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Synthesis and Biological Evaluation of Antibiotics Targeting the Ribosome

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Chemistry

by

Richard James Fair

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2014

The dissertation of Richard James Fair is approved, and it is acceptable in quality and

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Chair

University of California, San Diego

2014

DEDICATION

For my parents, Patricia and Rick Fair, and my sister, Megan Fair.

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ABBREVIATIONS

2-DOS	2-Deoxystreptamine
AAC	Aminoglycoside N-acetyltransferase
ACN	Acetonitrile
AHL	Acyl-homoserine-lactone
AIDS	Acquired immunodeficiency syndrome
AME	Aminoglycoside modifying enzyme
ANT	Aminoglycoside O-nucleotidyltransferase
APH	Aminoglycoside O-phosphotransferase
ATP	Adenosine triphosphate
Boc	*tert*-butoxycarbonyl
CA-MRSA	Community acquired methicillin resistant *Staphylococcus aureus*
Cbz	Benzyl carbamate
CDC	Center for Disease Control and Prevention
CF	Cystic fibrosis
COSY	Correlation spectroscopy
DCM	Dichloromethane
DIPEA	Diisopropylethylamine
DMB	2,4-Dimethoxybenzyl
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
EARS-Net	European Antimicrobial Resistance Surveillance Network
ECDC	European Center for Disease Prevention and Control
ESBL	Extended spectrum β-lactamase
EU	European Union
FDA	Food and Drug Administration
Fmoc	Fluorenylmethyloxycarbonyl
FRET	Förster resonance energy transfer
GAIN Act	Generating Antibiotic Incentives Now Act

HAART	Highly active anti-retroviral therapy
HABA	L-hydroxyaminobutyramide
HAI	Healthcare associated infection
HATU	1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
IDSA	Infectious Diseases Society of America
KPC	*Klebsiella pneumoniae* carbapenemase
MBL	Metallo-β-lactamase
MDR	Multi-drug resistant
MHB	Mueller-Hinton broth
MIC	Minimum inhibitory concentration
MRSA	Methicillin reistant *Staphylococcus aureus*
MS	Mass spectrometry
NDM-1	New Delhi metallo-β-lactamase 1
NMM	N-methylmorpholine
NMR	Nuclear magnetic resonance spectroscopy
NPV	Net present value
OD	Optical density
OXA	Oxacillinase
PBP	Penicillin binding protein
PDR	Pan-drug resistant
PTC	Peptidyl transferase center
PVL	Panton valentine leukocidin
R&D	Research and development
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
rt	Room temperature

SAM	S-adenosyl-L-methionine
SAR	Structure activity relationship
STAAR Act	Strategies to Address Antimicrobial Resistance Act
TB	Tuberculosis
TEA	Triethylamine
TFA	Trifluroacetic acid
THF	Tetrahydrofuran
TIPS	Triisopropyl silane
TLC	Thin layer chromatography
TPS	2,4,6-Triisopropylbenzenesulfonyl
TSST-1	Toxic shock syndrome toxin 1
US	United States
VISA	Vancomycin intermediate *Staphylococcus aureus*
VRE	Vancomycin resistant *Enterococci*
VRSA	Vancomycin resistant *Staphylococcus aureus*
WHO	World Health Organization
XDR-TB	Extensively drug resistant tuberculosis

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Fair, R. J.; McCoy, L. S.; Hensler, M. E.; Nizet, V.; Tor, Y. Singly Modified Amikacin and Tobramycin Derivatives Show Increased A-site Binding and Higher Potency against Resistant Bacteria, *In Preparation*.

ABSTRACT OF THE DISSERTATION

Synthesis and Biological Evaluation of Antibiotics Targeting the Ribosome

by

Richard James Fair

Doctor of Philosophy in Chemistry

University of California, San Diego, 2014

Professor Yitzhak Tor, Chair

Dangerous, antibiotic resistant bacteria have been observed with increasing regularity over the past several decades. Though bacterial evolution of antibiotic resistance mechanisms is a naturally occurring process, evidence strongly suggests that human mismanagement of antibiotics has greatly accelerated this process. A concurrent decline in research and development by the pharmaceutical industry has led to a deficiency of new antibacterial agents to fight these emerging threats.

The semi-synthetic modification of members of existing antibiotic classes is one approach that has been successfully used to generate new antibacterial agents. Three families of modified aminoglycosides were synthesized. Their affinities for the primary intracellular target of aminoglycosides, the ribosomal A-site, were evaluated using an in vitro Förster resonance energy transfer (FRET)-based assay. Their antibacterial efficacy

was quantified by determining minimum inhibitory concentration (MIC) values against resistant bacterial strains.

Singly or doubly modified guanidino-aminoglycosides were synthesized by selectively converting aminoglycoside primary alcohols or primary amines into guanidinium groups. They exhibited superior A-site binding in almost all cases and in some cases greater antibacterial efficacy as compared to their parent aminoglycosides. Particularly promising was an amikacin derivative modified at the 6'' position.

Aminoglycoside amines were globally converted to guanidinium groups to produce guanidinoglycosides. Guanidinoglycosides mostly showed comparable A-site affinities to their parent compounds, but their antibacterial activity was completely compromised.

Tobramycin and amikacin, two of the most clinically used aminoglycosides, were selectively modified with various hydrogen bonding moieties at their 6'' positions. Almost all of these analogs had greater affinities for the A-site. Tobramycin derivatives showed overall disappointing antibacterial activity, but several amikacin analogs showed potent and broad-spectrum antibacterial activity against resistant bacteria.

Aromatic analogs of the lead compound TAN-1057 were synthesized. Some analogs were evaluated in the previously described manner and also in eukaryotic and prokaryotic in vitro translation assays. The analogs showed inconclusive or poor activity in all assays. An alternate synthesis of the surrogate TAN-1057 side chain, β^3-lysine was also devised. An inability to resolve racemic β^3-lysine has thus far stymied the utility of this method, however.

Chapter 1

Bacterial Resistance and Antibiotic Development

1.1 The Rise of Antibiotic Resistance

Bacterial resistance to antibiotics has been a recognized reality almost since the dawn of the antibiotic era, but only within the past twenty years has the emergence of dangerous, resistant strains occurred with a disturbing regularity. This escalating evolution of resistance coupled with a diminished antibiotic pipeline has led some to claim that a post-antibiotic era is eminent.[1] Given that the three main causes of death in pre-antibiotic America were tuberculosis, pneumonia, and gastrointestinal infections, which combined accounted for 30% of all deaths, this is a frightening prospect.[2] Though we are still far from that scenario becoming reality, the trend in the field of antibiotics has decidedly been negative for some time now. The annual impact of resistant infections is estimated to be $20 billion in excess health care costs and 8 million additional hospital days in the United States (US)[3] and over 1.6€ billion and 2.5 million additional hospital days in the European Union (EU).[4] Antimicrobials currently account for over 30% of hospital pharmacy budgets in the US.[5]

A waning interest in antibiotics by the pharmaceutical industry is one factor that has contributed to an increased occurrence of hard to treat bacterial infections. In 2004 for example, only 1.6% of drugs in clinical development by the world's 15 largest drug companies were antibiotics. This reduced output of antibiotics has several causes.[6]

Antibiotics regimens are administered only for very limited durations making them far less profitable than drugs used to treat chronic ailments. Further, newly approved drugs for most other ailments are immediately prescribed to any patients that would benefit, whereas new antibiotics are typically held in reserve and only prescribed for infections that more established antibiotics can't treat. This policy helps delay resistance, but it also limits initial investment return. A market saturated with generic competitors and the inevitable growth of bacterial resistance exacerbates this profit disparity as compared to other drugs in the long term.

Regulatory hurdles have also muted the interest of major pharmaceutical companies. The tolerance of adverse side effects has recently been decreased for many drug classes, including antibiotics. Approval requirements during clinical trials have escalated in most cases from demonstration of noninferiority to superiority, and at times a lack of clear trial guidelines for antibiotics in particular have stifled development.[7] Pharmaceutical companies are presented with a paradox wherein federal agencies issue calls for more antibiotic development while concomitantly other federal agencies enact policies limiting the appeal of that very development.

These factors have made investment in antibiotics too high risk, and cost at an estimated $1.7 billion per drug, with too little potential reward for many large pharmaceutical companies.[8] A metric called net present value (NPV) has been developed for pharmaceutical companies to determine the best avenues of investment at a given time. NPV is a risk adjusted measure of the projected future revenues of a drug discounting initial development investment and other projected future expenses. A

characteristic NPV for an injectable gram positive antibiotic may be around 100, which is somewhat unattractive compared to a typical cancer drug, around 300, or a neuroscience drug around 720.[8a]

Since 1998 AstraZeneca, GlaxoSmithKline, Merck, Johnson & Johnson, and Pfizer/Wyeth are the only major pharmaceutical companies to develop an antibiotic past phase I clinical trials.[9] Sanofi Aventis, Eli Lilly, Bristol-Myers Squibb, GlaxoSmithKline, Proctor & Gamble, Roche, and Wyeth have all greatly curtailed, eliminated, or spun off their antibiotic R&D divisions.[5] In fact, as of 2013 there are only four multinational pharmaceutical companies with antibiotics divisions left.[9] No government has ever successfully discovered and developed an antibiotic and there have been no indications that any will contribute the resources necessary for such an endeavor anytime in the near future.[5] As a consequence much of what is currently being done in antibiotic development in the western world is done in small pharmaceutical companies, biotechs, and academic institutions. A number of large pharmaceutical companies still play a central role in antibiotic development in Japan, however.[9, 10]

Policies have recently been enacted and incentives offered in an effort to reverse this exodus from antibiotic R&D. Agencies including the World Health Organization (WHO), the European Center for Disease Prevention and Control (ECDC), the Infectious Diseases Society of America (IDSA), and even the US Congress have gotten involved.[4,7,9,11,12] In the 111th congress the Generating Antibiotic Incentives Now (GAIN) Act and the Strategies to Address Antimicrobial Resistance (STAAR) Act were introduced.[13] In 2011 the US government gave $94 million in government funding for the

development of Anacor's GSK-052 (though it's clinical trials were subsequently halted in 2012) and $67 million for Teatraphase's TP-434 (eravacycline), currently in phase III trials.[9] Even the FDA has recently publically acknowledged that there is an antibiotic crisis.[14]

The other factor fueling antibiotic resistance is the evolution and dissemination of resistance factors within bacterial populations. There are a plethora of means by which humans have inadvertently accelerated the evolution of bacterial resistance. The over prescription of antibiotics by doctors for symptoms that in many cases may not be caused by bacteria has historically been one such problematic policy. In recent years steps have been taken to limit antibiotic over prescription, however. In surveys of doctor's visits in 1995 compared to 2005, the percentage that resulted in antibiotic prescriptions decreased universally for symptoms including ear infections, colds, bronchitis, sore throats, and sinusitis.[3] Despite these positive trends the Center for Disease Control and Prevention (CDC) recently estimated that approximately 50% of antibiotics are still prescribed unnecessarily in the US at a yearly cost of $1.1 billion.[3]

In the hospital setting antibiotic stewardship programs are becoming more commonplace and have been correlated in many cases to significant reductions in some strains of resistant bacteria.[15] Despite these successes only 48% of US hospitals have adopted stewardship policies to date and numbers are unquestionably even lower in the majority of developing countries.[16] Varied methodologies in measuring antibiotic consumption in US hospitals has been an undermining factor even where stewardship policies are enacted though.[17] Along with overall reductions to antibiotic usage, cycling

usage between antibiotic classes, using combination therapies, and avoiding use of broad spectrum and last resort antibiotics whenever possible have also been implemented as strategies to avoid the evolutionary pressure that accelerates resistance.[18]

Overly long or improper treatment regimens may also in some cases exert unnecessary evolutionary pressure on bacteria.[19] This can lead to acquired drug resistance in which a minority resistant bacterial phenotype can find themselves in a less competitive, and therefore more advantageous environment as a phenotypically sensitive majority is killed off.[5] Outpatient antibiotic use has been directly tied to macrolide resistance in *Streptococcus pyogens* and penicillin resistance in *Streptoccous pneumoniae*.[20] More restrictive policies regarding outpatient regimes has resulted in declines of certain resistant isolates in both Finland and France.[21]

A lack of public knowledge about antibiotics has also led to their overuse. In a 2009 European survey, of those who had taken antibiotics within the last year, 20% claimed to have taken them for influenza, a viral malady, and only 36% of those surveyed answered correctly that antibiotics do not kill viruses.[22] This particular variety of misuse is especially problematic in countries where antibiotics can be obtained without prescriptions.[23] Europe has instituted an Antibiotics Awareness Day annually on November 18th in an effort to raise public knowledge.[16]

The use of antibiotics in animal feed stocks has also exacerbated the spread of resistance. Especially egregious is their use for non-curative reasons such as prophylaxis, metaphylaxis, and growth promotion which by one estimate accounted for 25 – 50% of all antibiotic consumption in the early 2000s.[18] Other estimates within the US during the

same time period estimated agricultural use to be much greater at 24.6 million pounds of antibiotics being given to animals for non-therapeutic purposes, 2 million pounds being used therapeutically on animals, and 3 million pounds being used in humans per year.[24] Antibiotic use for growth promotion has been banned in the European Union (EU) since 2003[25] and finally in 2012 the FDA banned the use of antibiotics in livestock without a veterinary prescription.[26] There are still many countries where this practice remains unlegislated, however.

There is strong evidence that the use fluoroquinolones in food animals has led to the emergence of fluroquinolone resistant *E. coli*,[27] *Salmonella*, and *Campylobacter*.[28] The emergence of vancomycin resistant *Enterococci* (VRE) in Europe was tied to the use of the glycopeptide avoparcin in food animals.[29] Avoparcin was banned in the EU in 1997, which resulted in a reduction in VRE there,[30] but many members of critical antibiotic classes are still used for veterinary purposes. In a survey by the European Medicines Agency there was actually an increase in veterinary sales of fluoroquinolones and fourth generation cephalosporins from 2005 to 2009.[31] The food industry's use of antibiotics has not been strictly limited to livestock either. In the US, in 1996 for example, 300,000 pounds of streptomycin and oxytetracyline were sprayed prophylactically on apples and pears.[32] Waste runoff containing resistant bacteria or antibiotics from large corporate farms or agro-industrial plants is also a concern.[16] This serves as a mobile means of exposure to antibiotics and the terrestrial locale provides an ideal environment for dissemination of resistance elements from both pathogenic bacteria and potentially soil bacteria as well.[16]

Though there is undoubtedly a significant human contribution to resistance, there is also resistance that has occurred in nature absent human interference.[33] Resistances to first in class antibiotics such as penicillin and streptomycin, discovered during the golden age of antibiotics, were observed shortly after their initial isolation.[34] Though this is not always the case, this phenomenon is typical when examining the antibiotic arsenal as a whole.[32] With the advent of cloning and sequencing it was possible to trace β-lactamases to a large number of homologous, but distinct genes that were transferred vertically and horizontally throughout many microbial communities, directly between bacteria and indirectly mediated by the many bacteriophages that infect them.[35] Resistance genes can associate in clusters and be transferred together as well.[36] This kind of genetic diversity couldn't have arisen in the time frame since penicillin's discovery and indeed phylogenetic analysis suggested a more ancient root evolution of these enzymes.[37]

Resistance elements have even been found in bacterial DNA that was isolated for 30,000 years in permafrost.[38] Estimates based on the genetic divergence of antibiotic biosynthetic genes have suggested that some antibiotics could have evolved hundreds of millions of years ago.[39] Taken together this evidence suggests that bacteria have likely had a very long time to evolve resistance to many, if not all, natural product antibiotics, and therefore, resistance is highly likely to exist long before their discovery by man. Most soil bacteria exhibit some form of antibiotic resistance and many of them exhibit many resistances even to antibiotics that they do not naturally produce.[40] It could be argued that these samples could be contaminated in a variety of ways including antibiotic runoff. However, this evidence is also supported by a number of studies have found antibiotic

resistant (in some cases highly resistant), commensal bacteria on both humans and animals from remote locales that have never been exposed to antibiotics through unnatural means.[41] Evolution of bacteria to antibiotics therefore is a natural process and would exist even absent human mismanagement.

Human use (and misuse) of antibiotics has clearly put unnatural selective pressure on bacteria which has accelerated their evolutionary process to the detriment of everyone. To address this problem, faster development of new antibiotics and more responsible use of currently antibiotics is necessary.

1.2 Emergent Bacterial Threats

There are many species of dangerous gram positive and gram negative bacteria. A sampling of some of the most problematic pathogens and their most alarming resistances are reviewed (Table 1.1). In the 1990s resistant gram positive bacteria materialized as a major threat with methicillin (MRSA) and vancomycin (VRSA) resisant *Staphylococcus aureus*, VRE, penicillin resistant *Streptococcus pneumonia*, and multi-drug resistant (MDR) *Clostridium difficile* dominating headlines. *S. aureus* is a gram positive, facultative anaerobic pathogen with both hospital and community acquired strains. Though traditionally opportunistic, many *S. aureus* strains are now aggressively pathogenic.[32] It is the most common skin bacteria with 60% of humans being intermittent carriers and 20% being persistent carriers, chronically harboring at least one strain.[42]

S. aureus has evolved an arsenal of extracellular proteins and defense factors unassociated with antibiotic resistance. These include hemolysins, proteases,

Table 1.1: Emergent Bacterial Threats

Bacterium	Gram Stain	Respiration	Problem Resistances
Staphylococcus aureus	+	Facultative anaerobe	β-lactams, glycopeptides
Enterococci	+	Facultative anaerobe	β-lactams, glycopeptides, aminoglycosides
Streptococcus pneumoniae	+	Aerotolerant anaerobe	β-lactams, macrolides, quinolones
Clostridium difficile	+	Obligate anaerobe	β-lactams, quinolones
Mycobacterium tuberculosis	+	Aerobe	Rifamycins, quinolones, aminoglycosides
Escherichia coli	-	Facultative anaerobe	β-lactams, quinolones, aminoglycosides
Pseudomonas aeruginosa	-	Facultative anaerobe	All classes except polymyxins
Acinetobacter	-	Facultative anaerobe	All classes
Klebsiella pneumoniae	-	Facultative anaerobe	β-lactams, quinolones, aminoglycosides
Enterobacter	-	Facultative anaerobe	β-lactams, quinolones
Neisseria gonorrhoeae	-	Aerobe	β-lactams, quinolones, tetracyclines, macrolides

hyaluronidase, collagenase and an enterotoxin that causes gastroenteritis. Approximately 25% of strains express the exotoxin toxic shock syndrome toxin (TSST-1), and in 5% of strains the exotoxin Panton-Valentine leukocidin (PVL) which causes necrotic hemmorhagic pneumonia.[43] PVL is encoded by a bacteriophage now found commonly in community acquired MRSA (CA-MRSA).[44] These toxins have made effective protein translation inhibiting antibiotics particularly desirable in the treatment of some *S. aureus* strains.[45] Additionally, the pigment that gives this bacterium is golden color is staphyloxanthin, a carotenoid, antioxidant, virulence factor that aids in immune system evasion.[46]

S. aureus also frequently causes chronic infections by forming biofilms. It is the leading cause of chronic infections associated with indwelling medical devices.[47] Methicillin resistance is also highly prevalent and though numbers can vary widely by country, its incidence is high in almost all countries where such data exists, and it is the single most commonly observed drug resistance in both the US and Europe.[4, 5] MRSA was recently estimated to be responsible for 60 – 89% of nosocomial infections leading to 19,000 deaths and over $3 billion in health care costs per year in the United States.[7,13,48,49] It was reported in 2009 that MRSA infections kill more people in US hospitals than HIV/AIDS and tuberculosis combined.[45] β-lactam resistance in MRSA is primarily due to expression of the *mecA* gene which encodes the low affinity penicillin binding protein (PBP 2a).[50]

The glycopeptides, vancomycin and teicoplanin, are common treatments for MRSA, however, resistance has now developed towards them as well. Vancomycin intermediate *S. aureus* (VISA), which is also usually insensitive to teicoplanin as well evolved a less permeable cell wall that traps these antibiotics.[51] VISA was first isolated in 1996 in Japan, but has since been encountered globally.[32] VRSA was first reported in 2002 and is far less common.[52] It is caused primarily by an acquired resistance from the VRE *vanA* gene, which alters the terminal sequence of cell wall precursors, making them poor substrates for vancomycin and teicoplanin.[51] VISA and VRSA strains are not strictly opportunistic, making them even more dangerous.[53]

Resistant *Enterococci* are comprised of primarily two species, *E. faecalis* and *E. faecium,* both of which are gram-positive, facultative anaerobic, opportunistic pathogens.

Enterococci are particularly environmentally tolerant with the ability to withstand a wide range of temperatures and pHs, as well as high salt concentration.[54] They are also capable of colonizing a wide range of locales including the gut, skin, and inanimate surfaces. Both have high level (30 – 50%) resistance rates against the aminoglycosides gentamicin and streptomycin.[55] *E. faecium* is usually inherently resistant to β-lactam antibiotics also, making it particularly difficult to treat when it develops vancomycin resistance, which it much more commonly does than *E. faecalis*.[54] The streptogramin combination, quinupristin / dalfopristin is an effective treatment for *E. faecium*, but is ineffective against *E. faecalis*.[56]

Some VRE isolates express enterococcal surface protein, which allows for the production of thicker, more drug resistant biofilms. Like VRSA, these traits make VRE common in healthcare-associated infections (HAIs), and particularly in the colonization of indwelling medical devices.[57] Vancomycin resistance in *E. faecium* is common in the US at 61% in 2002, however, this resistance is far less common in the EU.[4, 16] Though VRE is known to produce several resistance genes, the most common form of vancomycin resistance, as with VRSA, is *vanA*.[50]

S. pneumoniae is a gram-positive, aerotolerant, anaerobic, opportunistic pathogen. It is the leading cause of bacterial pneumonia, but it can also cause otitis media, sinusitis, and meningitis among other pathologies.[58] It has a polysaccharide capsule that makes it naturally resistant to phagocytes. It also produces hydrogen peroxide to kill other bacteria.[59] Approximately 40% of strains are no longer susceptible to penicillin, and its penicillin resistance often correlates with resistances to macrolides, sulfamides, older

tetracyclines, and early generation cephalosporins.[60] Even absent β-lactam resistance,

macrolide resistances caused by upregulated efflux encoded by *mef* or *erm* genes is

increasing in *S. pneumoniae*.[61] Resistance to the third-generation fluoroquinolone,

levofloxacin, has also been observed recently.[62] Though resistance isn't as prevalent as in

some other gram-positive pathogens, the pathologies associated with *S. pneumoniae*

infection make the prospects of increased resistance worth particular consideration.

    C. difficile is a gram-positive, obligate anaerobic, spore forming opportunistic

pathogen. Spores are highly environmentally tolerant. They are resistant to heat, changes

in pH, alcohol based hand sanitizers, and many traditional cleaning products that don't

contain bleach.[63] *C. difficile* can be community acquired, but has a particularly high rate

of acquisition in hospitals. Patients hospitalized for over four weeks have an

approximately 50% chance of contracting *C. difficile*.[64] It is probably best known for

causing antibiotic associated diarrhea. This pathology results when *C. difficile* is

contracted and antibiotics that it is resistant to kill all other bacteria in the gastrointestinal

tract. This subsequently causes *C. difficle* overgrowths as they spread to inhabit these

now vacant niches. It produces an enterotoxin (toxin A) and a cytotoxin (toxin B) which

play a role in the resultant symptoms and can lead to colitis, as well as life threatening

complications.[65] Prophylactic cephalosporin use in surgeries has been linked to this

condition and their use for this purpose is now restricted in certain at risk patient

populations.[32] In 2005 a hypervirulent strain of fluoroquinolone resistant *C. difficile*

emerged and quickly spread across North America.[66] As serious *C. difficile* infections

rarely emerge without the use of antibiotics, eliminating unnecessary usage becomes especially important with the advent of resistant strains of this pathogen.

These gram-positive threats are still widespread and destructive, but in recent years resistance rates have stabilized or decreased for many of them, including MRSA and VRE, according to the European Antimicrobial Resistance Surveillance Network (EARS-Net).[11b] Unfortunately, within the past ten years, as antibiotic development has focused on these threats,[67] drug resistant tuberculosis and a wave of new gram-negative strains just as perilous as their gram-positive counterparts have evolved. At least one analysis has suggested that the resistant gram-negatives may now be even more costly then gram-positives, MRSA included.[68] Also, in a European survey a compilation of some of the most common gram-negative infections were found to slightly outnumber common gram-positive infections.[4] In some ways the new resistant gram-negative pathogens are even more worrying, as their more difficult to penetrate outer membranes and higher prevalence of efflux pumps make them naturally resistant to many antibiotics. The main gram-negative threats are multi- (MDR) and pan- (PDR) drug resistant *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter*, *Klebsiella pneumoniae*, *Enterobacter*, and most recently *Neisseria gonorrhoeae*.[69]

Mycobacterium tuberculosis is a highly aerobic, pathogenic bacteria that is the main cause of tuberculosis (TB). Though it doesn't typically gram stain because of a high lipid content in its cell wall, it's considered a gram-positive bacteria because of the lack of an outer membrane present in gram-negative bacteria. This atypical cell wall protects it from macrophage digestion and gives it an inherent resistance to many antibiotics.[70] An

estimated one third of the world's population is infected with latent TB. Many will not

have the disease progress to an active state, however enough do to make TB

unquestionably one of the greatest bacterial threats.[71] TB is second only to HIV/AIDS as

the greatest killer worldwide due to a single infectious agent with 1.3 million deaths from

8.6 million new infections in 2012 largely in developing countries.[71] MDR-TB, resistant

to the first line combination therapy of rifamycin, isoniazid, and pyrazinamide is

becoming fairly commonplace with about 450,000 people in the world developing cases

in 2012. Of those cases 9.6% are estimated to be extensively drug resistant (XDR-TB),

which is further resistant to at least one second line fluoroquinolone and

aminoglycoside.[71, 72] XDR-TB sometimes requires a two year course of antibiotics at a

staggering average cost of $483,000 and can be fatal even with proper treatment.[3, 73]

E. coli are gram-negative, facultative anaerobes that are most commonly

commensal, but can also be pathogenic. Pathogenic strains can produce potentially

deadly toxins including enterohemmorhagic verotoxin (Shiga-like toxin) which causes

hemolytic-uremic syndrome and renal failure.[74] This toxin was originally gained from a

prophage.[75]

Traditionally *E. Coli* have been one of the most widely antibiotic susceptible of

the *Enterobacteriaceae* family. Recently though horizontal gene transfer has allowed for

the rise of highly resistant strains.[76] *E. coli* resistance is worrying because they are the

most common gram-negative bacterial infections in humans and occurrence of strains

with extended spectrum β-lactamases (ESBLs) conferring resistance to third generation

cephalosporins has been steadily rising in Europe.[4] ESBL positive strains in bacteraemias

have also shown high cross resistance to fluoroquinolones (>80%) and gentamicin (>40%).[76] Although still fairly uncommon, *E. coli* on multiple continents have also acquired the New Delhi Metallo-β-lactamase-1 (NDM-1) enzyme from *K. pneumoniae*, which confers a broad resistance to all β-lactams including carbapenems with the exception of the monobactam, aztreonam.[77, 78] Fluoroquinolone resistance is also common among *E. coli*.[79] *E. coli* overexpressing *FomA* and *FomB* plasmidic genes are capable of inactivating fosfomycin through phosphorylation.[80] *E. coli* are also the most commonly zoonotic pathogens discussed herein. *E. coli* O157:H7, an enterohemmorhagic strain, has been associated with many zoonotic outbreaks and incidences of food borne illness including a 1999 outbreak in the US that infected at least 127 people.[81] Another enterohemmorhagic strain, E. coli O104:H4, infected over 3,800 people in Germany in 2011 causing 54 fatalities.[82]

P. aeruginosa is a gram-negative, facultative anaerobic, opportunistic pathogen. It is the most common cause of chronic lung infections in cystic fibrosis (CF) patients.[83] These strains are frequently highly resistant and it is no longer uncommon to see CF related infections that are resistant to all antibiotics except polymyxins.[84] *P. aeruginosa* employs a type III secretion system to extrude a host of potent cytotoxins directly into host cells.[85] It has a high environmental tolerance especially with respect to nutritional requirements and has been known to survive in such diverse environments as jet fuel and disinfectant.[86] *P. aeruginosa* naturally has a host of siderophores and pigments that allow it to evade the innate immune system. Additionally it has particularly discriminating outer membrane porins that make its outer membrane impermeable and thus naturally

resistant to many antibiotics, and a high propensity to form biofilms that can increase resistances to antibiotics 100 to 1000 fold.[87]

Further antibiotic resistance occurs thorough a wide variety of mechanisms. Some strains have acquired a variety of β-lactamases including ESBLs, *K. pneumoniae* carbapenemase (KPC), and metallo-β-lactamases (MBLs).[88] *P. aeruginosa* also has an extremely comprehensive efflux pump systems. Mutations resulting in loss of the OprD porin coupled with upregulation of MexEF-OprN efflux pumps result in resistance to carbapenems and fluoroquinolones. MexCD-OprJ upregulation also results in resistance to fluorquinolones and some β-lactams. MexAB-OprM upregulation confers resistance to sulfonamides, β-lactams, cephalosporins, fluoroquinolones, macrolides, novobiocin, tetracycline, chloramphenicol, and some detergents. MexXY-OprM results in aminoglycoside efflux.[89] Fluoroquinolone resistance can also occur through DNA gyrase and topoisomerase IV mutations. While rare, mutations to both are found in many persistent infections.[90] Pan-resistant *P. aeruginosa* susceptible only to polymyxins strains have been isolated. In one case the isolated strain produced AmpC β-lactamases, decreased OprD porin expression, and upregulation of MexXY efflux.[91] Another strain produced an MBL, AmpC β-lactamase, and two aminoglycoside acetylating enzymes (AACs).[92]

Resistant *P. aeruginosa* and *Acinetobacter* could be especially dangerous in the long term because of their intrinsic resistance to some gram-negative antibiotics and their ready acquisition of DNA from other bacteria ensuring the spread of additional resistances.[93] In a survey of select European countries they currently have the highest

resistance rates to both carbapenems and aminoglycosides, two traditionally last resort antibiotics.[79] *P. aeruginosa* resistance has been stabilizing in the US, while unfortunately *Acinetobacter* resistance has been increasing.[94] The mortality rate for the latter is notably higher as well.[95]

The most common resistant *Acinetobacter* species is *A. baumannii*, a gram-negative, facultative anaerobic, opportunistic pathogen. This bacterium is also colloquially referred to as "Iraqibacter" because of its rapid emergence as a problem pathogen in wounded soldiers during the Iraq war. Many gram-negative bacteria are known for environmental persistence and *Acinetobacter* are particularly adept. With especially thick cell walls that protect them from dry conditions and high tolerance to temperature, pH, and nutrient changes, they are capable of surviving for up to 5 months on inanimate objects.[96] *A. baumannii* is naturally resistant to many antibiotics due to both poor membrane penetration and active efflux pumps as well.[96a] Overexpression of the AdeABC efflux pump causes broad resistances to cephalosporins, fluoroquinolones, and tigecycline, while the AbeM efflux pump leads to aminoglycoside and fluoroquinolone resistance.[97] More specific efflux pumps, Tet(A) and Tet(B) for tetracyclines and CmlA for chloramphenicols also exist.[96a] Further hindering uptake, 30% of *A. baumannii* isolates produce an exopolysaccharide capable of forming biofilms.[98] It also expresses a powerful, epithelial cell targeting cytotoxin that aids in its colonization.[99]

MDR *A. baumannii* has two main modes of resistance. The first is the aforementioned efflux pumps, which also impart resistance to ammonia based disinfectants.[96a] The second is a variety of β-lactamases including ESBLs and

carbapenemases including imipenem MBLs and oxacillinases (OXAs).[100] These antibiotic resistances factors coupled with *Acinetobacter* natural resistances have combined to produce *A. baumannii* strains with resistance to all known antibiotics including colistin.[96a] MDR *Acinetobacter* are also already particularly common with greater than 60% of all hospital acquired *Acinetobacter* strains resistant to multiple drugs including carbapenems.[101] Moreover this resistance emerged over a remarkably short time period with a greater than 30% increase in carbapenem resistant *A. baumannii* strains from 1995 and 2004, which coincides closely with the rapid spread of OXAs.[102] During the same time period resistance to amikacin, ciprofloxacin, and piperacillin-tazobactam also increased steadily.[97] MDR *Acinetobacter* and *Klebsiella* are so dangerous that their outbreaks have resulted in hospital ward closures on multiple occasions.[103]

K. pneumoniae is a gram-negative, facultative anaerobic, primarily opportunistic bacterium that can be nosocomial or community acquired. Community acquired *K. pneumoniae* most commonly causes pneumonia, like *S. pneumoniae*, there are a variety of other pathologies it can cause as well and only about 5% of pneumonia cases are caused by *K. pneumoniae*. *K. pneumoniae* has a thick polysaccharide capsule that acts as an antiphagocytic factor.[104] They were the first species that *qnr* quinolone resistance genes were isolated from.[105]

This species also commonly acquires MDR determinants, and in particular an impressive array of β-lactamases. Most worrying are ESBLs, KPC, and most recently NDM-1. The latter two have caused multiple epidemics and even more troublingly are

capable propagation to other species.[77a,100, 106] Carbapenem resistances are a serious

problem since carbapenems were highly resistant to most other β-lactamases prior to the

advent of KPC and were often used as drugs of last resort for serious gram-negative

infections.[88] NDM-1 genes have commonly been found on plasmids and since the first

identification of NDM-1 in 2007, producer strains have quickly spread around the

world.[69b, 78] NDM-1 producing strains are typically highly resistant with other resistance

mechanisms commonly including ESBLs, AAC AMEs and ribosomal methylases for

aminoglycoside resistance, and fluoroquinolone resistant topoisomerase mutations,

among others. The majority of these strains are only susceptible to tigecycline and

colistin.[69b, 107] Pan-resistant strains have even been reported.[108] Though NDM-1

producing strains have so far remained relatively rare, [78] their rapid globalization coupled

with their extreme resistance profiles warrants close monitoring in years to come.

    Enterobacter is a genus of gram-negative, facultative anaerobic, opportunistic

pathogens. They are mainly known to exhibit antibiotic resistance through expression of

an extensive variety of ESBLs and carbapenemases including, KPC, OXA, and several

MBLs.[109] They are also the pathogen to most commonly have *qnr* quinolone resistance

genes at over 30% occurrence in isolates.[110] Their outlook is less grim then some of the

aforementioned pathogens, however. Colistin, tigecycline, amikacin, and some

fluoroquinolones remain options even for most MDR strains.

    N. gonorrhoeae is a gram-negative, aerobic, fastidious, sexually transmitted

pathogen. N. gonorrhoeae has several methods of avoiding the immune system including

Opa proteins that bind immune cell receptors to prevent a response and antigenic

variation which prevents the host from developing immunological memory against them.[111] Like *E. coli*, *N. gonorrhoeae* has traditionally been an easy to treat pathogen, but progressive accumulation of resistance mechanisms has gradually led to highly resistant strains. Penicillin and ciprofloxacin resistances acquired by plasmid, are now widespread, with azithromycin and some cephalosporin resistances becoming increasingly common.[112] High level tetracycline resistance via TetM efflux proteins is also common.[113] Most recently a MDR *N. gonorrhoeae* strain with high level resistances to the third-generation cephalosporins cefixime and ceftriaxone has been identified as well.[101] MDR *N. gonorrhoeae* is particularly worrying as it is community acquired and very commonly pathogenic, infecting 700,000 per year in the United States alone.[3]

Bacteria are responsible for approximately 90% of all HAIs.[101] HAIs are a major problem in the industrialized world having 5% and 7.1% incidence rates in the US and the EU, respectively.[114] In developing countries where sterile practices are less stringent the problem is much worse, with an estimated 15.5% incidence.[114b] Additionally, the often immuno-compromised patients that these infections target obviously have higher mortality rates than those with healthy immune systems. The risk of fatality associated with infections caused by resistant bacteria as compared to antibiotic sensitive bacteria is much higher as well. In most cases this is not because of any increased virulence, but rather because of prolonged bacterial exposure due to delayed, or a lack of an appropriate therapy.[5, 115]

1.3 A History of Established Antibiotic Classes

The number of antibacterial agents has decreased steadily in the United States over the last several decades.[7, 116] Historically there has been a higher chance of success with the development of compounds that belong to already established antibiotic classes.[117] Developmental risks are lower because of already proven microbiological assays to determine efficacy, known toxicological issues, and established regulatory routes for approval.[118]

Some scaffolds have been used particularly extensively. Between 1981 and 2005 cephalosporins, penicillins, quinolones, and macrolides accounted for 73% of all new antibiotics.[119] There is also a lack of diversity in the cellular target of all known antibiotics. Almost all clinically used antibiotics inhibit DNA, RNA, protein or cell wall synthesis, and there are less than twenty-five molecular targets for that account for their activity. Approximately half of all antibiotics target the cell wall.[120] In some cases structurally distinct antibiotics, even from separate gene clusters, are known to bind the same sites.[121]

Synthetic antibiotics are still extremely rare with the sulfa drugs, quinolones, oxazolidinones, and diarylquinilines as the only examples. All remaining antibiotics classes are composed of natural products and their semi-synthetic derivatives. Approximately two thirds of natural product antibiotics are isolated from *Streptomyces* soil bacteria.[122] Multiple classes of antibiotics are even known to be encountered within the same gene clusters.[123]

Improving biophysical techniques have garnered a wealth of information about cellular targets and binding modes of many established antibiotics making the rational design of semi-synthetic analogs of natural products a fruitful exercise in many cases. Precursor directed biosynthesis, mutasynthesis, and chemoenzymatic approaches are also increasingly being investigated to diversify certain established scaffolds as well.[124] These modifications to circumvent bacterial resistance mechanisms have allowed many scaffolds to continue to be useful long after clinical resistance has become predominant to early members of the classes.

The majority of the antibiotics introduced within the last 30 years are semi-synthetically derived. There are now semi-synthetic members of most antibiotic classes that are founded on natural products and there are many examples of highly utilized or extremely promising antibiotics that are semi-synthetically derived. These include the β-lactams meropenem (**5**) and tazobactam, the aminoglycoside amikacin (**10**), the macrolide azithromycin (**12**), the tetracycline tigecycline (**17**), the rifamycin rifampicin (**18**), the glycopeptide telavancin (**20**), and the streptogramin combination quinupristin (**25**) / dalfoprisitin (**26**).[119]

The following are profiles of the major established classes of antibiotics. What are thought to be emerging classes are also discussed even though some may only have one currently clinically approved member. Discussion of many structurally unique antibiotics has been avoided for practical considerations. Promising antibiotics in clinical trials are also discussed with a focus primarily on phase II and III candidates.

Sulfonamides

Prontosil (**1**)　　　　Sulfamethoxazole (**2**)
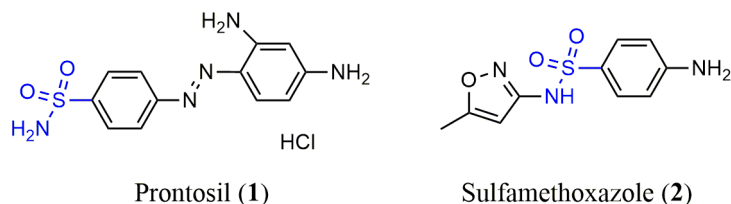
Figure 1.1: Sulfonamides. Sulfonamide moiety highlighted in blue.

Sulfonamides are a structurally diverse class of antibiotics that all have an aryl sulfonamide moiety in common (Figure 1.1). The first sulfonamide discovered was prontosil (**1**) in 1932. Sulfonamides were first used clinically in 1936.[125] They are synthetic antimetabolites that inhibit dihydropteroate synthetase, an enzyme totally absent human cells used in folic acid metabolism. Inhibition of this enzyme ultimately leads to repressed DNA replication in aerobic gram-positive and negative bacteria. Due to their broad spectrum activity sulfonamides were once popular antibiotics.[126] Increases in resistance, allergic reactions, and rare, but serious side effects including Stevens-Johnson syndrome and blood dyscrasias led to declines in their use many years ago.[127] However, interest has recently been rekindled in the use of sulfamethoxazole (**2**) in a combination therapy with trimethoprim, a compound that inhibits DNA replication by binding dihydrofolate reductase, another enzyme involved in folic acid metabolism. Trimethoprim has been found to have an up to 100 fold synergistic effect in combination with sulfonamides.[32] This combination has good activity against some MRSA strains and evidence has suggested that resistance has actually decreased to these compounds in recent years likely because of many years of infrequent usage.[128]
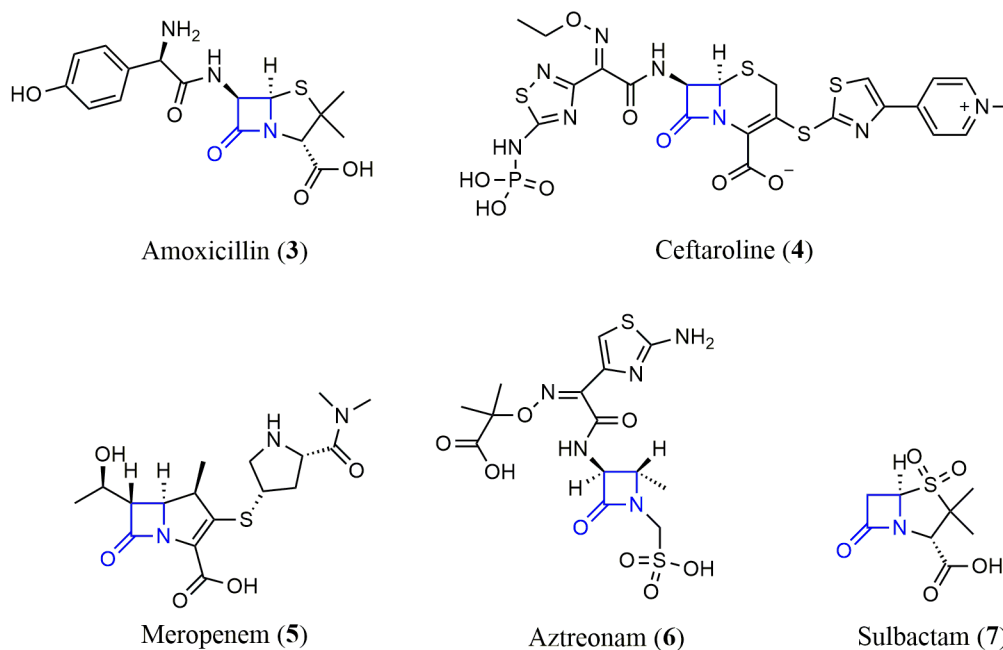
β-lactams

Figure 1.2: β-lactams: amoxicillin (**3**) (penicillin), ceftaroline (**4**) (cephalosporin), meropenem (**5**) (carbapenem), aztreonam (**6**) (monobactam), and sulbactam (**7**) (β-lactamase inhibitor). β-lactam moiety highlighted in blue.

β-lactam antibiotics are diverse in their structure, but they share a common four-membered β-lactam ring, which functions as the active pharmacophore for this class (Figure 1.2). The first β-lactam antibiotic to be discovered was benzylpenicillin (penicillin G) in 1928 though it wasn't used clinically until 1938.[125] β-lactams are the class with by far the most FDA approved members. They are also the most populous class on the WHOs list of critically important antibiotics to human medicine. There are 28 members, including antibiotic / β-lactamase inhibitor combinations, from three subclasses: penicillins, cephalosporins, and carbapenems that are listed as critically important.[129] They exhibit antibacterial activity by acting as suicide substrates for penicillin binding proteins (PBPs) (transpeptidases) inhibiting cell wall biosynthesis,

specifically maintenance of peptidoglycan. This leads to cell stress responses that result in cell lysis.[130] Many currently used β-lactams have very broad spectrum activity against most aerobic and anaerobic gram-positive and negative bacteria as well as low toxicity profiles making them popular first line antibiotics.[32] Resistance to older members of this class, especially the penicillin subclass, has proliferated dramatically though.

Resistance usually occurs via hydrolysis of the β-lactam ring mediated by a wide range of β-lactamases. These enzymes have been divided into four classes by the Ambler classification system: class A (KPCs and most ESBLs), class B (MBLs), class C (AmpC β-lactamases), and class D (OXAs). Class A includes many enzymes that can hydrolyze penicillins and cephalosporins as well as some that can hydrolyze monobactams and KPCs that are capable of hydrolyzing carbapenems.[131] The ESBLs from this class are plasmid mediated which has aided in their intra- and interspecies diffusion.[132] MBLs use divalent cations such as zinc as cofactors. Many are encoded in class 1 integrons, typically on gene cassettes also coding for aminoglycoside modifying enzymes (AMEs), found on transposons facilitating their spread.[133] MBLs inactivate many β-lactams including carbapenems and there are no currently improved inhibitors for them, but they have no activity against aztreonam, a monobactam.[134] AmpC β-lactamases are typically chromosomally encoded. AmpC and other class C β-lactamases can inactivate many β-lactams including aztreonam with preferential activity against cephalosporins, but they have no activity against carbapenems.[131, 135] Many OXAs are encoded on integrons.[136] Class D which is solely comprised of OXAs can hydrolyze cephalosporins and aztreonam and some have carbapenemase activity as well.[131, 137] Though their activity isn't as great

as MBLs they are the most commonly found β-lactamase in *Acinetobacter*, which makes them particularly problematic.[132]

Altered PBPs, especially in *Streptococci*, also occur.[138] Methicillin and other β-lactam resistances in MRSA is caused by production of low affinity PBP2a in greater than 90% of isolates.[139] Likewise in *S. pneumoniae* resistance to β-lactams is commonly caused by expression of a variety of low affinity PBPs.[32] Efflux by RND and ABC efflux pumps,[140] and outer membrane impermeability[141] can also cause resistance to β-lactams.

Progressive generations of β-lactams have largely advanced through semi-synthetic modification. Within the penicillin subclass some early modifications were focused increasing stability to early penicillinases through the attachment of bulky side chains as in the cases of methicillin and oxacillin. Other modifications were made to increase spectrum activity, from penicillin G, which is comparatively narrow spectrum, especially against gram-negatives, as compared to other β-lactams. Examples of this include the aminopenicillins such as ampicillin and amoxicillin (**3**), and ureidopenicillins like piperacillin. Despite dramatic proliferation of resistance to this class many penicillins remain important first line antibiotics.[32]

The first cephalosporin was cephalosporin C, which was discovered in 1948. Early generations semi-synthetic cephalosporins largely sought to improve pharmacokinetics and increase spectrum of activity against gram-negative pathogens primarily through increased cellular penetration. Later generations have become increasingly focused on combating β-lactam resistance.[32] Now in their fifth generation,

excellent safety profiles and increased spectrum of activity have made modern

cephalosporins some of the most highly utilized first line antibiotics.

