CONFORMATIONALLY CONSTRAINED AMINOGLYCOSIDES: RIBOSOMAL BINDING PROBES AND POTENTIAL ANTIBIOTICS

A Thesis

Submitted to the Graduate School
of the University of Notre Dame
in Partial Fulfillment of the Requirements
for the Degree of

Master of Science

by

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Notre Dame, Indiana
April 2006
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ACKNOWLEDGMENTS

I owe a great deal of thanks to many people who I have worked with over the past two and a half years. First on that list is my advisor, Dr. Shahriar Mobashery. Without his support, scientific expertise and management skills the highs would not have been nearly as high and the lows much more difficult to recover from. Your unique combination of skills has helped me tremendously in wading through some very challenging chemistry and I am a better scientist for it. Thank you Shahriar. I will miss being apart of your group.

To Dusan, Mijoon and Jed I wish to also extend my deepest thanks. Dusan, your suggestions have been instrumental in teaching me how to solve some of the numerous problems aminoglycoside chemists face. I have appreciated working alongside you these few years. Continue to enjoy the Florida sun. Mijoon, I am sorry you were the first person I always turned to for reaction advice. You were very patient with all of us inexperienced scientists in the lab though, and personally I am extremely grateful. Best of luck with your future career path. I hope everything works out for you. Finally Jed I would like to thank you. You were the scifinder of our group when both university accounts were being used (and to be quite honest, even when they weren’t). One would almost swear the publishers sent you emails directly with respect to work that was pertinent to our group. At the same time I also came to appreciate the breadth of organic/medicinal chemistry knowledge you possess. I will miss our discussions. My
thanks also to the National Institute of Health for funding the research presented in this thesis.

In conclusion, I would like to thank my wonderful wife Kristi. You have made even the longest of days/weeks in the lab forgettable and I have been able to maintain my focus as a result. Asking you to play war at the Dow Center was the best decision I have ever made and I cannot imagine life without you. There is nothing more valuable to me than our relationship.
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<td>carbon 13</td>
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<tr>
<td>$^1$H</td>
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<tr>
<td>Å</td>
<td>angstrom ($10^{-10}$ meters)</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>$N,N$-dimethylformamide</td>
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</tr>
<tr>
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<td>( R_t )</td>
<td>retention time</td>
</tr>
<tr>
<td>s</td>
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<tr>
<td>δ</td>
<td>lower case delta (chemical shift)</td>
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<td>μ</td>
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CHAPTER ONE

THE BACTERIAL RIBOSOME: A TARGET FOR ANTIBIOTICS

1.1 The Path of Gene Expression

Though generally oversimplified by those outside of the scientific community as 
strictly physical resemblances uniting parents to offspring (having something to do with 
DNA), the transmission of visible traits between generations is obviously a grossly 
incomplete picture for the role of genes in living organisms. Indeed research in the past 
60 years has revealed that gene expression is far more complex than even those in the 
field of science previously thought (for example, the majority of all protein molecules 
found within prokaryotes and eukaryotes are built upon the blueprints found in DNA). In 
an attempt to understand the molecular details that give proper justice to this natural 
phenomenon, scientists in biology, physics and chemistry have been hard at work 
investigating and uncovering its truths for decades. The additive nature of these data has 
resulted in a revolutionized view of genetic continuity and evolution in recent years, 
comparable only to the scientific turning point put in motion by Darwin’s *Origin of 
Species* 150 years ago. How then is the genetic information found in DNA ultimately 
articulated in the amino acid sequence of proteins and how can this biological pathway be 
manipulated for the benefit of human health?

Transfer of information stored in a gene begins inside the nucleus of a cell with 
production of an RNA strand from a DNA template, a process referred to as
transcription. During this process DNA is unraveled from its double helical form by a complex enzyme system as one strand gets transcribed in a base complementary fashion to form another nucleotide strand (RNA). The resulting polynucleotide chain will become one of three possible RNA molecules (controlled by posttranscriptional processing that will not be discussed herein). Ribosomal RNA (rRNA), which eventually becomes a major structural constituent of the ribosome, is the first. Second, there are those that end up as transfer RNA (tRNA; Figure 1-1). These strands function not only to carry individual amino acids to the ribosome for addition to a growing peptide chain, but also have a responsibility to recognize the information encoded in the final RNA type (mRNA) through pairing of a three base nucleotide triplet in tRNA (anticodon) with a complementary three base nucleotide triplet (codon) on mRNA within the ribosome (detail will be given to this process later). Finally, messenger RNA (mRNA) may result from posttranscriptional processing. The role of the mRNA strand is to encode the information of an amino acid sequence for synthesis of proteins by the ribosome; a process known as translation. RNA identity (of the three possibilities described) is dictated by the first 30-70 bases, known as the promoter region, of the polynucleotide strand. Depending on the base sequence of this promoter, unique processing enzymes will bind the strand for appropriate functional modifications. Regardless of this RNA identity, however, all three structures have a part to play in protein biosynthesis.

1.2 Ribosome Structure and Function

Elucidating the role of ribosomes in gene expression was one of the most significant contributions to come out of molecular biology in the 1960s. Translation of mRNA encoded genetic information into the polypeptide chain of a protein, work for
which the ribosome is accountable, provides the key link between a species genotype and its phenotype. This single organelle thus bears an enormous responsibility; one which must be carried out at a marvelous pace and with great fidelity.\textsuperscript{2}

All ribosomes consist of two unequally sized subunits. In bacteria these two units are referred to as the 50S (large) and 30S (small) subunits according to their sedimentation rates by centrifugation. The 30S subunit contains a strand of rRNA labeled 16S RNA and roughly 20 proteins, whereas the 50S subunit houses 23S RNA, 5S RNA and nearly 30 proteins.\textsuperscript{3} Association of the large and small subunits through a network of intermolecular bridges\textsuperscript{3} results in the fully functional 70S ribosome of approximately 2.5 MD (Figures 1-2 and 1-3); a ribonucleoprotein macromolecule for which recent X-ray crystallographic efforts have yielded extensive structural data.\textsuperscript{5-11}
Figure 1-2: Structural insights from a 5.5 Å crystal structure of the *T. Thermophilus* ribosome. Panels (A) through (D) represent successive 90 rotations about the vertical axis of (A). (A) View from the back of the 30S subunit in which H represents the head, P the platform, N the neck and B the body. (B) View from the right showing the small subunit on the left (light blue) and the large subunit on the right (grey). The proteins of the small subunit (dark blue) and the large subunit (purple) are seen here to mostly occupy the exterior of the ribosome. (C) View from the back of the 50S subunit. (D) View from the left of (A).

Transfer RNA (tRNA) is the substrate that the ribosome consumes during protein synthesis. Every cell contains a population of tRNAs that differ in the amino acid they “transfer”, but each has similar molecular mass (~ 25,000 D) and the same L shaped tertiary structure (Figure 1-1). At the far end of the long arm of this L there is a three base anticodon sequence that is complementary to an mRNA nucleoside base triplet (codon).
The mRNA, which weaves its way through the ribosome near the subunit interface, uses this base triplet to recognize and gather appropriate tRNAs from the cytoplasm. At the distal end of the tRNA’s short arm is a base sequence to which the amino acid specified by the anticodon is attached. It is the job of the so-called “synthetases” within the cytosol to enzymatically attach each amino acid to the appropriate tRNA through formation of ester bonds between the carboxylate group of the amino acid and the terminal adenine residue of tRNA (Figure 1-1 and 1-4). Hence there is at least one tRNA molecule for every amino acid used in protein synthesis.

Three unique tRNA binding sites within the ribosome have been found. These are designated the A (for “aminoacyl”; the site for binding aminoacyl tRNA), P (for “peptidyl”; the site for tRNA with the growing polypeptide chain) and E (for “exit”; where deacylated tRNA resides prior to its release from the ribosome) sites. During translation the 30S subunit holds mRNA and the anticodon stem loop (ASL) of the correct (cognate) tRNA in place such that the three nucleic acid codon sequence of
Figure 1-4: Example of enzymatic attachment of the amino acid cysteine to tRNA by tRNA synthetase. One equivalent of ATP is consumed during this reaction.

mRNA is paired with the anticodon of tRNA. Meanwhile the aminoacylated end of tRNA moves into position within the 50S subunit. It is here, within the 50S subunit, that peptide bond formation is catalyzed and transfer of an amino acid from the A-site tRNA to the nascent polypeptide chain within the P-site takes place (Figure 1-5).

