cyclopentyl adenosine (26)
HSQC NMR of 2', 3'-O-Isopropylidene-5'-deoxy-5'-benzylamino-\(N^6\)-cyclopentyl adenosine (26)
$^1$H NMR of 2', 3'-O-Isopropylidene-5'-deoxy-5'-phenethylamino-$N^6$-cyclopentyl adenosine (27).
$^{13}$C NMR of 2', 3'-O-isopropylidene-5'-deoxy-5'-phenethylamino-$N^6$-cyclopentyl adenosine (27).
COSY NMR of 2', 3'-O-Isopropylidene-5'-deoxy-5'-phenethylamino-\(N^6\)-cyclopentyl adenosine (27).
HSQC NMR of 2', 3'-O-Isopropylidene-5'-deoxy-5'-phenethylamino-N6-cyclopentyl adenosine (27).
$^1$H NMR of 2', 3'-O-Isopropylidene-5'-deoxy-5'-propylphenylamino-N$^6$-cyclopentyl adenosine (28).
$^{13}$C NMR of 2', 3'-O-isopropylidene-5'-deoxy-5'-propylphenylamino-\textit{N}^6-cyclopentyl adenosine (28).
COSY NMR of 2', 3'-O-Isopropylidene-5'-deoxy-5'-propylphenylamino-\(\text{N}^6\)-cyclopentyl adenosine (28).
HSQC NMR of 2',3’-O-isopropylidene-5'-deoxy-5'-propylphenylamino-\textsuperscript{N\textdegree}-cyclopentyl adenosine (28).
$^1$H NMR of 2', 3'-O-Isopropylidene-5'-deoxy-5'-butylphenylamino-$N^6$-cyclopentyl adenosine (29).
$^{13}$C NMR of 2', 3'-O-Isopropylidene-5'-deoxy-5'-butylphenylamino-$N^6$-cyclopentyl adenosine (29).
COSY NMR of 2’, 3’-O-Isopropylidene-5’-deoxy-5’-butylphenylamino-N6-cyclopentyl adenosine (29).
HSQC NMR of 2', 3'-O-Isopropylidene-5'-deoxy-5'-butylphenylamino-N6-cyclopentyl adenosine (29).
$^1$H NMR of 5'-deoxy-5'-benzylamino-$N^6$-cyclopentyl adenosine (30).
$^{13}$C NMR of 5'-deoxy-5'-benzylamino-$N^6$-cyclopentyl adenosine (30).
COSY NMR of 5'-deoxy-5'-benzylamino-N^6-cyclopentyl adenosine (30).
HSQC NMR of 5'-deoxy-5'-benzylamino-N^6-cyclopentyl adenosine (30).
$^1$H NMR of 5'-deoxy-5'-phenethylamino-$N^\epsilon$-cyclopentyl adenosine (31).
$^{13}$C NMR of 5'-deoxy-5'-phenethylamino-$N^6$-cyclopentyl adenosine (31).
COSY NMR of 5'-deoxy-5'-phenethylamino-N⁶-cyclopentyl adenosine (31).
HSQC NMR of 5’-deoxy-5’-phenethylamino-N^6-cyclopentyl adenosine (31).
$^1$H NMR of 5'-deoxy-5'-propylphenylamino-N$^6$-cyclopentyl adenosine (32).
$^{13}$C NMR of 5'-deoxy-5'-propylphenylamino-$N^6$-cyclopentyl adenosine (32).
COSY NMR of 5'-deoxy-5'-propylphenylamino-\(N^6\)-cyclopentyl adenosine (32).
HSQC NMR of 5’-deoxy-5’-propylphenylamino-\(N^6\)-cyclopentyl adenosine (32).
$^1$H NMR of 5'-deoxy-5'-butylphenylamino-$N^6$-cyclopentyl adenosine (33).
$^{13}$C NMR of 5'-deoxy-5'-butylphenylamino-N$^6$-cyclopentyl adenosine (33).
COSY NMR of 5'-deoxy-5'-butylphenylamino-\(\text{N}^6\)-cyclopentyl adenosine (33).
HSQC NMR of 5′-deoxy-5′-butylphenylamino-N6-cyclopentyl adenosine (33).