Fifth-generation cephalosporin, ceftaroline (**4**), approved by the FDA in 2010, has

shown increased activity against MRSA, but is no more potent against MDR gram-

negatives, likely because it is still susceptible to most ESBLs.[142] However, it has shown

promise in combination with the β-lactamase inhibitor, tazobactam, against many

resistant strains though this doesn't prevent inactivation via MBLs.[131] Cubist's

ceftolozane, in phase III trials, has shown complimentary activity. It is active against

many MDR gram-negatives including *E. coli* and *K. pneumoniae* strains, but has less

activity against MRSA. It will also likely be used in combination with tazobactam, which

broadens its range of activity against some ESBL producers.[143] Calixa's CXA-101, in

phase II trials, has shown broad spectrum activity, but its advantage is mainly in its

superior activity against *P. aeruginosa* including strains with AmpC β-lactamases and

upregulated efflux.[144]

Imipenem was the first carbapenem to be identified in 1976. Carbapenems show

enhanced activity against many anaerobic and gram-negative bacteria as compared to

other β-lactams mainly because of their resistance to most ESBLs. Many are susceptible

to the various carbapenemases that have more recently evolved though. Imipenem and

meropenem (**5**) resistance has also evolved in *P. aeruginosa* with loss of OprD porins and

MexAB-OprM efflux upregulation.[132] Doripenem, approved in Japan in 2005 and the US

in 2007, has resistance to certain KPCs and OXAs, but it is still susceptible to all

MBLs.[145] It shows similar activity against most bacteria as imipenem and is superior to

other carbapenems against *P. aeruginosa*, but it notably lacks activity against MRSA.[45, 146] Meropenem (**5**) and biapenem (approved in Japan) have shown acitivity against some imipenem resistant, MBL producing *P. aeruginosa*.[147] Razupenem, a carbapenem in phase II trials, has shown promising activity against ampicillin resistant *E. faecium*.[148]

The first, and currently only FDA approved monobactam, aztreonam (**6**), was first identified in 1981. Though it is useful against only gram-negative pathogens, it has the distinction of being the only β-lactam resistant to some of the most dreaded class B β-lactamases.[32, 134] Though only in phase I clinical trials, Basilea's BAL30072 is a very promising monobactam, which shows superior activity against MBL producing *P. aeruginosa* and *Acinetobacter*, as well as many KPC producing *Enterobacteriaceae*.[149] It has also showed promising synergistic activity with meropenem against *Acinetobacter*.[150]

Clavulanic acid, discovered in 1976, was the first identified β-lactamase inhibitor. Augmentin, a combination therapy of clavulanic acid and amoxicillin (**3**), is still one of the most successful antibiotics on market. Piperacillin and tazobactam are also a popular combination therapy particularly against some *P. aeruginosa* infections including those producing ESBLs.[125] There has also been some renewed interest in sulbactam (**7**), which has been used successfully in the past with ampicillin and has recently shown good synergistic activity in combination with meropenem (**5**) against a wide range of *A. baumannii* strains. Unfortunately this combination isn't yet clinically approved.[151] Avibactam is a newer β-lactamase inhibitor with broad spectrum activity against class A, C, and D β-lactamases.[152] There are quite a few β-lactam inhibitors, both with and without β-lactam structures, that are clinically approved or in clinical trials in

combination with β-lactam antibiotics. Many of these have activity against KPC, AmpC, and OXA β-lactamases.[153] There are very few with activity against MBLs, however, and none that are currently clinically approved.[154] Some combinations of inhibitors including ones that have siderophore activity have shown some promise against MBLs though.[131] Tricyclic competitive inhibitors of certain MBLs have also been isolated.[155]

Aminoglycosides
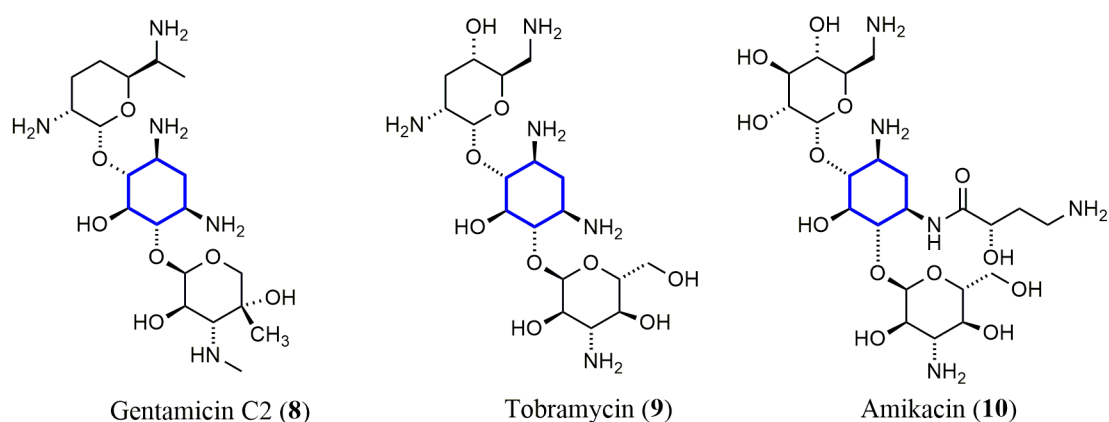
Figure 1.3: Aminoglycosides. 2-DOS ring highlighted in blue.

Aminoglycosides consist of amino-sugars connected through glycosidic bonds typically to a 2-deoxystreptamine (2-DOS) core (Figure 1.3). The first aminoglycoside to be discovered was streptomycin in 1943 and it was subsequently used clinically in 1946.[125] Six aminoglycosides are critically important according to the WHO.[129] They target the 30S ribosomal subunit, most commonly the A-site rRNA, leading to mistranslation of proteins. Some aminoglycosides are broad spectrum antibiotics with good activity against some aerobic gram-positive and most gram-negative species as well as *M. tuberculosis*. Their uptake is severely limited under certain anaerobic conditions, so

their efficacy can be severely diminished for certain facultative or obligate anaerobes. They are notably the only class of translation inhibiting antibiotics that is broadly bactericidal. The precise mechanism of their bactericidal activity isn't fully understood.[126] Insertion of flawed membrane proteins has been implicated though and this is known to promote further aminoglycoside uptake.[156]

They suffer from issues of nephrotoxicity and ototoxicity, which in most cases consigns them to the role of antibiotics of last resort rather than first line treatments. Also resistance, particularly common in gram-negatives, has developed through mechanisms including increased efflux by MexXY and ABC transporters, especially in *P. aeruginosa*, and methylated ribosomal A-sites, which decrease target affinity. Rmt and Arm methylases methylate the N7 position of rRNA G1405 conferring resistance to gentamicin (**8**) and kanamycin subclasses of aminoglycosides. The less common NpmA enzymes methylate N1 of A1408 causing broad resistance to the aforementioned subclasses as well as the neomycin subclass and apramycin.[157] The most common resistance mechanism is AMEs, however, consisting of N-acetyltransferases (AAC), O-nucleotidyltransferases (ANT) and O-phosphotransferases (APH). These are often encoded on mobile genetic elements and are readily disseminated between bacterial species via lateral gene transfer.[135]

Streptomycin was successfully used as a first line therapy for TB for many years until a mutation to the 30S ribosomal protein RpsL became commonplace, but it is still sometimes used as a second line therapy for MDR-TB.[158] Gentamicin (**8**), a natural product of *Micromonospora*, is the most widely used aminoglycoside. It is approved

infections caused by *Enterococci*, *Streptococci*, and *P. aeruginosa*. Tobramycin (**9**) has activity against many gram-negative pathogens, but is primarily used for the treatment of cystic fibrosis and resultant *P. aeruginosa* lung infections.[159] A large number of aminoglycosides are natural products, but several of the more recently developed members of this class are semi-synthetic. Amikacin (**10**) is a semi-synthetic designer derivative of kanamycin A clinically introduced in 1976. The L-hydroxyaminobutyramide (HABA) side chain of amikacin (**10**) blocks many AAC and APH enzymes, which increases its spectrum of activity considerably.[160] It is currently used mainly for the treatment of highly drug resistant gram-negative organisms including MBL producers and for MDR-TB.[159]

For the last several decades very little was done to advance new aminoglycosides into the clinic, but now plazomicin, a very promising, semisynthetic sisomicin derivative from Achaogen, is currently in phase II clinical trials. It was designed with several modifications, including a HABA side chain, to make it resistant to almost all AMEs and have lower toxicity than other aminoglycosides. It exhibits activity against fluroquinolone and aminoglycoside resistant pathogens as well as many β-lactamase producers.[9] It also shows impressive synergy with daptomycin and several β-lactam antibiotics.[161] It has good activity against many MRSA strains and carbapenemase producing *Enterobacteriaceae*, including KPC producing *K. pneumoniae*, but most NDM-1 producing isolates show resistance to it along with all other aminoglycosides. This is because these strains also typically produce ArmA and RmtC 16S rRNA

methylases.[9, 162] It also has lower activity against some *A. baumannii* and *P. aeruginosa* strains than currently used aminoglycosides.[163]

Amphenicols
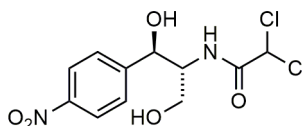
Figure 1.4: Chloramphenicol (**11**)

Amphenicols are a class of phenylpropanoid antibiotics. Chloramphenicol (**11**) was discovered in 1946 and introduced to the clinic in 1948 (Figure 1.4).[125] It is the only member of this class FDA approved for human consumption although there are other amphenicols that have been approved for use in other countries and for veterinary purposes. These antibiotics bind the peptidyl transferase center (PTC) of the 50S ribosomal subunit to inhibit the elongation step of translation. Chloramphenicol (**11**) has fairly broad spectrum bacteriostatic activity against some gram-positive and negative species including anaerobes.[126] They are bactericidal against *H. influenzae*, *N. meningitidis*, and *S. pneumoniae*, however.[164]

Despite their broad spectrum amphenicols have never been popular first line antibiotics in the developed world largely because of concerns regarding their safety. They are widely used in the developing world because they are inexpensive and readily available though.[165] Resistance can occur through target modification by the *cfr* gene encodes a methylase that methylates the C8 position of A2503 of the 23S rRNA causing resistance to amphenicols as well as many other PTC targeting antibiotics.[166]

Acetyltransferases are also a common resistance mechanism.[167] Efflux of amphenicols is also common with members in all of the major efflux pump families that recognize them.[32, 168]

Macrolides

Azithromycin (**12**)　　　　Roxithromycin (**13**)　　　　Telithromycin (**14**)
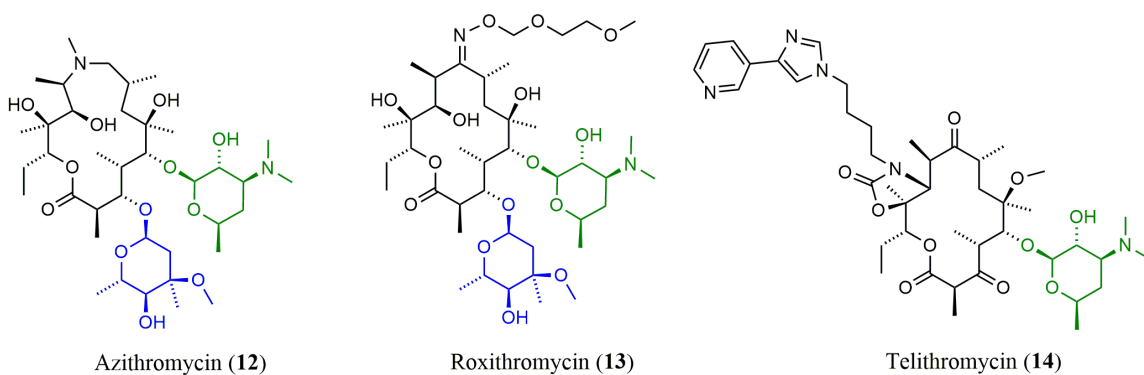
Figure 1.5: Macrolides and the ketolide telithromycin (**14**). The cladinose ring is highlighted in blue, deosamine in green.

Macrolides are macrocyclic lactones with deoxy-sugars, usually cladinose or desosamine, appended through glycosidic bonds (Figure 1.5). The first macrolide to be discovered was erythromycin in 1949 and it was introduced clinically in 1951.[125] Seven macrolides are designated as critically important by the WHO including the ketolide, telithromycin (**14**).[129] Macrolides bind the 50S ribosomal subunit blocking the peptide exit tunnel, inhibiting elongation of translation by causing premature disassociation of peptidyl tRNA from the ribosome.[169] They have fairly broad spectrum antibacterial activity against aerobic and anaerobic gram-positive and some gram-negative bacteria. They are bacteriostatic except at very high concentrations and in select situations, such as azithromycin (**12**), which is bactericidal against *H. influenzae*.[170]

Resistance to macrolides occurs via a variety of target modifications. Methylases encoded by the *erm* gene cause resistance. Mono-methylation of rRNA A2058 and occasionally A2509 in the N6 positions confers resistance many macrolides, but has no effect on the newer ketolide subclass. Di-methylation confers resistance to both macrolides and ketolides, however.[171] A2058G and A2059G mutations cause resistance although the effect is much diminished in ketolides. A2062C confers resistance selectively to 16-membered ring macrolides.[172] Mutations to ribosomal protein L22 and L4 can also result in resistance.[173] Macrolide modifying enzymes also exist.[174] As with many antibiotics, efflux is the main source of resistance, particularly in 14-membered ring macrolides.[32, 175]

Semi-synthetic members of this class azithromycin (**12**) and clarithromycin showed expanded spectrums of activity, better acid stability, and improved pharmacokinetics as compared to erythromycin making them popular first line antibiotics.[32] Azithromycin (**12**) was the most commonly prescribed outpatient antibiotic in the US in 2010.[176] Roxithromycin (**13**) another semi-synthetic macrolide is currently the only other member of this subclass on the US market.

Telithromycin (**14**), a semi-synthetic erythromycin derivative, was the first ketolide identified in 1997 and it is currently the only FDA approved member of this subclass. Telithromycin (**14**) shows improved activity against many strains with upregulated macrolide efflux and erm methylases including *Streptococci* and *S. Aureus* strains.[177] It was partially withdrawn on the US market after rare but serious side effects including blurred vision and liver failure were observed.[178] SAR studies have likely

pinpointed the structural origin of this toxicity, however, and ketolides currently in development including Advanced Life Science's cethromycin, in post-phase III trials, and Cempra's solithromycin, awaiting phase III trials, have sought to remedy this. Cethromycin has improved activity against *S. pneumoniae*, but is less effective against erm methylases producers.[179] Solithromycin has good activity against many gram-positive and some gram-negative pathogens, including some with erm methylase resistances, but reduced activity against some *S. aureus* strains.[180]

Tetracyclines

Tetracycline (**15**)   Eravacycline (**16**)   Tigecycline (**17**)
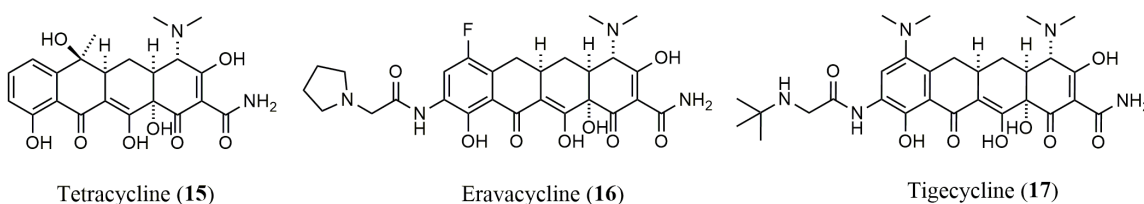
Figure 1.6: Tetracyclines and the glycylcycline, tigecycline (**17**).

Tetracyclines are an antibiotics class that shares a common octahydrotetracene skeleton (Figure 1.6). The first tetracycline was discovered in 1945 and was named chlorotetracycline. It was introduced clinically in 1952. They are broad spectrum, bacteriostatic antibiotics used against aerobic gram-positive and negative bacteria that bind the 30S ribosomal subunit and block aminoacyl tRNA access to the ribosome. Their typically low incidence of severe side effects has made them a first line therapy. Tetracycline resistance is most often due to efflux by SMR, RND, or ABC efflux pumps or by ribosomal modification. A tetracycline inactivating enzyme, TetX, has also been reported.[32, 181]

Early members of this class were natural products (tetracycline (**15**), oxytetracycline, and demeclocycline), but later members (doxycycline and minocycline) were semi-synthetic.[32] The semisynthetic derivatives have better pharmacokinetics.[182] Tetraphase has a fluorocycline, eravacycline (**16**), in phase III trials with broad spectrum activity against many MDR pathogens including MRSA, VRE, *C. difficile*, and KPC producing gram-negatives, but it has low activity against *P. aeruginosa* and some *Acinetobacter* strains.[9, 183] It circumvents several tetracycline resistance mechanisms including tetracycline specific efflux, tetracycline inactivating enzymes, and ribosomal modification.[9, 184] Also, Paratek's omadacycline, a derivative of minocycline, has passed phase II clinical trials. It has similar activity in many cases to the glycylcycline, tigecycline (**17**). Like both tigecycline (**17**) and eravacycline (**16**) it has little activity against *P. aeruginosa* though despite its advantages against many other species including highly resistant *N. gonnorhoeae*.[113, 179]

The only FDA approved glycylcycline, a new tetracycline subclass, is tigecycline (**17**). It is a derivative of minocycline, which was first identified in 1998 making it the first new tetracycline introduced in 30 years. It is also the only tetracycline designated as critically important by the WHO.[129] It overcomes previous tetracycline resistance mechanisms of ribosomal modification and efflux.[185] It exhibits broad spectrum activity, but most importantly it has good activity against MRSA, VRE, many MDR gram-negatives, including *A. baumannii* and ESBL producing *Enterobacteriaceae*. It notably has low activity against *P. aeruginosa* though. Low blood serum levels and some toxicity

are disadvantages. Also, efflux pumps have quickly evolved to recognize it, particularly *A. baumannii* AdeABC multi-drug efflux pumps. [186, 187]

Rifamycins
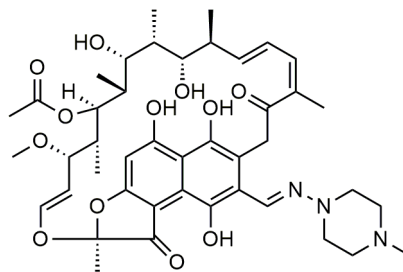
Figure 1.7: Rifampicin (**18**)

Rifamycins are ansamycin antibiotics possessing macrocyclic structures bridging an aromatic moiety. Rifampicin (**18**), the first rifamycin, was made as a semi-synthetic derivative of the *Nocardia* natural product rifamycin B in 1957 (Figure 1.7).[125] It quickly thereafter introduced to the clinic in 1958. These compounds exert antibacterial activity by binding the β-subunit of RNA polymerase inhibiting DNA dependent transcription. They are bactericidal against gram-positive bacteria and *M. tuberculosis*. They are bacteriostatic against some gram-negative bacteria, which has been attributed to their relatively lower cellular permeability.[188] Mutations to the β-subunit, most often on the side chains of residues 406 and 411, cause resistance. Efflux by VceB and Acr efflux pumps can also occur.[32]

Rifampicin (**18**) administered as a combination therapy with isoniazid and pyrazinamide is still a first line treatment for TB infections. Though less commonly used rifabutin and rifapentine are also primarily used for treating TB. These three compounds

are all designated as critically important by the WHO largely because of their efficacy

and common usage against TB.[129] The emergence of MDR- and more recently XDR-TB

strains, which are resistant to these treatments has necessitated an interest in the

development of next generation TB therapies, however.[189] Rifaximin is a newer

rifamycin approved by the FDA in 2004. It's only antibiotic indication is for treatment of

E. coli associated traveler's diarrhea, although it's spectrum of activity is considerably

broader.[190]

Glycopeptides

Glycopeptides are macrocyclic peptides with interspersed bridged aromatic

moieties and saccharide side chains linked through glycosidic bonds (Figure 1.8). The

first glycopeptide to be discovered was vancomycin (**19**) in 1952. It was then introduced

clinically in 1958.[125] In contrast to β-lactams, glycopeptides inhibit cell wall biosynthesis

in gram-positive bacteria by binding the terminal D-Ala-D-Ala dipeptide of

peptidoglycan units sterically inhibiting their use as substrates for PBPs and

Vancomycin (**19**)

Telavancin (**20**)
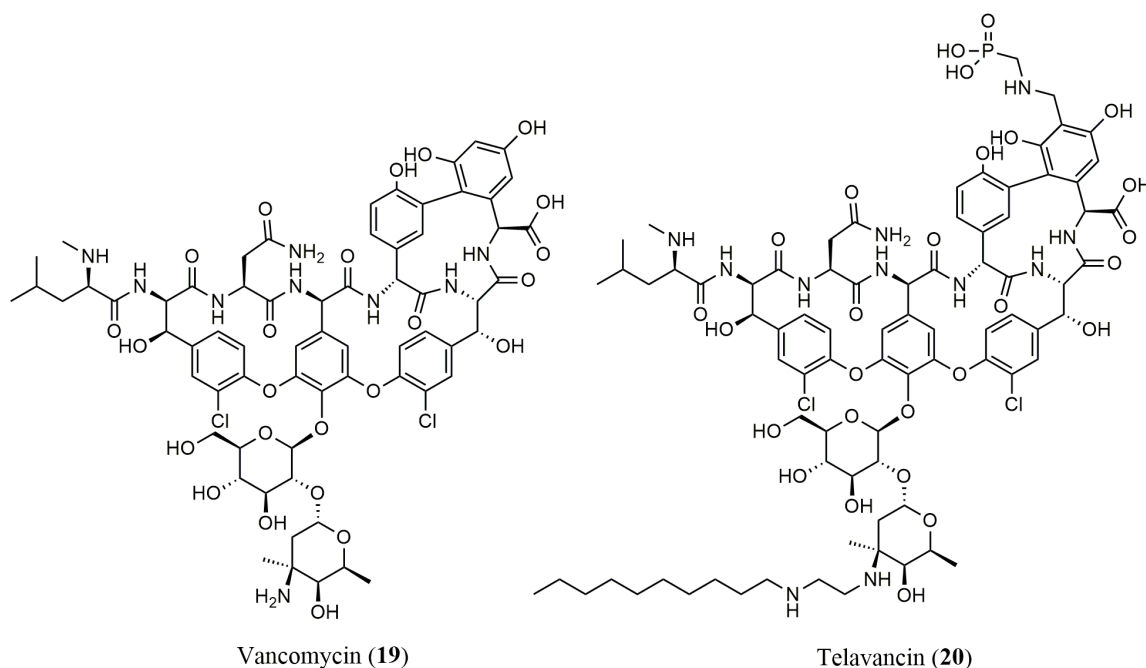
Figure 1.8: Glycopeptides

transglycosylases. Five vancomycin (**19**) resistant phenotypes (VanA-E), originating in

VRE, have altered peptidoglycan termini with lower affinities for vancomycin (**19**).[191]

VanH, VanR, VanS, and VanX are also proteins involved in the regulation and

reprograming of vancomycin (**19**) resistance. Efflux mediated resistance is rare for

glycopeptides, but AcrF efflux pumps have been known to cause resistance.[32]

Despite occasional resistance vancomycin (**19**) and the European approved

teicoplanin are both still designated as critically important by the WHO.[129] Vancomycin

stays free in the periplasm while teicoplanin is membrane anchored by a lipophilic side

chain.[192] Their spectrum of activity and efficacy similar in general, but this side chain

allows teicoplanin to overcome *vanB* encoded resistance that vancomycin (**19**) is

susceptible to.[193] In 2009 telavancin (**20**), a derivative of vancomycin (**19**), became the

first glycopeptide approved for use in the US since vancomycin (**19**). It has a secondary

mechanism of action, membrane depolarization, similar to daptomycin. It has shown

particularly good activity against MRSA, resistant *Enterococci*, and activity against

biofilm forming bacteria that vancomycin (**19**) lacks.[194] Oritavancin, a derivative of a

vancomycin precursor, has had a complicated development, but is currently undergoing

phase III trials under the management of The Medicines Co. It appears to have a higher

affinity for peptidoglycan and has a wider range of activity than vancomycin (**19**)

including activity against VRSA, *S. pneumoniae*, and an impressive 1000 fold greater

activity against VRE.[195] Durata's dalbavancin, a teicoplanin analog, is undergoing phase

III clinical trials. It has a significantly higher activity than vancomycin (**19**) against

Staphylococci, but is not active against VRE. Its main advantage isn't increased activity

though, but once weekly dosing.[195a, 196]

Nanotherapeutics ramoplanin, a structurally similar lipoglycodepsipeptide,

isolated from *Actinoplanes*, has also generated a lot of interest and is currently in phase

III trials. Ramoplanin inhibits cell wall biosynthesis, but through a different mechanism

than glycopeptides. It inhibits the transglycosylation step by stopping lipid I

transformation into lipid II in gram-positive bacteria. It would primarily be used in the

treatment of MDR *C. difficle* if approved.[197]

Quinolones

Quinolone antibiotic possess a quinolone core that typically has a N linked cyclic

moiety and various substituents at the C(6) and/or C(7) positions (Figure 1.9). Nalidixic

acid, the first quinolone, was discovered in 1962. It wasn't until 1968 that a quinolone,
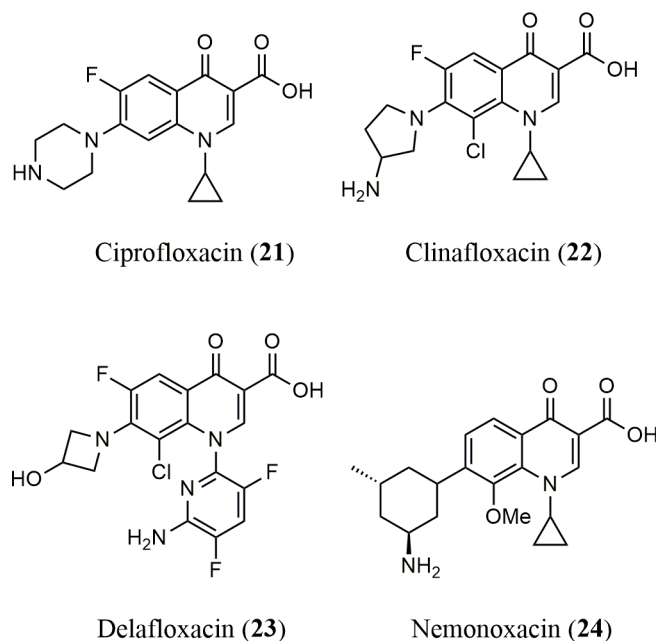
Figure 1.9: Quinolones

ciprofloxacin (**21**), which is a fully synthetic analog of nalidixic acid, was introduced

clinically.[125] This class has the second highest number WHO critically important

designees at 13, behind only the β-lactams.[129] Quinolones inhibit topoisomerases II

(DNA gyrase) and IV trapping the enzymes in the DNA cleavage stage, ultimately

inhibiting DNA synthesis among other things. Modern quinolones are bactericidal and

have broad spectrum activity that covers most aerobic gram-positive and negative

bacteria, some anaerobic gram-negatives, and *M. tuberculosis*. Most quinolones

preferentially target DNA gyrase or topoisomerase IV, though some, particularly later

generation compounds, target both equally.[32]

Resistance by target modification commonly occurs by mutations to genes *gyrA*

and *parC* in both *P. aeruginosa* and *A. baumannii* as well as the *grl* gene in *S. aureus*. [135,

198] The plasmid mediated *qnrA* gene, which produces a protein that protects DNA from

quinolone binding, has also been found primarily in *Enterobacter* and *Klebsiella*.[199] A number of other Qnr proteins have also been identified in gram-negative bacteria.[200] Fluoroquinolone efflux pumps, which can be intrinsic or acquired, commonly show broad activity against multiple antibiotic classes.[32, 201]

First generation quinolones are rarely used today because of poor spectrum of activity and biodistribution compared to more modern members of this class. Second generation drugs were characterized by expanded activity particularly against aerobic gram-negative bacteria, but were not broadly active against gram-positive bacteria.[202] Ciprofloxacin (**21**), a standout second generation fluoroquinolone, is still one of the most active quinolones against *P. aeruginosa* and has also garnered a lot of attention for its activity against extremely virulent bacteria such as *Bacillius anthracis* (anthrax) and *Yersinia pestis* (plague). Third generation compounds showed improved activity against gram-postiives.

The fourth generation of quinolones expanded activity even further especially in their coverage of anaerobic bacteria and bacteria that had developed resistances against this class. Some have also had more issues with toxicity than most second and third generation compounds though.[203] Fourth generation fluoroquinolones, sitafloxacin (approved in Japan) and clinafloxacin (**22**), overcome individual target modification resistances because they simultaneous target both DNA gyrase and topoisomerase IV. In some cases they are even active against double mutants in relevant organisms including *S. pneumoniae*, *E. faecium*, and *N. gonorrhoeae*.[204]

Three promising quinolones in development are Rib-X's delafloxacin (**23**),

TaiGen's nemonoxacin (**24**), and Furiex's avarofloxacin, which are all in phase III trials.

All have enhanced activity against gram-positive bacteria including *S. pneumoniae* and

MRSA strains including some that are ciprofloxacin (**21**) resistant.[205] MerLion's

finafloxacin, also in phase III trials, shows enhanced activity over other quinolones at low

pH and has particularly good activity against CA-MRSA and *A. baumannii* including

strains with ciprofloxacin resistance.[206]

Streptogramins

Quinupristin (**25**)   Dalfopristin (**26**)
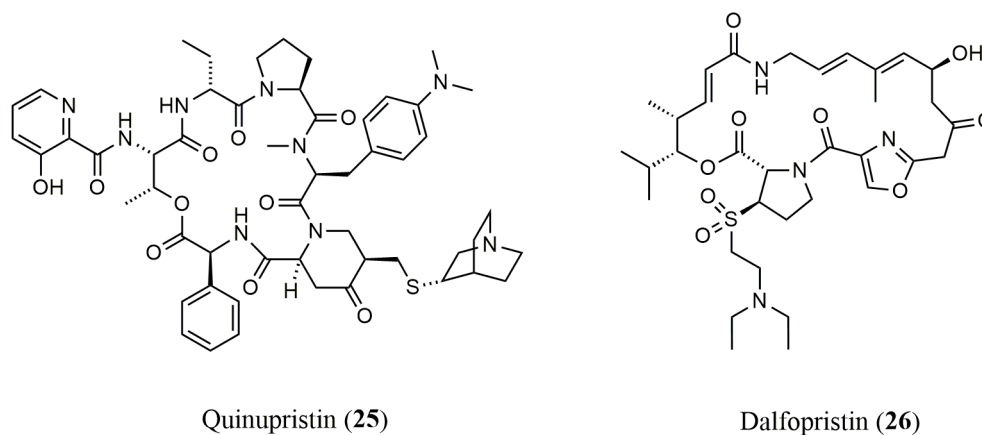
Figure 1.10: Streptogramins: quinupristin (**25**) (class B) and dalfopristin (**26**) (class A).

Streptogramins are divided into class A and class B based on their structures,

which also correlates with their mechanism of action. Class A streptogramins are 23-

membered unsaturated macrocycles containing peptide and lactone bonds. Class B

sreptogramins are 19-membered depsipeptides (Figure 1.10). Streptogramin B was

discovered in 1963, but it wasn't until 1999 that members of this class would be used

clinically.[125] They are typically administered clinically as pairs of molecules from each

class. Pristinamycin, itself a combination of class A and B molecules, and quinupristin (**25**) (class B) / dalfopristin (**26**) (class A), derivatives of the pristinamycins, are the only two approved drug combinations in this class. Both are designated as critically important by the WHO.[129]

Group A streptogramins bind the 50S ribosomal subunit at the PTC to inhibit initiation and translocation, whereas group B antibiotics bind the peptide exit tunnel to inhibit the elongation stage of translation. They have activity against gram-positive and in select cases gram-negative bacteria, but their overall narrow activity combined with poor aqueous solubility has limited the clinical use of many members of this class. They are usually bacteriostatic when administered alone. When they are administered as combinations of group A and B streptogramins they exhibit bactericidal activity.[126]

Quinupristin (**25**) / dalfopristin (**26**) and pristinamycin both show good bactericidal activity against MRSA, and the former also shows very high activity against vancomycin resistant *E. faecium*, but their activity becomes bacteriostatic in strains that exhibit erm methylases.[197] Erm methylases, which also produce resistance to macrolides, cause resistance in group B streptogramins.[207] Cfr methylases create resistance specifically to group A streptogramins.[208] There are now strains that have an *mlr* operon, which has both *erm* and *cfr* genes. These strains are resistant to all PTC targeting antibiotics.[209]

Polymyxins
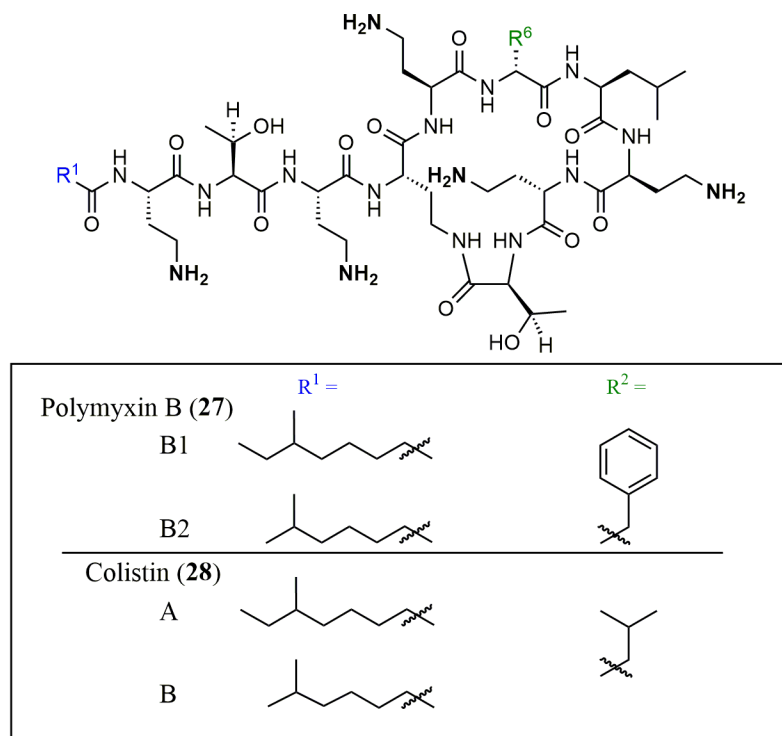
Figure 1.11: Polymyxins. Variable positions highlighted in blue and green.

Polymyxins are cyclic peptides with peptidyl side chains capped with a hydrophobic, saturated alkyl tail (Figure 1.11). Polymyxins A-E, natural product of

Bacillus, were discovered in 1947. Colistin (polymyxin E) (**28**) has been on the market since 1950. Primarily because of significant nephrotoxicity and neurotoxicity [210] it was infrequently used until recently, when interest was renewed in it and polymyxin B (**27**), as a drugs of last resort. Recent studies have shown that colistin nephrotoxicity may have been overstated possibly because of improper dosing or inferior formulation.[211] They have potent broad spectrum activity against most gram-negative bacteria although some strains of *E. Coli*, *Klebsiella*, *Enterobacter*, *M. tuberculosis*, and others have developed resistances.[212]

Polymyxins, which are polycationic, displace stabilizing magnesium and calcium ions to form electrostatic interactions with the anionic lipopolysaccharide (LPS) outer layer of gram-negative cell membranes. This disrupting interaction leads to increased cell membrane permeability, cell leakage, and rapid cell death.[213] Colistin (**28**) also has the added benefit of having potent anti-endotoxin activity also.[214] Resistance to polymyxins is fairly uncommon, although its frequency varies significantly by bacterial species and by geographic region.[215] Some gram-negative bacteria including *E. coli* and *P. aeruginosa* can exhibit resistance through expression of lower affinity modified LPS. *P. aeruginosa* can also upregulate membrane protein H1, which replaces divalent cations in the LPS, decreasing polymyxin affinity. *K. pneumoniae* increases production of its capsule polysaccharide, which limits polymyxin penetration to the LPS layer. Also, though not yet known to spread to pathogenic bacteria, strains of *B. polymyxa* are known to produce a degrading colistinase.[215]

Colistin (**28**) is now used in the treatment of MDR gram-negative pathogens with few other treatment options, particularly MDR *Pseudomonas*, *Klebsiella*, and *Acinetobacter* strains including NDM-1 producers.[97, 216] Cubist's CB-182,804 is a polymyxin B analog currently in phase I trials that shows activity against many MDR gram-negative bacteria and even some colistin resistant strains.[217] It was developed to not only have greater activity, but to have a more favorable toxicity profile though no evidence has been shown that this was successful.[218] Recently polymyxin B analogs with reduced overall positive charge have been shown to retain good antibacterial activity, while showing much improved in vitro toxicity profiles though. One of these molecules,

NAB739, is being actively developed in preclinical studies by Northern Antibiotics Ltd.[219]

Oxazolidinones
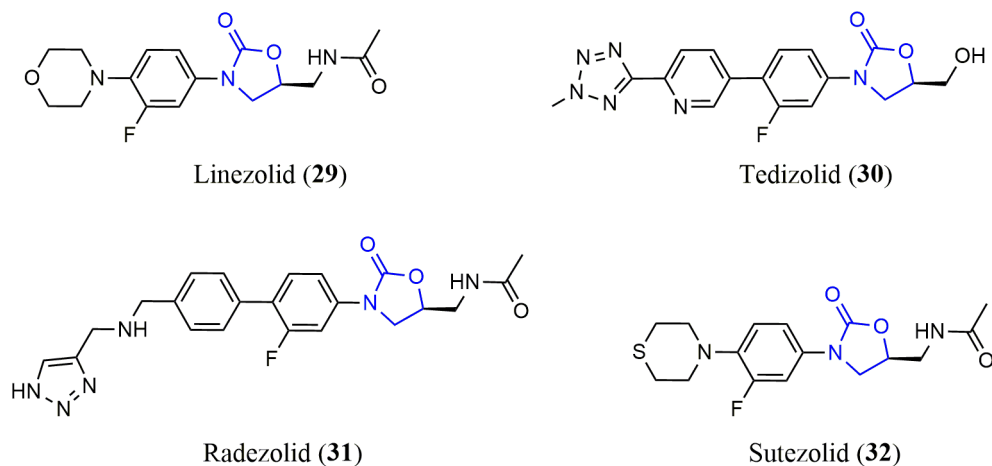
Figure 1.12: Oxazolidinones. The oxazolidinone rings are highlighted in blue.

Oxazolidinone antibiotics have a shared oxazolidinone core with various N-linked aryl and heterocyclic rings and short C(5) side chains (Figure 1.12). The first and currently only clinically approved oxazolidinone, linezolid (**29**), was first identified in 1995 and approved by the FDA in 2000, though the roots of this class go back to the 1970s.[220] Linezolid (**29**) has been designated as critically important by the WHO.[129] Oxazolidinones bind the PTC on the 50S ribosomal subunit blocking peptide bond formation to elicit bacteriostatic activity against gram-positive bacteria and *M. tuberculosis*. Resistance mechanisms to oxazolidinones are still somewhat rare. Target modification of the PTC by the G2576U mutation confers resistance in some *Enterococci* and *S. Aureus* strains, however.[221] U2500A and U2571C rRNA mutations, mutations to ribosomal proteins L3 and L4, and *cfr* encoded methylation of A2503 are also known to

result in resistance.[222] Although still rare, cfr methylases have spread to many countries and recently caused serious outbreaks of linezolid resistant *Staphylococci.* [223]

Linezolid (**29**) is useful against hard to treat gram-positive infections including those caused by MRSA and VRE.[224] Since linezolid's approval many oxazolidinones in development have been plagued by issues including poor solubility and pharmacokinetics, toxicity, and few improvements to activity. As a result most have failed in early developmental stages.[225] Trius's tedizolid (**30**), in phase III trials, and Rib-X's radezolid (**31**), in phase II trials have shown promise, however. They have both shown broadly improved activity and even activity against a wide range of linezolid resistant *Staphylococci* including MRSA strains.[226] Tedizolid (**30**) was found to have significantly greater activity against a variety of strains known to have point mutations or methylations that normally result in linezolid resistance.[227] The series of compounds that include radezolid (**31**) were designed based on computational models that were created using atomic level strucutres of linezolid (**29**). It was successfully designed to extend activity to include gram-negatives *H. influenzae* and *Moraxella catarrhalis*.[228] It also circumvents resistance mutations to L3 and L4, but it doesn't do as well as tedizolid (**30**) against rRNA point mutation resistances, or agasint cfr methylase producers.[226a, 228, 229] Pfizer's sutezolid and AstraZeneca's AZD5847, both in phase II trials, are being developed for use against MDR- and XDR-TB. Sutezolid (**32**) was designed to be potentially less toxic than linezolid (**29**) and was found to have broadly superior activity against *M. tuberculosis* isolates including those with resistance to isoniazid, rifampicin (**18**), ethambutol, and streptomycin.[230]

Lipopeptides
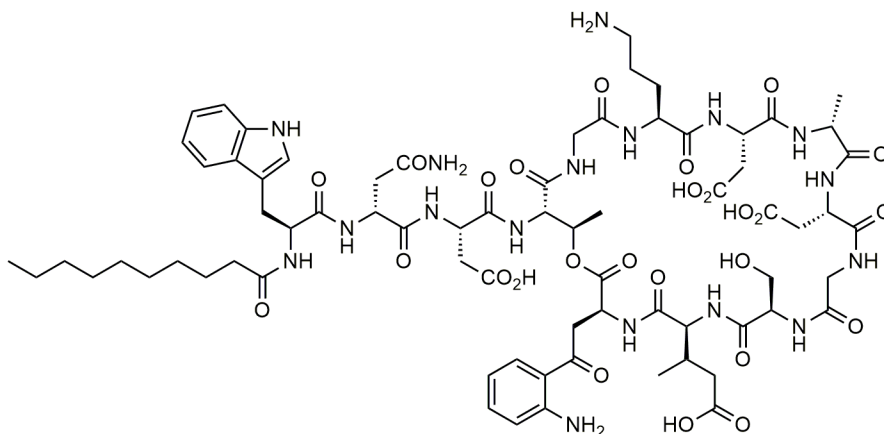
Figure 1.13: The lipopeptide, daptomycin (**33**).

Lipopeptides are cyclic depsipeptides with a peptidyl side chain capped with a saturated alkyl tail. Daptomycin (**33**), discovered in 1985, was the first lipopeptide antibiotic to be identified though it wasn't used clinically until 2003 (Figure 1.13).[125] It is currently the only clinically approved member of the lipopeptide class and has been designated as critically important by the WHO.[129] Lipopeptides work by inserting their lipid tails into the cytoplasmic membrane of gram-positive bacteria, which depolarizes the membrane leading to potassium efflux. This disrupts the structural integrity of the membrane resulting in cell lysis.

Daptomycin resistance is still rare and the mechanisms of its occurrence are not fully understood. Resistance in *Enterococci* has been linked to genes that alter cell envelope stress response and upregulation of cardiolipin synthase, an enzyme involved in cell membrane homeostasis.[231] *S. aureus* strains with thickened cell walls caused by increased production and D-alanylation of cell wall teichoic acids show daptomycin

resistance.[232] Single nucleotide polymorphisms in *S. aureus mprF* and *dltA-D* genes resulting in increased cell wall positive charge are also resistant.[233]

Daptomycin (**33**) displays good activity against many drug resistant gram-positive pathogens including MRSA and VRE.[197] Cubist's surotomycin is a lipopeptide in phase III trials for the treatment of *C. difficile*. So far it has been found to have a similar cure rate and a lower rate of relapse than vancomycin (**19**), the current standard treatment for this bacterium.[234]

Pleuromutilins
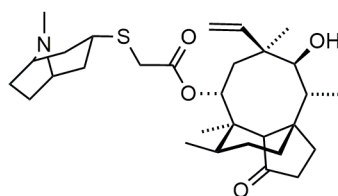
Figure 1.14: The pleuromutilin, retapmulin (**34**).

Pleuromutilins all have a common fused cyclo-octane / pentanone with a bridged cyclohexane ring system. They have a variety of ester linked side chains. Retapmulin (**34**) became the first clinically approved pleuromutilin in 2007 (Figure 1.14), though they were used extensively for several decades in veterinary medicine before that and some were discovered as early as the 1950s. Like many other antibiotics they bind the PTC on the 50S ribosomal subunit and thus inhibit translation. Upregulated *vga* genes that code for ABC efflux transporters confer resistance to pleuromutilins.[235] Target modifications including *cfr* mediated methylations, mutations to ribosomal protein L3, and point mutation to the 23S rRNA also cause resistance.[208, 236]

Retapmulin (**34**) has been approved for topical usage against gram-positive

bacteria including MRSA, reistant *Streptococci*, and erm methylase producers.[208]

Nabriva's BC-3781 is currently in phase II trials for non-topical applications. It shows

good activity against MRSA, VRE, and macrolide and quinolone resistant *S.*

pneumoniae, and has compared favorably to vancomycin (**19**) in trials.[237]

Macrolactones
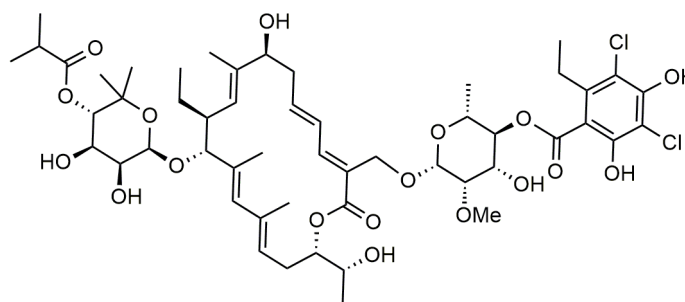
Figure 1.15: The macrolactone, fidaxomicin (**35**).

Antibacterial macrolactones have unsaturated macrolactone cores decorated with

deoxysugars and aromatic motifs. Macrolactones with antibacterial activity were first

discovered in the 1970s, but it wasn't until 2011 that the first and currently only

macrolactone, fidaxomicin (**35**), was approved for clinical use (Figure 1.15).[125]

Fidaxomicin (**35**) is an actinomycete natural product that inhibits RNA polymerase in

gram-positive and some gram-negative bacteria to elicit bactericidal activity. It has

almost no systemic bioavailability though which makes it unsuitable for the treatment of

many infections.[238]

Fidaxomicin (**35**) is a very narrow spectrum antibiotic with approval only for *C.*

difficile infections. As *C. difficile* associated diarrhea is a gastrointestinal affliction it has

been argued that it's narrow spectrum of activity is actually advantageous because of its low activity against beneficial commensal bacteria, which is thought to help prevent reoccurring infections. [238, 239]

Diarylquinolines
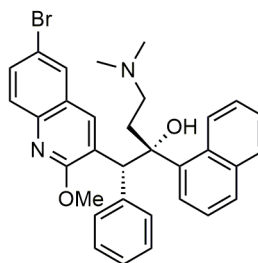
Figure 1.16: The diarylquinoline, bedaquiline (**36**).

Antibacterial diarylquinolines consist of a quinoline core with two other aryl groups linked through the C(3) position of the quinoline. Diarylquinolines with antibacterial activity were first discovered in 1997 through whole cell high throughput screening of synthetic molecules for direct antibacterial activity against the *M. tuberculosis* surrogate *M. smegmatis*.[240] Bedaquiline (**36**), the only clinically approved member of this class was first used in 2012. It inhibits F_1F_0-ATPase, the proton pump for ATP synthase (Figure 1.16).

Like fidaxomicin (**35**), bedaquiline (**36**) is a very narrow spectrum antibiotic. It has activity against *Mycobacteria*, and in particular *M. tuberculosis* making it the first new TB drug in more than forty years.[240] It will be used only for MDR-TB and XDR-TB. There was some controversy over its approval though as it was based on clearance of TB from sputum cultures rather than patient mortality.[241]

1.4 The Search for New Antibiotic Classes in the 21st Century

The development of new antibiotics in existing classes is an absolutely essential exercise that has been encouraged even by the IDSA, a principal entity in the push for new scaffold development.[9] However, new antibiotics that conform to established classes are often subject to at least some of the same resistances observed in previous members of the class. Therefore it is also necessary to develop new antibiotic classes. There have only been six first in class antibiotics with totally novel scaffolds approved since the 1960s and all of these have been introduced in the past fifteen years, a thirty year innovation gap (Figure 1.17). It is worth noting that all of these were developed to combat gram-positive pathogens including *M. tuberculosis* and they all have very little or no activity against gram-negatives. The innovation gap for totally novel antibiotics with potent gram-negative activity is therefore ongoing.
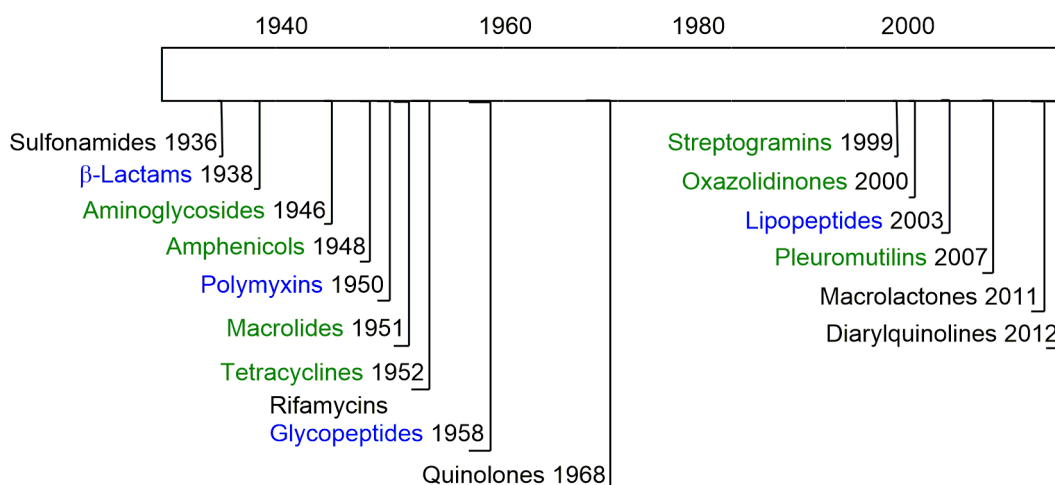
Figure 1.17: Timeline of first clinical introduction of antibiotic classes. Classes targeting the cell wall or membrane are highlighted in blue. Classes targeting the ribosome are highlighted in green.