Once this elongation step is carried out and the correct amino acid has been added to the growing peptide chain, the newly deacylated tRNA in the P site must be replaced by peptidyl-tRNA now found in the A site, and a new aminoacyl-tRNA from the cytosol must bind in the A site for addition of the next amino acid. Additionally, the tRNA contained within the E site must exit in order to create the necessary vacancy for

Figure 1-5: Representation of the peptidyl transferase reaction that occurs within the 50S subunit; the key step in peptide elongation.
deacylated P site tRNA. While these critical tRNA movements are still not completely understood,\textsuperscript{17-20} it is thought that a protein called elongation factor G helps facilitate the translocation (movement of tRNA from one site to the next) process by coupling the energy of GTP hydrolysis to conformational changes within the ribosome.\textsuperscript{21-22}

1.3 \textit{Specifics of Protein Synthesis}

One amino acid at a time, that is how even the largest of proteins (>1000 amino acids) is constructed. And though neither the activation of amino acids for addition to a growing polypeptide chain nor posttranslational processing (such as proper folding of the completed protein) are duties given to the ribosome, there is still enormous complexity in the operation of adding the correct amino acid to a growing polypeptide. To understand this complexity, translation of mRNA encoded genetic information into the proper protein is often broken down into three stages. These stages are known as initiation, propagation (or elongation) and termination/release\textsuperscript{23}; each stage requiring a very specific set of components from within the cell (Table 1-1).

Initiation in the bacterial ribosome begins when the 30S ribosomal subunit binds initiation factors 1 and 3 (abbreviated IF-1 and IF-3). These protein “factors” act as communicators of preparedness between ribosomal body parts in the beginning of protein synthesis. Messenger RNA binding to the 30S subunit then takes pace, and the start codon (AUG) on mRNA is guided to its proper position by a sequence of four to nine purine residues (the Shine-Dalgarno sequence).\textsuperscript{24} These residues are located roughly ten base pairs to the 5’ side of the initiation codon and base pair with a complementary pyrimidine-rich sequence at the 3’ end of 16S rRNA in the 30S subunit. Meanwhile IF-3 prevents the 50S subunit from prematurely combining with the 30S subunit.\textsuperscript{25}
The AUG start codon where the first aminoacylated tRNA (N-formylmethionyl-tRNA in bacterial systems) binds is positioned within the P site. N-formylmethionyl-tRNA represents the only amino acid-tRNA complex that binds first to the P site; the result of IF-1 blocking the A site. All remaining aminoacylated tRNAs will be guided to the A site codon during elongation. Upon complexation of this first tRNA substrate to its anticodon, GTP bound IF-2 joins the initiation complex. The combination of both IF-2 and N-formylmethionyl-tRNA being bound triggers addition of the 50S subunit. Finally, GTP from IF-2 is hydrolyzed to GDP, resulting in a conformational change within the ribosome that releases all three initiation factors (Figure 1-6). A fully functional 70S ribosome is now intact and ready for elongation.
Elongation represents the most elaborate stage of protein biosynthesis. To begin, the appropriate aminoacetylated-tRNA must bind a complex of elongation factor-tu (EF-Tu) and GTP. This complex of tRNA and EF-Tu is recognized by the 70S ribosome by both base pairing of the codon and anticodon triplets as well as by an overall shape recognition event of the intact complex with the ribosome.\textsuperscript{27-28} Once bound properly to the A site, GTP from EF-Tu is hydrolyzed and the complex is released from the ribosome, leaving only the aminoacylated tRNA behind. Peptide bond formation occurs by transfer of the N-formylmethionyl group within the P site to the amino group of the second amino acid (shown previously in Figure 1-3) in the A site, a process thought to be catalyzed by 23S rRNA.
The final step of elongation is known as translocation. As the ribosome prepares for the next aminoacyl-tRNA it must shift one codon (three bases) toward the 3’ end of mRNA. This movement not only vacates the A site for the next aminoacyl-tRNA, it also drags the peptidyl-tRNA into the P site and moves deacylated-tRNA from the P-site into the E site. Elongation factor G, bound near the A site, provides the energy needed for these movements through hydrolysis of GTP. Thus for each amino acid that is correctly added to a growing polypeptide, at least four GTP molecules (122 kJ/mol) are hydrolyzed.

Termination and release of a polypeptide represents the last step of protein synthesis for which the ribosome is responsible. In order for the ribosome to recognize that the elongation stage is over, one of three termination codons in mRNA (UAA, UAG, UGA) must occupy the A site. Once this happens, a series of three release factors (RF₁, RF₂, RF₃) have the role of properly dismantling the ribosome. To do this RF₁ and RF₂ must first recognize the stop codon in the A site and bind it. This recognition event induces the enzyme peptidyl transferase to transfer the polypeptide within the P site to a water molecule. At this point RF₃ is thought to release the 50S subunit from the intact ribosome and the unfolded polypeptide is left for posttranslational processing.²⁹-³⁰

1.4 Antibiotic Classes Targeting the Bacterial Ribosome

Due to its critical role in many biological processes within the cell, RNA has emerged as a prime target for small molecule binding.³¹ As with proteins, which have been therapeutic targets since the dawn of modern medicine, RNA structures contain rich functional diversity; the result of well defined secondary structures and unique three dimensional folds. This structural sophistication, combined with the negatively charged
pockets generated by unique RNA architectures, provides cavities with the potential for binding small molecules.\textsuperscript{32}

Given the fundamental importance of RNA to the ribosome and the centrality of protein biosynthesis to cellular function, it is not unusual that a host of natural and synthetic antibiotics target ribosomal RNA in one or more steps of this pathway. These “natural antibiotics” are the result of chemical warfare that takes place between different bacterial strains in an effort to survive tight environments, whereas the majority of synthetic antibiotics have come about from minor modifications being made to existing natural frameworks. Among these structural frameworks are antibiotics that target the 30S ribosomal subunit such as aminoglycosides, tetracyclines and pactamycin as well as those that target the 50S ribosomal subunit like the macrolides, lincosamides and oxazolidinones (Figure 1-7 and 1-8).

\textbf{Figure 1-7:} (Left) The 30S ribosomal subunit with the binding sites of common antibiotic classes shown by colored spheres. (Right) The 50S subunit and corresponding antibiotic binding sites.\textsuperscript{33}
Figure 1-8: A handful of common clinically relevant antibiotic scaffolds that act on the bacterial ribosome. Paromomycin has been chosen to represent the aminoglycosides.

It is clear from the observed error rates in protein biosynthesis, about one mistake in $10^4$ elongation cycles, that the ribosome relies on a high level of fidelity to carry out its primary function.\textsuperscript{34} Without it cell death is unavoidable. Therefore disrupting fidelity represents an enormous evolutionary advantage for microorganisms capable of producing the necessary natural antibiotic that will target and cripple this efficiency in nearby cells. One such antibiotic class is the aminoglycosides.\textsuperscript{35}

The discovery of streptomycin by Selman Abraham Waksman in 1944 ushered in a new era of clinically relevant antibacterials; work that was rewarded with the Nobel
Prize in medicine in 1952 when streptomycin was shown to be the first antibiotic effective against tuberculosis (Figure 1-9). Since that time several generations of aminoglycosides have come forward with potent bactericidal effects against gram-negative strains. Amongst the more notable structures in current clinical use are tobramycin, gentamicin and amikacin (Figure 1-10).36

Recent advances in X-ray crystallography and NMR-based techniques have revealed a great deal about the binding mode of aminoglycoside antibiotics.37-40 Such findings have led to a number of aminoglycosides with structures tailored to maximize interactions within the 30S subunit.41-42 Having a firm awareness of the binding proclivities of these compounds is therefore necessary in the development of potent structural variants in the future.

1.5 Characteristics of Aminoglycoside Binding

Structural evidence aimed at identifying the specific interactions between aminoglycosides and 16S ribosomal RNA has been accumulating steadily for nearly a
Figure 1-10: Clinically relevant aminoglycoside antibiotics. The rings of tobramycin have been labeled in red for reference.

decade. Seminal work carried out by Puglisi and coworkers described binding of paromomycin to a 27-nucleotide RNA fragment using nuclear magnetic resonance spectroscopy (NMR). These studies revealed that paromomycin binds an asymmetric loop (3:4) near the region of bases A\textsuperscript{1492} and A\textsuperscript{1493} (Figure 1-11) resulting in a bulging of A\textsuperscript{1492} away from helix 44. Interestingly, the vacancy created by this bulge is filled by ring I of paromomycin, which assumes a base-stacking role with G\textsuperscript{1491}. Ring II, on the other hand, spans the region of bases U\textsuperscript{1495} and G\textsuperscript{1494} forming hydrogen bonds with each. Finally, it was concluded based on minimal changes in chemical shift values for the final
two rings that each is involved in very weak interactions along the major groove.

Support for these findings was demonstrated a year later through a binding
comparison with the related aminoglycoside neomycin.⁴³

The functional insights gained from NMR studies were crucial in uncovering
some very basic truths behind aminoglycoside binding to the bacterial ribosome.
Naturally, though, the question of whether or not a 27-nucleotide fragment could
faithfully mimic the complete 30S subunit had to be answered. Are there nuances to this
short stem of RNA binding with paromomycin that do not exist when bound to the intact subunit? What role, if any, do neighboring RNA and protein moieties of the ribosome play in binding when the 30S subunit is whole? Answers to these questions have become much clearer recently with the outpouring of X-ray crystal structures containing aminoglycosides bound to the complete 30S ribosomal subunit.

Ramakrishnan and coworkers’ 3Å crystal structures of two different aminoglycosides (paromomycin and streptomycin) bound to the 30S subunit laid to rest many of the debates concerning the binding mode of aminoglycoside antibiotics. Paromomycin was found bound to the major groove of helix 44(H44), confirming mutagenesis studies that had been carried out previously (Figure 1-12). Furthermore, ring I of paromomycin was found to imitate a nucleotide base and stack with G1491 as Puglisi had described by NMR. Of additional note was the finding that ring I is involved

Figure 1-12: (Left) Zoomed out cartoon of paromomycin (gold) bound to the top of H44 (sky blue) within the 30S subunit. The anticodon stem loops of A-site tRNA (dark blue) and P-site tRNA (yellow) are also shown. (Right) Zoomed in version, highlighting mRNA (purple) and bases A1492 and A1493 (red) of H44.
in hydrogen bonding interactions with A1408, an interaction that was not well defined by NMR. It was also observed by NMR that binding of paromomycin to the short RNA fragment resulted in bulging of base A1492 away from helix 44. This finding revealed further support in the X-ray crystal structure with the determination that ring I of paromomycin is tightly bound to the phosphate backbone of A1493 and actually helps “lock” two bases, A1492 and A1493, into an extra-helical conformation. In contrast, each base appears to be dislocated from the helix to an even greater extent than NMR studies had revealed (Figure 1-13).

The X-ray crystal structure of paromomycin bound to the 30S subunit showed A1492 and A1493 pointing directly toward the minor groove of the A-site codon-anticodon helix, a significant conformational change compared to their original base stacked positions within helix 44. To complement this finding, both X-ray crystallography and methyl substitution studies, performed in the absence of aminoglycosides, have exposed the identity of two hydrogen bonds related to this base “flipping”, bonds hypothesized to be keys in binding of tRNA to the A-site. First, Puglisi and coworkers described the effects of N1 methylation on both adenine bases, making the observation that such modifications interfered with the binding of tRNA to the A-site.45 Moreover, mutation of either base was lethal in strains of E. coli, suggesting a functional role for this dynamic region of 16S rRNA.9 Secondly, Ramakrishnan proposed that A1492 and A1493 simultaneously hydrogen bond to two 2’ OH groups on each side of the codon-anticodon helix when in the “flipped out” position (again, absent of aminoglycoside). Since the distance between these OH groups is directly related to the base pairing geometry of codon and anticodon, each hydrogen bond would be sensitive to improper pairing.
Consequently, paromomycin’s bactericidal mode of action may come from its ability to lock the A-site into a conformation in which discrimination of cognate and near cognate tRNA is significantly diminished.

The work of Puglisi, Ramakrishnan and coworkers further shaped a growing understanding of how aminoglycosides bind the bacterial ribosome. Not surprisingly, this work led to a series of future investigations aimed at identifying interactions made between other aminoglycosides and the bacterial ribosome (including a higher resolution structure of paromomycin bound to an RNA fragment).\textsuperscript{38-39, 46} The accumulation of this data solidified some of the broad binding trends that had been reported in the past. For
instance, the multiple amino groups found on all aminoglycosides become protonated at physiological pH, functioning as polycationic binders to accessible regions of polyanionic 16S rRNA. The ammonium groups physically displace common biological cations (such as Mg$^{2+}$ and Ca$^{2+}$) that are normally bound within these regions of high anionic character. Furthermore, rings I and II of the paromomycin and neomycin families appear to be more critical for binding than either rings III or IV. Finally, the bactericidal activity of both natural and synthetic aminoglycosides has been traced to a single region of the ribosome, the bacterial decoding A-site.

Complementing the strictly physical nature of NMR and X-ray crystallography, a number of computational efforts have also come forward to describe the behavior of aminoglycoside binding to different RNA motifs. Such studies have not only been instrumental in modeling the interactions between aminoglycosides with RNA, they have also played a key role in bridging the gap between a strong understanding of target structure and the rational design of new drug candidates. For the purposes of discussion, two of these studies will be mentioned here.

Hermann and Westof were among the first to publish molecular modeling results based on the interaction of cationic antibiotics with RNA. Westof’s statement that, “The mere presence of positive charges in aminoglycosides does not explain how they are able, unlike many other oligocations, to discriminate between different RNA folding motifs.” drove modeling studies of aminoglycosides bound to the hammerhead ribozyme. This analysis led to the proposition that aminoglycosides offer a three-dimensional framework of ammonium groups which orient themselves in such a way that they exploit multiple ion (Mg$^{2+}$) binding sites, and therefore the origin of aminoglycoside binding affinity
likely arises from a complementarity between the orientation of these ammonium groups (governed by the rigid architecture of the aminoglycoside rings) and their unique fit to polyanionic three-dimensional folds in RNA.

In order to substantiate these initial claims, Hermann and Westof would later use Brownian dynamics (BD) simulations to map the electrostatic field gradient of different RNA molecules known to bind aminoglycosides.\textsuperscript{49} Localized areas of defined negative charge were then compared to regions known to actually bind aminoglycosides. Within the free (no drug) 16S rRNA oligonucleotide two negatively charged spherical pockets were immediately located. One of these pockets contained all the nucleotides, except for A\textsuperscript{1492}, necessary to make the intermolecular contacts with paromomycin reported in the NMR structure. Remarkably though, only rings III and IV of paromomycin were found occupying this area of high electron density. In contrast, rings I and II, which have a combined three ammonium groups, were found in regions of relatively low electron density within the region of bases G\textsuperscript{1491}-C\textsuperscript{1496} pointing to a very different binding mode for each pair of rings. While electrostatics appear to dominate interactions of the third and fourth rings, shape sensitive van der Waals contacts control binding of the first and second ring. This is very much in line with Puglisi’s NMR studies in that rings I and II impart specificity of antibiotic binding, whereas the other two rings make only weak contributions.

Building on the foundation of these studies, Haddad and coworkers used rings I and II of paromomycin (called paromomine) as the minimum structural motif in a computational search of over 273,000 compounds that might bind an A-site template.\textsuperscript{50} Results of this search were then narrowed based on steric and energetic demands. Finally,
an (S)-4-amino-2-hydroxybutyryl group was placed in position N1 of ring II as in
amikacin (Figure 1-10), a modification known to help confer resistance to
aminoglycoside modifying enzymes. Seven compounds were then targeted (Figure 1-15),
synthesized and measured for biological activity against a 27-nucleotide fragment of 16S
rRNA from *E. coli*. In every case low micromolar dissociation constants (*K*<sub>d</sub>) for these
derivatives were observed by titration, validating not only the NMR structure which these
models were built upon, but also the worth of computational simulations in arriving at
medicinally relevant molecules for the bacterial ribosome.