The new first in class antibiotics for human use are the streptogramin combination quinupristin / dalfopristin in 1999, the oxazolidinone linezolid in 2000, the lipopeptide daptomycin in 2003, the pleuromutilin retapmulin in 2007, the macrolactone fidaxomicin in 2011, and the diarylquiniline bedaquiline in 2012. Linezolid and bedaquiline are fully synthetic molecules and the others are natural products. Though they were only recently developed for approval these molecules or their leads were all discovered much earlier with the exception of the diarylquinilines. Streptogramins were discovered in the 1960s, the leads for linezolid in the 1970s, daptomycin in the 1980s, pleuromutilins were isolated in the 1950s, and macrolactones similar to fidxomicin were found in the 1970s.[10a]

The early successes of many of these newer antibiotic classes suggest that scaffolds originally discarded during the heyday of antibiotic discovery because of liabilities such as narrow spectrum of activity, like fidaxomicin and bedaquiline, or higher toxicity, like the recently resurrected polymyxins, may need to be revisited given the desperate situation we are in. It has even been argued that species specific antibiotics may offer some significant advantages.[48a] Their cellular targets are less likely to overlap with those of eukaryotic cells or mutualistic gut bacteria. Also, resistance will likely be slower to develop as resistance genes would likely have to originate in the target species since there would be no evolutionary pressure to produce resistance determinants in bacteria that are naturally resistant to the compound.

In response to this lack of innovation the IDSA issued the 10 x '20 initiative in 2010 that calls for the development of 10 novel, effective antibiotics by 2020.[242] A 2013

update showed little progress towards that goal though.[9] There is a particular need for antibiotics effective against gram-negative pathogens as they comprise most of the currently emerging threats and the majority of recently developed antibiotics are not effective against them.[4, 5] According to the IDSA, of the drugs currently in clinical trials there is in particular a deficiency in antibiotics that have good activity against MBL producing gram-negatives and broad activity against *Acinetobacter* strains.[9] The comparative difficulty of getting a totally novel antibiotic through clinical trials keeps their development relatively rare as compared to the development of new members of already established classes. In a 2011 survey of all potential antibiotics in clinical trials only two out of the twenty one had totally novel scaffolds.[10a]

Comparative analysis of bacterial genomes has indicated that there are around 300 essential, highly conserved proteins that could potentially be broad spectrum drug targets.[243] Studies have recently begun to identify many molecules with novel molecular targets that exhibit potent antibacterial action. Some of these new targets include secretion and signaling proteins SecA[244] and SPase 1,[245] cell division protein FtsZ,[246] and peptide deformylase[247] though antibacterial activity is insufficient for many of these to be developed without modification.

Synthetic Antibiotics

Given the length of time bacteria as a whole have likely had to develop resistance to many natural product antibiotics coupled with the apparent ease with which many resistance genes are disseminated, developing totally synthetic antibiotics would appear to be an attractive strategy. However, as mentioned previously, to date synthetic

antibiotics are still extremely rare with the sulfa drugs, quinolones, oxazolidinones, and diarylquinilines as the only examples. They are outnumbered two to one by natural product antibiotics and their semi-synthetic derivatives. [32, 248] with development over the past few decades has focused especially on the latter.[249] Historically all of these, with the exception of diarylquinilines, were originally discovered outside of traditional antibiotic discovery programs as well. Sulfa drugs were originally developed as dyes, the first quinolone was an intermediate in the synthesis of chloroquine, a malaria drug, and oxazolidinones were originally developed to treat foliage diseases in plants.[48a]

Without a doubt one of the greatest challenges to finding new synthetic scaffolds is the issue of bacterial cell penetration. This is particularly true of gram-negatives, which are naturally resistant to many antibiotics because of outer membranes that keep many amphipathic drugs out as well as inner membranes and highly active efflux pumps that often recognize highly hydrophilic molecules.[250] The difficulties of prokaryotic uptake often mean that antibiotics have to be administered at concentrations two to three orders of magnitude higher than for most other diseases. This leads to an additional issue of toxicity in many cases.[125]

The Lipinski rules are a series soft rules governing the likelihood of oral bioavailability and therefore how "drug like" a particular molecule is. These rules were designed in the context of treating eukaryotic maladies though. The majority of antibiotics do not strictly adhere to them, and in fact several major classes routinely break all of them. The establishment of a similar set of rules for bacteria would greatly aid in antibiotics rational design and in the formation libraries better suited for antibiotic

screening purposes. There are no rules that have been routinely applied as of yet; however, some insights have started to be noted. Relatively hydrophilic compounds with masses below 600 Da tend to have good penetrance probably because of their ability to pass through outer membrane porins.[251] MDR efflux pumps tend to recognize cations and hydrophobic compounds particularly well, whereas anions are generally not substrates.[252] The inclusion of atoms not usually found in nature like boron and fluorine have had success, possibly again because of efflux pump evasion.[253] Fluoroquinlones, at this point the most successful fully synthetic antibiotics, adhere to all of these observations.

Screening for synthetic leads has not conventionally been a successful method of discovery. Major high throughput synthetic screens and rational design campaigns of synthetic molecules have failed utterly in many cases to identify a single antibiotic.[254] The wide spread failure of cell free target based screens in particular, which were an industry standard, has been implicated as one of the major reasons for many major pharmaceutical companies movement away from antibiotics development.[254, 255] The majority of scaffolds found at major pharmaceutical companies are optimized more for human eukaryotic targets and are not up to the disparate challenges associated with prokaryotic cellular uptake and evasion of rampant bacterial efflux mechanisms, particularly in gram-negative bacteria. Therefore target based screens of synthetic molecules will often lead to hits with high potency, but no real world utility.[33, 48a] This is a drawback that rational design of synthetic molecules suffers from as well.[125] Whole cell screens do not suffer from this disadvantage though.

Taking note of fact that all current synthetic antibiotics were originally discovered for other purposes, whole cell screens of libraries originally created by non-antibiotics programs have been done recently. These have been used to identify some promising new leads.[256] These new screens include a sophisticated antisense RNA based whole cell assay that has been employed toward the discovery of several novel MurA inhibitors from a chemical library and in the identification of the mode of action of FtsZ and LpxC inhibitors.[246] Microbiotix identified through a whole cell based screen a series of bis-indoles that inhibit DNA and RNA synthesis and induce SOS response. One compound, MBX-1162 (**37**), is currently in phase I clinical trials and has shown good, broad spectrum activity against MDR *A. baumannii*, *K. pneumoniae*, VRE, and MRSA (Figure 1.18).[257] Many hits on whole cell screens may exhibit narrow or even genus specific activity, as in the case of bedaquiline though which notably is the only synthetic, clinically approved antibiotic to our knowledge that was discovered by high throughput

MBX-1162 (**37**)

Gemmacin (**38**)   Emmacin (**39**)
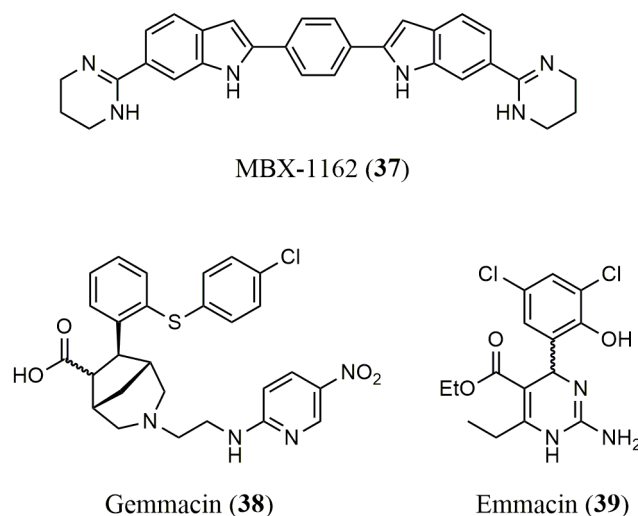
Figure 1.18: Totally synthetic potential antibiotics.

screens actually designed to identify antibiotics.[125] For some particularly hard to treat pathogens this may be acceptable at this point though.

Taking whole cell screens a step further, in vivo screens have also gained some interest with the rationalization that metabolically activated prodrugs, like sulfonamides, may be overlooked in traditional in vitro screens. Using animal models would of course be prohibitive in any large screening process for obvious reasons. However, *Caenorhabditis elegans*, a nematode, can be infected with human pathogens to making it a passable model organism for in vivo screens. Screens using *C. elegans* have had hits, including some that have no in vitro activity.[258]

Diversity oriented synthesis based approaches have been used to create promising totally synthetic molecules that more closely mimic microbial natural products.[259] Combinatorial chemistry can be done to create libraries around known priveledged scaffolds. Two encouraging examples of this are molecule based on a piperazine found to inhibit enoyl acyl carrier protein reductase, InhA in *M. tuberculosis* [260] and a biaryl molecule with wide gram-positive activity.[261] Another approach is to do unbiased diversity oriented synthesis. This approach coupled with subsequent SAR has been used to find gemmacin (**38**)[262] and emmacin (**39**),[263] two structurally distinct molecules with potent activity against clinically relevant MRSA strains.

Natural Product Antibiotics

Natural products are a historically successful and still a very much viable option as new antibiotics. During the "golden age" of antibiotics many of the current antibiotic

classes were discovered by a method of systematic screening of *Streptomyces* introduced by Selman Waksman in the 1940s. There is reason to believe that many natural products are still as yet undiscovered. One recent estimate put the number of discovered antibiotics as only 10% of the total from screened bacterial strains and only 1% from all microbes.[264] Historically the vast majority of natural product antibiotics have come from terrestrial soil actinomycetes.[32] Finding a useable antibiotic in the milieu of compounds produced by these organisms is no easy feat though, especially given that the most commonly produced antibacterial molecules for these particular bacteria have all likely been already identified. It was recently estimated that with current technology 10^7 actinomycete strains would have to be screened to discover the next novel antibiotics class.[265] Given that a strain collection at a large pharmaceutical company may be around 50,000 isolates, this is no longer a feasible approach.[125]

Exploration of bacteria from other ecological niches has recently yielded many promising new lead compounds, however. The producers of these include deep sea sediment actinomycetes,[266] marine sponges and seaweeds (though these seem to actually be made by bacteria colonizing them),[267] bacterial symbionts of insects, ascidians, fungi,[268] and myxobacteria.[269]

With the colossal advances in gene sequencing technology within last several decades, genomic prospecting has also begun. Genomic sequencing has in several cases identified silent operons that code for secondary metabolites within *Streptomyces*, some of which are not currently known to produce antimicrobial compounds.[270] The proper conditions for realizing expression of these potential antibiotics in cultures can be

difficult as antibiotic production may depend on a variety of factors including proper concentration of quorum sensing factors, which may be difficult to replicate.[271] Methods of manipulating these silent operons is an active area of research. These approaches have thus far never realized more than the identification of several lead compounds per year though. [270b, 272]

Natural products screens have been touted over synthetic molecule screens both for the obviously superior number of compounds available and the fact that natural products have already been "prescreened" by evolution.[125] It has traditionally been an often used approach, with whole cell empiric screening being the method of discovery of the majority of antibiotics used today.[255] Whole cell screening does not aid in identification of mode of action, however, and this approach can be expensive. It is made even more so in the realm of natural products screening, particularly for antibiotics, as many antibiotic producing bacteria are difficult to culture (an estimated 99% of microbial species are uncultured).[273]

Even more importantly, in current screens, many hits are actually already discovered compounds. This is because of the pervasiveness of lateral transfer of antibiotic producing genes amongst terrestrial soil bacteria. One study estimated that 1 in 100 actinomycete*s* produce streptomycin, 1 in 250 tetracycline, 1 in 66,000 vancomycin, and 1 in 200,000 erythromycin.[39] This phenomenon is not strictly limited to actinomycetes either.[274] Some members of the same antibiotic subclass can even be produced by extremely disparate organisms. Cephalosporins for example are produced by actinomycetes, proteobacteria, and fungi.[275] Several methods have been developed to

alleviate the problem of rediscovery. One strategy is to use strains resistant to commonly "rediscovered" antibiotics in the screening process. This has been done with wild type MRSA and with MDR *E. faecium*, which led to the discovery of many new promising compounds.[276] Similarly, recombinant strains with genes conferring resistance to the most frequently encountered antibiotics have been engineering for these assays.[277]

Target based natural products screens offer a useful counterpoint to whole cell screening. Target based screens do not suffer from all the drawbacks that these screens have when applied to synthetic molecules. Recently, several bioinformatics based, genome screening approaches have been used with some success.[278] Using these techniques a structurally novel glycosidic polyketide, ECO-0501 (**40**), with good activity against gram-positives was discovered from a strain of *Amycolatopsis orientalis* (Figure 1.19). [278c] It was previously mentioned that through genomic screening it has been

ECO-0501 (**40**)

Platensimycin (**41**)
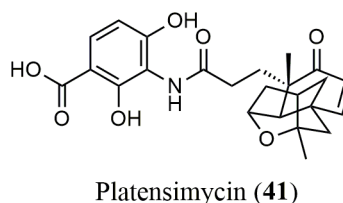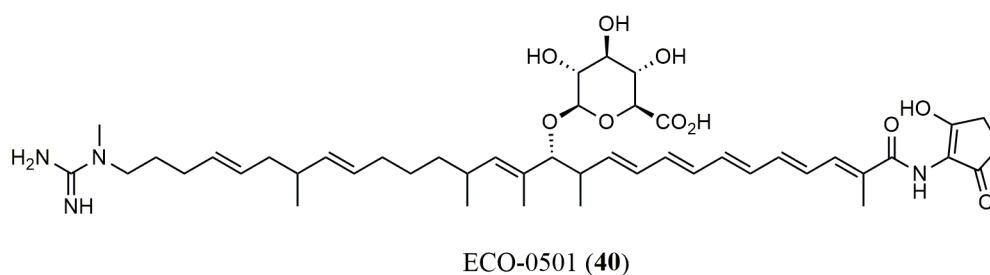
Figure 1.19: Natural product potential antibiotics.

estimated that there are hundreds of potential broad spectrum targets that no antibiotics have ever been developed for. Screens developed for these targets have the advantage that there aren't any false positives caused by already discovered antibiotics. Also, drugs developed for these targets may have less initial bacterial resistance than targets that already have selective pressure from many current antibiotics. This form of cell free screening has been successful in identification of the first inhibitor of FtsZ, a protein regulating cell division.[246]

Hybrids of whole cell and cell free target based assays now exist as well. Whole cell reporter assays or whole cell target-based assays employ either cells or conditions that are engineered to report specific molecular events at sub-bactericidal concentrations, unlike traditional whole cell screens that simply look at cell death upon introduction of a compound or isolate. Like the whole cell antisense approach that was used successfully to identify synthetic MurA inhibitors, antisense technology has also been used successfully to identify promising natural products. Particularly interesting was the discovery of platensimycin (**41**) and other potent fatty acid biosynthesis inhibitors through the use of these techniques.[279]

Combination Therapies

Taking a note from the drug cocktails used to combat HIV in the successful highly active anti-retroviral therapy (HAART) and the longtime strategy for combating *M. tuberculosis*, the use of antibiotics in combination therapies is becoming an increasingly attractive approach to combat resistance. Synergy can be such a powerful force that even molecules too weakly active on their own to be considered for

monotherapies can be administered in combination therapies to great effect.[280] Evidence

suggests that some antibiotic producing *Streptomyces* may also naturally employ

combination approaches to eliminate competition.[281] There are many combinations of

antibiotics that are known to exhibit synergy. Some antibiotics are almost always

administered as combination therapies even, such as streptogramins and rifamycins.

An extension of this strategy that is also being pursued is the production of

covalently linked hybrids of two antibiotic classes. Actelion's cadazolid (**42**), in phase III

trials, is structural hybrid of a fluoroquinolone and an oxazolidinone that primarily

inhibits translation in gram-positive bacteria (Figure 1.20).[218] It has activity against *C.*

difficile strains that are resistant to linezolid and moxifloxacin, so it is being developed

primarily to combat that pathogen.[282] A hybrid of two cell wall biosynthesis inhibiting

classes, Theravance's TD-1792 (**43**), in phase II trials, is a cephalosporin / glycopeptide

hybrid.[218] This hybrid principally targets the D-Ala-D-Ala terminus of peptidoglycan

units in gram-positive bacteria. It is being developed chiefly as a treatment for MRSA

although it also has activity against *C. difficile*.[283] The obvious advantage to this approach

is the potential added benefit of synergistic secondary antibiotic effects with the

administration of a single molecule. One disadvantage that has been noted, however, is

that gram-negative activity is commonly lost in hybrid molecules likely because of their

bulk, which is prohibitive of uptake.[284]

There are several compounds in development that reduce bacterial virulence.

Recently dipeptide inhibitors of *Pseudomonas* elastase, LasB, have been found. LasB is a

metalloprotease virulence factor that plays a role in tissue destruction, host
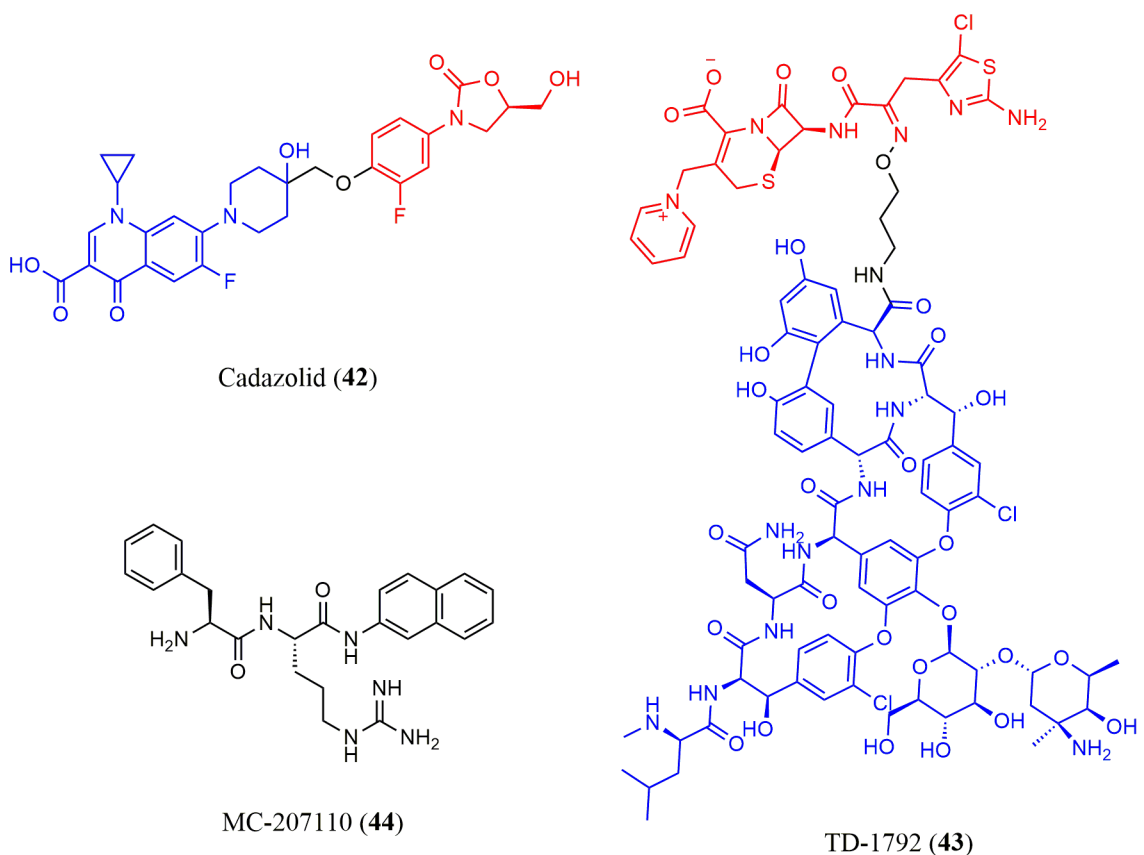
Figure 1.20: Potential combination therapies. Cadazolid (**42**), a quinolone (blue) and oxazolidinone (red) combination. TD-1792 (**43**), a glycopeptide (blue) and b-lactam (red) combination. MC-207110 (**44**) an efflux pump inhibitor.

immunomodulation, and in biofilm formation. The inhibitors were found to reduce

biofilms significantly especially when used in combination with antibiotics, making this a

promising adjunctive strategy.[285] Combinations of antibiotics and a potentiator to bypass

bacterial resistance have also shown promise. Many of the current antibiotic clinical trials

are actually on known antibiotic, potentiator combinations, particularly β-lactam / β-

lactamase inhibitor combinations.[9] β-lactamase inhibitors were thoroughly discussed in

the previous section. There has been interest for quite some time in inhibitors of

antibiotic modifying enzymes of other antibiotic classes also, though none but β-lactamase inhibitors have yet met much clinical success.[204]

Some interest has been shown in efflux pump inhibitors to resurrect indications of existing antibiotics.[16] Microcide's MC-207110 (**44**) was found to inhibit gram-negative resistance nodulation cell division (RND) efflux pumps including MDR *P. aeruginosa* MexAB-OprM pumps, which resensitized these strains to fluoroquinolones when administered in combination therapies. Unfortunately, issues of nephrotoxicity ended investigations into these compounds.[286] Unfortunately, the cationic nature of these molecules, which is the likely cause of their toxicity, also appears to be essential for their broad inhibition of RND efflux pumps.[125] Plants are known to produce inhibitors of gram-positive major facilitator (MF) MDR efflux pumps, but attempts at identifying more clinically relevant RND pump inhibitors were unsuccessful.[125] A variety of inhibitors of erm methylases have also been found. These include peptides that possibly act allosterically on the ErmC' methylase[287] and computationally designed small

molecules that bind either the S-adenosyl-L-methionine (SAM) binding pocket or the rRNA binding pocket of ErmC'.[288]

Interest has also increased in tackling the challenge of biofilm resistance.[87b] Traditionally antibiotic strategies have focused on targeting motile bacteria. This approach may be flawed, however, given that an estimated 80% of infections are biofilm mediated particularly in HAIs, and biofilm bacteria can exhibit up to 1000 fold resistance to some antibiotics as compared to motile counterparts.[47, 289] Some studies have been done on what current antibiotics used alone or in combination are best used against

biofilms.[290] There are also compounds being developed to inhibit biofilm formation and quorum sensing which plays a critical role both in biofilm formation and in various other processes related to bacterial pathogenesis including the production of virulence factors. LuxR/I quorum sensing systems utilize acyl-homoserine-lactone (AHL) autoinducers and are primarily found in gram-negative bacteria.[291] Halogenated furanones produced by a marine seaweed have been found to act as competitive inhibitors of AHL autoinducers.[292] A screen of synthetic compounds also found three molecules that inhibit AHL binding to the transcription factor LasR. Two were structural analogs of AHLs and one was structurally distinct.[293] Other high throughput screens have been used to find other structurally diverse inhibitors of biofilms including a triterpene,[294] iron salts,[295] and a hydrazide.[296] A computer aided drug design based virtual screen has also been used to identify a flavone with anti-biofilm activity.[297]

Many years of stagnant development and the alarming rise of bacterial resistance fueled by irresponsible policies and practices has created an undeniably dangerous quandary for the field of antibiotics research. Recent efforts by diverse groups including scientists, medical doctors, and even in some cases politicians, have shed light on this predicament, however. The approval of five new classes of antibiotics since the turn of the century to combat the emergent resistant gram-positive pathogens of the 1990s was a step in the right direction. Advances in scientific technology have provided the tools necessary for the discovery of new antibiotic classes and the improvement of already established ones to combat the largely unchecked rise of resistant gram-negative pathogens. It remains to be seen whether these encouraging developments will flower

with increases in funding and the backing of major pharmaceutical companies into an antibiotic renaissance or if they will wilt, paving the way for a dreaded "post-antibiotic" era.

Semi-synthetic modification of known antibiotic scaffolds has been successfully used to extend the usefulness of many established antibacterial classes. The aminoglycoside, amikacin, one of the most commonly clinically used members of this class, is one hallmark example of the efficacious implementation of this strategy. Several projects were undertaken to explore the effect of various modifications to aminoglycosides on their affinity for the ribosomal A-site and their antibacterial activity. Aminoglycosides were modified selectively with guanidinium groups at positions that were originally primary alcohols or primary amines in the parent structures. Non-selective, global guanidinylation of all aminoglycoside amines was also explored. Selective modification of the 6'' position of two of the most clinically utilized aminoglycosides, amikacin and tobramycin, with various charged and hydrogen bonding motifs was also undertaken.

The development of new classes of antibiotics is also a critical exercise in the war against resistant bacteria though this traditionally has a lower probability of success as compared to the development members of already established classes. TAN-1057 is a lead compound known to have potent activity against gram positive bacteria by inhibiting translation, one of the most common mechanisms of action of established antibiotics. Aryl analogs of this lead structure were synthesized and their biological properties, including their antibacterial activities were evaluated.

1.5 References

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[1] (a) P. C. Appelbaum, *J. Antimicrob. Chemother.* **2012**, 67, 2062. (b) J. Kluytmans, *Eur. Infect. Dis.* **2012**, 6, 19.

[2] Center for Disease Control and Prevention. Achievements in Public Health, 1900 – 1999: Control of Infectious Diseases. Available at: http://www.cdc.gov/mmwr/preview/mmwrhtml/mm4829a1.htm. Accessed 20 December **2013**.

[3] Center for Disease Control and Prevention. World Health Day: Media Fact Sheet. Available at: http://www.cdc.gov/media/releases/2011/f0407_antimicrobialresistance.pdf. Accessed 20 December **2013**.

[4] European Medicine Agency. The bacterial challenge: time to react. A call to narrow the gap between multidrug-resistant bacteria in the EU and the development of new antibacterial agents. Available at: http://www.ema.europa.eu/docs/en_GB/document_library/Report/2009/11/WC50000877 0.pdf. Accessed: 20 December **2013**.

[5] O. R. Sipahi, *Expert Rev. Anti. Infect Ther.* **2008**, 6, 523.

[6] D. M. Shlaes, *Antibiotics: The Perfect Storm*. The Netherlands: Springer; **2010**.

[7] B. Spellberg, R. Guidos, D. Gilbert, J. Bradley, H. W. Boucher, W. M. Scheld, J. G. Bartlett, J. Edwards Jr, *Clin Infect Dis* **2008**, 46, 155.

[8] (a) S. J. Projan, *Curr. Opin. Microbiol.* **2003**, 6, 427; (b) E. Power, *Clin. Microb Infect.* **2006**, 12(Suppl. 5), 25.

[9] H. W. Boucher, G. H. Talbot, D. K. Benjamin Jr, J. Bradley, R. J. Guidos, R. N. Jones, B. E. Murray, R. A. Bonomo, D. Gilbert, *Clin. Infect Dis.* **2013**, 56, 1685.

[10] (a) D. Jabes, *Curr Opin in Microbiol* **2011**, 14, 564; (b) R. C. Moellering, *Int J Antimicrob Agents* **2011**, 37, 2.

[11] (a) WHO: Department of Communicable Disease Surveillance and Response. WHO Global Strategy for Containment of Antimicrobial Resistance. Available at: http://www.who.int/csr/resources/publications/drugresist/en/EGlobal_Strat.pdf. Accessed: 20 December **2013**; (b) European Centre for Disease Prevention and Control. Communication from the Commission to the European Parliament and the Council. Action plan against the rising threats from Antimicrobial Resistance. Available at: http://ec.europa.eu/dgs/health_consumer/docs/communication_amr_2011_748_en.pdf. Accessed: 20 December **2013**.

[12] C. Nathan, *Nature* **2004**, 431, 899.

[13] B. Spellberg, M. Blaser, R. J. Guidos, H. W. Boucher, J. S. Bradley, *Clin Infect Dis* **2011**, 52(Suppl. 5), S397.

[14] (a) The Wall Street Journal. FDA chief focuses on antibiotic resistance. Available at: http://online.wsj.com/article/SB10001424052748703735804575536411612671060.html. Accessed 20 December 2013; (b) Brookings Institution. Facilitating antibacterial drug development 2012. Available at: http://www.brookings.edu/events/2012/05/09-antibacterialdrug-development. Accessed 20 December 2013.

[15] (a) P. Carling, T. Fung, A. Killion, N. Terrin, M. Barza, *Infect Control Hosp Epidemiol* **2003**, 24, 699; (b) M. K. Yong, K. L. Buising, A. C. Cheng, K. A. Thursky, *J Antimicrob Chemother* **2010**, 65, 1069; (c) N. Fishman, *Am J Med* **2006**, 119(6 Suppl. 1), S53.

[16] J N Pendleton, S P Gorman, B F Gilmore, *Expert Rev. Anti Infect. Ther.* **2013**, 11, 297.

[17] J T Jacob, R P Gaynes, *Expert Rev. Anti Infect. Ther.* 2010, 8, 893.

[18] S. R. Palumbi, *Science*, **2001**, 293, 1786.

[19] L. B. Rice, *Clin Infect Dis* **2008**, 46, 491.

[20] (a) H. Seppala, T. Klaukka, R. Lehtonen, E. Nenonen, P. Huovinen, *Clin. Infect. Dis.* **1995**, 21, 1378; (b) H. Goossens, M. Ferech, S. R. Vander, M. Elseviers, *Lancet*, **2005**, 365, 579.

[21] (a) H. Seppälä, T. Klaukka, J. Vuopio-Varkila, A. Muotiala, H. Helenius, K. Lager, *N Engl J Med* **1997**, 337, 441; (b) Anonymous, *Euro Surveill* **2008**, 13, 4.

[22] Special Eurobarometer 338 'Antimicrobial Resistance', 2009. Survey commissioned by the Directorate-General for Health and Consumers and coordinated by the Directorate-General Communication ('Research and Political Analysis' Unit). Available at: http://ec.europa.eu/public opinion/index fr.htm. Accessed 20 December **2013**.

[23] C. M. Kunin, *Ann. Intern. Med.* **1993**, 118, 557.

[24] S. L. Gorbach, *N. Engl. J. Med.* **2001**, 345, 1202.

[25] Official Journal of the European Union. Regulation (EC) No. 1831/2003 of the European Parliament and of The Council of 22 September 2003 on additives for use in animal nutrition. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2003:268:0029:0043:EN:PDF. Accessed: 20 December **2013**.

[26] FDA News Release. FDA takes steps to protect public health. Available at: http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm299802.htm. Accessed: 20 December **2013**.

[27] (a) X. Qin, Y. Razia, J. R. Johnson, J. R. Stapp, D. R. Boster, T. Tsosie, D. L. Smith, C. R. Braden, K. Gay, F. J. Angulo, *Antimicrob Agents Chemother* **2006**, 50, 3325; (b) A. E. van den Bogaard, N. London, C. Driessen, E. E. Stobberingh, *J Antimicrob Chemother* **2001**, 47, 763.

[28] P. S. Mead, L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, *Emerg Infect Dis* **1999**, 5, 607.

[29] H. C. Wegener, F. M. Aarestrup, L. B. Jensen, A.M. Hammerum, F. Bager, *Emerg Infect Dis* **1999**, 5, 329.

[30] H. C. Wegener *Curr Opin Microbiol* **2003**, 6, 439.

[31] European Medicines Agency. Trends in the sales of veterinary antimicrobial agents in nine European countries. Reporting period: 2005−2009. Available at: http://www.ema.europa.eu/docs/en_GB/document_library/Report/2011/09/WC500112309.pdf. Accessed: 20 December **2013**.

[32] C. Walsh, Antibiotics: *Actions, Origins, Resistance*. American Society for Microbiology (ASM) Press, Washington, DC, **2003**.

[33] G. D. Wright, H. Poinar, *Trends in Microbiol.* **2012**, 20, 157.

[34] J. Davies, D. Davies, *Microbiol. Mol. Biol. Rev.* **2010**, 74, 417.

[35] B. R. Lyon, R. Skurray, *Microbiol Rev* **1987**, 51, 88.

[36] S. Schaack, C. Gilbert, C. Feschotte, *Trends Ecol. Evol.* **2010**, 25, 537.

[37] B. G. Hall, M. Barlow, *J. Mol. Evol.* **2003**, 57, 255.

[38] V. M. D'Costa, C. E. King, L. Kalan, M. Morar, W. W. L. Sung, C. Schwarz, D. Froese, G. Zazula, F. Calmels, R. Debruyne, G. B. Golding, H. N. Poinar G. D. Wright, *Nature* **2011**, 477, 457.

[39] R. H. Baltz, *SIM News* **2005**, 55, 186.

[40] V. M. D'Costa, K. M. McGrann, D. W. Hughes, G. D. Wright, *Science* **2006**, 311, 374.

[41] L. Pallecchi, A. Bartoloni, F. Paradisi, G. M. Rossolini, *Expert Rev. Anti Infect. Ther.* **2008**, 6, 725.

[42] J. Kluytmans, A. van Belkum, H. Verbrugh *Clin. Microbiol. Rev.* **1997**, 10, 505.

[43] (a) K. Boubaker, P. Diebold, D. S. Blanc, F. Vandenesch, G. Praz, G Dupuis, N. Troillet, *Emerging Infect. Dis.* **2004**, 10, 121; (b) J. Parsonnet, M. A. Hansmann, M. L. Delaney, P. A. Modern, A. M. DuBois, W. Wieland-Alter, K. W. Wissemann, J. E. Wild, M. B. Jones, J. L. Seymour, *J. Clin. Microbiol.* **2005**, 43, 4628; (c) G. Lina, Y. Piémont, F. Godail-Gamot, M. Bes, M. O. Peter, V. Gauduchon, F. Vandenesch, J. Etienne, *Clin. Infect. Dis.* **1999**, 29, 1128; (d) F. Vandenesch, T. Naimi, M. C. Enright, G. Lina, G. R. Nimmo, H. Heffernan, N. Liassine, M. Bes, T. Greenland, M. E. Reverdy, *Emerging Infect. Dis.* **2003**, 9, 978.

[44] Y Gillet, B Issartel, P Vanhems, J. C. Fournet, G Lina, M Bes, F Vandenesch, Y Piemont, N Brousse, D Floret, *Lancet* **2002**, 359, 753.

[45] H. W. Boucher, G. H. Talbot, J. S. Bradley, J. E. Edwards, Jr, D. Gilbert, L. B. Rice, M. Scheld, B. Spellberg, J. Bartlett, *Clin. Infect. Dis.* **2009**, 48, 1.

[46] (a) G. Y. Liu, A. Essex, J. T. Buchanan, V. Datta, H. M. Hoffman, J. F. Bastian, J. Fierer, V. Nizet, *J. Exp. Med.* **2005**, 202, 209; (b) A. Clauditz, A. Resch, K. P. Wieland, A. Peschel, F. Götz, *Infect. Immun.* **2006**, 74, 4950.

[47] J. W. Costerton, P. S. Stewart, E. P. Greenberg, *Science* 1999, 284, 1318.

[48] (a) M. A. Fischbach, C. T. Walsh, *Science* **2009**, 325, 1089; (b) R. M. Klevens, *JAMA* **2007**, 298, 1763.

[49] G. H. Talbot, J. Bradley, J. E. Edwards Jr, D. Gilbert, M. Scheld, J. G. Bartlett, *Clin. Infect. Dis*. **2006**, 42, 657.

[50] A. Smith, Bacterial resistance to antibiotics. In: *Hugo and Russell's Pharmaceutical Microbiology.* S. P. Denyer, N. A. Hodges, S. P. Gorman (Eds). Blackwell Science Ltd, Oxford, UK, 220, **2007**.

[51] P. C. Appelbaum, *Int. J. Antimicrob. Agents*, **2007**, 30, 398.

[52] S. Chang, D. M. Sievert, J. C. Hageman, M. L. Boulton, F C Tenover, F P Downes S Shah J T Rudrik G R Pupp, W J Brown, *N. Engl. J. Med.* **2003**, 348, 1342.

[53] Department of Health, Standing Medical Advisory Committee, Sub-Group on Antimicrobial Resistance. The Path of Least Resistance. Available at: http://antibiotic-action.com/wp-content/uploads/2011/07/Standing-Medical-Advisory-Committee-The-path-of-least-resistance-1998.pdf. Accessed: 20 December **2013**.

[54] K. Fisher, C. Phillips, *Microbiology* **2009**, 155, 1749.

[55] D. M. Livermore, *J Antimicrob Chemother* **2008**, 62 Suppl(3), iii41.

[56] C. A. Arias, G. A. Contreras, B. E. Murray, *CMI* **2010**, 16, 555.

[57] W. J. Van Wamel, A. P. Hendrick, M. J. Bonten, J. Top, G. Posthuma, R. J. Willems, *Infect. Immun.* **2007**, 75, 924.

[58] S. H. Zinner, *Expert Rev. Anti Infect. Ther.* **2005**, 3, 907.

[59] G. Regev-Yochay, K. Trzcinski, C. M. Thompson, R. Malley, M. Lipsitch *J. Bacteriol.* **2006**, 188, 4996.

[60] (a) M. R. Jacobs, *Am. J. Med.* **2004**, 117 Suppl(3A), S3; (b) A. W. Karchmer, *Clin. Infect. Dis.* **2004**, 39 Suppl(3), S142.

[61] P. H. Edelstein, *Clin. Infect. Dis.* **2004**, 38 Suppl(4), S322.

[62] A. von Gottberg, K P Klugman, C Cohen, N Wolter, L de Gouveia, M du Plessis, R Mpembe, V Quan, A Whitelaw, R Hoffmann, *Lancet* **2008**, 371, 1108.

[63] H. Barth, K. Aktories, M. R. Popoff, B. G. Stiles, *Microbiol. Mol. Biol. Rev.* **2004,** 68, 373.

[64] C. R. Clabots, S. Johnson, M. M. Olson, L. R. Peterson, D. N. Gerding, *J. Infect. Dis.* **1992**, 166, 561.

[65] T. Planche, A. Aghaizu, R. Holliman, P. Riley, J. Poloniecki, A. Breathnach, S. Krishna, *Lancet Infect Dis.* **2008**, 8, 777.

[66] (a) J. Pepin, N. Saheb, M. A. Coulombe, M. E. Alary, M. P. Corriveau, S. Authier, M. Leblanc, G. Rivard, M. Bettez, V. Primeau, *Clin. Infect. Dis.* **2005**, 41, 1254; (b) L. C. McDonald, G. E. Killgore, A. Thompson, R. C. Owens, Jr., S. V. Kazakova, S. P. Sambol, S. Johnson, D. N. Gerding, *N. Engl. J. Med.* **2005**, 353, 2433.

[67] C. A. Loffler, C. MacDougall, *Exp. Rev. Anti Infect. Ther.* **2007**, 5, 961.

[68] F. Daxboeck, T. Budic, O. Assadian, M. Reich, W. Koller, *J. Hosp. Infect.* **2006**, 62, 214.

[69] (a) C. A. Arias, B. E. Murray , *N Engl J Med* **2009**, 360, 439; (b) K. K. Kumarasamy, M. A. Toleman, T. M. Walsh, J. Bagaria, F. Butt, R. Balakrishnan, U. Chaudhary, M. Doumith, C. G. Giske, S. Irfan, *Lancet Infect Dis* **2010**, 10, 597.

[70] (a) J. L. Flynn, J. Chan, *Curr. Opin. Immunol.* **2003**, 15, 450; (b) M. Niederweis, O. Danilchanka, J. Huff, C. Hoffmann, H. Engelhardt, *Trends in Microbiol.* **2009**, 18, 109.

[71] World Health Organization. Tuberculosis Fact Sheet. Available at: http://www.who.int/mediacentre/factsheets/fs104/en/index.html. Accessed: 20 December **2013**.

[72] World Health Organization. Extensively drug-resistant tuberculosis (XDR-TB): recommendations for prevention and control. *Wkly Epidemiol Rec* **2006**, 81, 430.

[73] (a) S. E. Dorman, R. E. Chaisson, *Nat. Med.* **2007**, 13, 295; (b) D. H. Kim, J. Hee, S. K. Park, S. J. Kong, S. Y. Kim, T. H. Kim, E. K. Kim, M. K. Lee, S. S. Lee, J. S. Park, *Am. J. Respir. Crit. Care Med.* **2008**, 178, 1075.

[74] J. B. Kaper, J. P. Nataro, H. L. Mobley HL. *Nat. Rev. Microbiol.* **2004**, 2, 123.

[75] A. D. O'Brien, J. W. Newland, S. F. Miller, R. K. Holmes, H. W. Smith, S. B. Formal, *Science* **1984**, 226, 694.

[76] D. M. Livermore, R. Hope, G. Brick, M. Lillie, R. Reynolds R. *J. Antimicrob. Chemother.* **2008**, 62, ii41.

[77] (a) L. Poirel, E. Lagrutta, P. Taylor, J. Pham, P. Nordmann, *Antimicrob. Agents Chemother.* **2010**, 54, 4914; (b) M. Solé, C. Pitart, I. Roca, A. Fabrega, P. Salvador, L. Munoz, I. Oliveira, J. Gascon, F. Marco, J. Vila, *Antimicrob. Agents Chemother.* **2011**, 55, 4402; (c) Y. Pfeifer, W. Witte, M. Holfelder, J. Busch, P. Nordmann, L Poirel, *Antimicrob. Agents Chemother.* **2011**, 55, 1318.

[78] P. Nordmann, L. Poirel, M. A. Toleman, T. R.Walsh, *J Antimicrob Chemother* **2011**, 66, 689.

[79] (a) M. Souli, I. Galani, H. Giamarellou, *Euro Surveill*. **2008**, 13, pii: 19045; (b) A. C. Rodloff, R. Leclercq, E. A. Debbia, R. Canton, B. A. Oppenheim, M. J. Dowzicky, *Clin. Microbiol. Infect*. **2008**, 14, 307.

[80] S. Kobayashi, T. Kuzuyama, H. Seto, *Antimicrob. Agents Chemother*. **2010**, 44, 647.

[81] New York State Department of Health. Health Commissioner Releases E. Coli Outbreak Report. Available at: http://www.health.ny.gov/press/releases/2000/ecoli.htm. Accessed 20 December **2013**.

[82] C. Frank, D. Werber, J. P. Cramer, M. Askar, M Faber, M an der Heiden, H Bernard, A Fruth, R Prager, A Spode, *N. Engl. J. Med.* **2011**, 365, 1771.

[83] C. A. Hart, C. Winstanley, *Br. Med. Bull.* **2002**, 61, 81.

[84] (a) A. Oliver, R Canton, P Campo, F. Baquero, J. Blazquez, *Science* **2000**, 288, 1251; (b) B. Henrichfreise, I Wiegand, W Pfister, B Wiedemann, *Antimicrob Agents Chemother* **2007**, 51, 4062.

[85] V T Lee, R S Smith, B Tummler, S *Infect. Immun.* **2005**, 73, 1695.

[86] F W Adair, S G Geftic, J Gelzer, *Appl. Microbiol.* **1969**, 18, 299.

[87] (a) H Nikaido, *Microbiol. Mol. Biol. Rev.* **2003**, 67, 593; (b) J L Fothergill, C Winstanley, C E James, *Expert Rev. Anti Infect. Ther.* **2012**, 10, 219.

[88] K Bush, *Curr. Opin. Microbiol.* **2010**, 13, 558.

[89] D. M. Livermore, *Clin. Infect. Dis.* **2002**, 34, 634.

[90] N. R. Morero, M. R. Monti, C. E. Argaraña, *Antimicrob. Agents Chemother.* **2011**, 55, 3668.

[91] A Deplano, O Denis, L Poirel, D. Hocquet, C. Nonhoff, B. Byl, P. Nordmann, J. L. Vincent, M. J. Struelens, *J Clin Microbiol* **2005**, 43, 1198.

[92] K Lolans, A M Queenan, K Bush, A Sahud, J P Quinn, *Antimicrob Agents Chemother* **2005**, 49, 3538.

[93] (a) H Nikaido, *Microbiol Mol Biol Rev* **2003**, 67, 593; (b) M P Crespo, N Woodford, A Sinclair, M E Kaufmann, J Turton, J Glover, J D Velez, C R Castaneda, M Recalde, D M Livermore, *J Clin Microbiol* **2004**, 42, 5094.

[94] A J Kallen, A Srinivasan, *Infect. Control Hosp. Epidemiol.* **2010**, 31 Suppl(1), S51.

[95] A Abbo, Y Carmeli, S Navon-Venezia, Y Siegman-Igra, M J Schwaber *Eur. J. Clin. Microbiol. Infect. Dis.* **2007**, 26, 793.

[96] (a) J Vila, S Martí, J Sánchez-Céspedes, *J. Antimicrob. Chemother.* **2007**, 59, 1210; (b) A Kramer, I Schwebke, G Kampf, *BMC Infect. Dis.* **2006**, 6, 130; (c) E T Houang, R T Sormunen, L Lai, C Y Chan, A S Leong, *J. Clin. Pathol.* **1998**, 51, 786.

[97] T D Gootz, A Marra, *Expert Rev. Anti Infect Ther.* **2008**, 6, 309.

[98] M L Joly-Guillou, *Clin. Microbiol. Infect.* **2005**, 11, 868.

[99] C H Choi, E Y Lee, Y C Lee Park, I Tae, H J Kim, S. H. Hyun, S. A. Kim, S. K. Lee, J. C. Lee, *Cell. Microbiol.* **2005**, 7, 1127.

[100] A M Queenan, K Bush, *Clin. Microbiol. Rev.* **2007**, 20, 440.

[101] D. M. Sievert, P. Ricks, J. R. Edwards, A. Schneider, J Patel, A Srinivasan, A Kallen B Limbago, S Fridkin, *Infect Control Hosp Epidemiol* **2013**, 34, 1.

[102] L S Munoz-Price, R A Weinstein, *N. Engl. J. Med.* **2008**, 358, 1271.

[103] (a) M B Macrae, K P Shannon, D M Rayner, A M Kaiser, P N Hoffman, G L French, *J. Hosp. Infect.* **2001**, 49, 183; (b) C Fontana, M Favaro, S Minelli, M C Bossa, G P Testore, F Leonardis, S Natoli, C Favalli, *BMC Infect. Dis.* **2008**, 8, 79.

[104] R Podschun, U Ullmann, *Clin. Microbiol. Rev.* **1998**, 11, 589.

[105] L Martinez-Martinez, A Pascual, G A Jacoby *Lancet* **1998**, 351, 797.

[106] D Yong, M A Toleman, C G Giske, H S Cho, K Sundman, K Lee, T R Walsh, *Antimicrob. Agents Chemother.* **2009**, 53, 5046.

[107] S P Hawser, S K Bouchillon, D J Hoban, R E Badal, P R Hsueh, D L Paterson, *Antimicrob Agents Chemother* **2009**, 53, 3280.

[108] A Elemam, J Rahimian, W Mandell, *Clin. Infect. Dis.* **2009**, 49, 271.

[109] M Castanheira, L M Deshpande, D Mathai, J M Bell, R N Jones, R E Mendes, *Antimicrob. Agents Chemother.* **2011**, 55, 1274.

[110] C H Park, A Robicsek, G A, Jacoby, D Sahm, D C Hooper, *Antimicrob. Agents Chemother.* **2006**, 50, 3953.

[111] A. Stern, M. Brown, P. Nickel, T. F. Meyer, *Cell*, **1966**, 47, 61.

[112] (a) S Yokoi, T Deguchi, T Ozawa, M Yasuda, S I Ito, Y Kubota, M Tamaki, S I Maeda, *Emerg Infect Dis* **2007**, 13, 1275; (b) H M Palmer, H Young, A Winter, J Dave, *J Antimicrob Chemother* **2008**, 62, 490.

[113] D M Livermore, *J. Antimicrob. Chemother.* **2009**, 64 Suppl(1), i29.