Numerous other design concepts have come forward in response to the growing
structural understanding of the ribosomal A-site.\textsuperscript{51-54} Hermann and coworkers, for
example, considered extensions at the primary alcohol position (6') of ring I of
paromomine in an effort to explore potential hydrogen bonding interactions of the
shallow groove.\textsuperscript{51} Conversely, Wong and coworkers took to surveying the major groove
through dimerization of neamine with either a polyglycol or polymethylene linker. The
length of this linker was then varied to exploit possible contacts within the major groove

![Figure 1-15: Common scaffold of the seven compounds synthesized by Mobashery and coworkers. The most common variation was shortening the carbon chain length of arm 2.](image)
of the RNA helix. Lastly, a series of investigations have been carried out recently to look at the role of aminoglycoside conformation in binding 16S rRNA.

Part of the effectiveness of aminoglycosides from a therapeutic standpoint comes from their ability to adopt multiple conformations; a result of the pseudoglycosidic bonds which unite most of their rings. The side effect of this freedom is that aminoglycosides tend to lack high RNA target selectivity, binding not only to 16S rRNA but also medicinally relevant ribozymes and the HIV-1 RNAs known as RRE and TAR. In addition, bacterial resistance enzymes that target these compounds take advantage of their conformational adaptability, binding aminoglycosides in a conformationally distinct matter. Such differences in binding mode led to the assumption that covalent linkage of individual rings may reduce the number of available conformations to such a degree that target selectivity could be altered.

Paromomycin and neomycin adopt a compact L-shaped conformation when bound to the A-site (Figure 1-16). In contrast, these compounds adopt a more extended conformation when bound to TAR and a surprisingly high energy conformation when bound to the modifying enzyme ANT(4’). Provided these structural differences two separate groups set out to covalently link rings I and III of neomycin (as shown in Figure 1-17). Molecular modeling was used to assess which RNA binding site the constrained neomycin analog would favor based on the known orientation of its unrestricted neomycin counterpart when bound to 16S rRNA, TAR and ANT(4’). These simulations revealed that the restricted derivative was energetically disfavored to bind either TAR or the resistance enzyme ANT(4’), but that the A-site RNA was still a viable target. Upon completing the synthesis of constrained neomycin, its biological activity
was tested in comparison with unrestricted neomycin. In the case of enzymatic modification, ANT(4’) adenylated the OH group at the 4’ position of neomycin within two minutes. A completely different behavior was exhibited by the constrained derivative however. After more than two hours with the enzyme, an excess of 95% remained unmodified. With regards to target promiscuity, the results were not as promising. Binding of restricted neomycin to TAR showed only an eleven-fold decrease in binding affinity compared to neomycin itself. Unfortunately this was accompanied by a twenty-two-fold decrease in binding affinity for the A-site, leading Tor to conclude that RNA binders may not be as nonselective as previously thought.\textsuperscript{61}
Figure 1-17: Constrained neomycin derivative with the covalent attachment shown in red.
CHAPTER TWO

RESULTS AND DISCUSSION

2.1 Introduction

Prior to the reports published by Tor, Barbaro and coworkers regarding the effects of placing conformational constraints on aminoglycoside antibiotics (discussed previously), investigations within our own group pointed to the potential for this class of derivatives. However, while the work published in these initial findings focused on the differences in aminoglycoside binding conformation between the bacterial ribosome and other binding sites (such as modifying enzymes and other RNA targets), our own thoughts on the subject of restricted aminoglycoside analogs originated from noticeable differences between the ribosome bound state of paromomycin in the NMR structures compared to the X-ray crystal structures. We thus set out to determine if these discrepancies were significant. Molecular modeling, based on the X-ray crystal structure of one of our own compounds (shown in Figure 1-15) bound to the A site, was used to explore the possibility of using synthetic methodologies to provide insight into these structural differences (Figure 2-1). Two molecules (1 and 2; Figure 2-2) based on the neamine subclass of aminoglycoside antibiotics were conceived from this work, and their synthesis is the subject of this chapter.

The spatial orientation of rings I and II in compounds 1 and 2 are identical by design and computational analysis, but the linkers that lock them in conformational space
**Figure 2-1:** X-ray structure of the antibiotic shown previously in Figure 1-15 at 2.6 Å resolution. The electron density of this compound is shown as a contour while the RNA backbone is shown in pink. For reference the hydroxyaminobutyrate is pointing directly upwards (12 o’clock). Bases A1492 and A1493 are flipped out of H44.

**Figure 2-2:** Restricted neamine derivatives conceived through molecular modeling.
are different (compound 1 $n = 4$, compound 2 $n = 2$; based on modeling). According to the NMR conformation, compound 1 should bind the A site pocket but the linker will prevent such binding according to the X-ray conformation. Conversely, X-ray data supports the idea that compound 2 will bind within the A site pocket but will not bind based on the NMR structure (again because of the unique linker). Thus, a comparison of the binding of these two molecules, or lack thereof, should help to address the obvious disparities between the NMR conformation and the X-ray conformation. In addition, the differences in the bound conformations of paromomycin and neomycin amongst known aminoglycoside binders (such as resistance enzymes, the bacterial ribosome, HIV-1 TAR RNA; discussed in chapter 1) also provided incentive to synthesize these molecules.

2.2 Synthesis

Two challenges immediately stood out in the retrosynthetic analysis of compound 1. The first issue was that of regioselectivity. Within the neamine core structure there are a total of eight possible nucleophiles. While four of these nucleophiles are far less reactive than the other four (amine $pK_a \sim 9-10$ whereas hydroxyl $pK_a \sim 16-18$), four amines still remain with similar reactivity. In order to tether the two rings of neamine together as shown in Figure 2-2, a regioselective protection strategy had to be employed. The second issue was that of chain length for the linker and the best approach for installing it between the two rings. Understanding the two challenges mentioned here, as well as the chemistry that has proven successful in other aminoglycoside transformations, four targets were conceived (Figure 2-3).

Synthesis of compound 3 started with methanolysis of commercially available neomycin B trisulfate (7; Scheme 1). Decomposition of the neamine (9) core can
Figure 2-3: The four target molecules which were conceived based on the linker found in compound 1. These molecules were expected to bind the A site according to the NMR conformation. Moreover, they should also bind preferentially to the bacterial ribosome over resistance enzymes in the locked conformation shown.

result from prolonged heating (seen through loss of the anomeric signal by NMR), and thus the reaction time was critical in controlling product yield. Protection of the N3 and N6\(^{\prime}\) amino groups (numbering scheme shown previously in Figure 1-17) using di-tert-butyldicarbonate was carried out using Cu\(^{2+}\) ions to impart regioselectivity (10). This protection strategy, which relies upon preferential chelation of Cu\(^{2+}\) ions to the N1 and
Scheme 1: Synthetic route to compound 3. The eight linear steps required to complete this synthesis have been shown along with the conditions used in each step.
N2’ regions of neamine, was investigated earlier within our group through measurement of the paramagnetic contributions of the cupric ion to T1 relaxation times by H-NMR spectroscopy. Remarkably this temporary protection strategy results in just two products; 10 and a tricarbamoylated neamine species (an N1, N3 and N6’ bi-product), which are easily separated by column chromatography. Subjecting compound 10 to benzyl chloroformate (Cbz) and aqueous base gave 11 in good yield. Thus, one of the key challenges pointed out in the initial retrosynthetic analysis had been overcome.