[114] (a) Centers for Disease Control and Prevention. Healthcare-associated Infections (HAIs) – The Burden. Available at: http://www.cdc.gov/HAI/burden.html. Accessed 20 December 2013. (b) B Allegranzi, S Bagheri Nejad, C Combescure, W Graafmans, H Attar, L Donaldson, D Pittet, *Lancet* **2011**, 377, 228.

[115] N. I. Paphitou, *Int. J. Antimicrob. Agents* **2013**, 42S, S25.

[116] L B Rice *J Infect Dis* **2008**, 197, 1079.

[117] L L Silver, *Clin Microbiol Rev* **2011**, 24, 71.

[118] D Andes, J Anon, M R Jacobs, W A Craig, *Clin Lab Med* **2004**, 24, 477.

[119] D. J. Newman, G. M. Cragg, *J. Nat. Prod.* **2007**, 70, 461.

[120] S B Singh, J F Barrett, *Biochem. Pharmacol.* **2006**, 71, 1006.

[121] (a) J L Hansen, P B Moore, T A Steitz, *J Mol Biol.* **2003**, 330, 1061; (b) P B Moore, T A Steitz, *Trends Biochem Sci.* **2005**, 30, 281.

[122] (a) T Weber, K Welzel, S Pelzer, A Vente, W Wohlleben, *J. Biotechnol.* **2003**, 106, 221; (b) R H Baltz, *Streptomyces. Trends Microbiol.* **1998**, 6, 76.

[123] M A Fischbach, *Curr. Opin. Microbiol.* **2009**, 12, 520.

[124] K Witting, R D Smith, *Curr. Drug Targets* **2011**, 12, 1547.

[125] K Lewis, *Nat. Rev. Drug Disc.* **2013**, 12, 371.

[126] M A Kohanski, D J Dwyer, J J Collins, *Nat. Rev. Microbiol.* **2010**, 8, 423.

[127] N Markowitz, E L Quinn, L D Saravolatz, *Ann. Intern. Med.* **1992**, 117, 390.

[128] (a) L Jemni, H Hmouda, A Letaief, *Clin. Infect. Dis.* **1994**, 19, 202; (b) P Huovinen, *Clin. Infect. Dis.* **2001**, 32, 1608.

[129] P. Collignon, J. H. Powers, T. M. Chiller, A. Aidara-Kane, F. M. Aarestrup, *Clin. Infect. Dis.* **2009**, 49, 132.

[130] A Tomasz, *Annu. Rev. Microbiol.* **1979**, 33, 113.

[131] M Bassetti, F Ginocchio, M Mikulska, L Taramasso, D Roberto Giacobbe, *Expert Rev. Anti. Infect. Ther.* **2011**, 9, 909.

[132] J A Torres, M V Villegas, J P Quinn, *Expert Rev. Anti Infect. Ther.* **2007**, 5, 833.

[133] T R Walsh, M A Toleman, L Poirel, P Nordmann, *Clin Microbiol Rev* **2005**, 18, 306.

[134] P Nordmann, L Poirel, *Clin Microbiol Infect* **2002**, 8, 321.

[135] R A Bonomo, D Szabo, *Clin. Infect. Dis.* **2006**, 42 Suppl(2), S49.

[136] (a) M M Navia, J Ruiz, J Vila, *Microb Drug Resist* **2002**, 8, 261; (b) I Vila, M Navia, J Ruiz, C Casals, *Antimicrob Agents Chemother* **1997**, 41, 2757; (c) L Poirel, P Gerome, C De Champs, J Stephanazzi, T Naas, P Nordmann, *Antimicrob Agents Chemother* **2002**, 46, 566; (d) L Poirel, D Girlich, T Naas, P Nordmann, *Antimicrob Agents Chemother* **2001**, 45, 447.

[137] T Naas, P Nordmann, *Curr. Pharm Des*, **1999**, 5, 865.

[138] R Hakenbeck, T Grebe, D Zahner, J B Stock, *Mol. Microbiol.* **1999**, 33, 673.

[139] H F Chambers, *Clin. Microbiol. Rev.* **1997**, 10, 781.

[140] A Mazzariol, G Cornaglia, H Nikaido, *Antimicrob. Agents Chemother.* **2000**, 44, 1387.

[141] R E W Hancock, *Trends Microbiol.* **1997**, 5, 37.

[142] (a) G J Noel, K Bush, P Bagchi, J Ianus, R S Strauss, *Clin. Infect. Dis*. **2008**, 46, 647; (b) A El Solh, *Expert Opin. Pharmacother*. **2009**, 10, 1675.

[143] H S Sader, P R Rhomberg, D J Farrell, R N Jones, *Antimicrob Agents Chemother* **2011**, 55, 2390.

[144] S Takeda, T Nakai, T Wakai, F Ikeda, K Hatano, *Antimicrob Agents Chemother* **2007**, 51, 826.

[145] A M Queenan, W Shang, R Flamm, K Bush, *Antimicrob. Agents Chemother*. **2010**, 54, 565.

[146] J Chastre, R Wunderink, P Prokocimer, M Lee, K Kaniga, I Friedland, *Crit. Care Med*. **2008**, 36, 1089.

[147] S Fujimura, Y Nakano, T Sato, K Shirahata, A Watanabe, *J. Infect. Chemother*. **2007**, 13, 147.

[148] D M Livermore, S Mushtaq, M Warner, *J. Antimicrob. Chemother.* **2009**, 64, 330.

[149] M G Page, C Dantier, E Desarbre, *Antimicrob. Agents Chemother*. **2010**, 54, 2291.

[150] T A Russo, M G Page, J M Beanan, R Olson, A M Hujer, K M Hujer, M Jacobs, S Bajaksouzian, A Endimiani, R A Bonomo, *J. Antimicrob. Chemother*. **2011**, 66, 867.

[151] C R V Kiffer, J L M Sampaio, S Sinto, C P Oplustil, P C M Koga, A C Arruda, P J Turner, C Mendes, *Diagn. Microbiol. Infect. Dis.* **2005**, 52, 317.

[152] D E Ehmann, H Jahic, P L Ross, R F Gu, J Hu, T F Durand-Reville, S Lahiri, J Thresher, S Livchak, N Gao, *J. Biol. Chem.* **2013**, 39, 27960.

[153] M S Butler, M A Blaskovich, M A Cooper, *J. Antibio.* **2013**, 66, 571.

[154] M Bassetti, E Righi, C Viscoli, *Expert Opin. Investig. Drugs*. **2008**, 17, 285.

[155] D J Payne, J A Hueso-Rodriguez, H Boyd, N O Concha, C A Janson, M Gilpin, J H Bateson, C Cheever, N L Niconovich, S Pearson, *Antimicrob. Agents Chemother.* **2002**, 46, 1880.

[156] B D Davis, L L Chen, P C Tai, *Proc. Natl Acad. Sci. USA* **1986**, 83, 6164.

[157] (a) J Wachino, K Shibayama, H Kurokawa, K Kimura, K Yamane, S Suzuki, N Shibata, Y Ike, Y Arakawa, *Antimicrob. Agents Chemother.* **2007**, 51, 4401; (b) J Wachino, Y Arakawa, *Drug Resist Updat.* **2012**, 15, 133.

[158] S H Gillespie, *Antimicrob. Agents Chemother.* **2002**, 46, 267.

[159] M L Avent, B A Rogers, A C Cheng, D L Paterson, *Int. Med. J.* **2011**, 41, 441.

[160] (a) M P Mingeot-Leclercq, Y Glupczynski, P M Tulkens, *Antimicrob. Agents. Chemother.* **1999**, 43, 727. (b) H Kawaguchi, T Naito, S Nakagawa, K Fujisawa, *J. Antibio.* **1972**, 25, 695.

[161] (a) G. Lin, L.M. Ednie, P.C. Appelbaum. *Antimicrob. Agents Chemother*. **2010**, 54, 2258; (b) G. A. Pankuch, G. Lin, A. Kubo, E S Armstrong, P C Appelbaum, K Kosowska-Shick, *Antimicrob. Agents Chemother*. **2011**, 55, 2463.

[162] D M Livermore, S Mushtaq, M Warner, J C Zhang, S Maharjan, M Doumith, N Woodford, *J Antimicrob Chemother* **2011**, 66, 48.

[163] (a) D Landman, P Kelly, M Bäcker, E Babu, N Shah, S Bratu, J Quale, *J. Antimicrob. Chemother*. **2011**, 66, 332; (b) D Landman, E Babu, N Shah, P Kelly, M Baecker, S Bratu, J Quale, *J. Antimicrob. Chemother*. **2010**, 65, 2123.

[164] J J Rahal Jr, M S Simberkoff, *Antimicrob. Agents Chemother.* **1979**, 16, 13.

[165] M E Falagas, P Kopterides, *Curr. Opin. Crit. Care* **2007**, 13, 592.

[166] C Kehrenberg, S Schwarz, L Jacobsen, L H Hansen, B Vester, *Mol. Microbiol.* **2005**, 57, 1064.

[167] I A Murray, W V Shaw, *Antimicrob. Agents Chemother*. **1997**, 41, 1.

[168] M A Arcangioli, S Leroy-Setrin, J L Martel, E Chaslus-Dancla, *FEMS Microbiol. Lett*. **1999**, 174, 327.

[169] T Tenson, M Lovmar, M Ehrenberg, *J. Mol. Biol.* **2003**, 330, 1005.

[170] F W Goldstein, M F Emirian, A Coutrot, J F Acar, *J. Antimicrob. Chemother.* **1990**, 25 Suppl(A), 25.

[171] M F Liu, S Douthwaite, *Antimicrob. Agents Chemother.* **2002**, 46, 1629.

[172] F Depardieu, P Courvalin, *Antimicrob. Agents Chemother.* **2001**, 45, 319.

[173] (a) H G Wittman, G Stoffler, D Apirion, L Rosen, K Tanaka, M Tamaki, R Takata, S Dekio, E Otaka, S Osawa, *Mol. Gen. Genet.* **1973**, 127, 175; (b) A Canu, B Malbruny, M Coquemont, T A Davies, P C Appelbaum, R Leclercq, *Antimicrob. Agents Chemother.* **2002**, 46, 125.

[174] O Chesneau, K Tsvetkova, P Courvalin, *FEMS Microbiol. Lett.* **2007**, 269, 317.

[175] (a) J Clancy, J Petitpas, F Dib Hajj, W Yuan, M Cronan, A V Kamath, J Bergeron, J A Retsema, *Mol. Microbiol.* **1996**, 22, 867; (b) R A Moore, D DeShazer, S REckseidler, A Weissman, D E Woods, *Antimicrob. Agents Chemother.* **1999**, 43, 465.

[176] L A Hicks, T H Taylor Jr., R J Hunkler, *N. Engl. J. Med.* **2013**, 368, 1461.

[177] D J Hoban, *J Chemother* **2002**, 3, 25.

[178] R M Echols, *Ann NY Acad Sci* **2011**, 1, 153.

[179] J A Sutcliffe, *Ann NY Acad Sci* **2011**, 1, 122.

[180] S D Putnam, H S Sader, D J Farrell, D J Biedenbach, M Castanheira, *Int J Antimicrob Agents* **2011**, 37, 39.

[181] M C Roberts, *FEMS Microbiol. Lett.* **1996**, 19, 1.

[182] K N Agwuh, A MacGowan, *J. Antimicrob. Chemother.* **2006**, 58, 256.

[183] J A Sutcliffe, W O'Brien, C Fyfe, T H Grossman, *Antimicrob. Agents Chemother.* **2013**, 57, 5548.

[184] T H Grossman, A L Starosta, C Fyfe, W O'Brien, D M Rothstein, A Mikolajka, D N Wilson, J A Sutcliffe, *Antimicrob. Agents Chemother.* **2012**, 56, 2559.

[185] C H Jones, P J Peterson, *Drugs Today* **2005**, 41, 637.

[186] (a) P J Petersen, N V Jacobus, W J Weiss, P E Sum, R T Testa, *Antimicrob Agents Chemother* **1999**, 43, 738; (b) E Tasina, A B Haidich, S Kokkali, M Arvanitidou, *Lancet Infect Dis* **2011**, 11, 834; (c) MHornsey, M J Ellington, M Doumith, S Hudson, D M Livermore , N Woodford, *J Antimicrob Chemother* **2010**, 65, 479; (d) A Ruzin, F W Immermann, P A Bradford, *Microb Drug Res Mech* **2010**, 16, 87; (e) T Horiyama, E Nikaido, A Yamaguchi, K Nishino, *J Antimicrob Chemother* **2011**, 66, 105.

[187] A Y Peleg, J Adams, D L Paterson, *Antimicrob. Agents Chemother.* **2007**, 51, 2065.

[188] P Sensi, *Rev. Infect. Dis.* **1983**, 5 Suppl(3), 402.

[189] P E Almeida Da Silva, J C Palomino, *J. Antimicrob. Chemother.* **2011**, 66, 1417.

[190] G R Lichtenstein, *Gastro. & Hepat.* **2007**, 3, 474.

[191] Y Cetinkaya, P Falk, C G Mayhall, *Clin. Microbiol. Rev.* **2000**, 13, 686.

[192] M A Cooper, D H Williams, *Chem. Biol.* **1999**, 6, 891.

[193] D. Kahne, C. Leimkuhler, W. Lu, C. Walsh, *Chem. Rev.* **2005**, 105, 425.

[194] J P Show, J Seroogy, K Kaniga, D L Higgins, M Kit, S Barriere, *Antimicrob. Agents Chemother.* **2005**, 49, 195.

[195] (a) J L Pace, G Yang, *Biochem. Pharmacol.* **2006**, 71, 968; (b) J F Barrett, *Curr. Opin. Investig. Drugs* **2001**, 2, 1039; (c) R C Mercier, L Hrebickova, *Expert Rev. Anti Infect. Ther.* **2005**, 3, 325.

[196] G Lin, K Credito, L M Ednic, P C Appelbaum, *Antimicrob. Agents Chemother.* **2005**, 49, 770.

[197] H Giamarellou, *Expert Rev. Anti Infect. Ther.* **2006**, 4, 601.

[198] (a) P A Lambert, *J. R. Soc. Med.* **2002**, 95 Suppl(41), 22; (b) D C Hooper, *Clin. Infect. Dis.* **2000**, 31 Suppl(2), S24.

[199] (a) A Robicsek, D F Sahm, J Strahilevitz, G A Jacoby, D C Hooper, *Antimicrob Agents Chemother* **2005**, 49, 3001; (b) P Nordmann, L Poirel, *Antimicrob Agents Chemother* **2005**, 56, 463.

[200] J Strahilevitz, G A Jacoby, D C Hooper, A Robicsek, *Clin. Microbiol. Rev.* **2009**, 22, 664.

[201] K Poole, *Antimicrob. Agents Chemother.* **2000**, 44, 2233.

[202] C M Oliphant, G M Green, *Am. Fam. Physician*, **2002**, 65, 455.

[203] P G Ambrose, R C Owens Jr, R Quintiliani, C H Nightingale, *Conn Med* **1997**, 61, 269.

[204] K Poole, *J. Pharm. Pharmacol.* **2001**, 53, 283.

[205] (a) S Lemaire, P M Tulkens, B F Van, *Antimicrob Agents Chemother* **2011**, 55, 649; (b) T L Lauderdale, Y R Shiar, J F Lai, H C Chen, C H King, *Antimicrob Agents Chemother* **2010**, 54, 1338; (c) B J Morrow, W He, K M Amslem, B D Foleno, M J Macielag, A S Lynch, K Bush, *Antimicrob Agents Chemother* **2010**, 54, 1955; (d) B J Morrow, D Abbanat, E Z Baum, S M Crespo-Carbone, T A Davies, W He, W Shang, A M Queenan, A S Lynch, *Antimicrob Agents Chemother* **2011**, 55, 5512.

[206] (a) P G Higgins, W Stubbings, H Wisplinghoff, H Seifert, *Antimicrob. Agents Chemother.* **2010**, 54, 1613; (b) Merlion Pharmaceuticals. Finafloxacin. Available at: http://www.merlionpharma.com/?q=node/16. Accessed 20 December **2013**.

[207] M C Roberts, *FEMS Microbiol. Lett.* **2008**, 282, 147.

[208] K S Long, J Poehlsgaard, C Kehrenberg, S Schwarz, B Vester, *Antimicrob. Agents Chemother.* **2006**, 50, 2500.

[209] L K Smith, A S Mankin, *Antimicrob. Agents Chemother.* **2008**, 52, 1703.

[210] J Koch-Weser, V W Sidel, E B Federman, P Kanarek, D C Finer, A E Eaton, *Ann. Intern. Med.* **1970**, 72, 857.

[211] (a) J P Ouderkirk, J A Nord, G S Turett, J W Kislak, *Antimicrob. Agents Chemother.* **2003**, 47, 2659; (b) A S MIchalopoulos, S Tsiodras, K Rellos, S Mentzelopoulos, M E Falagas, *Clin. Microbiol. Infect.* **2005**, 11, 115.

[212] (a) J Li, R L Nation, R W Milne, J D Turnidge, K Coulthard, *Int. J. Antimicrob. Agents* **2005**, 5, 11; (b) N Rastogi, M C Potar, H L David, *Ann. Inst. Pasteur Microbiol.*

1986, 137A, 45; (c) A C Gales, R N Jones, H S Sader, *Clin. MIcrobiol. Infect.* 2006, 12, 315.

[213] M Schindler, M J Osborn, *Biochemistry*, 1979, 18, 4425.

[214] S Senturk, *J. Vet. Pharmacol. Ther.* 2005, 28, 57.

[215] M E Falagas, P I Rafailidis, D K Matthaiou, *Drug Resist. Update* 2010, 13, 132.

[216] Y Koyama, A Kurosasa, A Tsuchiya, K Takakuta, *J. Antibiot.* 1950, 3, 457.

[217] J Quale, N Shah, P Kelly, E Babu, M Backer, G Rosas-Garcia, J Salamera, A George, S Bratu, D Landman, *Microb. Drug Res.* 2012, 18, 132.

[218] S Shapiro, *J. Antibiot.* 2013, 66, 371.

[219] (a) M Vaara, J Fox, G Loidl, O Siikanen, J Apajalahti, F Hansen, N Frimodt-Moller, J Nagai, M Takano, T Vaara, *Antimicrob. Agents Chemother.* 2008, 52, 3229; (b) F E Ali, G Cao, A Poudyal, T Vaara, R L Nation, M Vaara, J Li, *J. Antimicrob. Chemother.* 2009, 64, 1067; (c) M P Mingeot-Leclercq, P M Tulkens, S Denamur, T Vaara, M Vaara, Peptides 2012, 35, 248; (d) M Vaara, T Vaara, *Int. J. Antimicrob. Agents* 2013, 41, 292; (e) M Vaara, H S Sader, P R Rhomberg, R N Jones, T Vaara, *J. Antimicrob. Chemother.* 2013, 68, 636.

[220] R. B. Fugitt, R. W. Luckenbaugh. 1978. Patent 4,128,654.

[221] S Tsiodras, H S Gold, G Sakoulas, G M Eliopoulos, C Wennersten, L Venkataraman, R C Mollering, M J Ferraro, *Lancet* 2001, 358, 207.

[222] J A Sutcliffe, *Ann. N.Y. Acad. Sci.* 2011, 1241, 122.

[223] (a) K J Shaw, M R Barbachyn, *Ann NY Acad Sci* 2011, 1, 48; (b) G Morales, J J Picazo, E Baos, F J Candel, A Arribi, B Pelaez, R Andrade, M A de la Torre, J Fereres, M Sanchez-Garcia, *Clin. Infect. Dis.* 2010, 50, 821; (c) C Seral, Y Saenz, S Algarate, E Duran, P Luque, C Torres, F J Castillo, *Int. J. Med. Microbiol.* 2011, 301, 354; (d) B Gu, T Kelesidis, S Tsiodras, J Hindler, R M Humphries, *J. Antimicrob. Chemother.* 2013, 68, 4.

[224] (a) D J Diekema, R N Jones, *Lancet* 2001, 358, 1975; (b) M C Birmingham, C R Rayner, A K Meagher, S M Flavin, D H Batts, J J Schentag, *Clin. Infect. Dis.* 2003, 36, 159.

[225] K Bush, *Curr. Opin. Pharmacol.* 2012, 12, 527.

[226] (a) J B Locke, J Finn, M Hilgers, G Morales, S Rahawi, G C Kedar, J J Picazo, W Im, K J Shaw, J L Stein *Antimicrob. Agents Chemother.* 2010, 54, 5337; (b) I Rodriguez-

Avial, E Culebras, C Betriu, G Morales, I Pena, J J Picazo, *J. Antimicrob. Chemother.* **2012**, 67, 167.

[227] (a) J. B. Locke, J. Finn, M. Hilgers, G Morales, S Rahawi, G C Kedar, J J Picazo, W Im, K J Shaw, J Stein, L Jeffrey, *Antimicrob. Agents Chemother.* **2010**, 54, 5337; (b) K. J. Shaw, S. Poppe, R. Schaadt, V Brown-Driver, J Finn, C M Pillar, D Shinabarger, G Zurenko, *Antimicrob. Agents Chemother.* **2008**, 52, 4442.

[228] L. Lawrence, P. Danese, J. DeVito, F Franceschi, J A Sutcliffe, *Antimicrob. Agents Chemother.* **2008**, 52, 1653.

[229] J B Locke, J Finn, M Hilgers, G Morales, S Rahawi, G C Kedar, J J Picazo, W Im, K J Shaw, J L Stein, *Antimicrob. Agents Chemother.* **2010**, 54, 5337.

[230] J W C Alffenaar, T van der Laan, S Simons, T S Van der Werf, P J van de Kasteele, H de Neeling, D van Soolingen, *Antmicrob. Agents Chemother.* **2011**, 55, 1287.

[231] M Davlieva, W. Zhang, C A Arias, Y Shamoo, *Antimicrob. Agents Chemother.* **2013**, 57, 289.

[232] U. Bertsche, S J Yang, D Kuehner, S Wanner, N N Mishra, T Roth, M Nega, A Schneider, C Mayer, T Grau, *Plos One* **2013**, 8, 1.

[233] (a) S J Yang, B N Kreiswirth, G Sakoulas, M R Yeaman, Y Q Xiong, A Sawa, A S Bayer, *J. Infect. Dis.* **2009**, 200, 1916; (b) S J Yang, Y Q Xiong, P M Dunman, J Schrenzel, P Francois, A Peschel, A S Bayer, *Antimicrob. Agents Chemother.* **2009**, 53, 2636.

[234] Cubist Pharmaceuticals. Surotomycin (CB-315). Available at: http://www.cubist.com/products/cdad. Accessed 20 December **2013**.

[235] (a) K Kadlec, C F Pomba, N Couto, S Schwarz *J Antimicrob Chemother* **2010**, 65, 2692; (b) S Schwendener, V Perreten *Antimicrob Agents Chemother* **2011**, 55, 4900.

[236] K Miller, C J Dunsmore, W G Colin, I Chopra, *Antimicrob Agents Chemother* **2008**, 52, 1737.

[237] (a) Nabriva Pharmaceuticals. Developing a New Class of Antibiotics. Available at: http://www.nabriva.com/programs/development/. Accessed 20 December **2013**; (b) Drugs.com. Nabriva Reports Positive Results from Phase II Trial of Pleuromutilin Antibiotic BC-3781 in ABSSSI. Available at: http://www.drugs.com/clinical_trials/nabriva-reports-positive-results-phase-ii-trial-pleuromutilin-antibiotic-bc-3781-absssi-11577.html. Accessed 20 December **2013**.

[238] A Srivastava, M. Talaue, S Liu, D Degen, R Y Ebright, E Sineva, A Chakraborty, S Y Druzhinin, S Chatterjee, J Mukhopadhyay, Y W Ebright, A Zozula, J Shen, S Sengupta,

R R Niedfeldt, C Xin, T Kaneko, H Irschik, R Jansen, S Donadio, N Connell, R H Ebright, *Curr. Opin. Microbiol.* **2011**, 14, 532.

[239] T J Louie, J Emery, W Krulicki, B Byrne, M Mah, *Antimicrob. Agent Chemother.* **2009**, 53, 261.

[240] K Andries, P Verhasselt, J Guillemont, H W H Goehlmann, J M Neefs, H Winkler, J Van Gestel, P Timmerman, M Zhu, E Lee, *Science* **2005**, 307, 223.

[241] J Avorn, *JAMA*, **2013**, 309, 1349.

[242] Infectious Diseases Society of America, *Clin. Infect. Dis.* **2010**, 50, 1081.

[243] (a) B J Akerley, E J Rubin, V L Novick, K Amaya, N Judson, J J Mekalanos, *Proc. Natl Acad. Sci. USA* **2002**, 99, 966; (b) C A Hutchinson III, S N Peterson, S R Gill, R T Cline, O White, C M Fraser, H O Smith, J C Venter, *Science* **1999**, 286, 2165; (c) Y Ji, B Zhang, S F Van Horn, P Warren, G, Woodnutt, M K R Burnham, M Rosenberg, *Science* **2001**, 293, 2266; (d) K Kobayashi, S D Ehrlich, A Albertini, G Amati, K K Andersen, M Arnaud, K Asai, S Ashikaga, S Aymerich, P Bessieres, *Proc. Natl Acad. Sci. USA* **2003**, 100, 4678; (e) R A Forsyth, R J Haselbeck, K L Ohlsen, R T Yamamoto, H Xu, J D Trawick, D Wall, L Wang, V Brown-Driver, J M Froelich, *Mol. Microbiol.* **2002**, 43, 1387.

[244] K Young, H Jayasuriya, J G Ondeyka, K Herath, C Zhang, S Kodali, A Galgoci, R Painter, V Brown-Driver, R Yamamoto, *Antimicrob. Agents Chemother.* **2006**, 50, 519.

[245] (a) P Kulanthaivel, A J Kreuzman, M A Strege, M D Belvo, T A Smitka, M Clemens, J R Swartling, K L Minton, F Zheng, E L Angleton, *J. Biol. Chem.* **2004**, 279, 36250; (b) M Paetzel, J J Goodall, M Kania, R E Dalbey, M G, *J. Biol. Chem.* **2004**, 279, 30781.

[246] J Wang, A Galgoci, S Kodali, K B Herath, H Jayasuriya, K Dorso, F Vicente, A Gonzalez, D Cully, D Bramhill, *J. Biol. Chem.* **2003**, 278, 44424.

[247] M Chu, R Mierzwa, L He, L Xu, F Gentile, J Terracciano, M Patel, L Miesel, S Bohanon, C Kravec, *Tetrahedron Lett*. **2001**, 42, 3549.

[248] J Clardy, M A Fischbach, C T Walsh, *Nat Biotechnol.* **2006**, 24, 1541.

[249] D J Newman, G M Cragg, *J. Nat. Prod.* **2007**, 70, 461.

[250] (a) O Lomovskaya, K Lewis, *Proc. Natl Acad. Sci. USA* **1992**, 89, 8938; (b) X Z Li, H Nikaido, *Drugs* **2004**, 64, 159.

[251] (a) R O'Shea, H E Moser, *J. Med. Chem.* **2008**, 51, 2871; (b) L L Silver, *Expert Opin. Drug Discov.* **2008**, 3, 487.

[252] (a) P C Hsieh, S A Siegel, B Rogers, D Davis, K Lewis, *Proc. Natl Acad. Sci. USA* **1998**, 95, 6602; (b) F R Stermitz, P Lorenz, J N Tawara, L A Zenewicz, K Lewis, *Proc. Natl Acad. Sci. USA* **2000**, 97, 1433; (c) K Lewis, F M Ausubel, *Nature Biotech.* **2006**, 24, 1504.

[253] F L Rock, W Mao, A Yaremchuk, M Tukalo, T Crepin, H Zhou, Y K Zhang, V Hernandez, T Akama, S J Baker, J. J. Plattner, L Shapiro, S A Martinis, S J Benkovic, S Cusack, M R K Alley, *Science* **2007**, 316, 1759.

[254] D J Payne, M N Gwynn, D J Holmes, D L Pompliano, *Nature Rev. Drug Discov.* **2007**, 6, 29.

[255] S B Singh, K Young, L Miesel, *Expert Rev. Anti Infect. Ther.* **2011**, 9, 589.

[256] (a) M S Boguski, K D Mandl, V P Sukhatme, *Science* **2009**, 324, 1394; (b) K Andries, P Verhasselt, J Guillemont, H W. H. Göhlmann, J M Neefs, H Winkler, J Van Gestel, P Timmerman, M Zhu, E Lee, P Williams, D de Chaffoy, E Huitric, S Hoffner, E Cambau, C Truffot-Pernot, N Lounis, V Jarlier, *Science* **2005**, 307, 223.

[257] M M Butler, J D Williams, N P Peet, D T Moir, R G Panchal, S Bavari, D L Shinabarger, T L Bowlin, *Antimicrob. Agents Chemother*. **2010**, 54, 3974.

[258] (a) T I Moy, A R Ball, Z Anklesaria, G Casadei, K Lewis, F M Ausubel, *Proc. Natl Acad. Sci. USA* **2006**, 103, 10414; (b) E J O'Rourke, A L Conery, T I Moy, *Methods Mol. Biol.* **2009**, 486, 57.

[259] W R J D Galloway, A Bender, M Welch, D R Spring, *Chem. Commun.* **2009**, 2446.

[260] X. Hea, A. Alian , P. R. O. de Montellano, *Bioorg. Med. Chem.* **2007**, 15, 6649.

[261] G. C. Look, C. Vacin, T. M. Dias, S. Ho, T. H. Tran, L. L. Lee, C. Wiesner, F. Fang, A. Marra, D. Westmacott, A. E. Hromockyj, M. M. Murphy, J. R. Schullek, *Bioorg. Med. Chem. Lett.* **2004**, 14, 1423.

[262] A. Robinson, G. L. Thomas, R. J. Spandl, M. Welch, D. R. Spring, *Org. Biomol. Chem.* **2008**, 6, 2978.

[263] E. E. Wyatt, W. R. J. D. Galloway, G. L. Thomas, M. Welch, O. Loiseleur, A. T. Plowright, D. R. Spring, *Chem. Commun.* **2008**, 4962.

[264] M. G. Watve, R. Tickoo, M. M. Jog, B. D. Bhole, *Arch. Microbiol*. **2001**, 176, 386.

[265] R H Baltz, *SIM News* **2005**, 55, 186.

[266] B. Bister, D Bischoff, M Stroebele, J Riedlinger, A Reicke, F Wolter, A T Bull, H Zaehner, H P Fiedler, R D Suessmuth, *Angew. Chem. Int. Ed.* **2004**, 43, 2574.

[267] (a) M S Laport, O C Santos, G Muricy, *Curr. Pharm. Biotechnol.* **2009**, 10, 86; (b) J G Burgess, E M Jordan, M Bregu, A Mearns-Spragg, K G Boyd *J. Biotechnol.* **1999**, 70, 27.

[268] (a) M. S. Donia, J. Ravel, E. W. Schmidt, *Nat. Chem. Biol.* **2008**, 4, 341; (b) L. P. Partida-Martinez, C. Hertweck, *Nature* **2005**, 437, 884; (c) J. Piel, *Nat. Prod. Rep*. **2009**, 26, 338; (d) J. J. Scott, D C Oh, M C Yuceer, K D Klepzig, J Clardy, C R Currie, *Science* **2008**, 322, 63.

[269] S. C. Wenzel, R. Muller, *Curr. Opin. Drug Discov. Dev.* **2009**, 12, 220.

[270] (a) S D Bentley, K. F. Chater, A. M. Cerdeño-Tárraga, G. L. Challis, N. R. Thomson, K. D. James, D. E. Harris, M. A. Quail, H. Kieser, D. Harper, A. Bateman, S. Brown, G. Chandra, C. W. Chen, M. Collins, A. Cronin, A. Fraser, A. Goble, J. Hidalgo, T. Hornsby, S. Howarth, C. H. Huang, T. Kieser, L. Larke, L. Murphy, K. Oliver, S. O'Neil, E. Rabbinowitsch, M. A. Rajandream, K. Rutherford, S. Rutter, K. Seeger, D. Saunders, S. Sharp, R. Squares, S. Squares, K. Taylor, T. Warren, A. Wietzorrek, J. Woodward, B. G. Barrell, J. Parkhill, D. A. Hopwood, *Nature* **2002**, 417, 141; (b) H Ikeda, J Ishikawa, A Hanamoto, M Shinose, H Kikuchi, T Shiba, Y Sakaki, M Hattori, S Omura, *Nature Biotech.* **2003**, 21, 526.

[271] (a) Y Ohnishi, S Kameyama, H Onaka, S Horinouchi, *Mol. Microbiol.* **1999**, 34, 102; (b) A S Khoklov, I. I. Tovarova, L. V. Borisova, S. A. Pliner, L. A. Schevchenko, E. Y. Kornitskaya, N. S. Ivkina, I. A. Rapport, *Dokl. Akad. Nauk SSSR* **1967**, 177, 232; (c) S Horinouchi, *Front. Biosci.* **2002**, 7, 2045.

[272] (a) R H Baltz, *J. Ind. Microbiol. Biotechnol.* **2010**, 37, 759; (b) J P Gomez-Escribano, M J Bibb, *Microb. Biotechnol.* **2011**, 4, 207; (c) M Komatsu, T Uchiyama, S Omura, D E Cane, H Ikeda, *Proc. Natl Acad. Sci. USA* **2010**, 107, 2646; (d) R H Baltz, *J. Ind. Microbiol. Biotechnol.* **2011**, 38, 657.

[273] (a) M S Rappe, S J Giovannoni, *Annu. Rev. Microbiol.* **2003**, 57, 369; (b) P D Schloss, J Handelsman, *Microbiol. Mol. Biol. Rev.* **2004**, 68, 686.

[274] (a) C Freiberg, N A Brunner, G Schiffer, T Lampe, J Pohlmann, M Brands, M Raabe, D Habich, K Ziegelbauer *J Biol Chem.* **2004**, 279, 26066; (b) J Needham, M T Kelly, M Ishige, R J Andersen *J Org Chem*. **1994**, 59, 2058; (c) A Fredenhagen, S Y Tamura, P T Kenny, H Komura, Y Naya, K Nakanishi, K Nishiyama, M Sugiura, H Kita *J Am Chem Soc.* **1987**, 109, 4409; (d) R A Long, D C Rowley, E Zamora, J Liu, D H Bartlett, F Azam *Appl Environ Microbiol*. **2005**, 71, 8531; (e) M Jin, M A Fischbach, J Clardy *J Am Chem Soc*. **2006**, 128, 10660.

[275] (a) P Liras, J F Martin *Int Microbiol.* **2006**, 9, 9; (b) P Liras, A Rodriguez-Garcia, J F Martin *Int Microbiol.* **1998**, 1, 271.

[276] (a) N M Haste, V R Perera, K N Maloney, D N Tran, P Jensen, W Fenical, V Nizet, M E Hensler, *J. Antibiot. (Tokyo)* **2010**, 63, 219; (b) W Li, J E Leet, H A Ax, D R Gustavson, D M Brown, L Turner, K Brown, J Clark, H Yang, J Fung-Tomc, *J. Antibiot. (Tokyo)* **2003**, 56, 226.

[277] R H Baltz, *Microbe* **2007**, 2, 125.

[278] (a) G. L. Challis, *J. Med. Chem.* **2008**, 51, 2618; (b) J. B. McAlpine, *J. Nat. Prod.* **2009**, 72, 566; (c) A. H. Banskota, J B McAlpine, D Sorensen, A Ibrahim, M Aouidate, M Piraee, A M Alarco, C M Farnet, E Zazopoulos, *J. Antibiot. (Tokyo)* **2006**, 59, 533.

[279] J Wang, S M Soisson, K Young, W Shoop, S Kodali, A Galgoci, R Painter, G Parthasarathy, Y S. Tang, R Cummings, S Ha, K Dorso, M Motyl, H Jayasuriya, J Ondeyka, K Herath, C Zhang, L Hernandez, J Allocco, Á Basilio, J R. Tormo, O Genilloud, F Vicente, F Pelaez, L Colwell, S H Lee, B Michael, T Felcetto, C Gill, L L. Silver, J D. Hermes, K Bartizal, J Barrett, D Schmatz, J W. Becker, D Cully, S B. Singh, *Nature* **2006**, 441, 358.

[280] L Ejim, M A Farha, S B Falconer, J Wildenhain, B K Coombes, M Tyers, E D Brown, G D Wright, *Nat. Chem. Biol.* **2011**, 7, 348.

[281] G L Challis, D A Hopwood, *Proc. Natl. Acad. Sci. U.S.A.* **2003**, 100 Suppl(2), 14555.

[282] M U Rashid, H M Lozano, A Weintraub, C E Nord, *Anaerobe*, **2013**, 20, 32.

[283] (a) J Blais, S R Lewis, K M Krause, B M Benton, *Antimicrob. Agents Chemother.* **2012**, 56, 1584; (b) K L Tyrrell, D M Citron, Y A Warren, E J Goldstein *Antimicrob. Agents Chemother.* **2012**, 56, 2194.

[284] V Pokrovskaya, T Baasov, *Expert Opin. Drug Discov.* **2010**, 5, 883.

[285] G R Cathcart, D Quinn, B Greer, P Harriott, J F Lynas, B F Gilmore, B Walker, *Antimicrob. Agents Chemother.* **2011**, 55, 2670.

[286] (a) T E Renau, R Leger, E M Flamme, J Sangalang, M W She, R Yen, C L Gannon, D Griffith, S Chamberland, O Lomovskaya, S J Hecker , V J Lee , T Ohta , K Nakayama, *J. Med. Chem.* **1999**, 42, 4928; (b) O Lomovskaya, M S Warren, A Lee, J Galazzo, R Fronko, M Lee, J Blais, D Cho, S Chamberland, T Renau, R Leger, S Hecker, W Watkins, K Hoshino, H Ishida,V J Lee, *Antimicrob. Agents Chemother.* **2001**, 45, 105.

[287] R B Giannattasio, B Weisblum, *Antimicrob. Agents Chemother.* **2000**, 44, 1961.

[288] M Feder, E Purta, L Koscinski, S Cubrilo, G M Vlahovicek, J M Bujnicki, *ChemMedChem* **2008**, 3, 316.

[289] (a) D Davies *Nat. Rev. Drug Discov.* **2003**, 2, 114; (b) H Ceri, M E Olson, R J Turner *Expert Opin. Pharmacother.* **2010**, 11, 1233.

[290] (a) A F Widmer, R Frei, Z Rajacic, W Zimmerli *J. Infect. Dis.* **1990**, 162, 96; (b) M Monzón, C Oteiza, J Leiva, B Amorena *J. Antimicrob. Chemother.* **2001**, 48, 793.

[291] V Sperandio, *Expert Rev. Anti Infect. Ther.* **2007**, 5, 271.

[292] T B Rasmussen, M Manefield, J B Andersen, L Eberl, U Anthoni, C Christophersen, P Steinberg, S Kjelleberg, M Givskov, *Microbiol.* **2000**, 146(Pt 12), 3237.

[293] (a) U Muh, M Schuster, R Heim, A Singh, E R Olson, E P Greenberg, *Antimicrob. Agents Chemother.* **2006**, 50, 3674; (b) U Muh, B J Hare, B A Duerkop, M Schuster, B L Hanzelka, R Heim, E R Olson, E P Greenberg, *Proc. Natl. Acad. Sci. USA* **2006**, 103, 16948.

[294] J. F. Hu, E. Garo, M. G. Goering, M. Pasmore, H. D. Yoo, T. Esser, J. Sestrich, P. A. Cremin, G. W. Hough, P. Perrone, Y. S. L. Lee, N. T. Le, M. O'Neil-Johnson, J. W. Costerton, G. R. Eldridge, *J. Nat. Prod.* **2006**, 69, 118.

[295] D. J. Musk, D. A. Banko, P. J. Hergenrother, *Chem. Biol.* **2005**, 12, 789.

[296] L. M. Junker, J. Clardy, *Antimicrob. Agents Chemother.* **2007**, 51, 3582.

[297] Z. Zeng, L. Qian, L. Cao, H. Tan, Y. Huang, X. Xue, Y. Shen, S. Zhou, *Appl. Microbiol. Biotechnol.* **2008**, 79, 119.

Chapter 2

Selectively Guanidinylated Aminoglycosides as Antibiotics

2.1 Introduction

The discovery of antibiotics, small molecules of natural or synthetic origin that specifically interfere with vital processes in bacteria can be viewed as one of the major medical breakthroughs of the 20th century. Indeed, antibiotics facilitated the cure of previously untreatable life-threatening infectious diseases. However, many of the originally identified antibiotics are no longer clinically useful as they are compromised by bacterial resistance mechanisms, which include modification of the drug molecules, mutation of the molecular drug targets, or increased cellular drug efflux by small-molecule transporters and biofilm formation.[1] The emergence of pathogens resistant to nearly all antibiotics in current use is of particular concern to clinicians. While infections caused by Gram-positive organisms, such as methicillin-resistant *Staphylococcus aureus* (MRSA), remain a major problem worldwide, the emergence within the last decade of multiple-drug-resistant Gram-negative organisms, such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Klebsiella pneumonia*, is equally worrisome.[2] In fact, Gram-negative resistance to drugs of last resort, such as colistin, has become alarmingly more commonplace in the clinical setting.[3] The widespread and frequently indiscriminate use of antibiotics in human and veterinary medicine has further accelerated the

emergence of these resistant, highly pathogenic bacteria that can cause life-threatening infections.[4] Unfortunately, the surge seen in the appearance of resistant bacteria has not been met by a parallel development of new effective, broad-spectrum antibiotics, and only two novel classes of antibacterial agents, fluoroquinolones and oxazolidinones, have been identified over the past few decades.[5] It is apparent, therefore, that new potent antibiotics are required to complement or even replace currently used drugs, whose utility is ever increasingly compromised by bacterial resistance.

Diverse approaches to the discovery of new antibiotics exist. High-throughput screening campaigns of novel natural products and synthetic libraries can, in principle, lead to the identification of potent novel agents.[6] Such screening strategies can involve empirical, whole-cell testing or target-based in vitro assays for known or underutilized, genomically identified targets with the former approach having the potential to identify antibacterial agents with distinct modes of action.[7] Unfortunately, such efforts have resulted in minimal output and lead compound discovery. Alternatively, modifications of existing and perhaps retired antibiotics can revive some of their utility through systematic structural modifications.[8] While incremental, such efforts rely on established bioactive scaffolds targeting known bacterial pathways, with the prospective of generating potent agents, which can potentially evade deactivation by prevailing resistance mechanisms. Here, we address the selective modification of aminoglycoside antibiotics, a large family of natural products active primarily against Gram-negative bacteria.[9]

Aminoglycosides are polycationic antibiotics, and most bind to the 16S ribosomal A-site RNA, leading to diminished translational fidelity.[10] Being a particularly well-

studied antibacterial class, a wealth of information exists regarding their interactions with their intracellular target and bacterial resistance mechanisms.[1a,b,d,11] This vast knowledge makes the aminoglycoside scaffold an ideal starting point for the development of new, potentially more effective antibiotics.[12] Indeed, semisynthetic, second-generation aminoglycosides such as amikacin have proven very successful in the clinic.[9] With this in mind, we set out to make minor structural modifications to selected members of these known antibiotics in an effort to retain or even improve upon their affinity for the 16S A-site, while potentially decreasing their susceptibility to the most prevalent modes of bacterial deactivation.

Here we disclose the synthesis of a small focused library of aminoglycoside derivatives selectively modified at one or two positions. We strategically replace amine or hydroxy functionalities with a guanidine group in tobramycin, amikacin, kanamycin A, neomycin B neamine, paromomycin, and apramycin (Figure 2.1).[12a,c] Most of the newly synthesized guanidino-aminoglycosides displayed enhanced affinity for the ribosomal A-site, the biological target of the parent derivatives, as determined by an in vitro Förster resonance energy transfer (FRET)-based binding assay. The results of antibacterial tests on a diverse collection of regular and resistant strains illustrate that certain analogues exhibit improved potency against resistant strains, including MRSA. An amikacin analogue shows particular promise with activities greater or equal to those of the parent antibiotic in the majority of strains tested.

2.2 Results
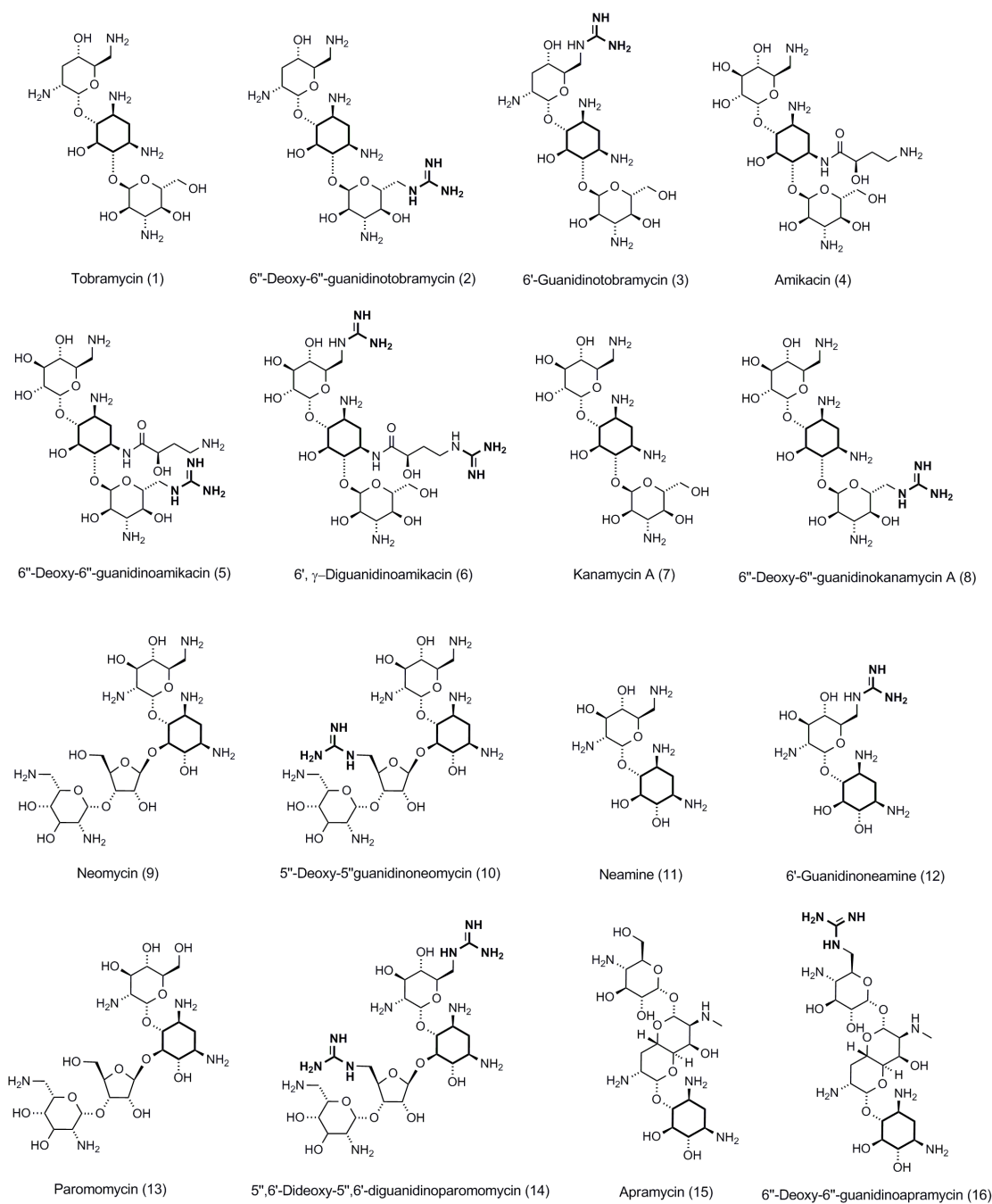
Figure 2.1: Parent aminoglycosides and guanidino-aminoglycosides. The guanidine and 2-deoxystreptamine (2-DOS) moieties are highlighted in bold.