Deprotection of the Boc groups at N3 and N6’ proceeded smoothly using a 25% solution of trifluoroacetic acid in dichloromethane (12). Dilution of the resulting residue with anhydrous ether led to the precipitation of a white solid. This white solid was then dissolved in neat pyridine (creating a 5 mM solution) before adding one equivalent of succinic anhydride. Compound 13 (or its N3 equivalent) was confirmed using LCMS, as visualized by a single peak, but was not isolated. Instead, EDC was added directly to the reaction mixture and stirring continued for two days (monitored again by LCMS). Upon removal of pyridine by rotoevaporation, precipitation of compound 14 was achieved by the addition of water. Product purity was confirmed at this point using both NMR spectroscopy and LCMS to be in excess of 95%, but the experiments necessary to nail down connectivity were not carried out until the final deprotection step. Removal of the two remaining Cbz groups at positions N1 and N2’ proceeded under standard palladium catalyzed hydrogenation conditions to yield 15 in 92% yield (a 16% overall yield from neomycin trisulfate). A combination of homonuclear decoupling experiments, two-dimensional NMR and IR spectroscopy were then used to establish connectivity of the linker element uniting the two rings.
With 15 in hand, and the best length for the linker worked out, it seemed that compound 5 was then next logical target to go after. At first glance, reduction of the lactam functionalities found in 15 appeared to be the quickest, and potentially most obvious, method to achieve target 5. Thus, 15 was submitted to a 1 M solution of borane in THF (6-7 equivalents; Scheme 2) at varying reaction times. Somewhat expectedly (amides/lactams are not always susceptible to these mild reductive conditions), no reduction product was observed each time this reaction was performed. Keeping in mind that more reactive reagents such as lithium aluminum hydride can be detrimental to aminoglycoside cores, more forceful conditions were still sought. Such conditions were achieved by dissolving compound 15 in diglyme and adding sodium borohydride under high heat.\(^6\)\(^5\) Unfortunately, heating the diglyme solution at 100 °C for 12 hours gave only starting material by LCMS and mass spectral analysis (FAB+). Temperatures were subsequently elevated to 150 °C and then 130 °C, but in each case complete decomposition of the neamine core was observed. This reduction strategy was therefore abandoned and alternative techniques were sought.

Based on the failed attempts to reduce either amide bond in the linker of compound 15, later attempts to synthesize 5 focused on installing a linker that would not require this chemical modification. To accomplish this, N-alkylation strategies were explored first (Scheme 3). In order to avoid multiple alkylations on the free amines found in compound 12 it was necessary that each be protected in such a way that deprotonation of the amide nitrogen was not only possible, but more likely relative to the Cbz carbamates at N1 and N2’ (pK\(_a\) ~ 21-25) and the four unprotected alcohols (pK\(_a\) ~ 16-18). Fukuyama’s work using o-nitrobenzenesulfonamides and p-nitrobenzenesulfonamides
Scheme 2: Two reduction procedures attempted in order to arrive at target 5.

(so-called nosyl groups; $pK_a \sim 10-12$) for the protection of amines provided this $pK_a$ boost.\textsuperscript{66} Alternatively, using a less bulky protecting group such as trifluoroacetamide might be possible, though $pK_a$’s (~ 17) become much more competitive with the unprotected alcohols in this case.

Attempts to protect the N3 and N6’ amines with 2-nitrobenzenesulfonyl chloride resulted in the addition of just one nosyl protecting group. Despite extended reaction times (> 2 days) the second amine (most like the less reactive N3 amine) simply did not react. Heating the mixture to 50 °C did little to remedy the problem, which most likely involved steric interference of the first sulfonamide with reaction of the second equivalent. As a result of these steric concerns, trifluoroacetic anhydride was used as an alternative (Scheme 4). Treatment of 12 with trifluoroacetic anhydride provided 18 in
Scheme 3: Reaction scheme considered for the N-alkylation linker strategy. Neither compounds 16 or 17 were able to be synthesized. All reactions were run at room temperature greater than 90% yield, evidence that steric bulk probably played a key role in failing to protect both amines with nosyl groups. However, every attempt to alkylate the two amides failed. Compound 18 was treated with either cesium carbonate or sodium hydride in the presence of 1,4-dibromobutane for a period of 12 hours. LCMS revealed the presence of a single peak corresponding to starting material, while extended reaction times (24 hrs) gave the same result. Changing to a more reactive electrophile such as allyl bromide, providing for a potential cross metathesis route, also failed. These findings are particularly perplexing considering the multiple alkylation sites that are found on 18, not to mention the relatively high reactivity of allyl bromide towards alkylations of this sort.
Scheme 4: Reaction scheme representing the second attempt at N-alkylation of 12. The generically defined base in the second reaction was either cesium carbonate or sodium hydride. All reactions were run at room temperature.

Additionally, excess base (10 equivalents) was used in each of these reactions so that even if alcohol deprotonation did take place there was ample base left over to also deprotonate the activated amide. Regardless, a different pathway had to be developed in order to overcome these early setbacks. Based on reactions that have been successfully employed in the synthesis of other aminoglycoside derivatives, reductive amination seemed like a solid strategy.67

As a means to test the validity of using reductive amination chemistry on compound 12, neamine (9) was used in initial investigations (due to the smaller synthetic investment; two steps to neamine versus five steps to 12) to test various aldehydes (Scheme 5). Three different aldehydes were chosen for these pilot reactions based on
their unique reactivity differences, and all were submitted to identical reductive amination conditions on a 100 mg scale. Mass spectroscopy was used to identify the presence of product because of the difficulties associated with using thin layer chromatography (TLC) to monitor reaction progress (a problem associated with the high polarity of neamine). Interestingly, mass spectroscopy revealed that the least reactive aldehyde (p-anisaldehyde based on electronics) was the only one of the three different aldehydes that yielded product (22). Both the p-nitrobenzaldehyde and butyraldehyde derivatives gave awful mixtures of multiple peaks which could not be identified; even if multiple additions had taken place there were still no matches to the peaks seen by mass spectroscopy. Hence, a short discussion of these peculiar results is necessary.

Imine formation in reductive amination chemistry is clearly the most significant hurdle to overcome if one is to achieve success. In order to drive formation of this intermediate one of two approaches are typically taken. The first is addition of some sort of Bronsted acid such as acetic acid (this method was used in the three reactions shown in Scheme 5). A second approach relies on removal of water (by using molecular sieves for example) in order to drive the equilibrium of the reaction towards imine formation. With respect to using a sodium acetate buffer (pH 6) as solvent (the first set of conditions used in this reaction), the second approach to forcing imine formation obviously did not come into play. Comparatively those conditions outlined in scheme 5, supported by literature precedent in the synthesis of other aminoglycoside analogs, also relied upon acid promoted conditions. Both set of conditions led to similar results; successful reductive amination of p-anisaldehyde with neamine and complicated mixtures with butyraldehyde and p-nitrobenzaldehyde. It is still unclear from these early
Scheme 5: Three reactions used to test the feasibility of using reductive amination chemistry on compound 12. Sodium acetate buffer (pH 6) was also used in place of the methanol/acetic acid solvent system, but the results were the same and thus are not shown.
investigations what led to such mixed results between the three aldehydes. The use of sodium triacetoxyborohydride as an alternative reductant did not help to resolve these reactivity problems.

At this point many of the reactions that looked to be the most promising in obtaining target 5 had been exhausted. Any sort of linkage that required a subsequent amide reduction (Scheme 2) was problematic. Additionally, N-alkylation through either a nitrobenzenesulfonamide or trifluoroacetamide had not worked (Schemes 3 and 4). The steric bulk of Fukuyama’s nosyl protecting group was too much for the addition of a second equivalent whereas deprotonation/alkylation issues arose in the latter route with trifluoroacetamide protection of N3 and N6’. Finally, reductive aminations using multiple aldehydes, and two different solvent systems for each system, were inconsistent at best (Scheme 5). This, along with the fact that synthesis of a suitable four-carbon dialdehyde gave low yields (24, Scheme 6), ended exploration of this type of transformation to achieve 5.

One last route was considered before completely abandoning the idea of synthesizing target 5. Up until this point each of the linker installation strategies relied upon one of two electrophiles (a reactive carbonyl functionality or a halogen) to unite

![Scheme 6: DIBAL reduction of a methylester to the corresponding aldehyde.](image)

\[
\begin{align*}
\text{H}_3\text{CO} & \quad \text{OCH}_3 \\
\text{OCH}_3 & \quad \text{OCH}_3 \\
\text{1. DIBAL, CH}_2\text{Cl}_2 & \quad \text{4hrs} \\
\text{2. MeOH, sat. Na}_2\text{SO}_4 & \quad \text{10\%} \\
\text{H}_3\text{CO} & \quad \text{OCH}_3 \\
\end{align*}
\]
rings I and II of neamine. However, epoxides were ignored almost entirely. One advantage that epoxides offer over either of the two electrophiles mentioned above is that they typically will not react without the presence of an acid or a base. Thus, in the absence of either acid or base their electrophilicity can be masked, while another electrophile at the opposite end of the linker entity may freely react with either of the amines in 12. Conversely, nucleophilic epoxide ring opening could take place preferentially over displacement of a weaker electrophile at the opposing end. It was the last of these two tactics that was utilized in our final effort (Schemes 7 and 8).

Treatment of 4-bromobutene with excess m-CPBA in dichloromethane resulted in the clean conversion of 25 to the corresponding epoxide 26. This epoxide was then added to a solution of 12 in neat pyridine for a period of 12 hours under room temperature (Scheme 8). Surprisingly, the complete disappearance of 12 was seen by both LCMS and mass spectroscopy within these 12 hours. In its place emerged a single peak with a mass matching that calculated for 28. Since the conditions set by the epoxide ring-opening reaction were suitable for the final ring closure, 28 was not isolated. Instead the solution was warmed to approximately 60 °C.

Scheme 7: m-CPBA oxidation of 4-bromobutene to the corresponding epoxide.
and left stirring for more than three days. LCMS traces were taken at roughly 12 hour intervals, but only the peak representing starting material was ever observed. It is difficult to understand why installation of the fully reduced linker found in target 5 became such a large synthetic obstacle, especially when one considers the relative ease with which the unsaturated equivalent was made (8 steps; > 16% overall yield from neomycin trisulfate). As outlined above several very different strategies were used in order to arrive at compound 5. Due to the specific barriers (solubility,

**Scheme 8:** Use of 4-bromooxirane in arriving at a neamine derivative related to 5. The first step shown here was successful, but ring closure could not be achieved despite heating 28 to 60°C.
purification and characterization) that aminoglycosides confer (similar to those that make carbohydrate chemistry so challenging) these failures are not so much surprising as they are disappointing. Work to complete the remaining targets will undoubtedly continue in the future with the successes and failures of this early work in mind.

2.3 Biological Profile

To conclude, the biological activity of compound 15 was tested against the aminoglycoside-modifying enzyme APH(3’) to see whether the conformational constraints put in place by the linker provided any resistance to phosphorylation for this enzyme (Figure 2-3). Compound 15 had a turnover ($k_{cat}/K_m$) value that was more than four orders of magnitude slower than neamine itself ($(2.0 \pm 0.3) \times 10^3$ M$^{-1}$s$^{-1}$ versus $(2.4 \pm 2.0) \times 10^7$ M$^{-1}$s$^{-1}$) for APH(3’). This large difference in turnover has been substantiated by the work of Asensio and coworkers who reported a more than sixty-fold difference in the turnover of ANT(4’) for a contrained neomycin analog compared to neomycin itself. However, much like the restricted derivative reported by Blount and coworkers, a substantial loss in activity as an antibiotic (presumably due to reduced affinity for the target ribosome or attenuated transport into the cytoplasm) was found for 15. This effect was manifested in a large drop in minimum inhibitory concentration (MICs; > 250µM) compared to neomycin (the analysis with APH(3’) was carried out by Mr. Choonkeun Kim and MIC values were determined by Dr. Sergei Vakulenko). Finally, attempts to synthesize targets 4 and 6 never took place because of the failure to develop the necessary synthetic route to form the linker found in 6.
Figure 2-4: Steady state kinetic profile of 15 (DLK-9) for APH(3’). Turnover ($k_{cat}/K_m$) was found to be more than four orders of magnitude slower than that previously reported for neamine. 

2.4 General

All reagents were purchased from commercial sources and were used without further purification. Solvents were purchased anhydrous and were used exclusively unless otherwise noted. Column chromatography was carried out using Whatman Purasil (60A) 230-400 mesh silica gel. Melting points were determined with a Haake Buchler Variable Heat Melting Point Apparatus. Infrared spectra were obtained with a Thermo Nicolet 200 IR spectrometer. LC/MS analyses were conducted on a Waters ZQ instrument consisting of chromatography module Alliance HT, photodiode array detector 2996, and mass spectrometer Micromass ZQ, using a 3 x 50 mm Pro C18 YMC reverse
phase column (mobile phases: 10mM ammonium acetate in HPLC grade water (A) and HPLC grade acetonitrile (B); gradient was formed from 5% to 80% of B in 10 minutes at 0.7 mL/min with the MS electrospray source operating at capillary voltage 3.5 kV and a desolvation temperature of 300°C). $^1$H and $^{13}$C NMR spectra were recorded with a Varian INOVA-500 spectrometer (operating at 500MHz and 125MHz respectively). Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (0 ppm) and coupling constants are reported in Hz. Multiplicities are represented using standard notation; singlet (s), doublet (d), triplet (t) and multiplet (m). A combination of 2D NMR and homo decoupling experiments were used to establish appropriate assignment of $^1$H and $^{13}$C chemical shifts and connectivity. Mass spectra were obtained on a JEOL JMS-AX505HA mass spectrometer.

2.5 Experimental

**Neamine (9).** A solution of 20 g (22 mmol) of neomycin B sulfate in 1 L of anhydrous methanol was refluxed for 6 hours under a nitrogen atmosphere with 215 ml of 1.8 N methanolic hydrogen chloride. The flask was then cooled to room temperature before placement in a refrigerator overnight. Refrigeration caused the precipitation of 5.6 g of an amorphous white solid which was isolated via vacuum filtration (methanolysis product 1). The mother liquor from the precipitation was concentrated to 300 ml and diluted with 200 ml anhydrous ether to afford another 1.8 g of tan colored solid (methanolysis product 2).

Crystallization of methanolysis product 1 was achieved by taking 5.6 g of the solid hydrochloride up in 6.3 ml of concentrated ammonium hydroxide and diluting it
with 350 ml of methanol. Ammonia gas was then passed through the solution until crystallization began. This solution was then refrigerated overnight. The following day crystals were collected via vacuum filtration on a Buchner funnel, washing three times with 20 ml portions of cold anhydrous methanol and drying in vacuo. This resulted in the isolation of 5.1 g (16 mmol) of neamine free base; a 72% overall yield from neomycin B sulfate. Attempts were made to crystallize methanolysis product 2, but only marginal purity was observed by NMR.\textsuperscript{62,63} \textsuperscript{1}H (D\textsubscript{2}O, 500 MHz) \delta 5.12 (d, J = 4 Hz, 1H, H1'), 3.60 (m, 1H, H5'), 3.39 (m, 2H, H3', H4), 2.97-3.2 (m, 3H, H4', H5, H6), 2.84 (dd, \omega = 16 Hz, J = 13.5 Hz, 2.5 Hz, 1H, H6'), 2.52-2.73 (m, 4H, H1, H2', H3, H6'), 1.81 (ddd, \omega = 21 Hz, assigned as a 1:2:1:1:2:1 6 line m, J\textsubscript{2eq-2ax} = 13 Hz, J\textsubscript{2eq-1} = J\textsubscript{2eq-3} = 4 Hz, 1H, H2eq), 1.04 (ddd, \omega = 37 Hz, assigned as a 1:3:3:1 4 line m, J\textsubscript{2ax-2eq} = 13 Hz, J\textsubscript{2ax-1} = J\textsubscript{2ax-3} = 12 Hz, 1H, H2ax); \textsuperscript{13}C NMR (D\textsubscript{2}O, 500 MHz) \delta 100.6 (C1'), 86.7 (C5), 77.4 (C6), 76.1 (C4), 73.6 (C3'), 72.5 (C5'), 71.5 (C4'), 55.3 (C2'), 50.5 (C1), 49.4 (C3), 41.6 (C2), 35.5 (C6'). MS (FAB\textsuperscript{+}): M/Z = 323 [M+H]\textsuperscript{+}, calculated for C\textsubscript{12}H\textsubscript{26}N\textsubscript{4}O\textsubscript{6} is 322.3. MP = 255-256 °C, Lit. 256 °C.