Design strategy

The bacterial A-site is a highly discriminating RNA target that is not tolerant of major structural changes to its cognate ligands.[12n,13] Therefore, we decided to selectively and strategically functionalize aminoglycosides at positions that are less likely to perturb binding and, at the same time, are synthetically accessible.[12a,c] A relatively small modification, which would retain or enhance the overall charge of the RNA-targeting antibiotic, could be achieved by replacing a hydroxy or amine group with a guanidine functionality. In contrast to amines, the planar guanidine functional group is highly basic and can participate in well-defined directional hydrogen bonds. To probe this strategy, we derivatized several aminoglycoside antibiotics, including neamine, kanamycin A, tobramycin, paromomycin, neomycin B, amikacin, and apramycin, by converting selected primary alcohols into guanidine groups, or turning an existing aminomethyl group into the corresponding guanidine derivative. We hypothesized that beyond yielding a greater affinity for the A-site, functional group changes at some sites could potentially lead to decreased recognition by aminoglycoside-modifying enzymes, one of the major bacterial resistance mechanisms.

Synthesis

A general synthetic approach for the conversion of aminoglycoside primary alcohols to guanidinium groups is illustrated using tobramycin (**1**) as an example (Scheme 2.1). First, all amines were globally *tert*-butyloxycarbonyl (Boc)-protected using di-*tert*-butyl dicarbonate. The single primary alcohol of (Boc)$_5$tobramycin (**17**) was then
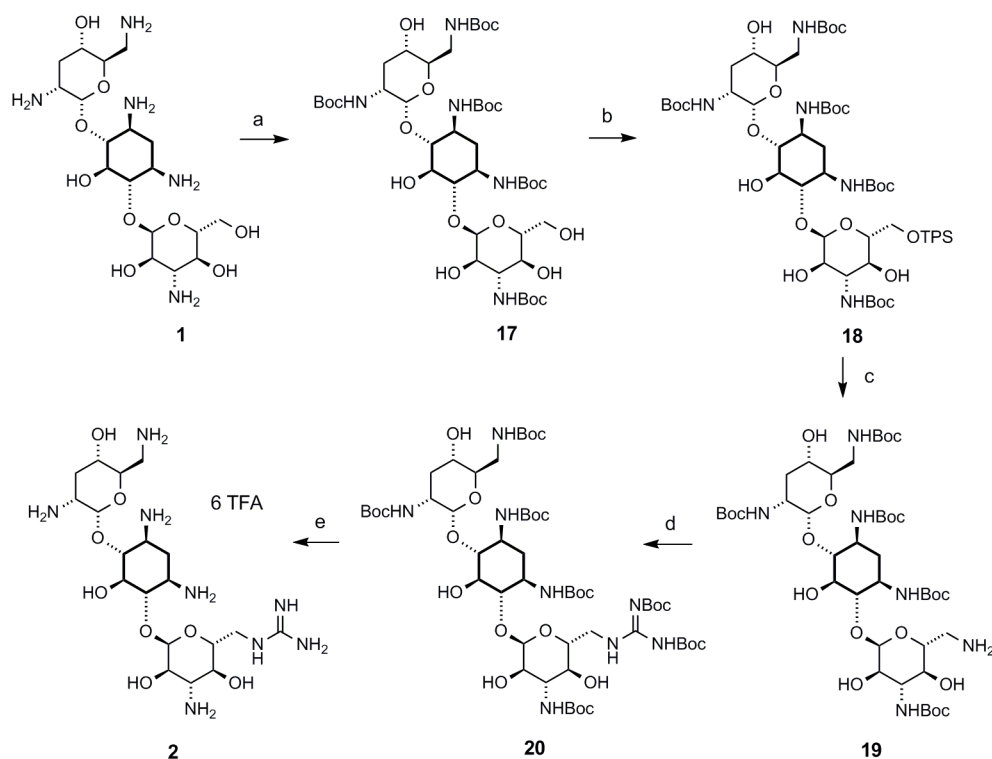
Scheme 2.1: Primary alcohol to guanidinium conversions. *Reagents and conditions:* a) Boc₂O, Et₃N, H₂O, DMF, 55 °C, 4–6 h, 91–97 %; b) TPSCl, pyridine, RT, 2 days, 51–66 %; c) NH₃, MeOH, 80 °C, 2–2.5 days, 57–93%; d) 1,3-di-Boc-2-(trifluoromethylsulfonyl)guanidine, Et₃N, CH₂Cl₂, MeOH, RT, 3 days, 71–85 %; e) TFA, TIPS, CH₂Cl₂, RT, 2.5–3 h, 79–96 %.

selectively converted to a sterically demanding sulfonate by treatment with 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl) in pyridine.[12f] Reflux in methanolic ammonia then afforded 6''-deoxy-6''-amino(Boc)₅tobramycin (**19**). This three-step process, converting primary alcohols into amines, has been previously used in our laboratory for the synthesis of other modified aminoglycosides.[14] Treatment of the single free amine with 1,3-di-Boc-2 (trifluoromethylsulfonyl) guanidine in the presence of triethylamine gave fully Boc-protected guanidino-aminoglycoside, 6''-deoxy-6''-guanidino(Boc)₇tobramycin (**20**). Acidic deprotection of all Boc groups using a one to one mixture of trifluoroacetic acid (TFA) and tri-*iso*-propyl silane (TIPS) in

dichloromethane, followed by HPLC purification, afforded the analytically pure 6''-deoxy-6''-guanidinotobramycin (**2**).
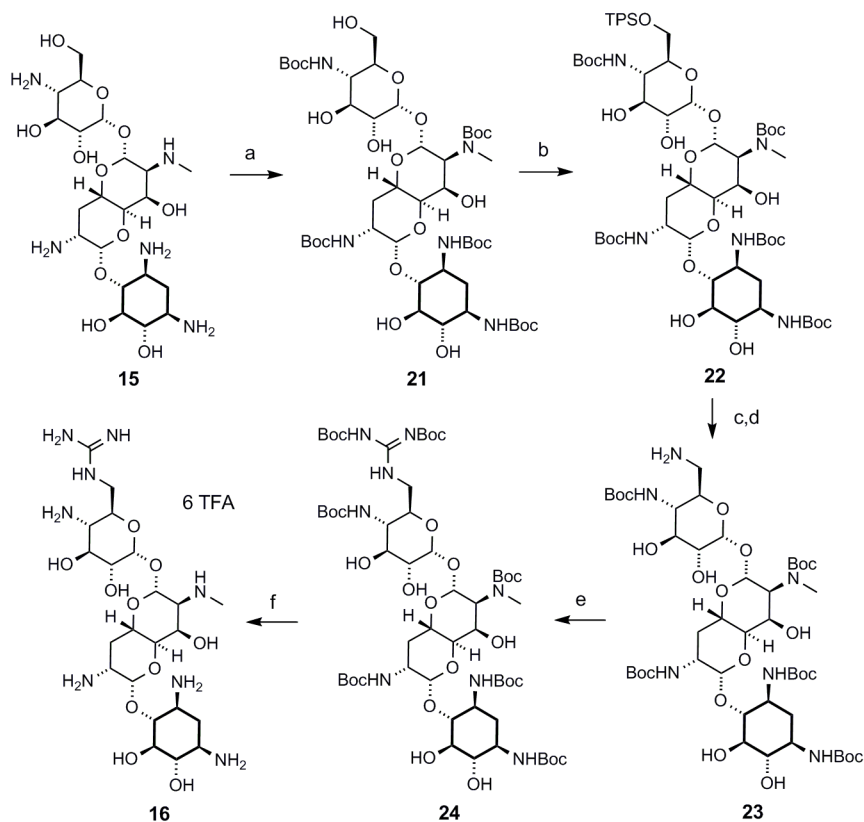
Scheme 2.2: 6''-Deoxy-6''guanidinoapramycin synthesis. *Reagents and conditions:* a) Boc$_2$O, Et$_3$N, H$_2$O, DMF, 55 °C, 8 h, 92%; b) TPSCl, pyridine, RT, 1.5 days, 31%; c) NaN$_3$, DMF, 55 °C, 2 days; d) Pd/C, H$_2$, MeOH, RT, overnight, 80% (two steps); e) 1,3-di-Boc-2-(trifluoromethylsulfonyl)guanidine, Et$_3$N, CH$_2$Cl$_2$, MeOH, RT, 3 days, 74%; f) TFA, TIPS, CH$_2$Cl$_2$, RT, 2 h, 78%.

A slightly different approach was employed for the synthesis of 6''-deoxy-6'' guanidinoapramycin (**16**). Care had to be taken in preparing the sulfonate to prevent activation of multiple hydroxyl groups. This was achieved by using fewer equivalents of the sulfonyl chloride (Step b, Scheme 2.2), in comparison with the other aminoglycosides. In addition, unlike other aminoglycoside derivatives, 6''-deoxy-6''-triisopropylbenzylsulfonyl(Boc)$_5$-apramycin (**22**) was found to degrade in refluxing

methanolic ammonia. Instead, it was converted to the amino intermediate via a two-step process wherein the sulfonyl functionality was first substituted for an azide using sodium azide and then subsequently reduced in a palladium-catalyzed hydrogenation to give 6''-deoxy-6''-guanidino(Boc)$_7$apramycin (**23**).
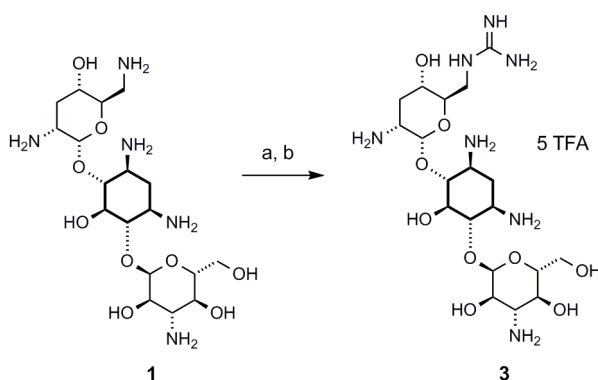
Scheme 2.3: Amine to guanidinium conversions. *Reagents and conditions:* a) 1,3-di-Boc-2-(trifluoromethylsulfonyl)guanidine, Et$_3$N, CH$_2$Cl$_2$, MeOH, RT, 5 days; b) TFA, TIPS, CH$_2$Cl$_2$, RT, 2 h, 12–22% (two steps).

A third protocol was used for selectively converting aminomethyl groups in aminoglycosides to the corresponding guanidine derivatives, relying on their higher nucleophilicity compared with the other more sterically hindered amines. Treatment of unprotected tobramycin with sub-stoichiometric quantities of 1,3-di-Boc-2-(trifluoromethylsulfonyl)guanidine followed by deprotection in a one to one mixture of TFA in dichloromethane provided desired derivative **3** (Scheme 2.3). Similar protocols were applied to amikacin and neamine. Note, all guanidino-aminoglycoside derivatives were first converted to their free-base form by exposure to a strong basic anion (OH−) exchange resin (Monosphere 550A, Dowex) prior to their evaluation in any A-site binding assays or antibacterial experiments.

Affinity for the bacterial 16S A-site RNA construct

To determine the affinity of all derivatives to the bacterial 16S A-site, we used a modified version of a FRET-based assay that was previously developed in our lab.[15] In this assay, a coumarin–aminoglycoside conjugate placeholder binds to a Dy-547-labeled 16S A-site construct. Coumarin acts as a FRET donor to its matched Dy-547 acceptor. The affinity of unlabeled ligands for the A-site can be measured in a competition experiment, where the compound of interest is titrated in and displaces the coumarin–aminoglycoside placeholder, resulting in a decreased sensitized acceptor emission. Different coumarin–aminoglycoside conjugates can be used to cover distinct affinity ranges of putative competitor antibiotics.

Initial titrations were performed with a coumarin–kanamycin derivative, the lowest affinity placeholder aminoglycoside conjugate (Table 2.1). Potent A-site binders, such as neomycin and paromomycin derivatives **10** and **14**, respectively, were titrated against a coumarin–neomycin derivative (Table 2.2). In all cases, titration curves were generated by plotting the fractional fluorescence saturation of the acceptor against the concentration of the molecule of interest (for a representative example, see Figure 2.2).

In general, derivatives with primary alcohols converted to guanidinium groups were found to have significantly higher affinities for the A-site in comparison with their parent aminoglycosides. In particular, 6''-deoxy-6''-guanidinoamikacin (**5**) and 6''-
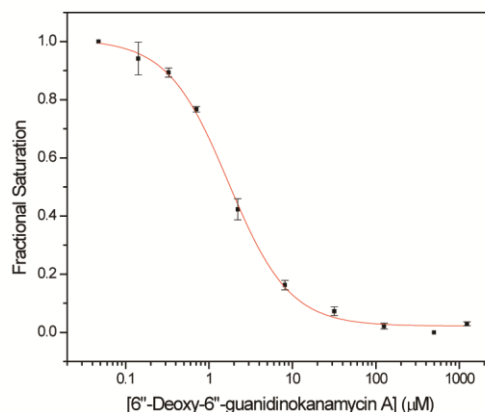
Figure 2.2: Representative A-site competitive displacement curve

Table 2.1: IC$_{50}$ Values for Competing Off Kanamycin-Coumarin[a]	
Compound	IC$_{50}$ (µM)
Tobramycin (**1**)	1.6 ± 0.2
6''-Deoxy-6''-guanidinotobramycin (**2**)	1.0 ± 0.1
6'-Guanidinotobramycin (**3**)	1.6 ± 0.1
Amikacin (**4**)	6.7 ± 0.7
6''-Deoxy-6''-guanidinoamikacin (**5**)	1.4 ± 0.04
6',γ-Diguanidinoamikacin (**6**)	3.2 ± 0.2
Kanamycin A (**7**)	7.0 ± 0.7
6''-Deoxy-6''-guanidinokanamycin A (**8**)	1.8 ± 0.1
Neamine (**11**)	4.5 ± 0.4
6'-Guanidinoneamine (**12**)	4.4 ± 0.5
Apramycin (**15**)	1.7 ± 0.1
6''-Deoxy-6''-guanidinoapramycin (**16**)	1.9 ± 0.2
[a] Conditions: A-site RNA (1 µM), kanamycin-coumarin (0.53 µM), cacodylate buffer pH 7.0 (20 mM), NaCl (100 mM), EDTA (0.5 mM)	

deoxy-6''-guanidinokanamycin A (**8**) showed marked improvements compared with their

parent compounds, which were the weakest binders tested. 5''-Deoxy-5''-

guanidinoneomycin (**10**) and 5'',6'-dideoxy-5'',6'-diguanidinoparomomycin (**14**) both showed higher affinities for the A-site than neomycin (**9**), which was the highest affinity binder among the natural aminoglycosides. The only exception to this trend was 6''-deoxy-6''-guanidinoapramycin (**16**), which has a similar affinity to the unmodified antibiotic. Most of the aminoglycoside derivatives, where amines at primary carbon centers are replaced with guanidinium groups, show comparable affinity to their parent antibiotics. This is not surprising since the overall positive charge is unlikely to drastically change; this is in contrast to derivatives where primary alcohols were converted to a guanidine group, which would have increased positive character.

Table 2.2: IC$_{50}$ Values for Competing Off Neomycin-Coumarin[a]	
Compound	IC$_{50}$ (μM)
Neomycin (**9**)	4.4 ± 0.3
5''-Deoxy-5''-guanidinoneomycin (**10**)	1.5 ± 0.1
Paromomycin (**13**)	19.6 ± 2.0
5'',6'-Dideoxy-5'',6'-diguanidinoparomomycin (1**4**)	1.8 ± 0.1
[a] Conditions: A-site RNA (1 μM), neomycin-coumarin (0.53 μM), cacodylate buffer pH 7.0 (20 mM), NaCl (100 mM), EDTA (0.5 mM)	

Antibacterial activities

While the structure–activity relationship (SAR) data generated is intriguing, the ultimate test is the actual efficacy against bacteria. To assess the relative antibacterial activities of the synthetic derivatives, minimum inhibitory concentration (MIC) values of both the modified and parent antibiotics were determined against a wide variety of bacterial strains (Table 2.3). The compounds were first tested against the antibacterial

Table 2.3: MIC Values (µg ml^{-1})[a]

Compound	*E. coli* (ATCC25922)	*S. aureus* (ATCC33591)	*P. aeruginosa* (PA01)	*P. aeruginosa* (GNR0697)
1	0.78 – 1.56	3.125	0.39	0.78
2	3.125 – 6.25	**1.56**	6.25	25
3	25 – 50	25	50	>50
4	1.56 – 3.125	12.5 – 25	3.125	6.25
5	*1.56 – 3.125*	**3.125**	**1.56**	**3.125**
6	>50	>50	>50	>50
7	6.25	25	>50	>50
8	*6.25*	**3.125 – 6.25**	>50	>50
9	0.78	1.56 – 3.125	3.125 – 6.25	12.5 – 25
10	1.56	**0.78 – 1.56**	6.25 – 12.5	>50
13	6.25	12.5 – 25	>50	>50
14	12.5	**6.25 – 12.5**	**25 – 50**	>50
15	6.25	25 – 50	6.25	12.5
16	12.5 – 25	>50	50	>50
Compound	*K. pneumoniae* (GNR0713)	*K. pneumoniae* (GNR1100)	*A. baumannii* (GNR0717)	*A. baumannii* (GNR1753)
1	>50	>50	6.25 – 12.5	0.78
2	>50	>50	>50	1.56
3	>50	>50	12.5	25
4	25	25	1.56	0.39
5	25 – 50	*25*	3.125	*0.39*
6	>50	>50	6.25	6.25
7	>50	>50	12.5	>50
8	>50	>50	*12.5*	>50
9	>50	6.25	0.39 – 0.78	6.25
10	>50	*6.25*	3.125 – 6.25	*6.25*
13	>50	3.125	0.39	>50
14	>50	>50	6.25	**6.25 – 12.5**
15	>50	>50	6.25	0.39
16	>50	>50	25	*0.39*

[a] Italics = MIC equal to parent, Bold = MIC lower than parent.

agent-susceptible control *E. coli* strain ATCC25922. We also used the clinically relevant Gram-positive MRSA strain ATCC33591. Most of the guanidino-aminoglycosides showed improved efficacy over their respective parent aminoglycosides against MRSA. The two compounds showing the greatest improvements were 6''-deoxy-6''-guanidinokanamycin A (**8**) with an MIC value dropping to 3.125–6.25 mg mL^{-1} from 25 mg mL^{-1} for the parent compound, and 6''-deoxy-6''-guanidinoamikacin (**5**), which improved to 3.125 mg mL^{-1} from a parent MIC value of 12.5–25 mg mL^{-1}.

All synthetic derivatives were also tested against a variety of antibacterial-drug-resistant, Gram-negative, clinical isolates including strains of *P. aeruginosa*, *K. pneumoniae*, and *A. baumannii*. Compound **5** showed the broadest spectrum activity, with potency greater or equal to its parent compound in six out of the eight strains tested. For example, the MIC values of compound **5** against the two *P. aeruginosa* strains were 1.56 mg mL^{-1} and 3.125 mg mL^{-1}, respectively, compared with 3.125 mg mL^{-1} and 6.25 mg mL^{-1} for the parent compound—a twofold increase in potency in both cases. With the exception of the aforementioned derivative, the guanidino-aminoglycosides did not perform as well in general against these strains. Surprisingly, 5'',6'-dideoxy-5'',6'-diguanidinoparomomycin (**14**) showed a vast improvement against the A. baumannii strain GNR1753 compared with paromomycin (**13**), dropping from an MIC value of greater than 50 mg mL^{-1} to 6.25–12.5 mg mL^{-1}.

2.3 Discussion

Aminoglycoside derivatives with guanidinylated aminomethyl groups or primary alcohols selectively converted to guanidinium groups were synthesized. Analogues with guanidinylated amines showed modest, if any, improvement in affinity for the bacterial A-site RNA. These analogues did not show any improvement over their parent compounds when tested for antibacterial activity. In contrast, analogues with primary alcohols converted to guanidinium groups, particularly those of the kanamycin class, consistently showed marked increases in A-site affinity, which was coupled, in most cases, with improved antibacterial activity.

The marked increase in the affinity of the kanamycin class of derivatives upon replacing the 6'' hydroxy with a guanidinium group is interesting since the 6'' hydroxy group in the parent compounds in not involved in hydrogen bonding with the A-site, at least not in the published co-crystal structures, but the 6'' hydroxy group is in close proximity to U1406 and C1407. This could suggest that the new guanidine group in these derivatives, in addition to its overall electrostatic contribution, might be extended far enough to make new contacts with these RNA nucleobases (Figure 2.3).[11b,c,e] The increased binding affinity of 5'',6'-dideoxy-5'',6'-diguanidinoparomomycin (**14**) is most likely due to the increased overall charge and perhaps also replacement of the hydroxy-based hydrogen bonds observed for the parent molecule paromomycin (**13**), which is known to make A-site contacts at both the 5'' and 6' positions,[9d] with stronger (charged) hydrogen bonds. The affinity of 6''-deoxy-6''-guanidinoapramycin (**16**) can also be

6"-Deoxy-6"-guanidinokanamycin A (8)

6"-Deoxy-6"-guanidinoapramycin (16)
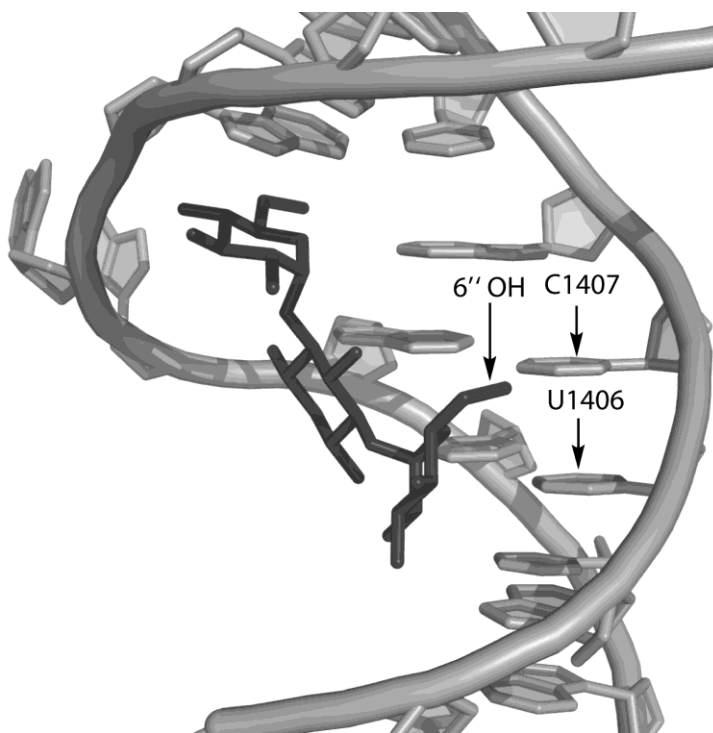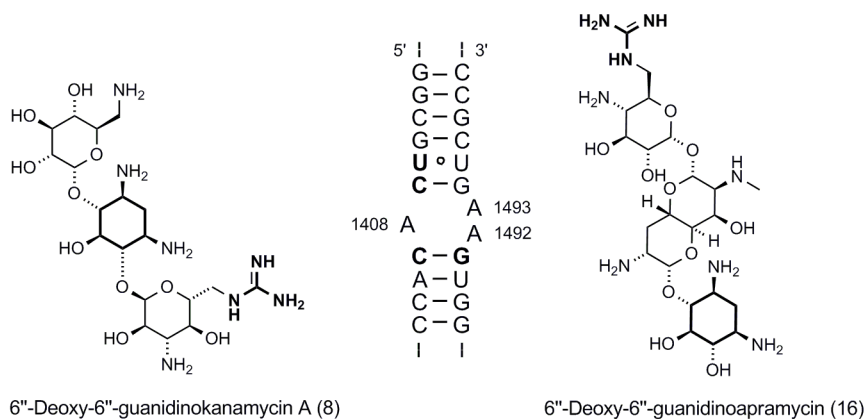
Figure 2.3: Proposed interactions between guanidinium groups of guanidino-aminoglycosides and A-site RNA bases. A) 6''-Deoxy-6''-guanidinokanamycin A (**8**) interactions with U1406 and C1407 and 6''-Deoxy-6''-guanidinoapramycin (**16**) interactions with C1409 and G1491 bolded. B) Kanamycin A (**7**) crystal structure with 6'' OH modification site and potential new contacts of 6''-Deoxy-6''-guanidinokanamycin A (**8**) highlighted.

potentially rationalized by examining the crystal structure of the parent aminoglycoside.

A crystal structure of apramycin (**15**) with the 16S A-site shows that the 6''-hydroxy

group forms a unique hydrogen-bonding interaction in which it functions as both a donor

and an acceptor along the edge of the G1491–C1409 base pair.[11a] Disrupting these interactions could explain why an alteration at that site was not as well tolerated (Figure 2.3).

The lack of improvement in binding seen for derivatives with modifications to the 6' amine is not entirely surprising. We recognize that the guanidine groups, being highly basic, can also somewhat attenuate the pKa of neighboring ammonium groups, leading to derivatives with similar overall charge.[12a] Additionally, all of these derivatives are modified at the 6' amine, which is known to make critical contacts with A1408 within the A-site binding pocket.[11b,e] 6',γ-Diguanidinoamikacin (**6**) was the only derivative of this class to show statistically significant improvements in affinity compared with the parent compound. However, 6',γ-diguanidinoamikacin (**6**) still showed weaker affinity than 6''-deoxy-6''-guanidinoamikacin (**5**), the corresponding derivative with a guanidinium group replacing an alcohol.

When analyzing the potency and MIC values, it is important to remember that affinities to the A-site do not necessarily correlate with antibacterial potency.[16] It is interesting to note that all but two of the synthesized compounds, 6''-deoxy-6''-guanidinoamikacin (**5**) and 6''-deoxy-6''-guanidinokanamycin A (**8**), showed inferior antibacterial activity against the control *E. coli* strain ATCC25922, suggesting that improvement in activity against resistant strains is at least partially due to overcoming bacterial resistance mechanisms. This makes the broad spectrum improvement of 6''-deoxy-6''-guanidinoamikacin (**5**) a particularly intriguing observation given that amikacin is a semi-synthetic aminoglycoside structurally derived from kanamycin A with

an amino 2-hydroxybutyryl (AHB) side chain, which lowers its susceptibility to aminoglycoside-modifying enzymes. It is possible that the AHB and guanidinium modifications operate synergistically to further decrease its affinity for modifying enzymes. Derivatives where amines were guanidinylated generally show poor antibacterial activity, in all cases inferior to the parent aminoglycosides, which could suggest that the amine at position 6' plays an important role in antibacterial activity. This is somewhat surprising, since the majority of aminoglycoside-modifying enzymes make alterations to ring I including the AAC(6') enzyme which directly modifies 6' amines.[1a] In contrast, 5'',6'-dideoxy-5'',6'-diguanidino-paromomycin (**14**), which contains a 6' hydroxy functionality, exhibits several improved antibacterial activities.

2.4 Conclusions

A series of guanidino-aminoglycosides, selectively modified aminoglycosides, was synthesized. In almost all cases, these derivatives have proven to be superior binders of the bacterial A-site compared with their parent antibiotics when tested in an in vitro FRET-based assay. Some of the compounds showed potent antibacterial activity, frequently performing as well or even better than the parent aminoglycosides. In particular, 6''-deoxy-6'' guanidinoamikacin (**5**) proved to be particularly promising; showing equal or better activity than amikacin (**4**) against almost all of the bacterial strains tested, including several clinical isolates.

2.5 Experimental Section

Materials

Unless otherwise specified, materials purchased from commercial suppliers were used without further purification. All aminoglycosides were obtained from Sigma–Aldrich as their sulfate salts. Tobramycin sulfate was converted to the trifluoroacetic acid (TFA) salt by first passing it over an anion (OH^-) exchange resin (Monosphere 550A, Dowex) to get the free base, then stirring in 0.1% $TFA/H2O$. Neamine hydrochloride was made by methanolysis of commercially available neomycin sulfate.[17] 1,3-Di-Boc-2-(trifluoromethylsulfonyl)guanidine was synthesized according to an established procedure.[18] Anhydrous NH_3 was purchased from Airgas. All other anhydrous solvents and reagents, and ion exchange resins were purchased from Sigma–Aldrich. NMR solvents were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). The Dy-547-labeled A-site construct was purchased from Thermo Scientific and purified by gel electrophoresis. Kanamycin–coumarin and neomycin–courmarin conjugates were synthesized and purified according to established procedures.[15] Chemicals for preparing buffer solutions (enzyme grade) were purchased from Fisher Biotech. Autoclaved water was used in all fluorescence titrations. Mueller–Hinton broth used for sensitivity testing was obtained from Hardy Diagnostics (Santa Maria, CA, USA). Polystyrene 96-well microplates for MIC testing were purchased from Corning Inc. (Corning, NY, USA). Bacterial strains for sensitivity testing included two reference strains from the American Type Culture Collection (Manassas, VA, USA): hospital-associated MRSA strain 33591 rendered resistant to rifampicin by serial passage and *E. coli strain* 25922. *P. aeruginosa strain* PA01 was used as a general antibiotic-sensitive *P. aeruginosa* strain.[19] Other

Gram-negative strains used were clinical isolates obtained from a tertiary academic hospital in the New York metropolitan area; these were: *P. aeruginosa* strain GNR0697 (blood isolate), *K. pneumoniae* strain GNR0713 (blood isolate), *K. pneumoniae* strain GNR1100 (respiratory isolate), *A. baumannii* strain GNR0717 (urine isolate), and *A. baumannii* strain GNR1753 (wound isolate).

Instrumentation

NMR spectra were recorded on Varian Mercury 300 and 400 MHz, Varian VX 500 MHz, and Jeol ECA 500 MHz spectrometers. All two-dimensional NMR spectra were recorded on a Jeol ECA 500 MHz spectrometer and processed using the Delta NMR Processing and Control Software (version 4.3.6). Mass spectra (MS) were recorded at the University of California, San Diego Chemistry and Biochemistry Mass Spectrometry Facility, utilizing an Agilent 6230 HR-ESI-TOF mass spectrometer. Reverse-phase HPLC (Vydac C18 column) purification and analysis were carried out using an Agilent 1200 series instrument. Products were lyophilized utilizing a Labconco FreeZone 2.5 freeze drier. Steady-state fluorescence experiments were carried out in a microfluorescence cell with a path length of 1.0 cm (Hellma GmH & Co KG, Mullenheim, Germany) on a Jobin Yvon Horiba FluoroMax-3 luminescence spectrometer. A background spectrum (buffer) was subtracted from each sample. A VersaMax plate reader (Molecular Devices, Mountain View, CA, USA) set at 600 nm wavelength was used for MIC assays.

Synthesis

Primary Alcohol to Guanidinium Conversions
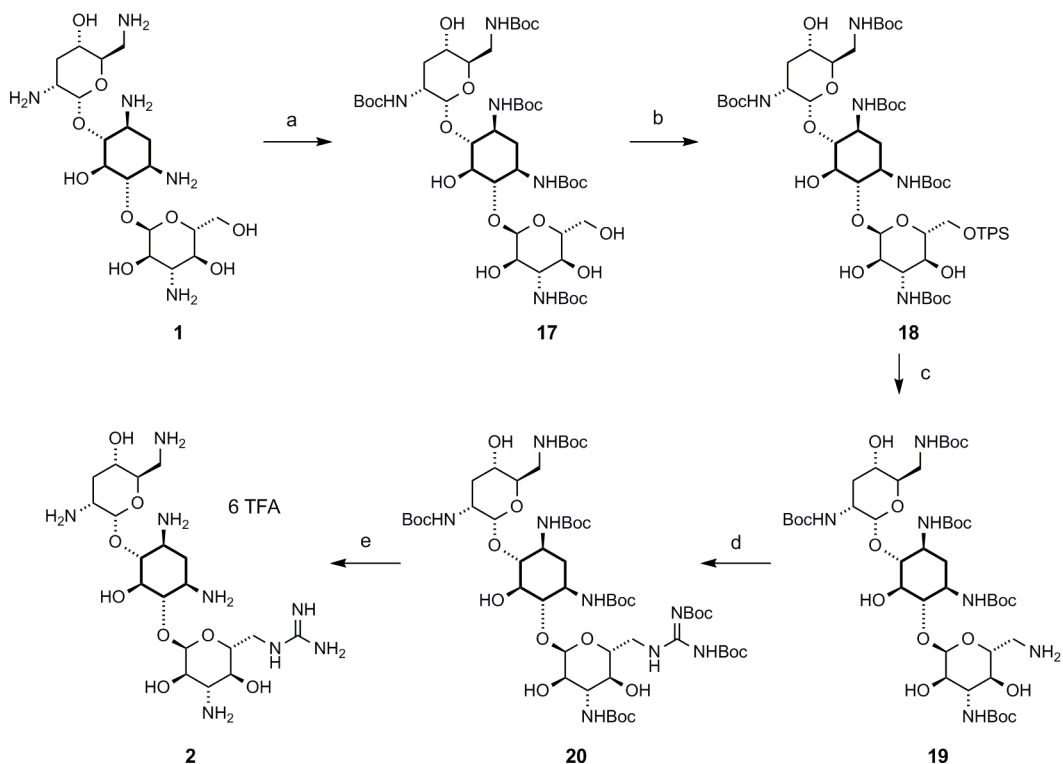
Scheme 2.4: 6"-Deoxy-6"-guanidinotobramycin synthesis. (a) Boc$_2$O, TEA, H$_2$O, DMF. (b) TPSCl, Pyridine. (c) NH$_3$, MeOH, (d) 1,3-Di-boc-2-(trifluoromethylsulfonyl)guanidine, TEA, DCM, MeOH. (e) TFA, TIPS, DCM.

6''-Deoxy-6''-amino-(Boc)₅tobramycin (19). Synthesis and characterization of

precursors 17 and 18 previously reported.[20] Anhydrous methanol (18 mL) was added to

6''-deoxy-6''-triisopropylbenzylsulfonyl-(Boc)₄tobramycin (18) (411 mg, 0.33 mmol) in

a pressure tube. The yellow solution was cooled to 0 °C and anhydrous ammonia was

bubbled into the solution for 10 mins. The vessel was capped and heated to 80 °C for 2.5

days. The vessel was cooled to 0 °C and opened. After 5 mins DOWEX® Monosphere®

550A ion exchange resin (⁻OH form) was added. The reaction was stirred for 12 hours at

rt and filtered. The solvent was removed under reduced pressure and the resulting solid

was dissolved in DCM and washed with a 2 M sodium bicarbonate solution. The organic

layer was dried over sodium sulfate and the solvent was removed under reduced pressure.

Product: White solid (184 mg, 0.19 mmol, 57% yield). ^{1}H-NMR (400 MHz, CD$_3$OD): δ

5.11 (s, 2H), 4.21 (dd, J$_1$ = 5.6 Hz, J$_2$ = 2 Hz, 1H), 4.20 – 4.11 (m, 1H), 4.01 – 3.89 (m,

2H), 3.71 – 3.33 (m, 7H), 3.19 – 3.02 (m, 2H), 2.95 (t, J = 7.2 Hz, 1H), 2.80 (q, J = 7.2

Hz, 1H), 2.76 – 2.68 (m, 1H), 2.18 – 1.91 (m, 3H), 1.64 (q, J = 12.4 Hz, 1H), 1.56 – 1.10

(m, 43H), 0.96 – 0.90 (m, 2H); HR-ESI-MS calculated for C$_{43}$H$_{79}$N$_6$O$_{18}$ [M+H]$^+$

967.5445, found 967.5450

6''-Deoxy-6''-guanidino-(Boc)$_7$tobramycin (20). DCM (3.3 mL), methanol (0.1 mL),

and TEA (37 μL, 0.26 mmol) were added to 6''-deoxy-6''-amino-(Boc)$_4$tobramycin (19)

(169 mg, 0.18 mmol). 1,3-Di-boc-2-(trifluoromethylsulfonyl)guanidine (685 mg, 1.75

mmol) was added. The light yellow solution was stirred for 3 days. The solvent was

removed under reduced pressure. The product was isolated by flash chromatography (0 –

7% methanol in DCM). Product: White solid (169 mg, 0.14 mmol, 85% yield). ^{1}H-NMR

(400 MHz, CD$_3$OD): δ 5.13 (s, 1H), 5.09 (s, 1H), 4.16 – 4.11 (m, 1H), 3.91 – 3.86 (m,

1H), 3.79 – 3.19 (m, 14H), 2.17 – 1.95 (m, 3H), 1.57 – 1.51 (m, 13H), 1.51 – 1.41 (m,

42H); HR-ESI-MS calculated for C$_{49}$H$_{89}$N$_8$O$_{20}$ [M+H]$^+$ 1109.6188, found 1109.6186

6''-Deoxy-6''-guanidinotobramycin· 6 TFA (2). DCM (3.75 mL) and TIPS (0.2 mL)

were added to 6''-deoxy-6''-guanidino-(Boc)$_6$kanamycin A (20) (150 mg, 0.12 mmol).

TFA (3.75 mL) was added. The yellow solution was stirred for 2.5 hours. Toluene (8 mL)

was added and the solvent was removed under reduced pressure. The remaining white

solid was dissolved in water and purified by reverse phase HPLC (0 – 0.1% ACN in water (0.1% TFA) over 13 min) eluted after 10.2 min, then lyophilized. Product: White powder (117 mg, 0.098 mmol, 79% yield). ^{1}H-NMR (400 MHz, D$_2$O): δ 5.74 (d, J = 3.2 Hz, 1H), 5.10 (d, J = 3.6 Hz, 1H), 4.05 – 3.90 (m, 4H), 3.83 – 3.66 (m, 4H), 3.64 – 3.47 (m, 6H), 3.41 (dd, J$_1$ = 14 Hz, J$_2$ = 3.6 Hz, 1H), 3.27 (q, J = 7 Hz, 1H), 2.54 (dt, J$_1$ = 12.8 Hz, J$_2$ = 4.2 Hz, 1H), 2.29 (dt, J$_1$ = 12.4 Hz, J$_2$ = 4.4 Hz, 1H), 2.03 (q, J = 11.6 Hz, 1H), 1.92 (q, J = 12.8 Hz, 1H); ^{13}C-NMR (100 MHz, D$_2$O): δ 163.63 (J = 35 Hz), 158.48, 116.93 (J = 290 Hz), 101.42, 94.74, 84.25, 78.17, 74.73, 71.84, 71.10, 68.61, 66.41, 64.91, 55.29, 50.49, 48.94, 48.32, 41.86, 40.32, 29.82, 28.35; HR-ESI-MS calculated for C$_{19}$H$_{41}$N$_8$O$_8$ [M+H]$^+$ 509.3042, found 509.3041
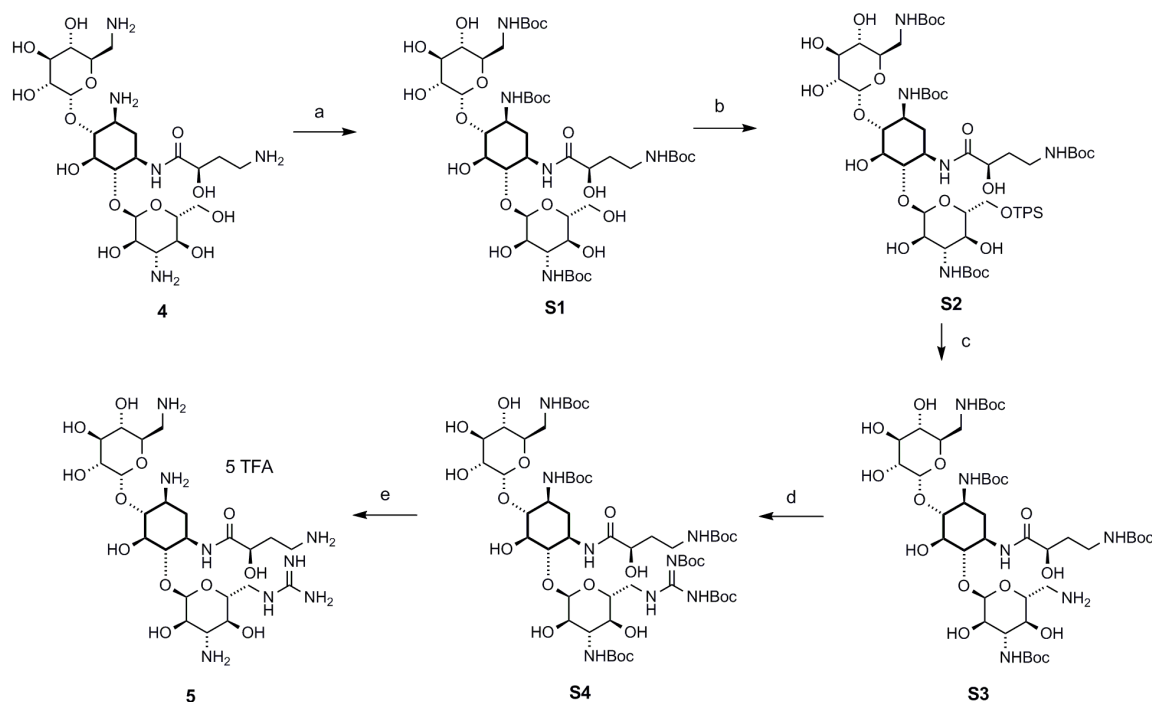
Scheme 2.5: 6"-Deoxy-6"-guanidinoamikacin synthesis. (a) Boc$_2$O, TEA, H$_2$O, DMF. (b) TPSCl, Pyridine. (c) NH$_3$, MeOH, (d) 1,3-Di-boc-2-(trifluoromethylsulfonyl)guanidine, TEA, DCM, MeOH. (e) TFA, TIPS, DCM.

(Boc)₄Amikacin (S1). Water (2.1 mL), DMF (2.5 mL), and TEA (1.3 mL, 12.81 mmol) were added to amikacin sulfate (5) (500 mg, 0.85 mmol). The reaction was heated to 55 °C and di-tert-butyl dicarbonate (1.12 g, 5.12 mmol) dissolved in DMF (8 mL) was added slowly. The pale yellow solution was stirred for 6 hours. The solvent was removed under reduced pressure and the resulting solid was suspended in warm water. The solid was filtered and washed thoroughly with water. The product was dissolved in ACN and the solvent was removed under reduced pressure. Product: White solid (816 mg, 0.83 mmol, 97% yield). ^{1}H-NMR (400 MHz, CD₃OD): δ 5.12 (s, 1H), 5.04 (d, J = 4.4 Hz, 1H), 4.06 − 4.00 (m, 1H), 3.98 (dd, J_1 = 8.8 Hz, J_2 = 3.6 Hz, 1H), 3.80 − 3.59 (m, 7H), 3.50 − 3.17 (m, 13H), 2.16 − 2.09 (m, 1H), 1.98 − 1.90 (m, 1H), 1.80 − 1.71 (m, 1H), 1.50 − 1.33 (m, 36H), 1.31 (t, J = 7.2 Hz, 1H); HR-ESI-MS calculated for $C_{42}H_{75}N_5O_{21}Na$ [M+Na]$^+$ 1008.4847, found 1008.4833.

6''-Deoxy-6''-triisopropylbenzylsulfonyl-(Boc)₄amikacin (S2). Anhydrous pyridine (11.3 mL) was added to (Boc)₄amikacin (S1) (816 mg, 0.83 mmol). Triisopropylbenzylsulfonyl chloride (3.41 g, 11.27 mmol) was added. The orange solution was stirred for 2 days. The solvent was removed under reduced pressure. The product was isolated by flash chromatography (0 − 5% methanol in DCM). Product: White solid (530 mg, 0.42 mmol, 51% yield). ^{1}H-NMR (300 MHz, CD₃OD): δ 7.27 (s, 2H), 5.49 (s, 2H), 4.40 − 4.28 (m, 3H), 4.19 − 4.08 (m, 4H), 4.00 − 3.82 (m, 3H), 3.76 − 3.31 (m, 8H), 3.28 − 3.16 (m, 4H), 2.99 − 2.86 (m, 3H), 2.08 − 1.90 (m, 3H), 1.52 − 1.38

(m, 30H), 1.38 – 1.13 (m, 22H), 0.98 – 0.86 (m, 3H); HR-ESI-MS calculated for

$C_{57}H_{97}N_5O_{23}SNa$ [M+Na]$^+$ 1274.6187, found 1274.6190

6''-Deoxy-6''-amino-(Boc)$_4$amikacin (S3). Anhydrous methanol (7.5 mL) was added to

6''-deoxy-6''-triisopropylbenzylsulfonyl-(Boc)$_4$amikacin (S2) (480 mg, 0.39 mmol) in a

pressure tube. The yellow solution was cooled to 0 °C and anhydrous ammonia was

bubbled into the solution for 10 mins. The vessel was capped and heated to 80 °C for 2

days. The vessel was cooled to 0 °C and opened. After 5 mins DOWEX® Monosphere®

550A ion exchange resin ($^-$OH form) was added. The reaction was stirred for 12 hours at

rt and filtered. The solvent was removed under reduced pressure and the resulting solid

was dissolved in DCM and washed with a 2 M sodium bicarbonate solution. The organic

layer was dried over sodium sulfate and the solvent was removed under reduced pressure.

Product: Tan solid (360 mg, 0.37 mmol, 93% yield). ^{1}H-NMR (400 MHz, CD$_3$OD): δ

5.12 (s, 1H), 5.08 (s, 1H), 4.53 – 4.44 (m, 2H), 4.00 – 3.84 (m, 3H), 3.72 – 3.59 (m, 5H),

3.51 – 3.46 (m, 2H), 3.30 – 3.08 (m, 6H), 2.88 – 2.81 (m, 2H), 2.14 (t, J = 7.6 Hz, 1H),

2.08 – 1.89 (m, 2H), 1.80 – 1.68 (m, 1H), 1.62 – 1.40 (m, 15H), 1.36 – 1.19 (m, 18H),

0.94 – 0.84 (m, 3H); HR-ESI-MS calculated for $C_{42}H_{76}N_6O_{20}Na$ [M+Na]$^+$ 1007.5007,

found 1007.5002

6''-Deoxy-6''-guanidino-(Boc)$_6$amikacin (S4). DCM (2 mL), methanol (0.2 mL), and

TEA (65 μL, 0.47 mmol) were added to 6''-deoxy-6''-amino-(Boc)$_4$amikacin (S3) (150

mg, 0.151 mmol). 1,3-Di-boc-2-(trifluoromethylsulfonyl)guanidine (616 mg, 1.58 mmol)

was added. The yellow solution was stirred for 3 days. The solvent was removed under reduced pressure. The product was isolated by flash chromatography (0 – 7% methanol in DCM). Product: Tan solid (144 mg, 0.117 mmol, 78% yield). ^{1}H-NMR (400 MHz, CD$_3$OD): δ 5.12 (s, 1H), 5.01 (s, 1H), 4.60 (s, 1H), 4.35 – 4.28 (m, 1H), 4.20 – 4.08 (m, 3H), 4.06 – 3.95 (m, 2H), 3.90 – 3.42 (m, 8H), 3.20 – 3.12 (m, 3H), 2.95 (t, J = 7.2 Hz, 2H), 2.10 – 1.91 (m, 3H), 1.78 – 1.69 (m, 1H), 1.55- 1.50 (m, 6H), 1.48 – 1.38 (s, 27H), 1.32 – 1.23 (m, 21H); HR-ESI-MS calculated for C$_{53}$H$_{94}$N$_8$O$_{24}$Na [M+Na]$^+$ 1249.6273, found 1249.6259

6''-Deoxy-6''-guanidinoamikacin· 5 TFA (5). DCM (0.9 mL) and TIPS (40 μL) were added to 6''-deoxy-6''-guanidino-(Boc)$_6$amikacin (S4) (43 mg, 0.033 mmol). TFA (0.9 mL) was added. The yellow solution was stirred for 2.5 hours. Toluene (2 mL) was added and the solvent was removed under reduced pressure. The remaining white solid was dissolved in water and purified by reverse phase HPLC (0 – 6% ACN in water (0.1% TFA) over 12 min) eluted after 5.8 min, then lyophilized. Product: White powder (34 mg, 0.028 mmol, 85% yield). ^{1}H-NMR (500 MHz, D$_2$O): δ 5.47 (d, J = 3.8 Hz, 1H), 5.11 (d, J = 3.8 Hz, 1H), 4.24 – 4.20 (m, 2H), 4.07 – 4.03 (m, 1H), 3.98 – 3.95 (m, 1H), 3.84 – 3.68 (m, 5H), 3.63 (dd, J$_1$ = 10 Hz, J$_2$ = 4 Hz, 1H), 3.55 – 3.42 (m, 4H), 3.39 – 3.31 (m, 3H), 3.17 (q, J = 7 Hz, 1H), 3.11 (t, J = 7.5 Hz, 2H), 2.18 – 2.09 (m, 2H), 1.93 – 1.86 (m, 1H), 1.74 (q, J = 12.6 Hz, 1H); ^{13}C-NMR (125 MHz, D$_2$O): δ 176.15, 163.63 (J = 34 Hz), 158.36, 116.96 (J = 290 Hz), 98.60, 97.82, 73.61, 72.72, 71.48, 71.20, 71.03, 70.10,

69.36, 68.57, 66.98, 55.78, 49.46, 48.69, 41.94, 40.81, 37.51, 31.44, 30.81; HR-ESI-MS

calculated for $C_{23}H_{47}N_8O_{12}$ [M+H]$^+$ 627.3308, found 627.3306
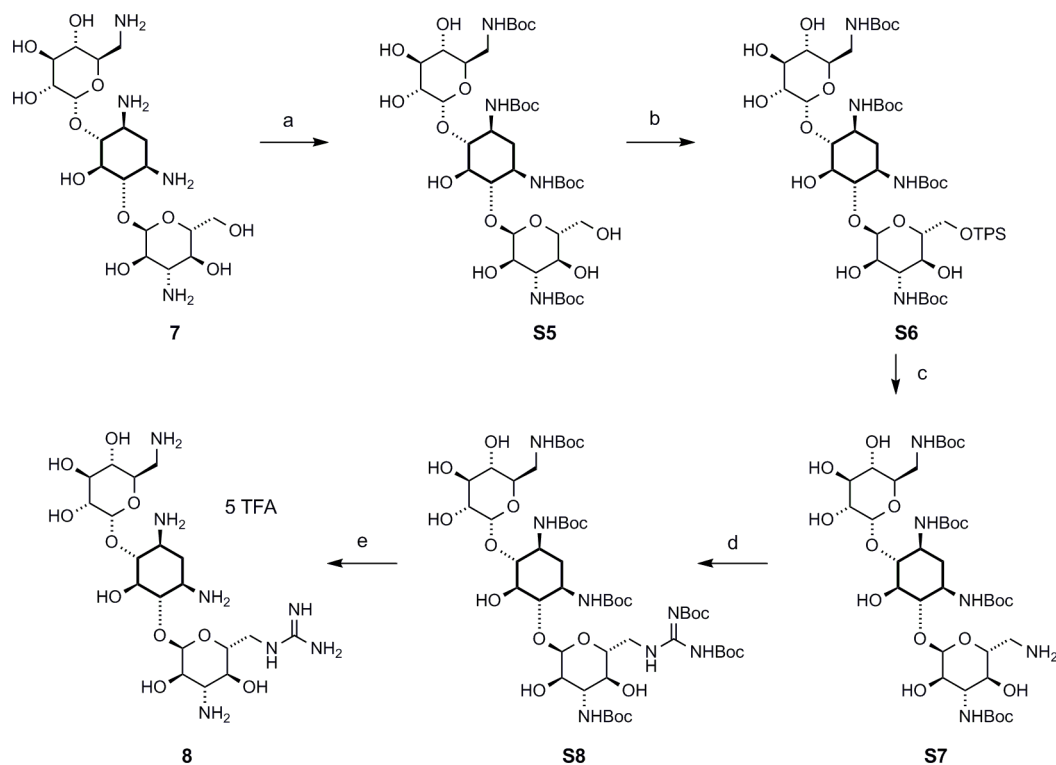
Scheme 2.6: 6"-Deoxy-6"-guanidinotkanamycin A synthesis. (a) Boc$_2$O, TEA, H$_2$O, DMF. (b) TPSCl, Pyridine. (c) NH$_3$, MeOH, (d) 1,3-Di-boc-2-(trifluoromethylsulfonyl)guanidine, TEA, DCM, MeOH. (e) TFA, TIPS, DCM.

6''-Deoxy-6''-amino-(Boc)₄kanamycin A (S7). Synthesis and characterization of

precursors S5 and S6 were previously reported.[20] Anhydrous methanol (10 mL) was

added to 6''-deoxy-6''-triisopropylbenzylsulfonyl-(Boc)₄kanamycin A (S6) (325 mg,

0.28 mmol) in a pressure tube. The yellow solution was cooled to 0 °C and anhydrous

ammonia was bubbled into the solution for 10 mins. The vessel was capped and heated to

80 °C for 2 days. The vessel was cooled to 0 °C and opened. After 5 mins DOWEX$^®$

Monosphere$^®$ 550A ion exchange resin ($^-$OH form) was added. The reaction was stirred

for 12 hours at rt and filtered. The solvent was removed under reduced pressure and the

resulting solid was dissolved in DCM and washed with a 2 M sodium bicarbonate solution. The organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure. Product: White solid (227 mg, 0.26 mmol, 91% yield). ^{1}H-NMR (400 MHz, CD$_3$OD): δ 5.12 (s, 1H), 5.08 (s, 1H), 4.20 − 3.80 (m, 3H), 3.71 − 3.40 (m, 7H), 3.23 − 3.07 (m, 5H), 3.01 − 2.94 (m, 1H), 2.62 (q, J = 7 Hz, 1H), 2.16 (dt, J$_1$ = 18.4 Hz, J$_2$ = 8.4 Hz, 1H), 2.08 − 1.98 (m, 1H), 1.63 − 1.40 (m, 21H), 1.38 − 1.17 (m, 12H), 0.94 − 0.85 (m, 3H); HR-ESI-MS calculated for C$_{38}$H$_{69}$N$_6$O$_{18}$Na [M+Na]$^+$ 906.4530, found 906.4527

6''-Deoxy-6''-guanidino-(Boc)$_6$kanamycin A (S8). DCM (3 mL), methanol (0.6 mL), and TEA (65 µL, 0.45 mmol) were added to 6''-deoxy-6''-amino-(Boc)$_4$kanamycin A (S7) (200 mg, 0.23 mmol). 1,3-Di-boc-2-(trifluoromethylsulfonyl)guanidine (885 mg, 2.26 mmol) was added. The light yellow solution was stirred for 3 days. The solvent was removed under reduced pressure. The product was isolated by flash chromatography (0 − 7% methanol in DCM). Product: White solid (202 mg, 0.18 mmol, 79% yield). ^{1}H-NMR (400 MHz, CD$_3$OD): δ 5.12 (s, 1H), 5.06 (s, 1H), 3.88 − 3.82 (m, 1H), 3.77 − 3.65 (m, 3H), 3.63 − 3.30 (m, 12H), 3.13 (q, J = 6.6 Hz, 1H), 2.07 − 2.00 (m, 1H), 1.60 − 1.38 (m, 16H), 1.30 − 1.14 (m, 39H); HR-ESI-MS calculated for C$_{49}$H$_{88}$N$_7$O$_{22}$ [M+H]$^+$ 1126.5977, found 1126.5973

6''-Deoxy-6''-guanidinokanamycin A· 5 TFA (8). DCM (1.5 mL) and TIPS (0.1 mL) were added to 6''-deoxy-6''-guanidino-(Boc)$_6$kanamycin A (S8) (67 mg, 0.060 mmol).

TFA (1.5 mL) was added. The yellow solution was stirred for 2.5 hours. Toluene (3 mL) was added and the solvent was removed under reduced pressure. The remaining white solid was dissolved in water and purified by reverse phase HPLC (0 – 7% ACN in water (0.1% TFA) over 15 min) eluted after 6 min, then lyophilized. Product: White powder (56 mg, 0.051 mmol, 82% yield). ^{1}H-NMR (500 MHz, D_2O): δ 5.53 (d, J = 4 Hz, 1H), 5.05 (d, J = 4.2 Hz, 1H), 4.01 (dt, J_1 = 9.8 Hz, J_2 = 3.4 Hz, 1H), 3.97 – 3.94 (m, 1H), 3.90 (dd, J_1 = 10.9 Hz, J_2 = 3.8 Hz, 1H), 3.86 – 3.81 (m, 2H), 3.75 – 3.68 (m, 2H), 3.64 (dd, J_1 = 10 Hz, J_2 = 3.7 Hz, 1H), 3.58 – 3.45 (m, 5H), 3.40 – 3.34 (m, 2H), 3.20 (q, J = 6.8 Hz, 1H), 2.50 (dt, J_1 = 12.6 Hz, J_2 = 4 Hz, 1H), 1.87 (q, J = 12.6 Hz, 1H); ^{13}C-NMR (125 MHz, D_2O): δ 163.62 (J = 36 Hz), 158.37, 116.96 (J = 290 Hz), 101.42, 98.64, 84.25, 80.02, 73.95, 72.54, 71.78, 71.47, 71.17, 69.38, 68.70, 66.85, 55.41, 50.63, 48.63, 42.12, 40.78, 28.25; HR-ESI-MS calculated for $C_{19}H_{40}N_7O_{10}$ [M+H]$^{+}$ 526.2831, found 526.2826
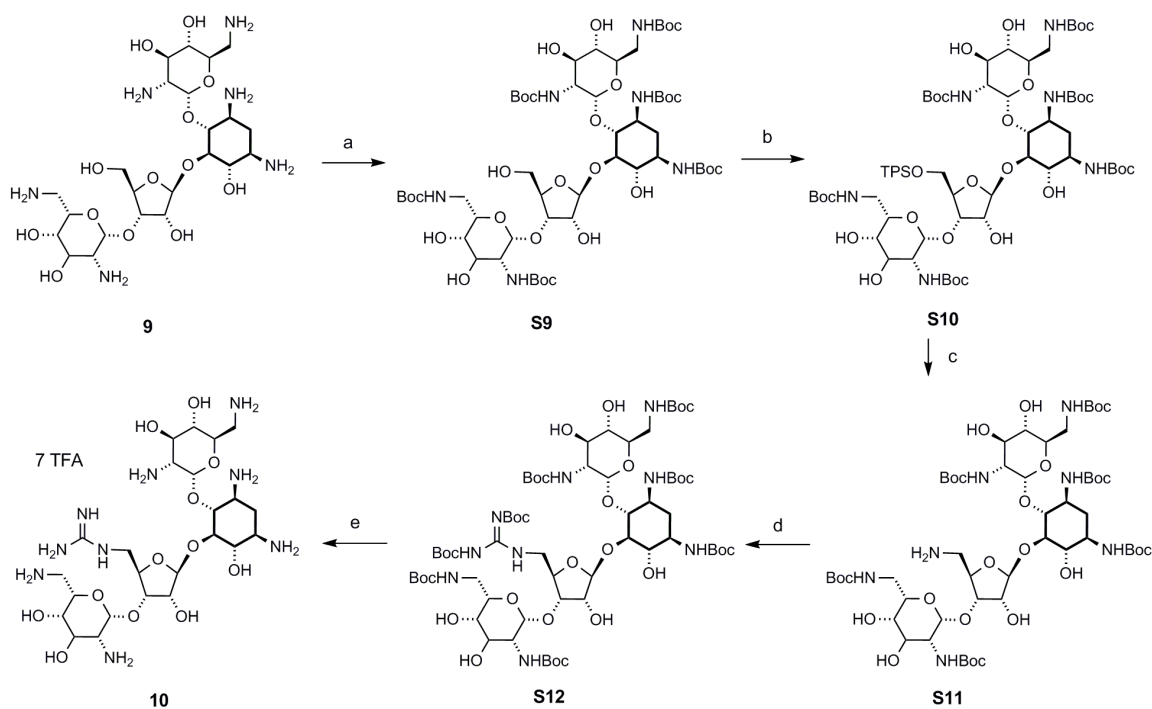
Scheme 2.7: 5"-Deoxy-5"-guanidinoneomycin synthesis. (a) Boc$_2$O, TEA, H$_2$O, DMF. (b) TPSCl, Pyridine. (c) NH$_3$, MeOH, (d) 1,3-Di-boc-2-(trifluoromethylsulfonyl)guanidine, TEA, DCM, MeOH. (e) TFA, TIPS, DCM.

5''-Deoxy-5''-guanidino-(Boc)₈neomycin (S12). Synthesis and characterization of

precursors S9 – S11 were previously reported.[21] DCM (2.5 mL), methanol (0.1 mL), and

TEA (40 μL, 0.28 mmol) were added to 5''-deoxy-5''-amino-(Boc)₆neomycin (S11) (226

mg, 0.19 mmol). 1,3-Di-boc-2-(trifluoromethylsulfonyl)guanidine (728 mg, 1.86 mmol)

was added. The yellow solution was stirred for 3 days. The solvent was removed under

reduced pressure. The product was isolated by flash chromatography (0 – 7% methanol in

DCM). Product: White solid (218 mg, 0.149 mmol, 80% yield). ^{1}H-NMR (400 MHz,

CD$_3$OD): δ 5.50 (s, 1H), 5.29 (s, 1H), 5.15 (s, 1H), 4.39 – 4.11 (m, 3H), 4.05 – 3.97 (m,

2H), 3.90 – 3.72 (m, 5H), 3.65 – 3.43 (m, 8H), 3.40 – 3.26 (m, 4H), 3.23 – 3.16 (m, 2H),

2.00 – 1.94 (m, 1H), 1.55 (s, 9H), 1.50 – 1.30 (m, 64H); HR-ESI-MS calculated for

C$_{64}$H$_{114}$N$_9$O$_{28}$ [M+H]$^+$ 1456.7768, found 1456.7771

5''-Deoxy-5''-guanidinoneomycin· 7 TFA (10). DCM (1.26 mL) and TIPS (65 μL) were added to 5''-deoxy-5''-guanidino-(Boc)$_8$neomycin (S12) (73 mg, 0.05 mmol). TFA (1.26 mL) was added. The yellow solution was stirred for 3 hours. Toluene (3 mL) was added and the solvent was removed under reduced pressure. The remaining white solid was dissolved in water and purified by reverse phase HPLC (5 – 8% ACN in water (0.1% TFA) over 10 min) eluted after 6 min, then lyophilized. Product: White powder (70 mg, 0.05 mmol, 96% yield). ^{1}H-NMR (500 MHz, D$_2$O): δ 6.08 (d, J = 4 Hz, 1H), 5.42 (d, J = 2.9 Hz, 1H), 5.30 (d, J = 1.7 Hz, 1H), 4.46 (t, J = 5.3 Hz, 1H), 4.41 (t, J = 3.8 Hz, 1H), 4.34 – 4.30 (m, 2H), 4.23 (t, J = 3.1 Hz, 1H), 4.12 (t, J = 9.5 Hz, 1H), 4.03 (t, J = 9.6 Hz, 1H), 3.97 – 3.92 (m, 2H), 3.84 (t, J = 1.6 Hz, 1H), 3.69 (t, J = 9.7 Hz, 1H), 3.62 – 3.54 (m, 3H), 3.49 – 3.27 (m, 9H), 2.49 (dt, J$_1$ = 12.6 Hz, J$_2$ = 4.2 Hz, 1H), 1.89 (q, J = 12.7 Hz, 1H); ^{13}C-NMR (125 MHz, D$_2$O): δ 163.63 (J = 35 Hz), 157.91, 117.00 (J = 290 Hz), 111.15, 96.17, 94.62, 85.88, 79.63, 77.89, 75.39, 73.86, 72.96, 71.28, 70.76, 70.56, 68.70, 68.24, 68.11, 53.91, 51.46, 50.28, 49.20, 44.63, 41.13, 40.87, 28.53; HR-ESI-MS calculated for C$_{24}$H$_{50}$N$_9$O$_{12}$ [M+H]$^+$ 656.3573, found 656.3571
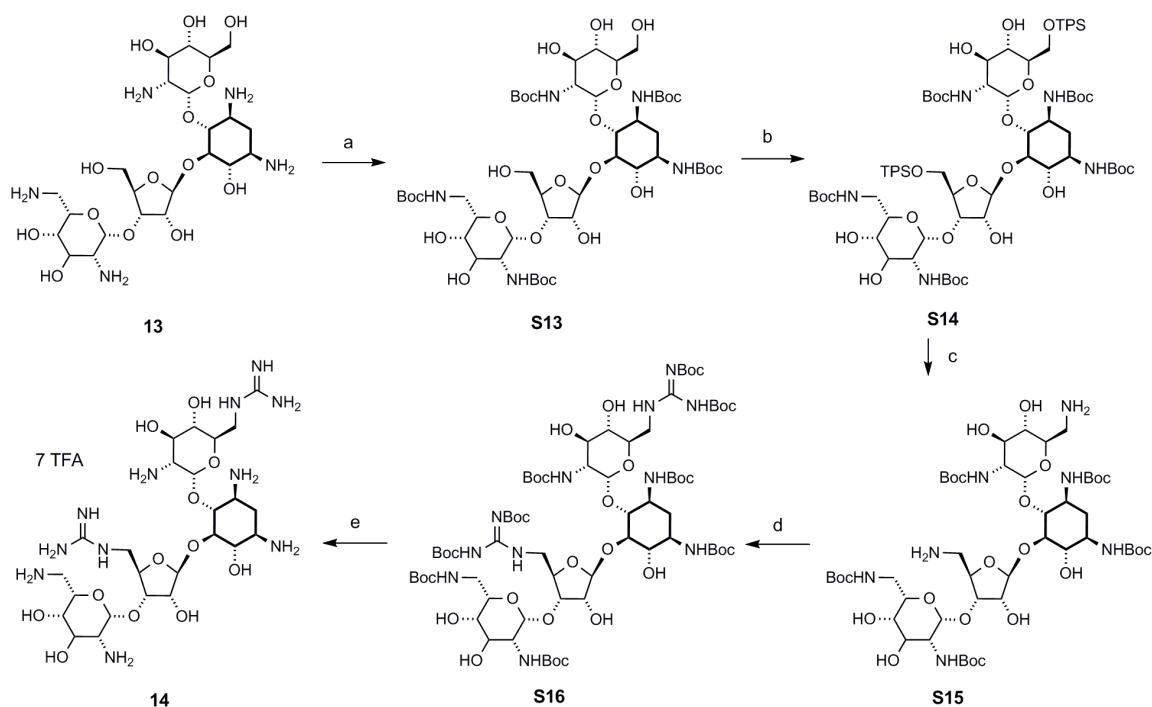
Scheme 2.8: 5'',6'-Dideoxy-5'',6'-diguanidinoparomomycin synthesis. (a) Boc_2O, TEA, H_2O, DMF. (b) TPSCl, Pyridine. (c) NH_3, MeOH, (d) 1,3-Di-boc-2-(trifluoromethylsulfonyl)guanidine, TEA, DCM, MeOH. (e) TFA, TIPS, DCM.

5'',6'-Dideoxy-5'',6'-di(triisopropylbenzylsulfonyl)-(Boc)₅paromomycin (S14).

Synthesis and characterization of precursor S13 was previously reported.[22] Anhydrous

pyridine (22 mL) was added to (Boc)₅paromomycin (S13) (1.63 g, 1.46 mmol).

Triisopropylbenzylsulfonyl chloride (8.84 g, 29.2 mmol) was added. The orange solution

was stirred for 2 days. The solvent was removed under reduced pressure. The product

was isolated by flash chromatography (0 – 4% methanol in DCM). Product: White solid

(1.36 g, 0.83 mmol, 57% yield). ^{1}H-NMR (400 MHz, CD₃OD): δ 7.31 (s, 2H), 7.30 (s,

2H), 5.43 (s, 1H), 5.17 – 5.13 (m, 2H), 4.40 – 4.08 (m, 11H), 4.01 – 3.95 (m, 1H), 3.87

(s, 1H), 3.82 – 3.72 (m, 2H), 3.63 – 3.20 (m, 11H), 3.15 (t, J = 6.8 Hz, 1H), 2.98 – 2.93

(m, 3H), 1.98 – 1.91 (m, 1H), 1.50 – 1.35 (m, 42H), 1.34 – 1.19 (m, 40H); HR-ESI-MS

calculated for $C_{78}H_{129}N_5O_{28}S_2Na$ [M+Na]$^+$ 1670.8158, found 1670.8154

5'',6'-Dideoxy-5'',6'-diamino-(Boc)₅paromomycin (S15). Anhydrous methanol (15.8

mL) was added to 5'',6'-dideoxy-5'',6'-di(triisopropylbenzylsulfonyl)-

(Boc)₅paromomycin (S14) (1.31 g, 0.83 mmol) in a pressure tube. The pale yellow

solution was cooled to 0 °C and anhydrous ammonia was bubbled into the solution for 10

mins. The vessel was capped and heated to 80 °C for 2 days. The vessel was cooled to 0

°C and opened. After 5 mins DOWEX® Monosphere® 550A ion exchange resin (⁻OH

form) was added. The reaction was stirred for 12 hours at rt and filtered. The solvent was

removed under reduced pressure and the resulting solid was dissolved in DCM and

washed with a 2 M sodium bicarbonate solution. The organic layer was dried over

sodium sulfate and the solvent was removed under reduced pressure. Product: Light

yellow solid (811 mg, 0.73 mmol, 88% yield). ^{1}H-NMR (400 MHz, CD₃OD): δ 5.48 (s,

1H), 5.22 – 5.11 (m, 2H), 4.26 – 4.08 (m, 5H), 3.98 – 3.86 (m, 4H), 3.76 (s, 2H), 3.70 –

3.65 (m, 1H), 3.60 – 3.45 (m, 6H), 3.17 – 2.95 (m, 4H), 2.86 – 2.79 (m, 1H), 2.67 (q, J =

6.8 Hz, 1H), 1.98 – 1.87 (m, 1H), 1.59 – 1.35 (m, 39H), 1.35 – 1.21 (m, 6H), 0.95 – 0.88

(m, 1H); HR-ESI-MS calculated for $C_{48}H_{88}N_7O_{22}$ [M+H]$^+$ 1114.5977, found 1114.5970

5'',6'-Dideoxy-5'',6'-diguanidino-(Boc)₉paromomycin (S16). DCM (9.2 mL),

methanol (0.5 mL), and TEA (0.29 mL, 2.05 mmol) were added to 5'',6'-dideoxy-5'',6'-

diamino-(Boc)₅paromomycin (S15) (761 mg, 0.68 mmol). 1,3-Di-boc-2-

(trifluoromethylsulfonyl)guanidine (1.80 g, 4.78 mmol) was added. The yellow solution

was stirred for 3 days. The solvent was removed under reduced pressure. The product

was isolated by flash chromatography (0 – 7% methanol in DCM). Product: White solid (770 mg, 0.482 mmol, 71% yield). ^{1}H-NMR (400 MHz, CD$_3$OD): δ 5.47 (s, 1H), 5.18 – 5.14 (m, 2H), 4.39 – 4.05 (m, 6H), 4.03 – 3.97 (m, 2H), 3.91 – 3.22 (m, 16H), 2.06 – 1.96 (m, 1H), 1.61 – 1.51 (m, 13H), 1.51 – 1.36 (m, 60H), 1.31 – 1.23 (m, 9H); HR-ESI-MS calculated for C$_{70}$H$_{124}$N$_{11}$O$_{30}$ [M+H]$^+$ 1598.8510, found 1598.8505

5'',6'-Dideoxy-5'',6'-diguanidinoparomomycin· 7 TFA (14). DCM (3 mL) and TIPS (0.15 mL) were added to 5'',6'-dideoxy-5'',6'-diguanidino-(Boc)$_9$paromomycin (S16) (183 mg, 0.12 mmol). TFA (3 mL) was added. The yellow solution was stirred for 3 hours. Toluene (6 mL) was added and the solvent was removed under reduced pressure. The remaining white solid was dissolved in water and purified by reverse phase HPLC (0 – 0.1% ACN in water (0.1% TFA) over 10 min) eluted after 8.5 min, then lyophilized. Product: White powder (149 mg, 0.10 mmol, 82% yield). ^{1}H-NMR (300 MHz, D$_2$O): δ 5.95 (d, J = 3.9 Hz, 1H), 5.38 (d, J = 3 Hz, 1H), 5.27 (s, 1H), 4.42 (t, J = 5.4 Hz, 1H), 4.37 – 4.35 (m, 1H), 4.32 – 4.24 (m, 2H), 4.19 (t, J = 3 Hz, 1H), 4.09 – 3.87 (m, 3H), 3.84 – 3.76 (m, 2H), 3.67 (t, J = 9.6 Hz, 1H), 3.60 – 3.47 (m, 5H), 3.45 – 3.25 (m, 6H), 2.46 (dt, J$_1$ = 12.6 Hz, J$_2$ = 4 Hz, 1H), 1.85 (q, J = 12.6 Hz, 1H); ^{13}C-NMR (125 MHz, D$_2$O): δ 163.60 (J = 37 Hz), 158.30, 157.95, 116.99 (J = 290 Hz), 110.82, 96.19, 94.99, 85.54, 79.58, 77.76, 75.75, 73.84, 72.91, 72.83, 70.77, 70.09, 69.14, 68.23, 68.09, 54.11, 51.48, 50.29, 49.28, 44.45, 42.14, 41.12, 28.52; HR-ESI-MS calculated for C$_{25}$H$_{52}$N$_{11}$O$_{12}$ [M+H]$^+$ 698.3791, found 698.3785
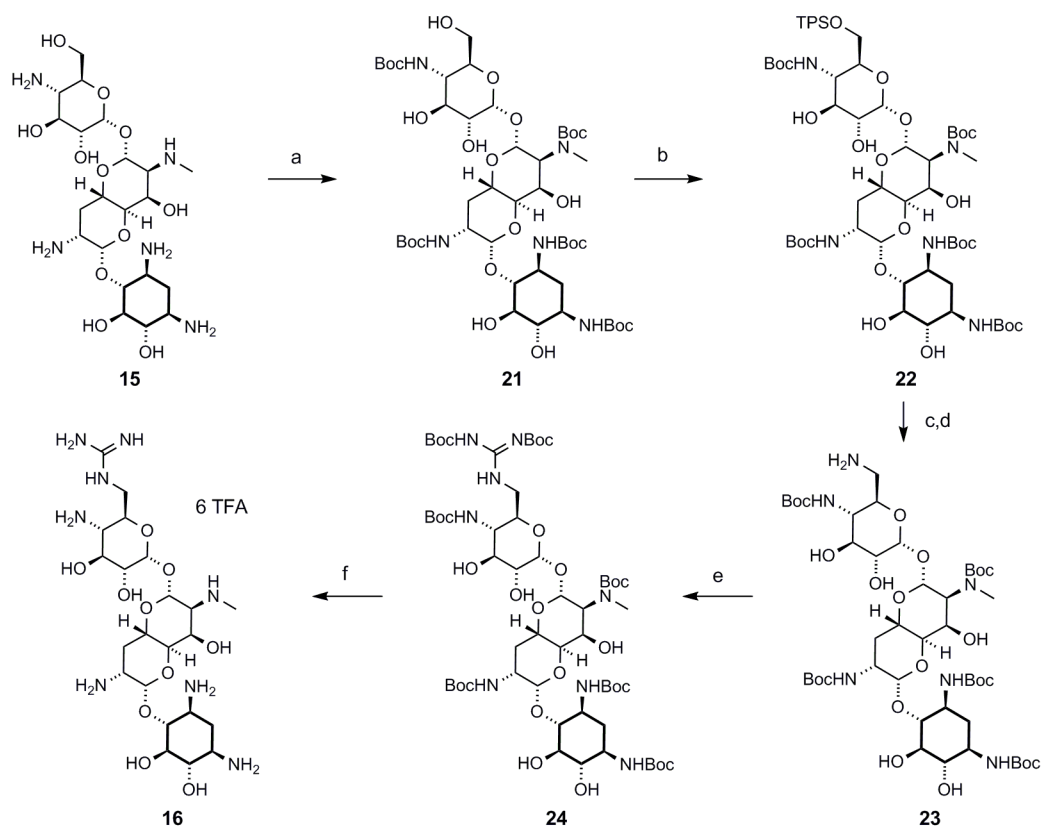
Scheme 2.9: 6"-Deoxy-6"-guanidinoapramycin synthesis. (a) Boc₂O, TEA, H₂O, DMF. (b) TPSCl, Pyridine. (c) NaN₃, DMF. (d) Pd/C, H₂, MeOH (e) 1,3-Di-boc-2-(trifluoromethylsulfonyl)guanidine, TEA, DCM, MeOH. (f) TFA, TIPS, DCM.

(Boc)₅Apramycin (21). Water (1.9 mL), DMF (2.1 mL), and TEA (1.2 mL, 11.76 mmol) were added to apramycin sulfate (15) (500 mg, 0.78 mmol). The reaction was heated to 55 °C and di-tert-butyl dicarbonate (1.03 g, 4.70 mmol) dissolved in DMF (7.5 mL) was added slowly. The yellow solution was stirred for 8 hours. The solvent was removed under reduced pressure and the resulting solid was suspended in warm water. The solid was filtered and washed thoroughly with water. The product was dissolved in ACN and the solvent was removed under reduced pressure. Product: White solid (749 mg, 0.72 mmol, 92% yield). ^{1}H-NMR (400 MHz, CD₃OD): δ 5.52 (s, 1H), 5.32 – 5.30 (m, 2H), 4.18 (s, 1H), 4.01 (d, J = 7.2 Hz, 1H), 3.81 (t, J = 7.6 Hz, 1H), 3.69 – 3.34 (m, 10H), 3.14 (t, J = 9 Hz, 2H), 3.05 (s, 3H), 2.06 – 2.01 (m, 1H), 1.98 – 1.92 (m, 1H), 1.71 (q, J = 11.6

Hz, 1H), 1.52 – 1.28 (m, 46H); HR-ESI-MS calculated for $C_{46}H_{81}N_5O_{21}Na$ $[M+Na]^+$ 1062.5316, found 1062.5319.

6''-Deoxy-6''-triisopropylbenzylsulfonyl-(Boc)₅apramycin (22). Anhydrous pyridine (4.6 mL) was added to (Boc)₅apramycin (21) (420 mg, 0.40 mmol). Triisopropylbenzylsulfonyl chloride (1.22 g, 4.03 mmol) was added. The orange solution was stirred for 36 hours. The solvent was removed under reduced pressure. The product was isolated by flash chromatography (0 – 5% methanol in DCM). Product: White solid (160 mg, 0.12 mmol, 31% yield). ^{1}H-NMR (500 MHz, CD₃OD): δ 7.29 (s, 2H), 5.54 (s, 1H), 5.29 (d, J = 9 Hz, 1H), 5.26 (d, J = 3.5 Hz, 1H), 4.20 – 4.12 (m, 3H), 4.03 (d, J = 7 Hz, 1H), 3.93 (d, J = 8 Hz, 1H), 3.82 (q, J = 9.5 Hz, 1H), 3.72 – 3.68 (m, 1H), 3.62 (q, J = 9.5 Hz, 1H), 3.53 – 3.36 (m, 4H), 3.17 (q, J = 10.5 Hz, 1H), 3.09 – 3.04 (m, 2H), 3.00 (s, 3H), 2.99 – 2.93 (m, 3H), 2.08 – 2.03 (m, 1H), 2.02 – 1.95 (m, 1H), 1.72 (q, J = 12 Hz, 1H), 1.54 – 1.50 (m, 8H), 1.50 – 1.44 (m, 29H), 1.40 – 1.37 (s, 9H), 1.28 – 1.26 (m, 18H); HR-ESI-MS calculated for $C_{61}H_{103}N_5O_{23}SNa$ $[M+Na]^+$ 1328.6657, found 1328.6649

6''-Deoxy-6''-amino-(Boc)₅apramycin (23). DMF (2.5 mL) was added to 6''-deoxy-6''-triisopropylbenzylsulfonyl-(Boc)₅apramycin (22) (160 mg, 0.12 mmol). Sodium azide (64 mg, 0.98 mmol) was added. The yellow solution was heated to 55 °C and stirred for 2 days. The solvent was removed under reduced pressure and the resulting solid was dissolved in DCM and washed with water. The organic layers were dried with sodium

sulfate and the solvent was removed under reduced pressure. Anhydrous methanol (1.1 mL) and acetic acid (10 µL) were added to the resulting white solid. The solution was degassed by bubbling through argon. Pd/C (10%, 14 mg, 0.013 mmol) was added and the reaction was stirred under atmospheric H_2 overnight. The solution was filtered through celite and the solvent was removed under reduced pressure. The product was isolated by flash chromatography (10% methanol, 1% TEA in DCM). Product: White solid (102 mg, 0.098 mmol, 80% yield). ^{1}H-NMR (400 MHz, CD_3OD): δ 5.47 (s, 1H), 5.30 (s, 1H), 5.27 (s, 1H), 4.24 – 4.14 (m, 3H), 4.02 – 3.94 (m, 2H), 3.86 – 3.31 (m, 6H), 3.17 – 3.09 (m, 2H), 3.03 (s, 3H), 2.94 (q, J = 6.6 Hz, 1H), 2.80 – 2.63 (m, 1H), 2.08 – 1.89 (m, 3H), 1.52 – 1.04 (m, 44H), 0.97 – 0.89 (m, 2H); HR-ESI-MS calculated for $C_{46}H_{83}N_6O_{20}$ $[M+H]^+$ 1039.5657, found 1039.5658

6''-Deoxy-6''-guanidino-(Boc)₇apramycin (24). DCM (0.7 mL), methanol (0.14 mL), and TEA (15 µL, 0.11 mmol) were added to 6''-deoxy-6''-amino-(Boc)₅apramycin (23) (55 mg, 0.05 mmol). 1,3-Di-boc-2-(trifluoromethylsulfonyl)guanidine (207 mg, 0.53 mmol) was added. The light yellow solution was stirred for 3 days. The solvent was removed under reduced pressure. The product was isolated by flash chromatography (0 – 6% methanol in DCM). Product: White solid (50 mg, 0.04 mmol, 74% yield). ^{1}H-NMR (400 MHz, CD_3OD): δ 5.39 (s, 1H), 5.28 (s, 1H), 5.13 (s, 1H), 4.22 – 4.15 (m, 2H), 4.02 – 3.96 (m, 2H), 3.86 – 3.80 (m, 2H), 3.70 – 3.35 (m, 6H), 3.22 – 3.14 (m, 3H), 2.97 (s, 3H), 2.08 – 1.95 (m, 3H), 1.74 – 1.68 (m, 1H), 1.55 (s, 6H), 1.49 – 1.39 (m, 36H), 1.32 –

1.26 (m, 21H); HR-ESI-MS calculated for $C_{57}H_{101}N_8O_{24}$ [M+H]$^+$ 1281.6923, found 1281.6925

6''-Deoxy-6''-guanidinoapramycin· 6 TFA (16). DCM (1 mL) and TIPS (50 μL) were added to 6''-deoxy-6''-guanidino-(Boc)$_6$kanamycin A (24) (50 mg, 0.039 mmol). TFA (1 mL) was added. The yellow solution was stirred for 2 hours. Toluene (2 mL) was added and the solvent was removed under reduced pressure. The remaining white solid was dissolved in water and purified by reverse phase HPLC ($0 - 0.1\%$ ACN in water (0.1% TFA) over 12 min) eluted after 9.6 min, then lyophilized. Product: White powder (38 mg, 0.030 mmol, 78% yield). ^{1}H-NMR (500 MHz, D$_2$O): δ 5.76 (d, J = 4 Hz, 1H), 5.59 (d, J = 3.5 Hz, 1H), 5.25 (d, J = 8.5 Hz, 1H), 4.61 (t, J = 2 Hz, 1H), 4.17 (dq, J$_1$ = 9.5 Hz, J$_2$ = 3 Hz, 1H), 3.99 – 3.92 (m, 3H), 3.80 (dd, J$_1$ = 9.5 Hz, J$_2$ = 2.3 Hz, 1H), 3.76 (dd, J$_1$ = 9.5 Hz, J$_2$ = 4 Hz, 1H), 3.71 – 3.67 (m, 3H), 3.60 – 3.54 (m, 3H), 3.42 (dd, J$_1$ = 8.5 Hz, J$_2$ = 3 Hz, 1H), 3.37 – 3.30 (m, 2H), 2.83 (s, 3H), 2.53 (dt, J$_1$ = 12.5 Hz, J$_2$ = 4 Hz, 1H), 2.40 (dt, J$_1$ = 11.5 Hz, J$_2$ = 4 Hz, 1H), 2.07 (q, J = 11.5 Hz, 1H), 1.89 (q, J = 12.5 Hz, 1H); ^{13}C-NMR (125 MHz, D$_2$O): δ 163.66 (J = 35 Hz), 158.58, 116.99 (J = 290 Hz), 96.23, 94.88, 93.60, 78.83, 75.77, 73.20, 70.63, 70.38, 69.45, 68.71, 66.83, 63.41, 60.15, 57.99, 52.50, 50.32, 49.09, 48.60, 30.73, 28.98, 27.44; HR-ESI-MS calculated for $C_{22}H_{45}N_8O_{10}$ [M+H]$^+$ 581.3253, found 581.3250

Amine to Guanidinium Conversions

6'-guanidinoneamine (12) was previously synthesized.[23]
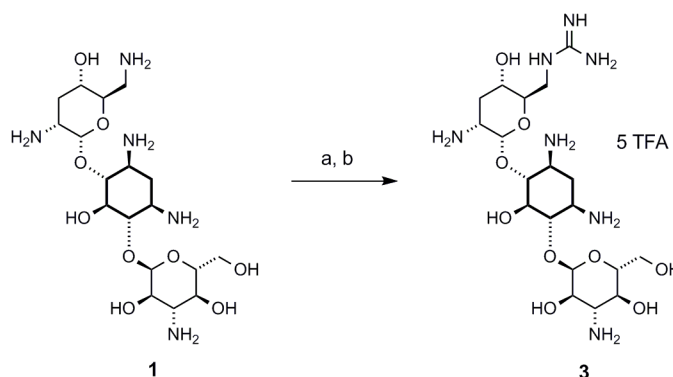
Scheme 2.10: 6'-Guanidinotobramycin synthesis. (a) 1,3-Di-boc-2-(trifluoromethylsulfonyl)guanidine, TEA, DCM, MeOH. (b) TFA, TIPS, DCM.

6'-Guanidinotobramycin· 5 TFA (3). Water (2.6 mL), methanol (3.4 mL), and TEA (38 μL, 0.29 mmol) were added to tobramycin · 5 TFA (1) (60 mg, 0.058 mmol). 1,3-Di-boc-2-(trifluoromethylsulfonyl)guanidine (16 mg, 0.041 mmol) was added. The light yellow solution was stirred for 5 days. The solvent was removed under reduced pressure. DCM (1.5 mL) and TIPS (80 μL) were added to the remaining solid. TFA (1.5 mL) was added. The pale yellow solution was stirred for 2 hours. Toluene (3 mL) was added and the solvent was removed under reduced pressure. The remaining white solid was dissolved in water and purified by reverse phase HPLC (0 – 0.1% ACN in water (0.1% TFA) over 14 min) eluted after 10.2 min, then lyophilized. Product: White powder (14 mg, 0.013 mmol, 22% yield). ^{1}H-NMR (400 MHz, D$_2$O): δ 5.64 (d, J = 4 Hz, 1H), 5.10 (d, J = 3.6 Hz, 1H), 3.97 – 3.88 (m, 3H), 3.87 – 3.82 (m, 3H), 3.79 – 3.70 (m, 4H), 3.68 – 3.53 (m 5H), 3.47 (t, J = 10.6 Hz, 1H), 2.55 (dt, J$_1$ = 12.8 Hz, J$_2$ = 4.2 Hz, 1H), 2.29 (dt, J$_1$ = 12 Hz, J$_2$ = 4.2 Hz, 1H), 2.02 – 1.88 (m, 2H); ^{13}C-NMR (100 MHz, D$_2$O): δ 163.63 (J = 35 Hz), 158.31, 116.95 (J = 290 Hz), 101.32, 95.54, 84.44, 78.88, 74.62, 73.57, 73.42, 68.60, 65.88, 63.90, 60.39, 55.45, 50.15, 48.96, 48.52, 41.83, 30.09, 28.40; HR-ESI-MS calculated for C$_{19}$H$_{40}$N$_7$O$_9$ [M+H]$^+$ 510.2882, found 510.2878
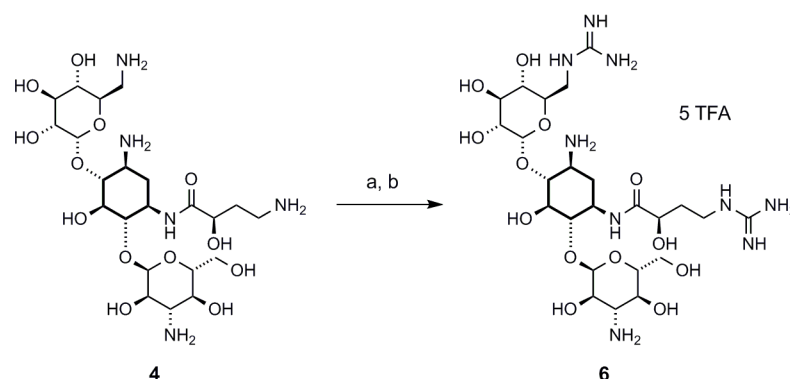
Scheme 2.11: 6',γ-Diguanidinoamikacin synthesis. (a) 1,3-Di-boc-2-(trifluoromethylsulfonyl)guanidine, TEA, DCM, MeOH. (b) TFA, TIPS, DCM.