3,6'-di-N-(t-Butoxycarbonyl)-neamine (10). Neamine free base (9) (5 g, 15 mmol) was dissolved in a minimal amount of water (10 ml) followed by the addition of 1 L DMSO. Copper (II) acetate monohydrate (12.4 g, 60 mmol) was then added and the blue solution was allowed to stir overnight before adding di-\textit{tert}-butyldicarbonate (7.43g, 34 mmol). The solution was stirred for an additional 24 h, after which time TLC analysis (CHCl\textsubscript{3}/MeOH/concentrated ammonia, 2:1:0.25) revealed the presence of 3 spots, one at R\textsubscript{f} = .7, one at R\textsubscript{f} = .5 and one at R\textsubscript{f} = .1 corresponding to the tricarbamoyl species,
dicarbamoyl species and monocarbamoyl species respectively. DMSO was removed by adding 800 ml ethyl ether, mixing vigorously for 15 minutes and decanting the upper layer. This process was repeated multiple times using fresh 500 ml portions of ether until a dark oily residue remained. The residue was then dissolved in 600 ml in MeOH/H₂O (4:1) and copper was precipitated by saturating the solution with hydrogen sulfide gas. Solid was filtered off through a layer of celite and the filtrate was evaporated to obtain a brown residue, which was taken up in a minimal amount of MeOH and chromatographed using the same solvent system as in the initial TLC. A mass of 3.94 g of title compound (2) was obtained providing an overall yield of 49%. ¹H NMR (CD₃OD, 500 MHz) δ 5.18 (d, J = 4 Hz, 1H, H1'), 3.66 (unresolved multiplet, 1H, H5'), 3.45 (overlapping multiplet, 3H, H3, H3', H4 or H5), 3.38 (t, J = 9 Hz, 1H, H4 or H5), 3.30 (broad, 2H, H6') 3.13 (t, J = 9.5 Hz, 1H, H4'), 3.06 (t, J = 9.5 Hz, 1H, H6), 2.55 (overlapping multiplet, 2H, H1, H2') 1.98 (ddd, ω = 21 Hz, assigned as a 1:2:1:2:1 6 line m, J₂eq-2ax = 13 Hz, J₂eq-1 = J₂eq-3 = 4 Hz, 1H, H2eq), 1.41 (s, 18H, OC(CH₃)₃), 1.29 (ddd, ω = 37, assigned as a 1:3:3:1 4 line m, J₂ax-2eq = 13 Hz, J₂ax-1 = J₂ax-3 = 12 Hz, 1H, H2ax); ¹³C NMR (CD₃OD, 500 MHz) δ 101.9 (C1'), 84.1 (C4), 78.6 (C6), 77.6 (C5), 74.7 (C3'), 72.6 and 72.4 (C4' and C5'), 57.1 (C2'), 52.4 (C1), 50.9 (C3), 42.1 (C6'), 36.3 (C2), 29.0 (OC(CH₃)₃). MS (FAB+): M/Z = 523 [M+H]+, calculated for C₂₂H₄₂N₄O₁₀ is 522 (+H = 523).

3, 6'-Di-N-(tert-butoxycarbonyl) 1, 2'-Di-N-benzylxycarbonylneamine (11).

Compound (10) (3.5 g, 6.7 mmol) was added to 300 ml of dioxane and stirred for 15 minutes to dissolve most of the solid. Benyl chloroformate (2.40 g, 14.1 mmol) and Na₂CO₃ (7.6 g, 20 mmol) were then added along with 100 ml of deionized water. The
reaction proceeded for 3 hours, after which time TLC (CHCl₃/MeOH/concentrated ammonia 4:1:0.1) revealed a single spot with Rₓ = .35. The solvent was removed and the remaining solid was washed 4 times with 150 ml portions of water. Product was obtained (3.85 g) in 72% yield. ¹H NMR (CD₃OD, 500 MHz) δ 7.25 (overlapping multiplet, 10H, Cbz), 5.18 (s, 1H, H1’), 4.96 (m, 4H, CH₂Ph), 3.62 (unresolved multiplet, 1H, H5’), 3.44-3.51 (overlapping multiplets, 2H, H3’, H5), 3.22-3.38 (overlapping multiplets, 6H, H1, H2’, H3, H4, H6’), 3.14 (t, J = 8.5 Hz, 1H, H4’), 3.06 (t, J = 10 Hz, 1H, H6), 1.92 (m, 1H, H2eq), 1.34 (s, 18H, OC(CH₃)₃), 1.25 (m, 1H. H2ax); ¹³C NMR (CD₃OD, 500 MHz) δ 157.9, 157.7, 157.2, 156.5 (C=O), 136.9, 128.1, 127.9, 127.6, 127.5 (C₆H₅), 98.8 (C1’), 80.6, 79.3, 77.4, 75.2 (C3’, C4, C4, C6), 71.2 (C5’) 70.9 (C4’) 66.3 and 66.0 (CH₂C₆H₅), 56.0 (C2’), 51.4 (C1), 49.6 (C3), 40.5 (C6’), 35.5 (C2), 27.5 and 27.4 (OC(CH₃)₃). MS (FAB+): M/Z = 813 [M + Na]⁺, calculated for C₃₈H₅₄N₄O₁₄ 790.9 (+ Na = 813). LC/MS (Scan ES+) provided a single peak at Rₓ = 7.68 min. corresponding to a mass of 791.

Melting point: 245 °C.

1, 2’-Di-N-benzyloxycarbonylneamine (12). To 1.47 g (1.9 mmol) of (11) was added 100 ml of 25% trifluoroacetic acid in CH₂Cl₂. This solution was stirred for a period of 1 hour at room temperature under an atmosphere of nitrogen, after which time the solvent was evaporated. Dioxane (200 ml) was then added, stirred for 5 minutes and removed. To the remaining golden brown residue was added 150 ml of diethyl ether. Precipitation of a white solid resulted. This solid was filtered onto a Buchner funnel and then dried by vacuum. ¹H NMR (DMSO, 500 MHz) δ 7.56, 8.10 and 8.25 (4H, NH’s), 6.90 (10H, Cbz), 5.18 (d, J = 4 Hz, 1H, H1’), 4.61 (m, 4H, Cbz CH₂Ph), 3.40 (s, 1H, H5’),
3.28 (overlapping multiplet, 3H, H3, H3', H4 or H5), 2.89-3.07 (overlapping multiplets, 7H, H1, H2’, H3 H4 or H5, H4’, H6, H6’), 1.98 (ddd, J = 21 Hz, assigned as a 1:2:1:2:1 6 line m, J2eq-2ax = 13 Hz, J2eq-1 = J2eq-3 = 4 Hz, 1H, H2eq), 1.29 (ddd, J = 37, assigned as a 1:3:3:1 4 line m, J2ax-2eq = 13 Hz, J2ax-1 = J2ax-3 = 12 Hz, 1H, H2ax).\(^{13}\)C NMR (DMSO, 500 MHz) \(\delta\) 128.6, 136.5 and 147.4 (C6H5), 115.3 and 117.6 (CH2Ph), 97.3 (C1’), 77.6 (C4), 75.0 (C6), 74.7 (C5), 71.2 (C3’), 70.1 (C4’), 68.9 (C5’), 67.4 (C2’), 54.0 (C1), 50.5 (C3), 49.4 (C6’), 40.3 (C2). MS (FAB+): M/Z = 613 [M + Na]⁺, calculated for C\(_{28}\)H\(_{38}\)N\(_{4}\)O\(_{10}\) is 590.6 (+ Na = 613). LC/MS (Scan ES+) provided a single peak at Rt = 4.3 min corresponding to a mass of 591. Melting point: 79-81 °C.

**(6-Benzylxycarbonylamino-4,5,17,18-tetrahydroxy-10,13-dioxo-2,20-dioxa-9,14-diaza-tricyclo[14.3.1.0\(^3,8\)]icos-19-yl)-carbamic acid benzyl ester (14).** To a flask containing 250 mg (0.3 mmol) of (12) was added 60 ml of pyridine. This mixture was stirred for a period of 15 minutes before the addition of 31 mg (0.3 mmol) succinic anhydride; stirring then continued for another 12 hours. LC/MS (Scan ES+) taken of the reaction mixture revealed a single peak at Rt = 4.5 min representing a mass of 691. (FAB+): M/Z = 691 [M + H]⁺, calculated for C\(_{32}\)H\(_{42}\)N\(_{4}\)O\(_{13}\) is 690.7. No purification was performed at this point. The crude compound (13) was used as is in the next step.