6', γ-Diguanidinoamikacin· 4 TFA (6). Water (15.5 mL), methanol (20.1 mL), and TEA (0.23 mL, 1.72 mmol) were added to amikacin sulfate (4) (300 mg, 0.34 mmol). 1,3-Di-boc-2-(trifluoromethylsulfonyl)guanidine (245 mg, 0.62 mmol) was added. The light yellow solution was stirred for 5 days. The solvent was removed under reduced pressure. DCM (8.6 mL) and TIPS (0.5 mL) were added to the remaining solid. TFA (8.6 mL) was added. The pale yellow solution was stirred for 2 hours. Toluene (17 mL) was added and the solvent was removed under reduced pressure. The remaining white solid was dissolved in water and purified by reverse phase HPLC (0 − 0.1% ACN in water (0.1% TFA) over 13 min) eluted after 8.2 min, then lyophilized. Product: White powder (48 mg, 0.042 mmol, 12% yield). ^{1}H-NMR (300 MHz, D$_2$O): δ 5.48 (d, J = 3.6 Hz, 1H), 5.15 (d, J = 3.6 Hz, 1H), 4.17 (dd, J$_1$ = 9.6 Hz, J$_2$ = 3.3 Hz, 1H), 4.11 − 4.05 (m, 2H), 3.93 − 3.60 (m, 10H), 3.53 − 3.47 (m, 3H), 3.42 − 3.31 (m, 4H), 2.20 (dt, J$_1$ = 12.6 Hz, J$_2$ = 4 Hz, 1H), 2.13 − 2.03 (m, 1H), 1.87 − 1.71 (m, 2H); ^{13}C-NMR (75 MHz, D$_2$O): δ 177.35, 163.60 (J = 38 Hz), 158.33, 157.46, 116.94 (J = 290 Hz), 98.01, 97.80, 80.54, 79.89, 73.62, 72.84, 71.94, 71.56, 70.27, 69.58, 68.65, 65.96, 65.55, 60.10, 55.86, 49.35, 48.84,

42.40, 38.38, 33.03, 30.85; HR-ESI-MS calculated for $C_{24}H_{48}N_9O_{13}$ [M+H]$^+$ 670.3366, found 670.3365

2-D NMR

Tobramycin (1), 6'-guanidinotobramycin (3), amikacin (4), and 6',γ-diguanidinoamikacin (6) were fully assigned by COSY and chemical shifts were compared to verify that only amines at primary sites were converted to guanidinium groups. It should be noted that all 2-D NMR spectra were performed on desalted aminoglycosides, unlike the 1-D ^{1}H NMR spectra, which were taken on TFA salts.
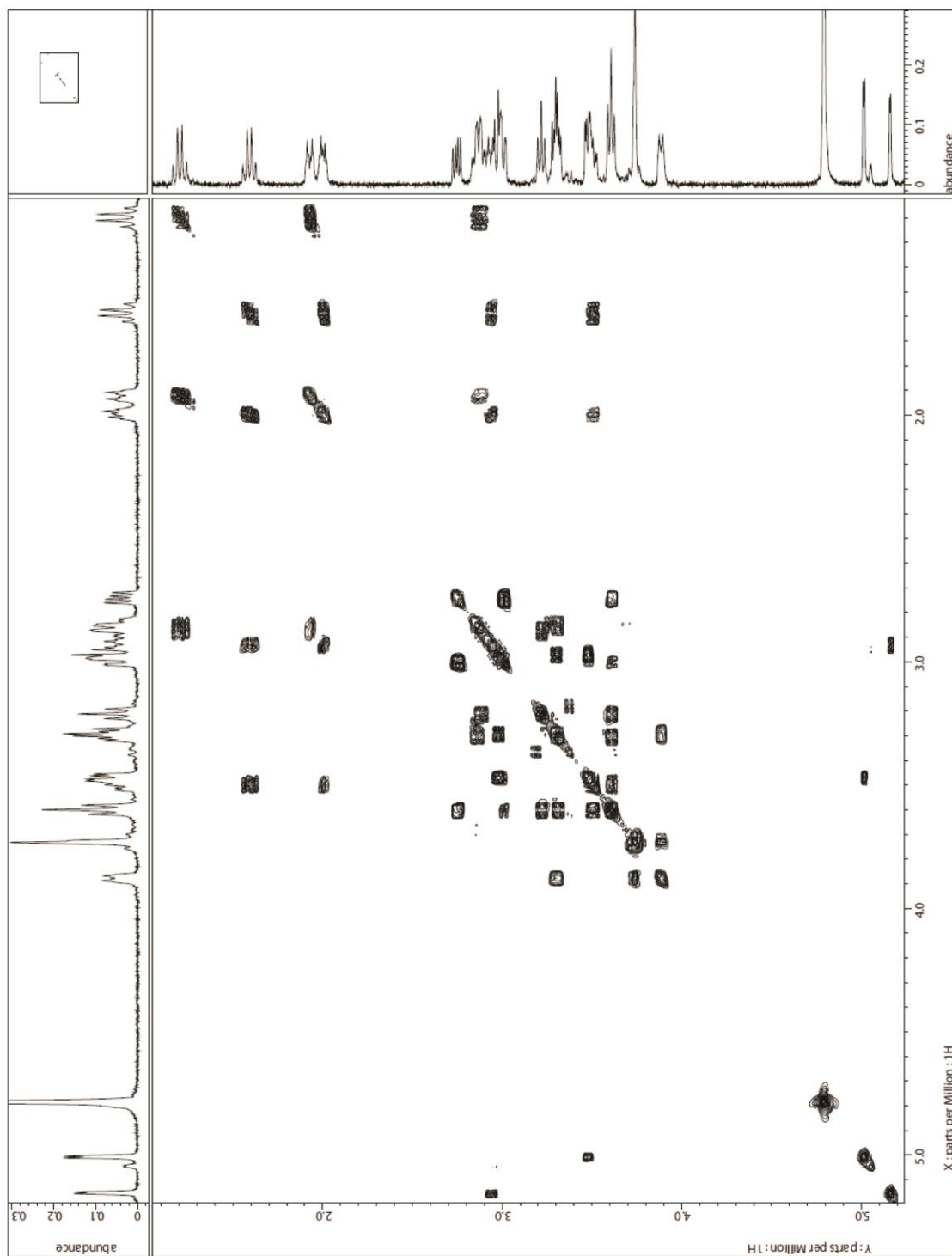
Figure 2.4: Tobramycin (1) COSY
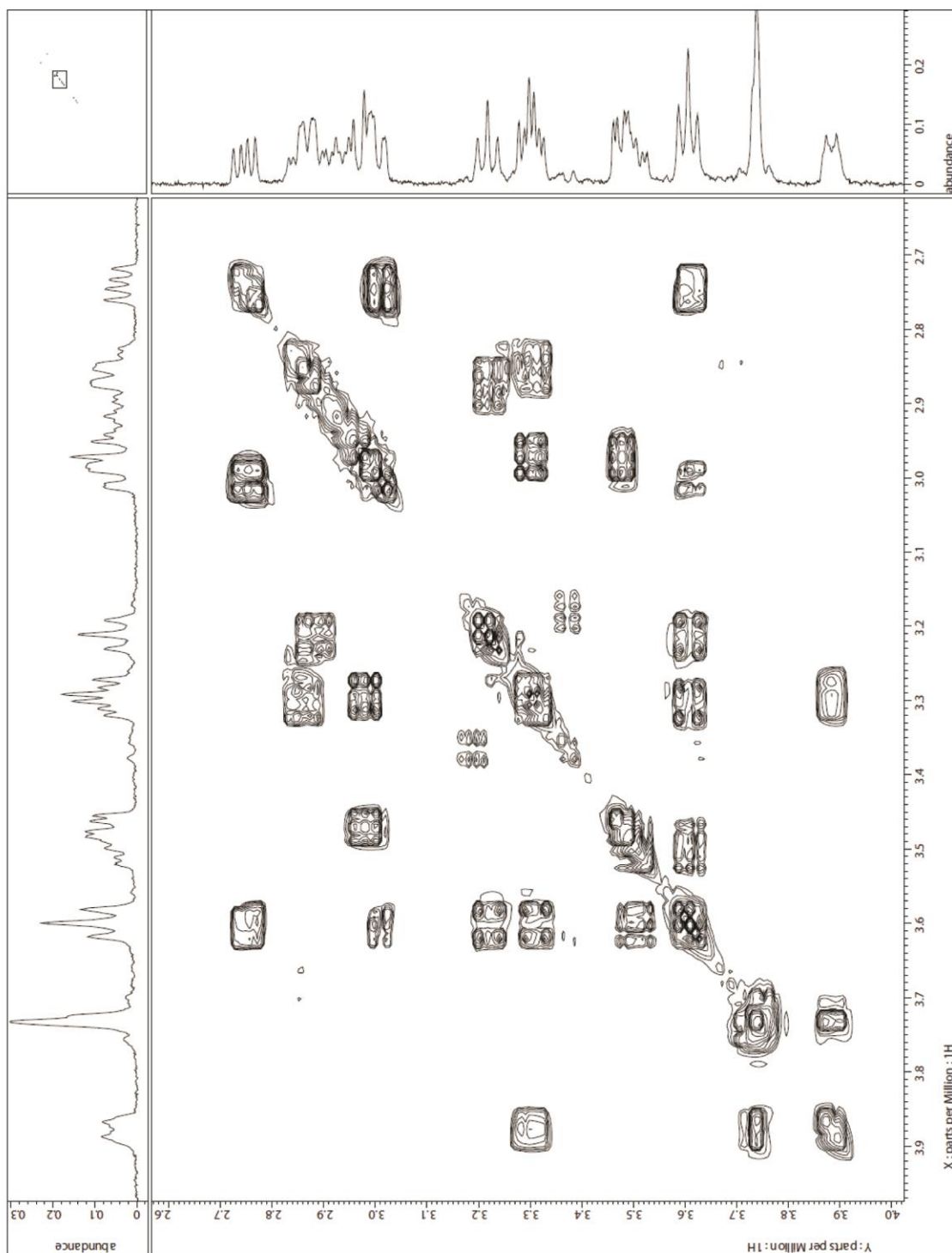
Figure 2.5: Tobramycin (1) COSY expanded
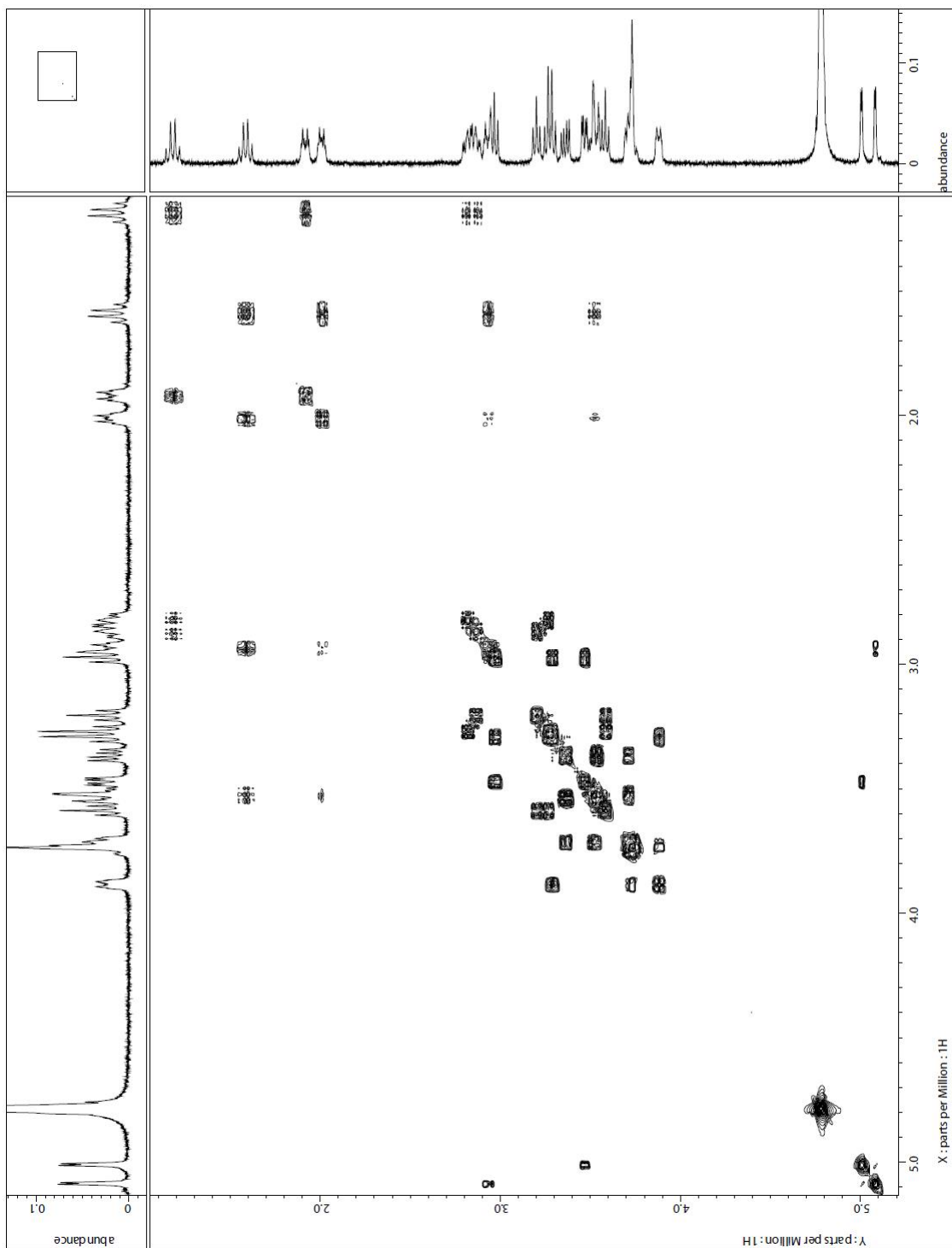
Figure 2.6: 6'-Guanidinotobramycin (3) COSY

Figure 2.7: 6'-Guanidinotobramycin (3) COSY expanded

Table 2.4: ^{1}H NMR Chemical Shift Comparison

Proton(s)	Tobramycin (1)	6'-Guanidinotobramycin (3)	Δ ppm
1'	5.14	5.09	-0.05
2'	2.92	2.94	0.02
3'	1.57	1.59	0.02
	1.98	2.01	0.03
4'	3.48	3.54	0.06
5'	3.58	3.72	0.14
6'	2.73	3.37	0.64
	2.99	3.55	0.56
1	2.84	2.85	0.01
2	1.39	1.20	-0.19
	1.90	1.92	0.02
3	2.86	2.87	0.01
4	3.19	3.22	0.03
5	3.59	3.59	0.00
6	3.28	3.27	-0.01
1"	5.00	5.01	0.01
2"	3.45	3.48	0.03
3"	2.96	2.97	0.01
4"	3.28	3.29	0.01
5"	3.87	3.89	0.01
6" (2H)	3.72	3.74	0.02

Figure 2.8: Amikacin (4) COSY

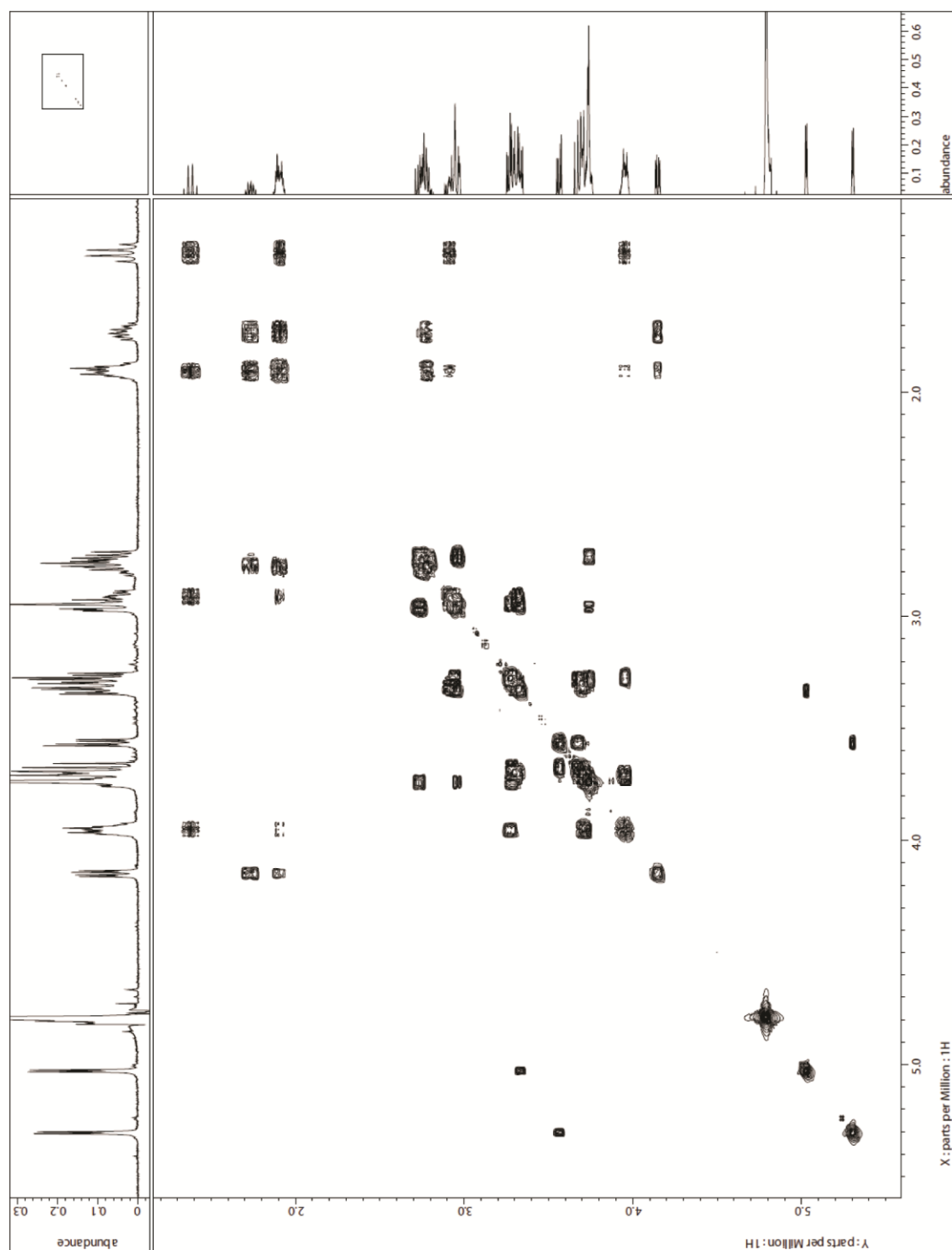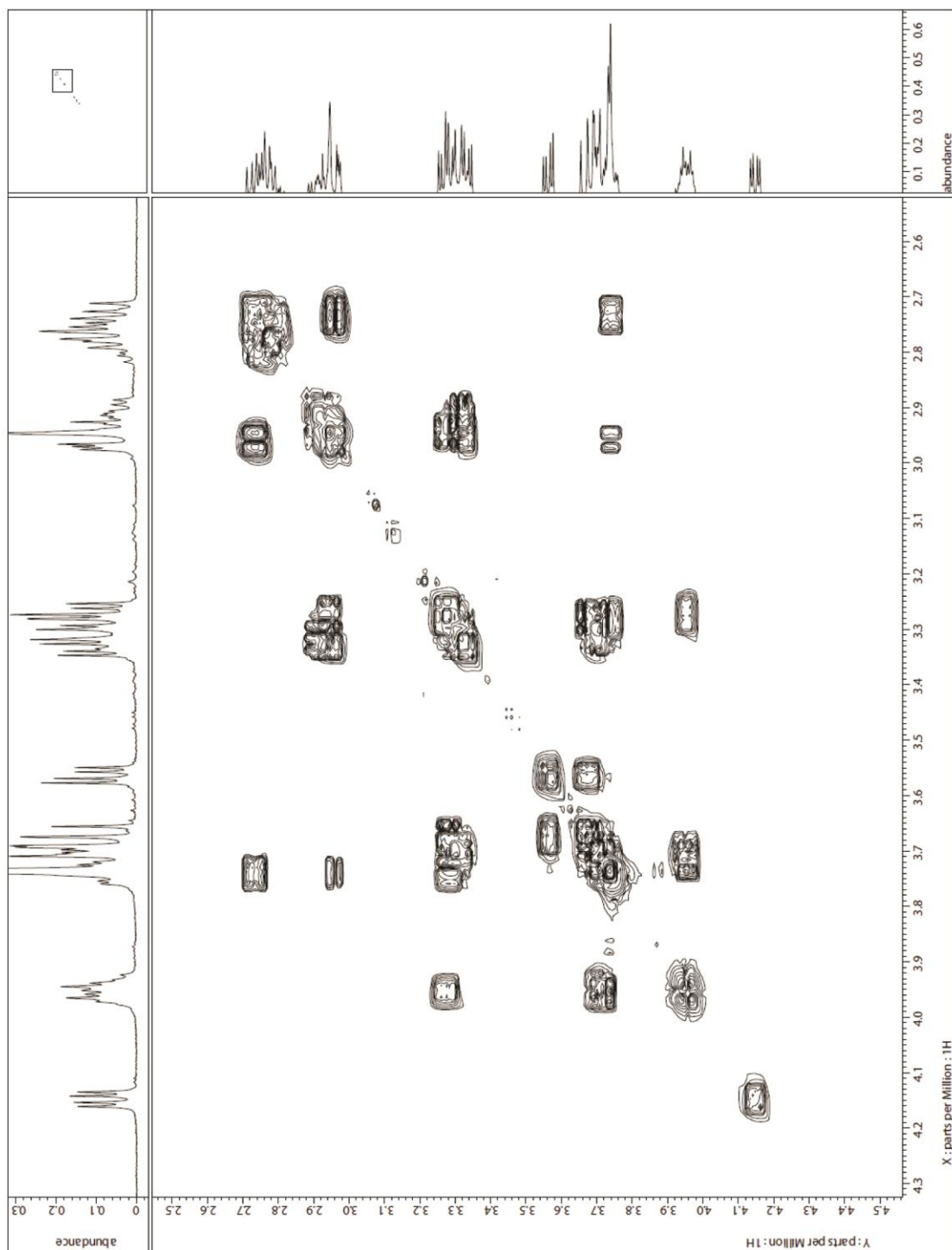**Figure 2.9:** Amikacin (4) COSY expanded
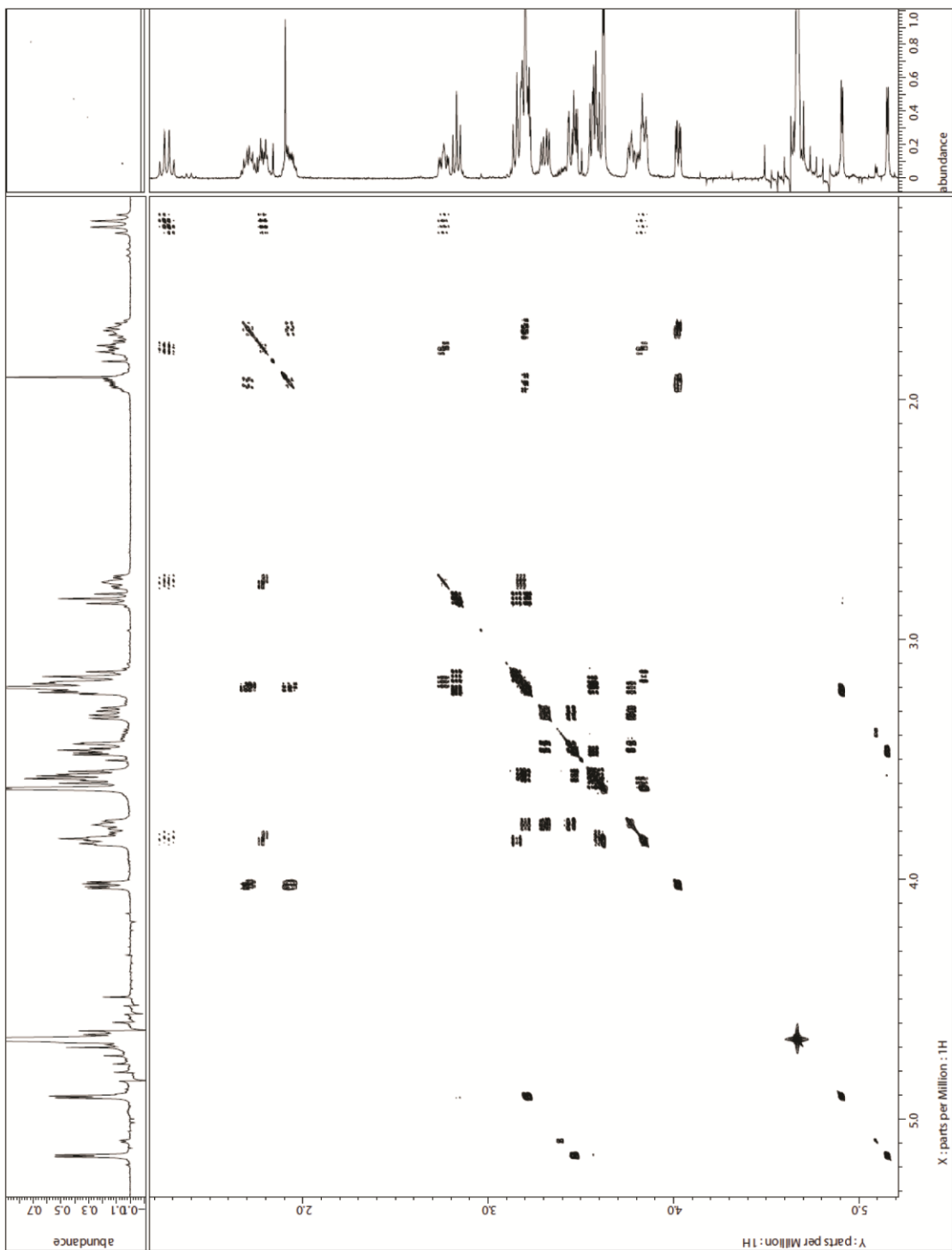
Figure 2.10: 6',γ-Diguanidinoamikacin (6) COSY
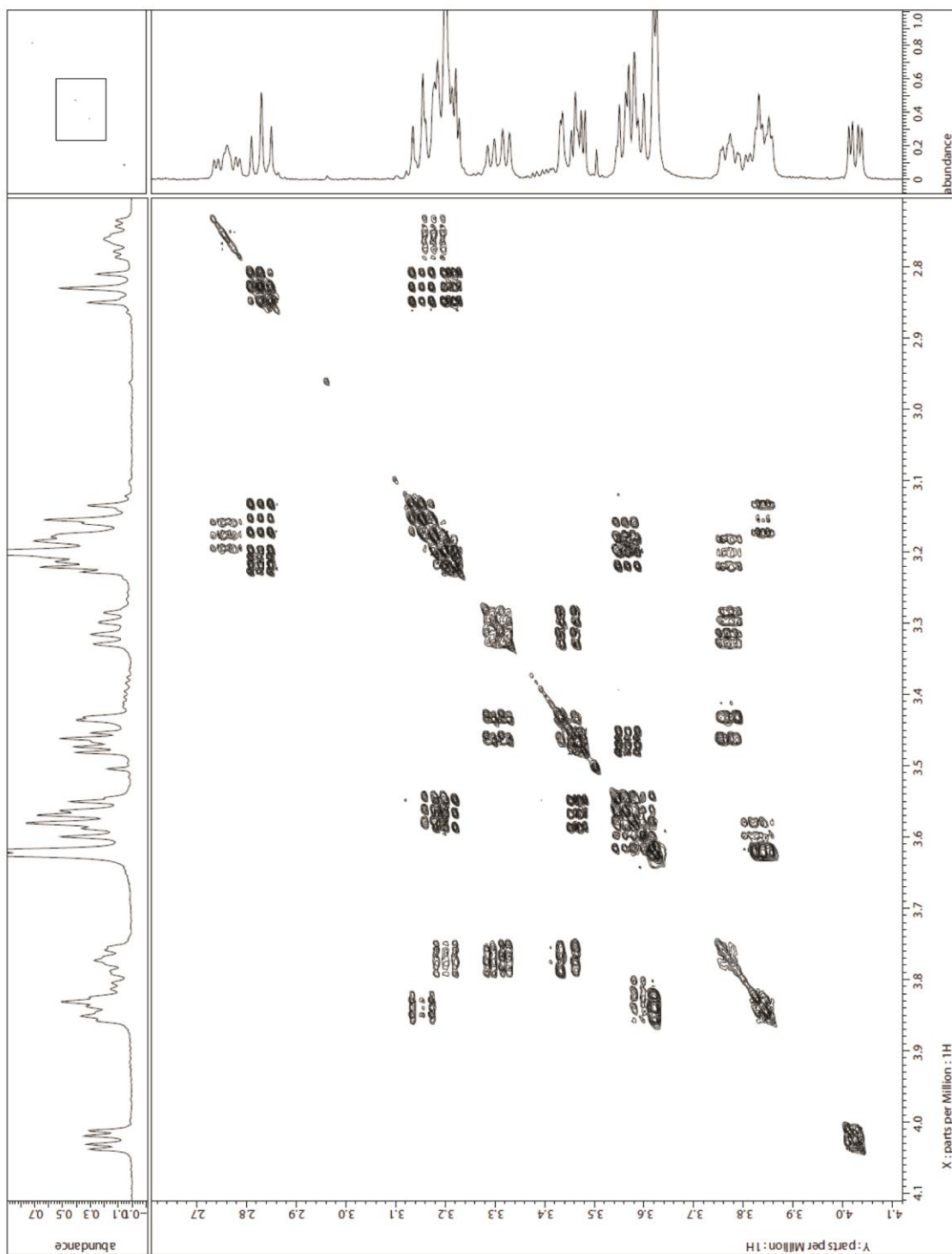
Figure 2.11: 6',γ-Diguanidinoamikacin (6) COSY expanded

Table 2.5: ^{1}H NMR Chemical Shift Comparison

Proton(s)	Amikacin (4)	6',γ-Diguanidinoamikacin (6)	Δ ppm
1'	5.30	5.28	-0.02
2'	3.57	3.60	0.03
3'	3.67	3.70	0.03
4'	3.29	3.33	0.04
5'	3.74	3.91	0.17
6'	2.74	3.43	0.69
	2.96	3.57	0.61
1	3.96	3.96	0.00
2	1.37	1.39	0.02
	1.90	1.92	0.02
3	2.91	2.88	-0.03
4	3.31	3.31	0.00
5	3.71	3.70	-0.01
6	3.72	3.74	0.02
1"	5.03	5.03	0.00
2"	3.34	3.34	0.00
3"	2.93	2.95	0.02
4"	3.28	3.28	0.00
5"	3.97	3.97	0.00
6" (2H)	3.72	3.75	0.03
α	4.14	4.15	0.01
β	1.73	1.83	0.10
	1.90	2.07	0.17
γ (2H)	2.78	3.33	0.55

Desalting

Guanidino-aminoglycoside·TFA (up to 40 mg) was dissolved in autoclaved H_2O (0.6 mL) in a sterile eppendorf tube. Monosphere 550 A (75 mg) was added, and the suspension was shaken lightly on a Fisher Vortex Genie 2 overnight. The resin was removed by centrifugal filtration and washed twice with autoclaved H_2O. The desalted solutions were lyophilized, and the removal of TFA counterions was confirmed by ^{13}C NMR spectroscopy.

A-Site Binding assay

Aminoglycoside Titrations

All titrations were performed with working solutions of 1 μM Dy-547 labeled A-site in 20 mM cacodylate buffer (pH = 7.0, 100 mM NaCl, 0.5 mM EDTA). The solutions were heated to 75 °C for 5 min, cooled to room temperature over 2 h, cooled to 0 °C for 30 min, then allowed to warm back to room temperature. Kanamycin-courmarin or neomycin-coumarin was added, to give a working concentration of 0.53 μM, just prior to aminoglycoside titrations. Steady state fluorescence experiments were carried out at ambient temperature (20 °C). Excitation and emission slit widths were 9 nm for kanamycin-coumarin experiments and 7 nm for neomycin-coumarin. The system was excited at 400 nm and changes in Dy-547 emission were monitored at 561 nm. Errors were generated from three sets of measurements. IC_{50} values were calculated using OriginPro 8.5 software by fitting a dose response curve (eq 1) to the fractional fluorescence saturation (F_s) plotted against the log of antibiotic (A) concentration.

$$F_s = F_{0 + (F_\infty}[A]^n)/([IC_{50}]^n + [A]^n) \qquad (1)$$

F_s is the fluorescence intensity at each titration point. F_0 and F_∞ are the fluorescence intensity in the absence of aminoglycoside or at saturation, respectively, and n is the Hill coefficient or degree of cooperativity associated with binding. A representative binding curve pictured and the raw fluorescence data used to generate the curve are pictured (Figure 2.12). The binding curves of all compounds are pictured (Figure 2.13 and 2.14).
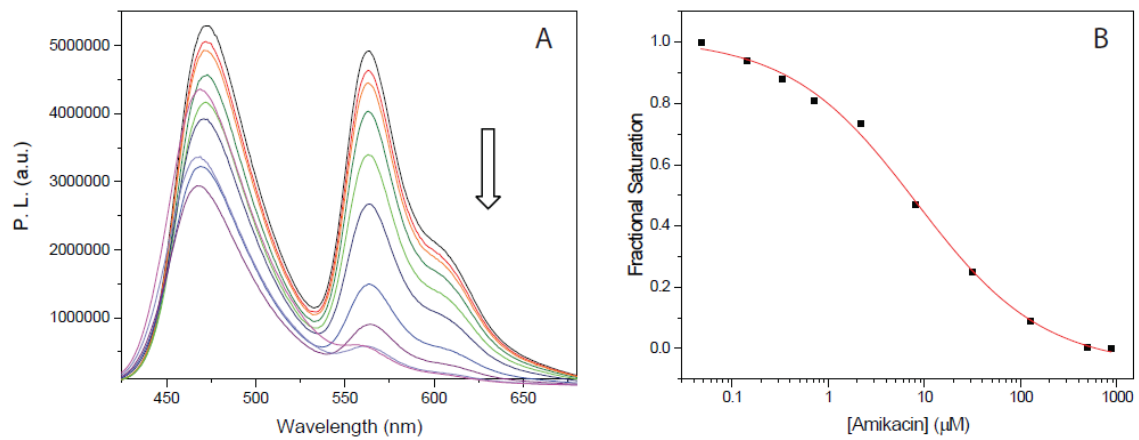
Figure 2.12: Raw fluorescence data (A) and normalized binding curve (B) for amikacin (**4**)

Binding Curves
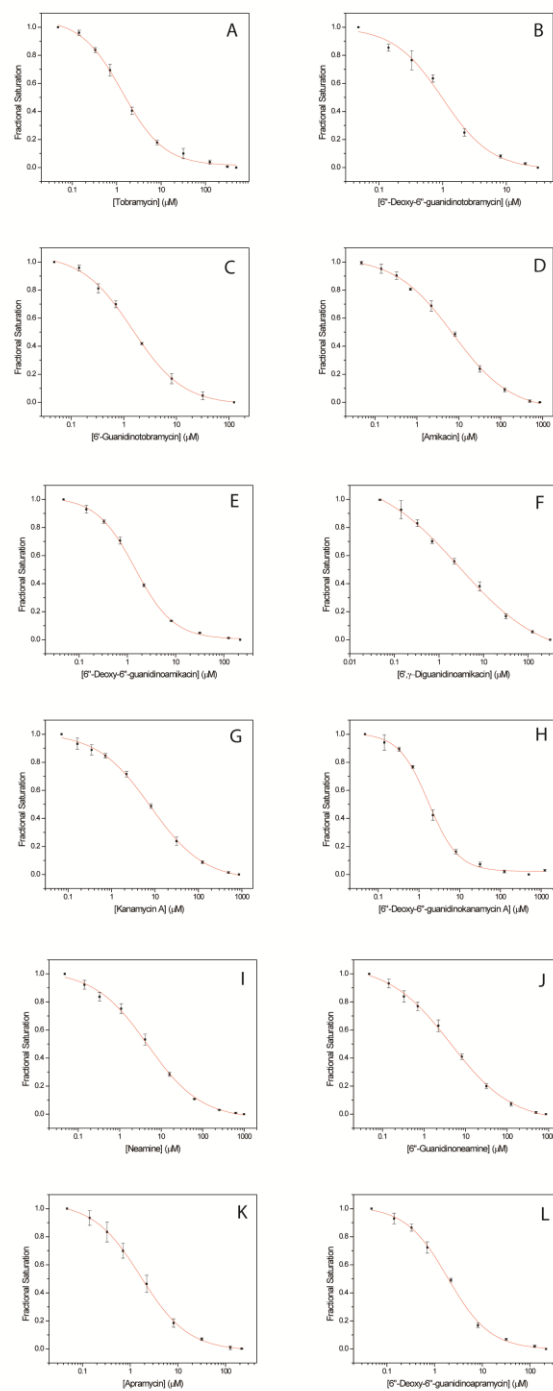
Figure 2.13: Kanamycin-Coumarin displacement curves. A = Tobramycin (1), B = 6''-Deoxy-6''-guanidinotobramycin (2), C = 6'-Guanidinotobramycin (3), D = Amikacin (4), E = 6''-Deoxy-6''-guanidinoamikacin (5), F = 6',γ-Diguanidinoamikacin (6), G = Kanamycin A (7), H = 6''-Deoxy-6''-guanidinokanamycin A (8), I = Neamine (11), J = 6'-guanidinoneamine (12), K = Apramycin (15), L = 6''-Deoxy-6''-guanidinoapramycin (16)
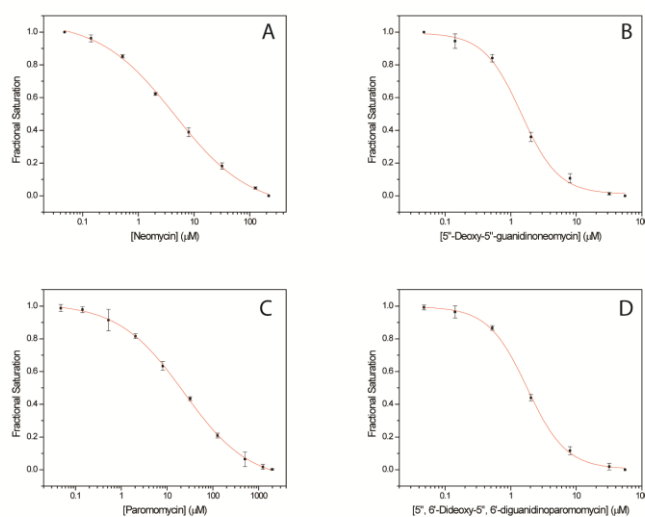
Figure 2.14: Neomycin-Coumarin displacement curves. A = Neomycin (9), B = 5''-Deoxy-5''-guanidinoneomycin (10), C = Paromomycin (13), D = 5'',6'-Dideoxy-5'',6'-diguanidinoparomomycin (14)

Minimum inhibitory concentration (MIC) determinations

MIC values for aminoglycosides were determined using broth microdilution in accordance with Clinical Laboratory Standards Institute guidelines.[24] Bacterial strains of interest were grown on Mueller-Hinton agar. A colony was selected and grown in an overnight liquid culture of cation adjusted Mueller-Hinton broth (MHB) (10 mg/mL CaCl$_2$ and 10 mg/mL MgCl$_2$) at 37 °C. 100 μL of the overnight culture was added to 10 mL of fresh cation adjusted MHB in a 20 mL test tube. This was shaken at 37 °C to an optical density (OD) value of 0.4 – 0.6 at 600 nm to give a log phase culture. This usually took 1.5 – 3.5 hours, for the strains used in this study, and varied between bacterial species and strain. The log culture was poured into a falcon tube and pelleted by centrifugation at 4,000 rpm for 8 minutes. The cation adjusted Mueller-Hinton broth was decanted off. The bacteria pellet was suspended in 0.5 mL PBS. In a 10 mL test tube, the

bacteria suspension was added step wise to 5 mL of PBS to give a final OD at 600 nm of 0.2 for *E. coli* and 0.4 for all other bacterial species used. Bacteria had to be diluted to to a final concentration of 5 x 10^5 cfu/mL just prior to addition to the 96-well test plate. *E. coli* strains were used directly. MRSA strains were diluted by addition of 0.5 mL of the OD = 0.4 solution to 9.5 mL of cation adjusted MHB. *K. pneumoniae* and *P. aeruginosa* strains were diluted by addition of 167 µL of OD = 0.4 solution to 9.83 mL of cation adjusted MHB. *A. baumannii* strains were diluted by addition of 833 µL of OD = 0.4 solution to 9.17 mL of cation adjusted MHB.

A 96-well compound dilution plate was prepared. The highest concentration well consisted of 90 µL of cation adjusted MHB and 10 µL of an aqueous stock solution at 5 mg/mL of compound. Serial dilutions in cation adjusted MHB were then made down the compound dilution plate.

Round bottom 96 well test plates were separately prepared. 80 µL of cation adjusted MHB was added to each well. 10 µL of the serial dilutions were transferred from the comound dilution plate using a multi-channel pipette. Each compound of interest was added to in two consecutive columns to give results in duplicate. In one column per test plate serial dilutions of a known antibiotic that the strain is sensitive to was added. For the strains used these were tobramycin for *P. aeruginosa*, tetracycline for *K. pneumoniae,* ciprofloxacin for *E. coli*, and vancomycin for MRSA. 10 mL of the 5 x 10^5 cfu/mL solution of bacteria were added to each well containing compound and also to three wells containing only media as a control. The plates were covered, parafilmed along the sides, and shaken at 37 °C overnight.

The 96-well plates were read at 600 nm using a plate reader. OD values at or below 0.065 were considered bacteria free. The first well that was cleared for a given compound is the MIC value. A representative table of raw data for the MIC assay is pictured (Table 4.5). Each compound of interested was tested minimally in quadruplicate. A difference of one serial dilution for separate runs was commonly observed. In this case the MIC is reported as a range.

Parent aminoglycoside crystal structures

All crystal structure representations were made using PyMOL Molecular Graphics Systems, Version 1.4.1, Schrödinger, LLC. All structures were adapted from PDB files: Tobramycin (1LC4), amikacin (2GSQ), kanamycin A (2E5I), neomycin (2ET4), neamine (2ET8), paromomycin (1J7T), apramycin (1YRJ).
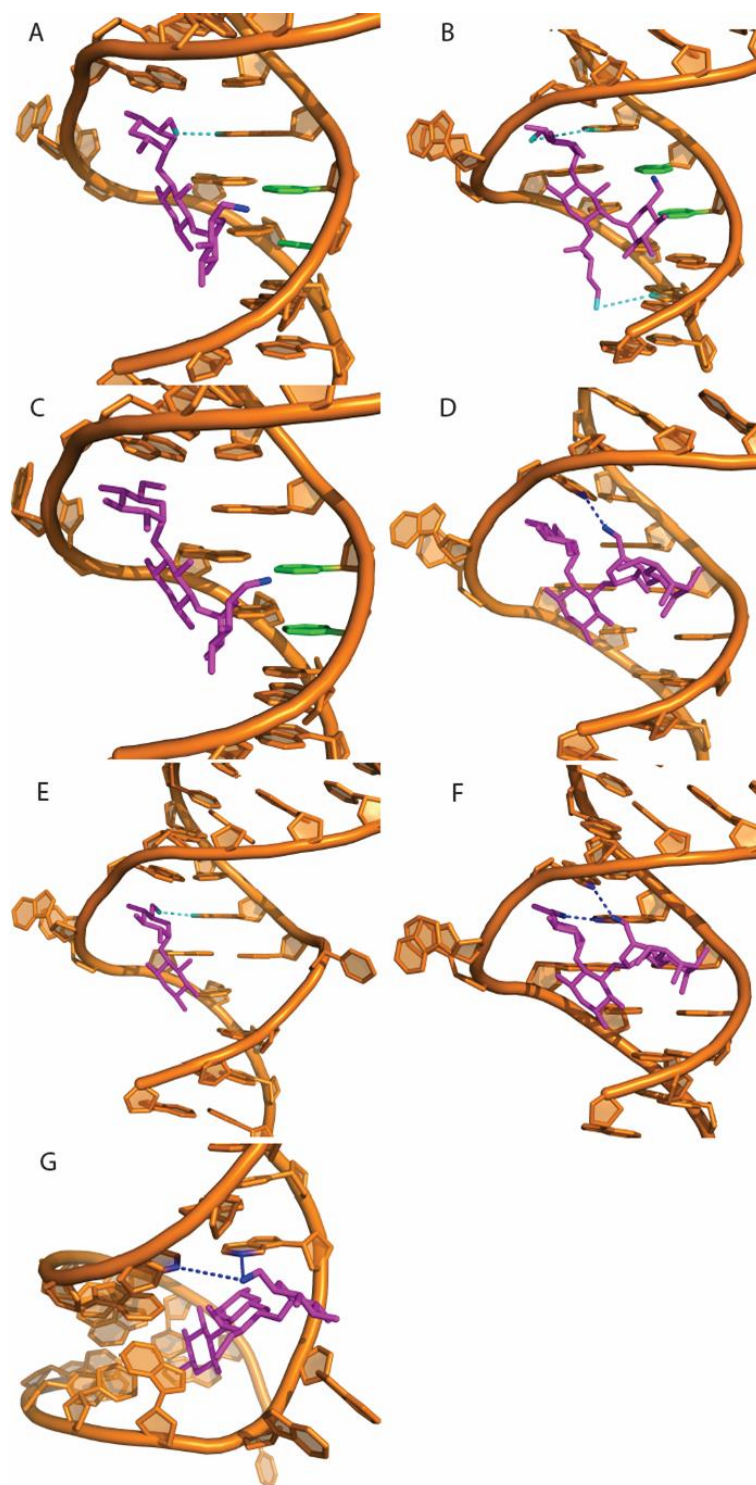
Figure 2.15: Parent aminoglycoside crystal structures. Orange = A-site RNA, Magenta = Aminoglycoside, Dark blue = Primary alcohols modification sites with hydrogen bonds, Light blue = Aminomethyl modification sites with hydrogen bonds, Green = Possible new contants for kanamycin class 6'' alcohol modifications. A = Tobramycin (1), B = Amikacin (4), C = Kanamycin A (7), D = Neomycin (9), E = Neamine (11), F = Paromomycin (13), G = Apramycin (15).

2.6 References

[1] a) M. P. Mingeot-Leclercq, Y. Glupczynski, P. M. Tulkens, *Antimicrob. Agents Chemother.* **1999,** *43,* 727–737; b) K. Poole, *J. Pharm. Pharmacol.* **2001,** *53,* 283–294; c) K. Lewis, *Antimicrob. Agents Chemother.* **2001,** *45,* 999–1007; d) S. B. Vakulenko, S. Mobashery. *Clin. Microbial. Rev.* **2003,** *16,* 430–450; e) G. F. Liou, S. Yoshizawa, P. Courvalin, M. Galimand, *J. Mol. Bio.* **2006,** *359,* 358–364.

[2] a) E. Durante-Mangoni, R. Zarrilli, *Future Microbiol.* **2011,** *6,* 407–422; b) A. Sanchez, S. Gattarello, J. Rello, *Semin. Respir. Crit. Care Med*. **2011,** *32,* 151–158.

[3] J. Li, C. R. Rayner, R. L. Nation, R. J. Owen, D. Spelman, K. E. Tan, L. Liolios, *Antimicrob. Agents Chemother*. **2006,** *50,* 2946–2950

[4] a) T. Strateva, D. Yordanov, *J. Med. Microbiol.* **2011,** *58,* 1133–1148; b) N. L. Werner, M. T. Hecker, A. K. Sethi, C. J. Donskey, *BMC Infect. Dis.* **2011,** *11,* 187–193.

[5] a) H. Liu, S. G. Mulholland, *Am. J. Med. Suppl.* **2005,** *118,* 14S–20S; b) K. L. Leach, S. J. Brickner, M. C. Noe, P. F. Miller, *Ann. NY Acad. Sci.* **2011,** *1222,* 49–54.

[6] a) D. J. Newman, G. M. Cragg, K. M. Snader, *J. Nat. Prod.* **2003,** *66,* 1022–1037; b) D. J. Newman, G. M. Cragg, *J. Nat. Prod.* **2007,** *70,* 461–477.

[7] a) D. J. Payne, M. N. Gwynn, D. J. Holmes, D. L. Pompliano, *Nat. Rev. Drug Discov.* **2007,** *6,* 29–40; b) W. R. J. D. Galloway, A. Bender, M. Welch, D. R. Spring, *Chem. Commun.* **2009,** *45,* 2446–2462; c) J. R. Miller, G. L. Waldrop, *Expert Opinion on Drug Discovery*. **2010,** *5,* 145–154.

[8] a) J. G. Silva, I. Carvalho, *Curr. Med. Chem.* **2007,** *14,* 1101–1119; b) M. E. Falagas, A. P. Grammatikos, A. Michalopoulos, *Expert Rev. Anti Infect. Ther.* **2008,** *6,* 593–600

[9] R. R. Edson, *Mayo Clin. Proc.* **1999,** *74,* 519–527.

[10] a) J. Davies, B. D. Davis. *Mol. Pharmacol.* **1965,** *1,* 93–106; b) J. Davies, B. D. Davis, *J. Biol. Chem.* **1968,** *243,* 3312–3316; c) D. Moazed, H. F. Noller, *Nature.* **1987,** *327,* 389–394; d) A. P. Carter, W. M. Clemons, D. E. Brodersen, R. J. Morgan-Warren, B. T. Wimberly, V. Ramakrishnan, *Nature.* **2000,** *407,* 340–348; e) J. M. Ogle, D. E. Brodersen, W. M. Clemons, M. J. Tarry, A. P. Carter, V. Ramakrishnan, *Science.* **2001,** *292,* 897–902.

[11] a) Q. Vicens, E. Westhof, *Structure.* **2001,** *9,* 647–668; b) Q. Vicens, E. Westhof, *Chem. Biol.* **2002,** *9,* 747–755; c) B. François, R. J. M. Russell, J. B. Murray, F. Aboul-ela, B. Masquida, Q. Vicens, E. Westhof, *Nucleic Acids Research.* **2005,** *33,* 5677–5690;

d) Q. Han, Q. Zhao, S. Fish, K. B. Simonsen, D. Vourloumis, J. M. Froelich, D. Wall, T. Hermann, *Angew. Chem*, **2005,** *117,* 2754–2760*; Angew. Chem. Int. Ed.* **2005,** *44,* 2694–2700; e) J. Kondo, B. François, R. J. M. Russell, J. B. Murray, E. Westhof, *Biochimie.* **2006,** *88,* 1027–1031.

[12] For examples of synthetic and semi-synthetic aminoglycoside analogs see: a) H. Wang, Y. Tor, *J. Am. Chem. Soc.* **1997,** *119,* 8734–8735; b) H. Wang, Y. Tor, *Bioorg. Med. Chem. Lett.* **1997,** *7,* 1951–1956; c) H. Wang, Y. Tor, *Angew. Chem.* **1998,** *110,* 117–120; *Angew. Chem. Int. Ed.* **1998,** *37,* 109–111; d) K. Michael, Y. Tor, *Chem. Eur. J.* **1998,** *4,* 2091–2098; e) Y. Tor, T. Hermann, E. Westhof, *Chem. Biol.* **1998,** *5,* R277–R283; f) K. Michael, H. Wang, Y. Tor, *Bioorg. Med. Chem.* **1999,** *7,* 1361–1371; g) K. Michael, Y. Tor, *RNA-Binding Antibiotics*, (Eds.: Schroeder & Wallis), Landes Bioscience, Austin **2001,** pp. 130–145; h) J. Haddad, L. P. Kotra, B. Llano-Sotelo, C. Kim, E. F. Azucena Jr., M. Liu, S. B. Vakulenko, C. S. Chow, S. Mobashery, *J. Am. Chem. Soc.* **2002,** *124,* 3229–3237; i) Y. Tor, *ChemBioChem.* **2003,** *4,* 998–1007; j) T. Hermann, Y. Tor, *Exper. Opin. On Therapeutic Patents.* **2005,** *15,* 49–62; k) F. Zhao, Q. Zhao, K. F. Blount, Q. Han, Y. Tor, T. Hermann, *Angew. Chem.* **2005,** *117,* 5463–5468; *Angew. Chem. Int. Ed.* **2005,** *44,* 5329-5334; l) M. Hainrichson, V. Pokrovskaya, D. Shallom-Shezifi, M. Fridman, V. Belakhov, D. Shachar, S. Yaron, T. Bassov, *Bioorg. Med. Chem.* **2005,** *13,* 5797–5807; m) K. Blount, Y. Tor, *ChemBioChem.* **2006,** *7,* 1612–1621; n) Y. Tor, *Biochimie.* **2006,** *88,* 1045–1051; o) V. Pokrovskaya, V. Belakhov, M. Hainrichson, S. Yaron, T. Baasov, *J. Med. Chem.* **2009,** *52,* 2243–2254; p) S. Bera, G. G. Zhanel, F. Schweizer, *Bioorg. Med. Chem. Lett.* **2010,** *20,* 3031–3035; q) J. Revuelta, T. Vacas, F. Corzana, C. Gonzales, A. Bastida, J. L. Asensio, *Chem. Eur. J.* **2010,** *16,* 2986–2991; r) V. Pokrovskaya, I. Nudelman, J. Kandasamy, T. Baasov, *Methods Enzymol.* **2010,** *478,* 437–462; s) L. Yang, X. S. Lee, *Curr. Top Med Chem.* **2010,** *10,* 1898–1926; t) R. B. Yan, M. Yuan, Y. Wu, X. You, X. S. Ye, *Bioorg. Med. Chem.* **2011,** *19,* 30–40; u) S. Bera, G. G. Zhanel, F. Schweizer, *Carbohydrate Research.* **2011,** *346,* 560–568; v) L. McCoy, Y. Xie, Y. Tor, *WIREs RNA.* **2011,** *2,* 209–232; w) T. Cottin, C. Pyrkotis, C. I. Stathakis, I. Mavridis, I. A. Katsoulis, P. Anastasopoulou, G. Kythreoti, A. L. Zografos, V. R. Nahmias, A. Papakyriakou, D. Vourloumis, *ChemBioChem.* **2011,** *12*, 71–87; x) V. Udumula, M. Chittapragada, J. B. Marble, D. L. Dayton, Y. W. Ham, *Bioorg. Med. Chem. Lett.* **2011,** *21,* 4713–4715; y) J. Szychowski, J. Kondo, O. Zahr, K. Auclair, E. Westhof, S. Hanessian, J. W. Keillor, *ChemMedChem.* **2011,** *6,* 1961–1966; z) L. Guan, M. D. Disney, *ACS Chem. Biol.* **2012,** *7,* 73–86.

[13] K. F. Blount, F. Zhao, T. Hermann, Y. Tor, *J. Am. Chem. Soc.* **2005,** *127,* 9818–9829.

[14] A. V. Dix, L. Fischer, S. Sarrazin, C. P. H. Redgate, J. D. Esko, Y. Tor, *ChemBioChem.* **2010,** *11,* 2302–2310.

[15] a) Y. Xie, A. V. Dix, Y. Tor, *J. Am. Chem. Soc.* **2009,** *131,* 17605–17614; b) Y. Xie, A. V. Dix, Y. Tor, *Chem. Comm.* **2010,** *46,* 5542–5544.