To the crude product (13) of the previous reaction was added 60 ml of pyridine along with 150 mg (.75 mmol, 2.5 eq.) of EDC. After 16 hours LCMS revealed a 50:50 mixture of starting material and product. The reaction was therefore stirred for an additional 32 hours (48 hours total) to run the reaction to completion. Pyridine was
removed to obtain a brown residue. Addition of 50 ml of water resulted in the
precipitation of a tan colored solid, which was recovered via vacuum filtration on a
Buchner funnel. Product was obtained (32 mg) in 34% yield. $^1$H (DMSO, 500 MHz)
$\delta$ 7.15, 7.19, 7.62 and 7.90 (4H, NH’s), 7.39 (m, 10H, C$_6$H$_5$), 5.18 (1H, H1’), 5.11 (m, 4H,
CH$_2$Ph), 3.77 (m, 1H, H3), 3.15-3.61 (overlapping multiplets, 12H, H2’ H3’ H4, H4’, H5,
H5’, H6, H6’ and 4 OH’s), 2.76 and 2.95 (m, 2H, H1, H6’), 2.38 (m, 4H, bridge CH$_2$’s),
2.1 (1H, H2$_{eq}$), 1.50 (1H, H2$_{ax}$); $^{13}$C (DMSO, 500 MHz) $\delta$ 155.7, 156.3, 170.7 and 171.0
(C=O), 127.6, 137.2 and 147.2 (C$_6$H$_5$), 121.5 and 125.0 (CH$_2$Ph), 100.9 (C1’), 85.2 (C4),
76.1 (C6), 73.4 and 74.1 (C3’ and C5), 72.3 (C4’), 71.2 (C5’), 56.9 (C2’), 51.1 (C1), 48.9
(C3), 42.2 (C6’), 34.8 and 33.2 (bridge CH$_2$’s), 32.2 (C2). (FAB+): M/Z = 673 [M+H]$^+$,
calculated for C$_{32}$H$_{40}$N$_4$O$_{12}$ is 672.7. LC/MS (Scan ES+) provided a single peak at R$_t =$
4.93 min corresponding to a mass of 673. Melting point: > 310$^\circ$C.

6,19-Diamino-4,5,17,18-tetrahydroxy-2,20-dioxa-9,14-diaza-tricyclo[14.3.1.0$_{3,8}$]icosane-10,13-dione (15). To a flask containing 82 mg of starting material
(14; 0.12 mmol) was added 30 ml of a DMF/MeOH/AcOH solution (50:48:2). The whole
system was then placed under an atmosphere of nitrogen before the addition of 50 mg
palladium (10%) on activated carbon. Evacuation (3X) was then carried out with
hydrogen atmosphere replacements each time; stirred overnight under approximately
1atm of hydrogen (balloon). The following day celite filtering agent (521) was used to
remove palladium, which was then washed 3X with MeOH (10 ml) and 1X with water
(10 ml). Upon evaporation of all the solvent a tan colored solid emerged with a net
weight of 44 mg, a 90% overall yield. $^1$H (D$_2$O, 500 MHz) $\delta$ 5.1 (d, J = 3.5 Hz, 1H, H1’),
3.75 (m, 1H, H3), 3.61 (overlapping multiplets, 3H, H4, H5', H6'), 3.46 (m, 2H, H6, H3' or H5), 3.38 (t, J = 10.5 Hz, 1H, H3' or H5), 3.19 (overlapping multiplets, 2H, H2', H4'), 3.09 (overlapping multiplets, 2H, H1, H6'), 2.43 (m, 4H, bridge CH2's), 2.1 (ddd, ω = 21 Hz, assigned as a 1:2:1:2:1 6 line m, J2eq-2ax = 13 Hz, J2eq-1 = J2eq-3 = 4 Hz, 1H, H2eq), 1.50 (ddd, ω = 38 Hz, assigned as a 1:3:3:1 4 line m, J2ax-2eq = 13 Hz, J2ax-1 = J2ax-3 = 12.5 Hz, 1H, H2ax), 13C (D2O, 500 MHz) δ 99.0 (C1'), 84.7 (C4), 75.6 (C6), 73.0 and 72.7 (C3' and C5), 71.7 (C4'), 69.6 (C5'), 55.2 (C2'), 50.0 (C1), 49.4 (C3), 40.1 (C6'), 33.7 and 32.1 (bridge CH2's), 30.4 (C2). FTIR (KBr pellet), 3395, 1654, 1560, 1412, 1050 cm⁻¹. MS (FAB+): M/Z = 405 [M+H]^+, calculated for C₁₆H₂₈N₄O₈ is 404. LC/MS (Scan ES+) provided a single peak at Rₜ = 0.85 min corresponding to a mass of 405. Melting point: 170-172 °C.

{2-[4-Benzyloxycarbonylamino-2,3-dihydroxy-6-(2,2,2-trifluoro-acetylamino)-cyclohexyloxy]-4,5-dihydroxy-6-[(2,2,2-trifluoro-acetylamino)-methyl]-tetrahydropyran-3-yl]-carbamic acid benzyl ester (18). To a flask containing 700 mg of 12 (0.85 mmol) was added 20 ml of pyridine and the whole system was placed under an atmosphere of nitrogen. Finally, 622 mg (3.0 mmol, 3.5 eq.) of trifluoroacetic anhydride was added. After stirring the mixture for a period of 4 hours LC/MS revealed the presence of a single peak at Rₜ = 6.9 min with a mass of 783, calculated for C₃₂H₃₆F₆N₄O₁₂ is 783. Water was added to quench the remaining trifluoroacetic anhydride before removal of both water and pyridine by rotoevaporation.
**General procedure for reductive amination reactions:** To a 3 ml reaction vial containing 100 mg of 9 was added 2 ml of a 10:1 MeOH/AcOH solution and 1.2 equivalents of aldehyde. The mixture stirred for a period of 30 minutes at room temperature before the addition of 1.2 equivalents sodium cyanoborohydride (or sodium triacetoxyborohydride). These reactions were then left to stir overnight at room temperature before submission to LCMS.

**4,4-Dimethoxybutyaldehyde (24)** To a flame dried three-neck round bottom flask was added 10 ml of dichloromethane and the solvent was cooled to -78°C under an atmosphere of nitrogen. A mass of 250 mg (1.5 mmol) of methyl 4,4-dimethoxybutyrate was then added followed by the addition of 2.3 mL (1 M solution in THF, 1.5 eq.) DIBAL. DIBAL was added slowly to prevent the temperature of the reaction from rising above -65 °C. This low temperature was maintained as the reaction stirred for 4 hours. After 4 hours 15 mL of dichloromethane was added followed by 200 µL of MeOH and 750 µL of saturated Na₂SO₄. This was then stirred for a period of 17 hours before the addition of 700 mg of anhydrous Na₂SO₄ solid. After one more hour of stirring the solid salts were filtered off and the solvent was removed via vacuum. NMR revealed the presence of very small amount of aldehyde(<5%) with the majority of the mixture being unreacted starting material. Extended reaction times (12 hours) did little to help the low yield (giving around 10%), nor did allowing the temperature to slowly rise to room temperature over these 12 hours (complex mixture resulted).
APPENDIX

SELECTED SPECTRA
Compound $^1$H NMR (500 MHz, D$_2$O)
Compound 10 $^{13}$C NMR (DMSO, 500 MHz)
Compound 11: $^{13}$C NMR (500 MHz, CD$_3$OD)
Compound 12 $^{13}$C NMR (D$_2$O, 500 MHz)
Compound 14 \(^1\)H NMR (500 MHz, DMSO)
Compound 15 \( ^1 \text{H NMR (500 MHz, D}_2\text{O) } \)
Compound 15 $^{13}$C NMR (500 MHz, D$_2$O)
REFERENCES