[16] M. Kaul, C. M. Barbieri, D. S. Pilch, *J. Am. Chem. Soc.* **2006,** *128,* 1261–1271

[17] J. D. Dutcher, M. N. Donin, *J. Am. Chem. Soc.* **1952,** *74,* 3420–3422

[18] T. J. Baker, M. Tomioka, M. Goodman, *Organic Synth.* **2002,** *78,* 91–94.

[19] W. M. Huston, M. P. Jennings, A. G. McEwan, *Mol. Microbiol.* **2002,** *45,* 1741–1750.

[20] K. Michael, H. Wang, Y. Tor, *Bioorg. Med. Chem*. **1999,** *7,* 1361-1371.

[21] (a) J. Boer, K. F. Blount, N. W. Luedtke, L. Elson-Schwab, Y. Tor, *Angew. Chem.* **2005,** *117,* 949-954; *Angew. Chem. Int. Ed.* **2005,** *44,* 927-932; (b) L. Elson-Schwab, O. B. Garner, M. Schuksz, B. E. Crawford, J. D. Esko, Y. Tor, *J. Biol. Chem.* **2007,** *282,* 13585-13591; (c) N. X. Wang, N. G. Yu, G. X. Wang, X. H. Zhang, Q. S. Li, *Synthesis.* **2007,** *8,* 1154-1158.

[22] K. F. Blount, Y. Tor, *ChemBioChem.* **2006,** *7,* 1612-1621.

[23] G. Jiao, O. Simo, M. Nagata, S. O'Malley, T. Hemscheidt, L. Cregar, S. Z. Millis, M. E. Goldman, C. Tang, *Bioorg. Med. Chem. Lett.* **2006,** *16,* 5183-5189.

[24] Performance standards for antimicrobial susceptibility testing; 19th informational supplement M100–S19. Clinical and laboratory standards institute, Wayne, PA, USA, **2008**.

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Chapter 3

RNA Binding and Antibacterial Activity of Guanidinoglycosides

3.1 Introduction

Diverse guanindinium-rich scaffolds have been used as molecular transport agents capable of effecting uptake cargos that exhibit poor cellular penetration alone.[1] Guanidinoglycosides, synthetic aminoglycoside derivatives wherein the amino moieties are globally converted to guanidinium groups, are one example of these guanindinium rich transporters. The cellular uptake properties of many guanidinoglycosides have been extensively studied in eukaryotic cells.[2] They are unique in that their cellular uptake is entirely dependent on heparan sulfate, a prolific glycosaminoglycan on most eukaryotic cell surfaces.[3]

Figure 3.1: Streptomycin (**1**)

Though there is an abundance of information regarding the cellular delivery properties of these molecules it was unknown if they retained the antibacterial activity of their parent aminoglycosides. It is worth noting that streptomycin (**1**) is an
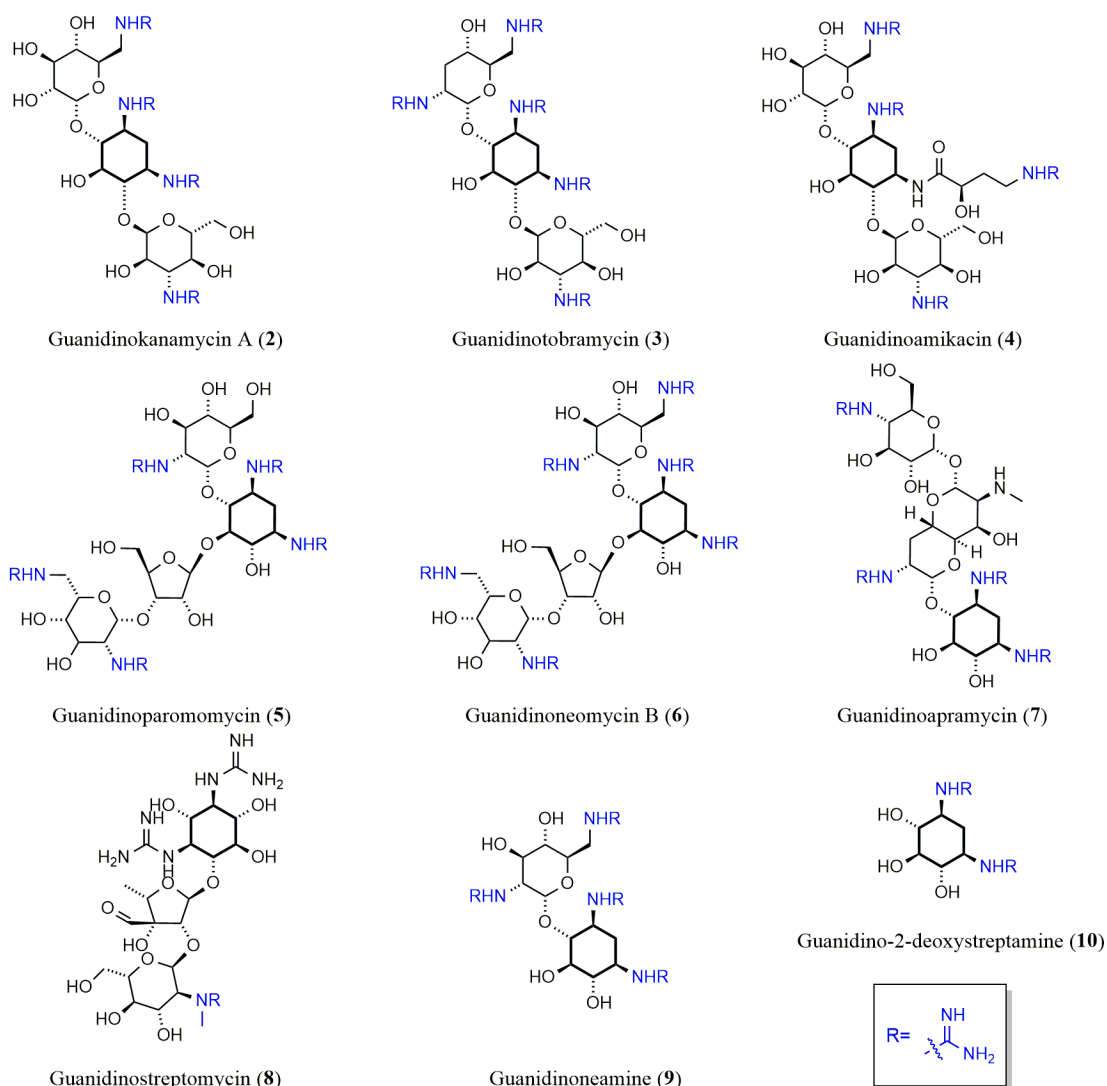

Figure 3.2: Guanidinoglycosides with guanidinium groups in blue and 2-deoxystreptamine (2-DOS) rings in bold.

aminoglycoside that naturally has two guanidinium moieties, so these groups are not completely foreign to this drug class (Figure 3.1). Here we detail the synthesis of a small library of guanidinoglycosides derived from members of several aminoglycoside

subclasses (Figure 3.2). Their ability to bind the primary intracellular target of most

aminoglycosides, the 16S ribosomal A-site, was tested with an in vitro Förster resonance

energy transfer (FRET) based assay. Their antibacterial efficacy was tested via minimum

inhibitory concentration (MIC) determinations against several bacterial isolates.

3.2 Results and Discussion

Synthesis

A general synthetic approach for the global conversion of amines to guanidinium

groups is illustrated for kanamycin A (**11**) (Scheme 3.1). Prolonged treatment with 1,3-

di-Boc-2-(trifluoromethylsulfonyl)guanidine in the presence of triethylamine gave the

fully Boc-protected guanidinoglycoside, (Boc)$_8$guanidinokanamycin A (**12**). Acidic

deprotection of all Boc groups using a one to one mixture of TFA and DCM with TIPS as

a cation scavenger, followed by HPLC purification, afforded the analytically pure

guanidinokanamycin A (**2**).
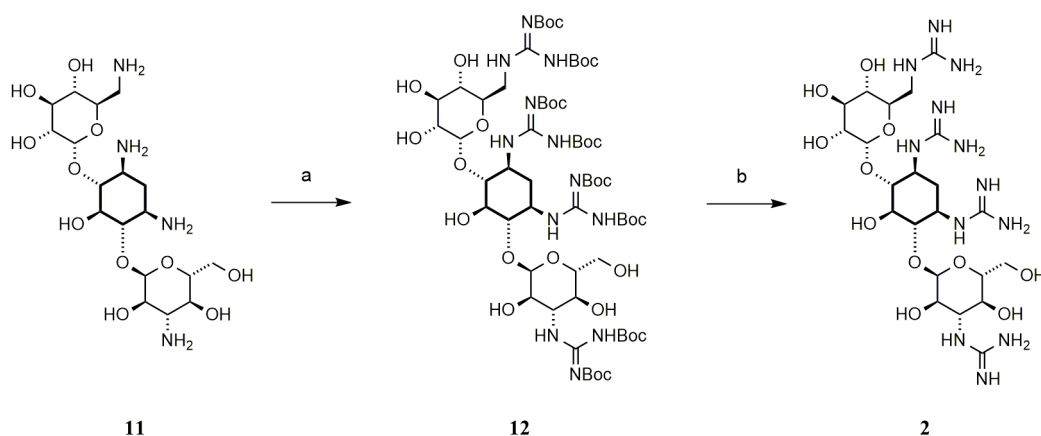
Scheme 3.1: Representative global amine to guanidinium conversion. Reagents and conditions: a) 1,3-di-Boc-2-(trifluoromethylsulfonyl)guanidine, TEA, DCM, MeOH, RT, 5 days, 82 - 94%; b) TFA, TIPS, DCM, RT, 2 h, 70–88 %.

The secondary amine of streptomycin (**1**) proved unreactive towards 1, 3-di-Boc-2-(trifluoromethylsulfonyl)guanidine. Reaction with 1H-Pyrazole-1-carboxamidine hydrochloride in water with sodium carbonate followed by HPLC purification provided free guanidinostreptomycin (**8**) in one step, however (Scheme 3.2). All guanidinoglycosides were first converted to their free-base form by exposure to a strong basic anion (OH⁻) exchange resin (Monosphere 550A, Dowex) prior to their evaluation in any A-site binding assays or antibacterial experiments.

1   **8**
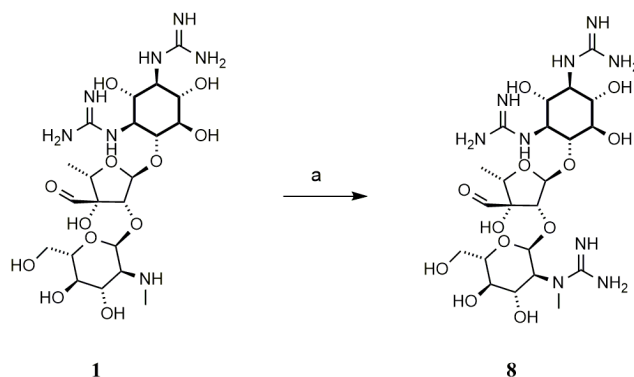
Scheme 3.2: Synthesis of guanidinostreptomycin (**8**). a) 1H-Pyrazole-1-carboxamidine HCl, Na_2CO_3, H_2O, RT, 3 days, 23%

Affinity for the bacterial 16S A-site construct

To determine the affinity of all derivatives to the bacterial 16S A-site, we used a version of a FRET-based assay discussed in the previous section that has been previously used in our lab to determine affinities of aminoglycosides for the A-site.[4] In this assay, a coumarin–aminoglycoside conjugate placeholder binds to a Dy-547-labeled 16S A-site construct. Coumarin acts as a FRET donor to its matched Dy-547 acceptor. The affinity of unlabeled ligands for the A-site can be measured in a competition experiment, where the compound of interest is titrated in and displaces the coumarin–aminoglycoside placeholder, resulting in a decreased sensitized acceptor emission. Binding curves can be

created and from them IC$_{50}$ values can be determined to give relative affinities. Titrations

were performed with a coumarin–kanamycin derivative, our lowest affinity placeholder

aminoglycoside conjugate (Table 3.1). In all cases, titration curves were generated by

plotting the fractional fluorescence saturation of the acceptor against the concentration of

the molecule of interest (for a representative example, see Figure 2.2).

Table 3.1: IC$_{50}$ Values for Competing Off Kanamycin-Coumarin[a]

Compound	IC$_{50}$ (µM)	Compound	IC$_{50}$ (µM)
Kanamycin A (**11**)	7.0 ± 0.7	Guanidinokanamycin A (**2**)	7.3 ± 0.5
Tobramycin	1.6 ± 0.2	Guanidinotobramycin (**3**)	1.4 ± 0.1
Amikacin	6.7 ± 0.7	Guanidinoamikacin (**4**)	5.9 ± 0.2
Paromomycin	1.1 ± 0.1	Guanidinoparomomycin (**5**)	1.1 ± 0.1
Neomycin B	1.0 ± 0.1	Guanidinoneomycin B (**6**)	0.9 ± 0.02
Apramycin	1.7 ± 0.1	Guanidinoapramycin (**7**)	2.9 ± 0.3
Streptomycin (**1**)	105 ± 11	Guanidinostreptomycin (**8**)	22.4 ± 2.4
Neamine	4.5 ± 0.4	Guanidinoneamine (**9**)	3.2 ± 0.2
2-DOS	> 5000	Guanidino-2-DOS (**10**)	> 5000

[a] Conditions: A-site RNA (1 µM), kanamycin-coumarin (0.53 µM), cacodylate buffer pH 7.0 (20 mM), NaCl (100 mM), EDTA (0.5 mM)

Most of the guanidinoglycosides exhibited A-site affinity within error of their

parent aminoglycosides and only guanidinoapramycin (**7**) was significantly worse than its

parent. This was somewhat surprising given that it has been shown that the A-site is a

highly discriminating target.[5] Guanidinoglycosides probably retain much of the

conformational flexibility of aminoglycosides and are more basic, and thus likely more

cationic at neutral pH than aminoglycosides. This may result in favorable electrostatic

interactions that could offset energetic penalties caused by unfavorable steric interactions.

Intriguingly, guanidinoneamine (**9**) and guanidinostreptomycin (**8**) show improved binding compared to parent compounds. In contrast to the other aminoglycosides tested, the primary ribosomal target of streptomycin (**1**) is not the A-site binding pocket. This makes the marked improved binding of guanidinestreptomycin (**8**) slightly less surprising. Neamine is the smallest of the aminoglycosides tested that shows affinity to the A-site, so the lesser bulk of guanidinoneamine (**9**) may be the reason for its success as an A-site binder.

Antibacterial activities

To test the antibacterial activity of our analogs MIC values were determined against gram positive MRSA (ATCC33591), and gram negative *E. coli* (ATCC25922), *P. aeruginosa* (PA01), and *K. pneumoniae* (GNR0713). None of the guanidinoglycosides showed any activity against any strain with the exception of guanidinoneomycin, which showed weak activity (MIC = 25 – 50 μg/mL) against *P. aeruginosa*. This almost total lack of antibacterial activity was somewhat surprising given the A-site affinities observed in the FRET assay.

There are two likely possibilities that could explain this lack antibacterial activity supposing that the guanidinoglycosides are not giving false positives by indiscriminately binding the A-site construct to cause conformational changes making the placeholder fall off. One possibility is that there is less intracellular accumulation due to decreased cellular uptake or increased efflux. This is certainly feasible given that their primary uptake mechanism in eukaryotes, heparan sulfate mediated endocytosis, is not present in bacterial cells. Alternatively, their highly cationic character could cause them to

nonspecifically bind nucleic acids and be sequestered by other RNAs. The former possibility could be tested by conducting uptake experiments on bacteria of the kind routinely performed in our lab on eukaryotic cells. The latter could be tested by conducting the FRET assay in the presence of scrambled RNAs or tRNAs and looking for increased IC_{50} values. As guanidinoglycosides have no antibacterial utility these experiment were deemed not worth pursuing, however.

3.3 Conclusions

A small library of guanidinoglycosides was synthesized in one or two steps from aminoglycoside precursors. Their affinity for the bacterial A-site was observed to be comparable to their parent aminoglycosides in an in vitro FRET based binding assay. They exhibited negligible antibacterial activity in MIC assays.

3.4 Experimental

Materials

Unless otherwise specified, materials purchased from commercial suppliers were used without further purification. All aminoglycosides were obtained from Sigma–Aldrich as their sulfate salts. Tobramycin sulfate was converted to the trifluoroacetic acid (TFA) salt by first passing it over an anion (OH⁻) exchange resin (Monosphere 550A, Dowex) to get the free base, then stirring in 0.1% TFA/H_2O. Neamine hydrochloride was made by methanolysis of commercially available neomycin sulfate.[6] 2-DOS hydrobromide was made by degradation of commercially available neomycin sulfate by heating in concentrated HBr.[7] 1,3-Di-Boc-2-(trifluoromethylsulfonyl)guanidine was synthesized according to an established procedure.[8] All other anhydrous solvents and

reagents, and ion exchange resins were purchased from Sigma–Aldrich. NMR solvents were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). The Dy-547-labeled A-site construct was purchased from Thermo Scientific and purified by gel electrophoresis. Kanamycin–coumarin and neomycin–courmarin conjugates were synthesized and purified according to established procedures.[9] Chemicals for preparing buffer solutions (enzyme grade) were purchased from Fisher Biotech. Autoclaved water was used in all fluorescence titrations. Mueller–Hinton broth used for sensitivity testing was obtained from Hardy Diagnostics (Santa Maria, CA, USA). Polystyrene 96-well microplates for MIC testing were purchased from Corning Inc. (Corning, NY, USA). Bacterial strains for sensitivity testing included two reference strains from the American Type Culture Collection (Manassas, VA, USA): hospital-associated MRSA strain 33591 rendered resistant to rifampicin by serial passage and *E. coli* strain 25922. *P. aeruginosa* strain PA01 was used as a general antibiotic-sensitive *P. aeruginosa* strain.[10] *K. pneumoniae* strain GNR0713 is a clinical blood isolate obtained from a tertiary academic hospital in the New York metropolitan area.

Instrumentation

NMR spectra were recorded on Varian Mercury 300 and 400 MHz, Varian VX 500 MHz, and Jeol ECA 500 MHz spectrometers. Mass spectra (MS) were recorded at the University of California, San Diego Chemistry and Biochemistry Mass Spectrometry Facility, utilizing an Agilent 6230 HR-ESI-TOF mass spectrometer. Reverse-phase HPLC (Vydac C18 column) purification and analysis were carried out using an Agilent 1200 series instrument. Products were lyophilized utilizing a Labconco FreeZone 2.5

freeze drier. Steady-state fluorescence experiments were carried out in a microfluorescence cell with a path length of 1.0 cm (Hellma GmH & Co KG, Mullenheim, Germany) on a Jobin Yvon Horiba FluoroMax-3 luminescence spectrometer. A background spectrum (buffer) was subtracted from each sample. A VersaMax plate reader (Molecular Devices, Mountain View, CA, USA) set at 600 nm wavelength was used for MIC assays.

Synthesis

Guanidinokanamycin A (**2**), guanidinotobramycin (**3**), guanidinoparomomycin (**5**), guanidinoneomycin B (**6**), guanidino-2-DOS (**10**), and their Boc-protected intermediates were previously synthesized.[11]

(Boc)$_8$Guanidinoamikacin. DCM (4.3 mL) and methanol (8.5 mL) were added to amikacin (100 mg, 0.17 mmol). TEA (0.42 mL, 2.1 mmol), then 1,3-di-boc-2-(trifluoromethylsulfonyl)guanidine (805 mg, 2.1 mmol) were added. The clear solution was stirred for 5 days. The solvent was removed under reduced pressure. The product was isolated by flash chromatography (0 – 7% methanol in DCM). Product: White solid (248 mg, 0.16 mmol, 94% yield). ESI-MS calculated for $C_{66}H_{115}N_{13}O_{29}$ [M+H]$^+$ 1554.80, found 1554.19.

Guanidinoamikacin · 4 TFA (4). DCM (4.1 mL) and TIPS (0.21 mL) were added to (Boc)$_8$guanidino-amikacin (248 mg, 0.16 mmol). TFA (4.1 mL) was added. The

yellow solution was stirred for 2 hours. Toluene (8 mL) was added and the solvent was removed under reduced pressure. The remaining white solid was dissolved in water and purified by reverse phase HPLC (0 – 14% ACN in water (0.1% TFA) over 14 min) eluted after 10.0 min, then lyophilized. Product: White powder (170 mg, 0.14 mmol, 88% yield). ^{1}H-NMR (400 MHz, D_2O): δ 5.45 (d, J = 3.6 Hz, 1H), 5.13 (d, J = 3.6 Hz, 1H), 4.15 (dd, J_1 = 9.6 Hz, J_2 = 3.2 Hz, 1H), 4.10 – 4.03 (m, 2H), 3.81 – 3.74 (m, 4H), 3.68 – 3.43 (m, 10H), 3.39 – 3.31 (m, 3H), 2.07 – 2.04 (m, 2H), 1.86 – 1.77 (m, 1H), 1.67 (q, J = 12.4 Hz, 1H); ESI-MS calculated for $C_{26}H_{51}N_{13}O_{13}$ [M+H]+ 754.38, found 754.31.

(Boc)$_8$Guanidino-apramycin. DCM (3.2 mL) and methanol (7.8 mL) were added to apramycin (100 mg, 0.16 mmol). TEA (0.38 mL, 1.9 mmol), then 1,3-di-boc-2-(trifluoromethylsulfonyl)guanidine (737 mg, 1.9 mmol) were added. The slightly cloudy mixture was stirred for 5 days. The solvent was removed under reduced pressure. The product was isolated by flash chromatography (0 – 7% methanol in DCM). Product: White solid (193 mg, 0.13 mmol, 82% yield). ^{1}H-NMR (400 MHz, CD_3OD): δ 5.49 (s, 1H), 5.37 (d, J = 3.6 Hz, 1H), 5.35 (d, J = 4 Hz, 1H), 4.38 – 4.27 (m, 2H), 4.20 (t, J = 2 Hz, 1H), 4.04 – 3.71 (m, 7H), 3.63 – 3.54 (m, 4H), 3.20 (t, J = 7.6 Hz, 1H), 3.01 – 2.98 (m, 1H), 2.74 (s, 3H), 2.30 – 2.24 (m, 1H), 2.22 – 2.16 (m, 2H), 1.69 – 1.29 (m, 73H); HR-ESI-MS calculated for $C_{65}H_{113}N_{13}O_{27}Na$ [M+Na]+ 1530.7761, found 1530.7768.

Guanidino-apramycin · 4 TFA (7). DCM (3.2 mL) and TIPS (0.17 mL) were added to (Boc)$_8$guanidino-apramycin (193 mg, 0.13 mmol). TFA (3.2 mL) was added. The

yellow solution was stirred for 2 hours. Toluene (7 mL) was added and the solvent was

removed under reduced pressure. The remaining white solid was dissolved in water and

purified by reverse phase HPLC (0 – 12% ACN in water (0.1% TFA) over 13 min) eluted

after 10.4 min, then lyophilized. Product: White powder (105 mg, 0.09 mmol, 70%

yield). ^{1}H-NMR (400 MHz, D_2O): δ 5.48 (d, J = 4 Hz, 1H), 5.45 (d, J = 3.2 Hz, 1H), 5.20

(d, J = 8.4 Hz, 1H), 4.45 (t, J = 2.6 Hz, 1H), 3.92 – 3.36 (m, 14H), 3.20 (dd, J_1 = 8.4 Hz,

J_2 = 3 Hz, 1H), 2.78 (s, 3H), 2.29 – 2.22 (m, 2H), 1.79 (q, J = 12.4 Hz, 1H), 1.64 (q, J =

12.8 Hz, 1H); ESI-MS calculated for $C_{25}H_{49}N_{13}O_{11}$ $[M+H]^+$ 708.38, found 708.25.

2''-Guanidinostreptomycin· 3 TFA (8). A 1 M sodium carbonate solution (1 mL) was

added to streptomycin sulfate (200 mg, 0.274 mmol). 1H-Pyrazole-1-carboxamidine

hydrochloride (135 mg, 0.903 mmol) was added. The reaction vessel was wrapped in

tinfoil and the clear solution was stirred for 3 days. TFA (0.1 mL) was slowly added and

the solution was stirred for 5 mins. The solvent was removed under reduced pressure. The

remaining solid was redissolved in water and reverse phase HPLC purified (0 – 0.1%

ACN in water (0.1% TFA) over 13 min) eluted after 11 min, then lyophilized. Product:

White powder (68 mg, 0.071 mmol, 26% yield). ^{1}H-NMR (400 MHz, D_2O): δ 5.67 (d, J

= 1.2 Hz, 1H), 5.55 (d, J = 3.2 Hz, 1H), 5.44 (d, J = 2 Hz, 1H), 4.57 (q, J = 6.4 Hz, 1H),

3.87 – 3.78 (m, 3H), 3.69 – 3.60 (m, 3H), 3.58 – 3.42 (m, 5H), 3.34 – 3.31 (m, 1H), 2.75

(s, 3H), 1.21 (d, J = 6.4 Hz, 3H); ^{13}C-NMR (100 MHz, D_2O): δ 163.60 (J = 36 Hz),

162.23, 158.71, 158.16, 116.87 (J = 291 Hz), 104.79, 98.78, 94.11, 81.66, 80.12, 77.92,

76.72, 73.48, 73.44, 71.99, 71.63, 69.73, 69.55, 60.87, 60.63, 59.17, 58.84, 31.32, 11.68;

HR-ESI-MS calculated for $C_{22}H_{42}N_9O_{12}$ $[M+H]^+$ 624.2947, found 624.2950

(Boc)₈Guanidinoneamine. DCM (4.4 mL) and methanol (10.6 mL) were added to neamine (100 mg, 0.21 mmol). TEA (0.52 mL, 2.6 mmol), then 1,3-di-boc-2-(trifluoromethylsulfonyl)guanidine (1 g, 2.6 mmol) were added. The cloudy mixture was stirred for 5 days. The solvent was removed under reduced pressure. The product was isolated by flash chromatography (0 – 7% methanol in DCM). Product: White solid (258 mg, 0.20 mmol, 93% yield). ^{1}H-NMR (400 MHz, CD$_3$OD): δ 5.80 (s, 1H), 4.48 (t, J = 10.4 Hz, 1H), 4.24 (d, J = 10.8 Hz, 1H), 4.10 (t, J = 10.4 Hz, 1H), 3.84 – 3.70 (m, 4H), 3.63 – 3.56 (m, 2H), 3.50 – 3.44 (m, 1H), 3.25 (t, J = 9.6 Hz, 1H), 2.28 – 2.22 (m, 1H), 1.67 – 1.41 (m, 72H), 1.33 – 1.29 (m, 1H); HR-ESI-MS calculated for C$_{56}$H$_{99}$N$_{12}$O$_{22}$ [M+H]$^+$ 1291.6991, found 1291.6997.

Guanidinoneamine · 4 TFA (9). DCM (5.1 mL) and TIPS (0.3 mL) were added to (Boc)₈guanidino-neamine (258 mg, 0.20 mmol). TFA (5.1 mL) was added. The yellow solution was stirred for 2 hours. Toluene (10 mL) was added and the solvent was removed under reduced pressure. The remaining white solid was dissolved in water and purified by reverse phase HPLC (0 – 11% ACN in water (0.1% TFA) over 17 min) eluted after 9.7 min, then lyophilized. Product: White powder (150 mg, 0.16 mmol, 80% yield). ^{1}H-NMR (400 MHz, D$_2$O): δ 5.50 (d, J = 3.2 Hz, 1H), 3.70 – 3.36 (m, 11H), 2.23 (dt, J$_1$ = 12.8 Hz, J$_2$ = 3.6 Hz, 1H), 1.63 (q, J = 12 Hz, 1H); ESI-MS calculated for C$_{16}$H$_{34}$N$_{12}$O$_6$ [M+H]$^+$ 491.28, found 491.17.

Desalting

Guanidinoglycoside·TFA (up to 40 mg) was dissolved in autoclaved H_2O (0.6 mL) in a sterile eppendorf tube. Monosphere 550 A (75 mg) was added, and the suspension was shaken lightly on a Fisher Vortex Genie 2 overnight. The resin was removed by centrifugal filtration and washed twice with autoclaved H_2O. The desalted solutions were lyophilized, and the removal of TFA counterions was confirmed by ^{13}C NMR spectroscopy.

A-Site Binding assay

All titrations were performed with working solutions of 1 μM Dy-547 labeled A-site in 20 mM cacodylate buffer (pH = 7.0, 100 mM NaCl, 0.5 mM EDTA). The solutions were heated to 75 °C for 5 min, cooled to room temperature over 2 h, cooled to 0 °C for 30 min, then allowed to warm back to room temperature. Kanamycin-courmarin or neomycin-coumarin was added, to give a working concentration of 0.53 μM, just prior to aminoglycoside titrations. Steady state fluorescence experiments were carried out at ambient temperature (20 °C). Excitation and emission slit widths were 9 nm for kanamycin-coumarin experiments and 7 nm for neomycin-coumarin. The system was excited at 400 nm and changes in Dy-547 emission were monitored at 561 nm. Errors were generated from three sets of measurements. IC50 values were calculated using OriginPro 8.5 software by fitting a dose response curve (eq 1) to the fractional fluorescence saturation (Fs) plotted against the log of antibiotic (A) concentration.

$$Fs = F_0 + (F_\infty[A]n)/([IC50]n + [A]n) \quad (1)$$

Fs is the fluorescence intensity at each titration point. F_0 and F_∞ are the fluorescence intensity in the absence of ligand or at saturation, respectively, and n is the

Hill coefficient or degree of cooperativity associated with binding. A representative set of

raw fluorescence data next to a normalized binding curve is previously pictured (Figure

2.12). The binding curves of parent aminoglycosides are previously pictured (Figure 2.13

and 2.14), the binding curves of the guanidinoglycosides are pictured (Figure 3.3).
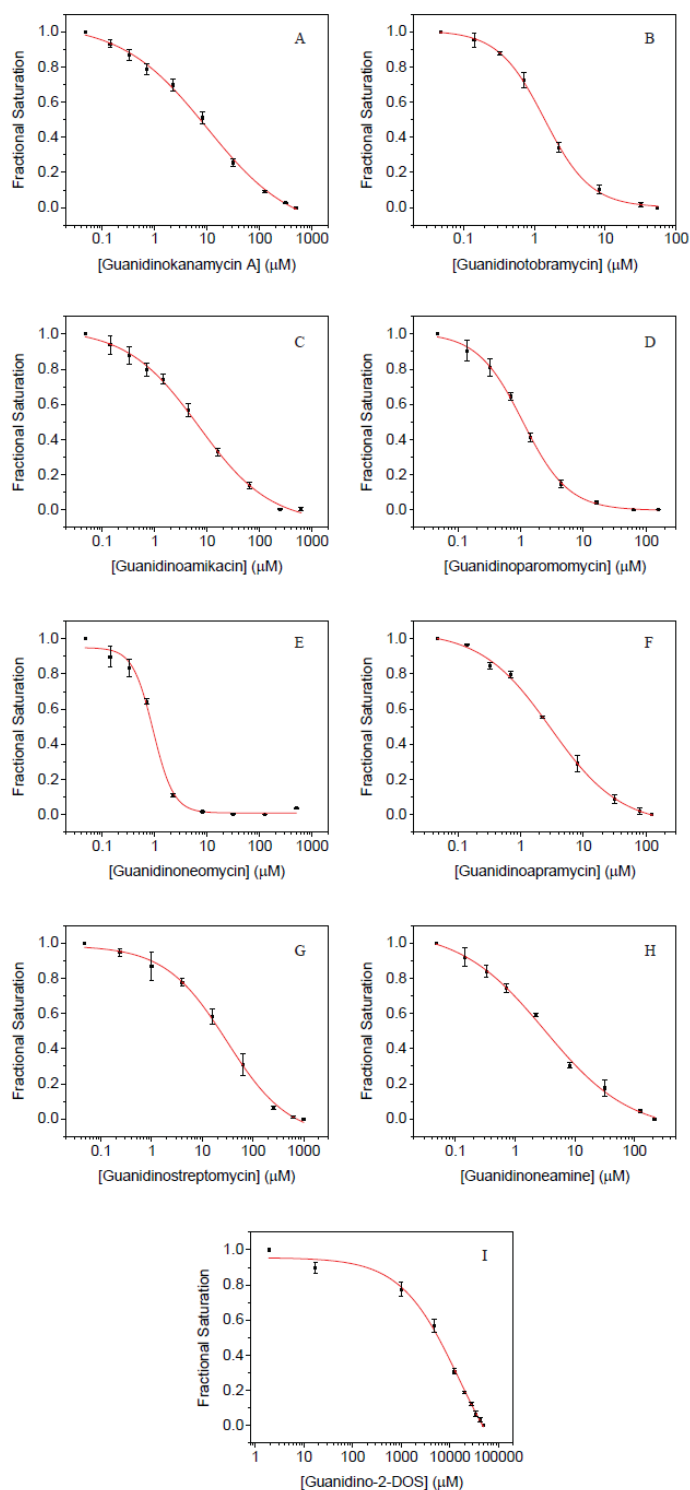
Figure 3.3: Kanamycin-Coumarin displacement curves. A = Guanidinokanamycin A (**2**), B = Guanidinotobramycin (**3**), C = Guanidinoamikacin (**4**), D = Guanidinoparomomycin (**5**), E = Guanidinoneomycin B (**6**), F = Guanidinoapramycin (**7**), G = Guanidinostreptomycin (**8**), H = Guanidinoneamine (**9**), I = Guanidino-2-DOS (**10**).

Minimum inhibitory concentration (MIC) determinations

MIC values for aminoglycosides were determined using broth microdilution in accordance with Clinical Laboratory Standards Institute guidelines.[12] Bacterial strains of interest were grown on Mueller-Hinton agar. A colony was selected and grown in an overnight liquid culture of cation adjusted Mueller-Hinton broth (MHB) (10 mg/mL $CaCl_2$ and 10 mg/mL $MgCl_2$) at 37 °C. 100 μL of the overnight culture was added to 10 mL of fresh cation adjusted MHB in a 20 mL test tube. This was shaken at 37 °C to an optical density (OD) value of 0.4 – 0.6 at 600 nm to give a log phase culture. This usually took 1.5 – 3.5 hours, for the strains used in this study, and varied between bacterial species and strain. The log culture was poured into a falcon tube and pelleted by centrifugation at 4,000 rpm for 8 minutes. The cation adjusted Mueller-Hinton broth was decanted off. The bacteria pellet was suspended in 0.5 mL PBS. In a 10 mL test tube, the bacteria suspension was added step wise to 5 mL of PBS to give a final OD at 600 nm of 0.2 for *E. coli* and 0.4 for all other bacterial species used. Bacteria had to be diluted to to a final concentration of 5 x 10^5 cfu/mL just prior to addition to the 96-well test plate. *E. coli* strains were used directly. MRSA strains were diluted by addition of 0.5 mL of the OD = 0.4 solution to 9.5 mL of cation adjusted MHB. *K. pneumoniae* and *P. aeruginosa* strains were diluted by addition of 167 μL of OD = 0.4 solution to 9.83 mL of cation adjusted MHB. *A. baumannii* strains were diluted by addition of 833 μL of OD = 0.4 solution to 9.17 mL of cation adjusted MHB.

A 96-well compound dilution plate was prepared. The highest concentration well consisted of 90 μL of cation adjusted MHB and 10 μL of an aqueous stock solution at 5

mg/mL of compound. Serial dilutions in cation adjusted MHB were then made down the compound dilution plate.

Round bottom 96 well test plates were separately prepared. 80 µL of cation adjusted MHB was added to each well. 10 µL of the serial dilutions were transferred from the comound dilution plate using a multi-channel pipette. Each compound of interest was added to in two consecutive columns to give results in duplicate. In one column per test plate serial dilutions of a known antibiotic that the strain is sensitive to was added. For the strains used these were tobramycin for *P. aeruginosa*, tetracycline for *K. pneumoniae,* ciprofloxacin for *E. coli*, and vancomycin for MRSA. 10 mL of the 5 x 10^5 cfu/mL solution of bacteria were added to each well containing compound and also to three wells containing only media as a control. The plates were covered, parafilmed along the sides, and shaken at 37 °C overnight.

The 96-well plates were read at 600 nm using a plate reader. OD values at or below 0.065 were considered bacteria free. The first well that was cleared for a given compound is the MIC value. A representative table of raw data for the MIC assay is pictured (Table 4.5). Each compound of interested was tested minimally in quadruplicate. A difference of one serial dilution for separate runs was commonly observed. In this case the MIC is reported as a range.

3.5 References

[1] (a) J. M. Gump, S. F. Dowdy, *Trends Mol. Med.* **2007**, 13, 443. (b) E. A. Dubikovskaya, S. H. Thorne, T. H. Pillow, C. H. Contag, P. A. Wender, *Proc. Natl. Acad. Sci. USA*

2008, 105, 12128. (c) T. B. Potocky, J. Silvius, A. K. Menon, S. H. Gellman, *ChemBioChem* **2007**, 8, 917. (d) C. B. Cooley, B. M. Trantow, F. Nederberg, M. K. Kiesewetter, J. L. Hedrick, R. M. Waymouth, P. A. Wender, *J. Am. Chem. Soc*. **2009**, 131, 16401. (e) K. K. Maiti, W. S. Lee, T. Takeuchi, C. Watkins, M. Fretz, D. C. Kim, S, Futaki, A. Jones, K. T. Kim, S. K. Chung, *Angew. Chem*. **2007**, 119, 5984; *Angew. Chem. Int. Ed*. **2007**, 46, 5880. (f) H. H. Chung, G. Harms, C. M. Seong, B. H. Choi, C. H. Min, J. P. Taulane, M. Goodman, *Biopolymers* **2004**, 76, 83. (g) F. S. Hassane, G. D. Ivanova, E. Bolewska-Pedyczak, R. Abes, A. A. Arzumanov, M. J. Gait, B. Lebleu, J. Gariepy, *Bioconjugate Chem*. **2009**, 20, 1523. (h) K. K. Maiti, O. Y. Jeon, W. S. Lee, S. K. Chung, *Chem. Eur. J***. 2007**, 13, 762. (i) Y. H. Wang, C. P. Chen, M. H. Chan, M. Chang, Y. W. Hou, H. H. Chen, H. R. Hsu, K. Liu, H. J. Lee, *Biochem. Biophys. Res. Commun*. **2006**, 346, 758.

[2] (a) A.V. Dix, L. Fischer, S. Sarrazin, C.P.H. Redgate, J. D. Esko, Y. Tor. *ChemBioChem* **2010,** 11, 2302 (b) S. Sarrazin, B. Wilson, W. S. Sly, Y. Tor, J. D. Esko. *Mol. Ther.* **2010,** 18, 1268. (c) L. Elson-Schwab, O. B. Garner, M. Schuksz, B. E. Crawford, J. D. Esko, Y. Tor. *J. Biol. Chem.* **2007,** 282, 13585. (d) N. W. Luedtke, P. Carmichael, Y. Tor. *J. Am. Chem. Soc.* **2003**, 125, 12375.

[3] L. Elson-Schwab, O. B. Garner, M. Schuksz, B. E. Crawford, J. D. Esko, Y. Tor, *J. Biol. Chem.* **2007**, 282, 13585.

[4] R. J. Fair, M. E. Hensler, W. Thienphrapa, Q. N. Dam, V. Nizet, Y. Tor, *ChemMedChem* **2012**, 7, 1273.

[5] K. Blount, Y. Tor. *ChemBioChem* **2006**, 7, 1612.

[6] J. D. Dutcher, M. N. Donin, *J. Am. Chem. Soc*. **1952**, 74, 3420.

[7] M. P. Georgiadis, V. Constantiou-Kokotou, G. Kokotos. *J. Carbohydrate Chem.***1991**,10, 739.

[8] T. J. Baker, M. Tomioka, M. Goodman, *Org. Synth.* **2002**, 78, 91; *Org. Synth.* **2004**, 10, 266.

[9] (a) Y. Xie, A. V. Dix, Y. Tor, *J. Am. Chem. Soc*. **2009**, 131, 17605. (b) Y. Xie, A. V. Dix, Y. Tor, *Chem. Commun.* **2010**, 46, 5542.

[10] W. M. Huston, M. P. Jennings, A. G. McEwan, *Mol. Microbiol*. **2002**, 45, 1741.

[11] T. J. Baker, N. W. Luedtke, Y. Tor, M. Goodman. *J. Org. Chem.* **2000**, 65, 9054.

[12] *Performance Standards for Antimicrobial Susceptibility Testing*, 19th Informational Supplement M100-S19, Clinical and Laboratory Standards Institute, Wayne, PA, USA, **2008**.

Chapter 4

Singly Modified Amikacin and Tobramycin Derivatives Show Increased A-site Binding and Higher Potency against Resistant Bacteria

4.1 Introduction

The discovery of penicillin, a β-lactam, and streptomycin, an aminoglycoside, in the 1940s launched the golden age of antibiotics. Many of the antibiotics discovered in the 1940s through the 1970s are used in the clinic today.[1] However, the flood of antibiotics into the environment via feedstock and human use has contributed to the increase in resistant pathogens. Horizontal gene transfer between bacteria via plasmids and other methods has played a significant role in conferring resistance.[2,3] Drug resistance bacteria, especially the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter s*pecies), *Clostridium difficile*, and *Escherichia coli*, have been commonly infecting not only immunocompromised hospital patients, but also otherwise healthy individuals.[4,5,6] This has led to rising healthcare costs, often due to length of stay in the hospital, and increased mortality.[7] Problematically, the number of new antibiotics approved by the US Food and Drug Administration has been steadily decreasing, and concurrently many pharmaceutical companies have been abandoning or downsizing their antibacterial research and development.[8,9]

On a positive note, there have been a few new classes of antibiotics in recent years: oxazolidanones,[10] lipopeptides,[11] diarylquinolines,[12] and macrocycles,[13] all of which target gram-positive bacteria. Nevertheless, the emergence of multi-drug resistant bacteria, especially gram negative bacilli with no new treatment options, has led to reexamination of drugs from the early years of antibiotic discovery.[14] Aminoglycosides, polycationic antibiotics, are effective against a broad range of bacteria, although the advent of safer, less toxic antibiotics resulted in their declined use. However, with the increase in resistant pathogens, especially severe gram negative infections, aminoglycosides remain useful for specific infections in the clinic.[15,16] Tobramycin (**1a**) is specifically used for *P. aeruginosa* infection in cystic fibrosis patients, amikacin (**2a**) is used for highly resistant gram negative infections, and gentamicin is used for more generally for preventative measures, as well as for sepsis (Figure 4.1).[15]

Most aminoglycosides bind to the ribosomal RNA (rRNA) A-site, the site of mRNA decoding, and cause translation infidelity.[17,18,19] The mode of action and resistance mechanisms have been well studied and the aminoglycoside scaffold has been established to bind RNA.[20] With this as a starting point, derivatives could lead to compounds that bind the A-site, and show activity against drug resistant bacteria by potentially evading resistance mechanisms. Additionally, modifications could possibly diminish toxicity effects. With this in mind, we have pursued the preparation and evaluation of minimally modified aminoglycoside in order to test their A-site affinity and, importantly, evaluate their effectiveness as potential antibiotics against many resistance bacterial strains.

Here we selectively modify two of the most common clinically used aminoglycoside antibiotics, amikacin and tobramycin. The primary alcohol in the 6" position on these molecules is accessible to modification, so we substituted it for a variety of hydrogen bond donors and acceptors of different sizes (Figure 4.1). Most of the compounds show an increase in vitro affinity to the A-site as determined by a fluorescence resonance energy transfer (FRET) binding assay. Additionally, some of the derivatives show equal to or better potency against certain resistant bacterial strains.
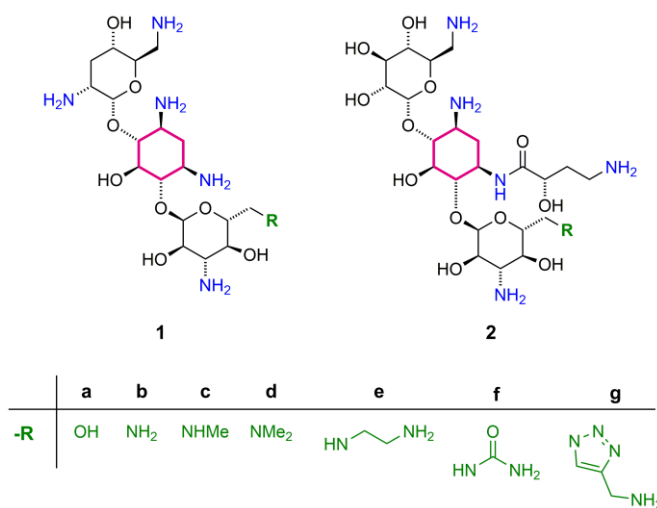

Figure 4.1: Tobramycin (**1a**), amikacin (**2a**) and derivatives prepared and studied. The 2-deoxystreptamine (2-DOS) ring is in pink. The 6" modification position is in green.

4.2 Results

Design Strategy

The 6" hydroxyl group is one of the few functional groups that appears to form no hydrogen bonds to the A-site RNA, neither direct or water mediated, in the crystal structures of tobramycin (**1a**) and amikacin (**2a**), though both are in close proximity to U1406 and C1407 (Figure 4.2).[21] Analogs with guanidinium groups replacing the 6" hydroxyl have shown to display increased A-site affinity and in some cases superior

antibacterial activity.[22] This suggests that certain modifications to the 6'' position may show increased affinity for the A-site and desirable antibacterial efficacy. We set out to test this hypothesis by making derivatives of both **1a** and **2a** with a variety of substituents differing in size, basicity, and in number of hydrogen bond donors and acceptors. More basic functional groups could increase the overall positive charge of the analogs, creating favorable electrostatic interactions with the polyanionic A-site rRNA. Hydrogen bond donors and acceptors could create new contacts to the A-site not observed in the parent compounds. Beyond imparting greater affinity for the A-site, some modifications could potentially lead to decreased recognition by aminoglycoside modifying enzymes, the
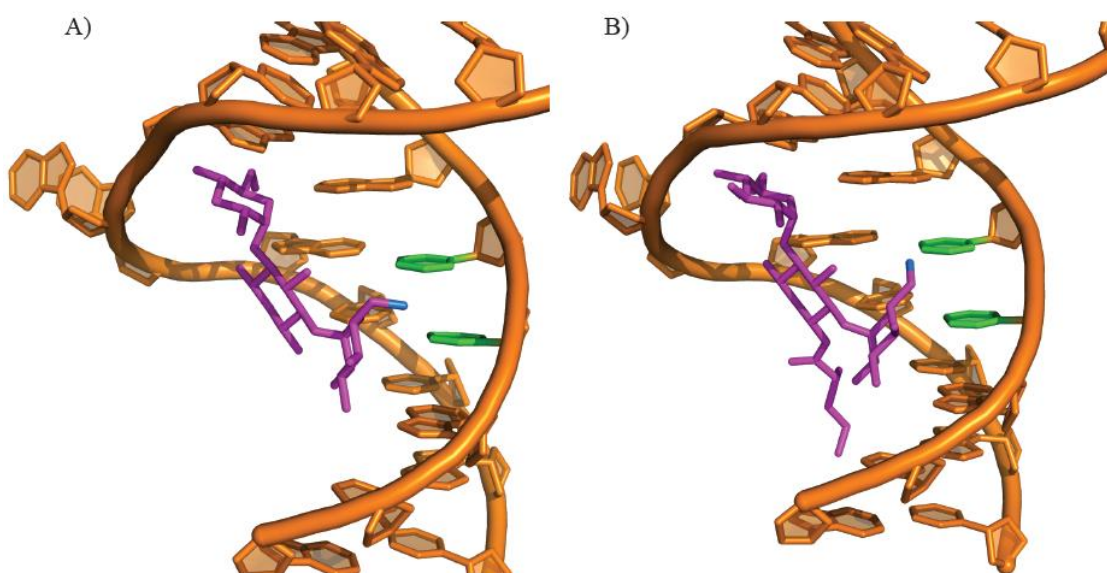

Figure 4.2: A) Crystal structure of tobramycin (**1a**) with A-site rRNA. **B)** Crystal structure of amikacin (**2a**) with A-site rRNA. RNA is in orange with U1406 and C1407 highlighted in green. Aminoglycosides are in magenta with 6'' alcohols highlighted in light blue. Figures were adapted from PDB files: tobramycin (1LC4), amikacin (2GSQ).[21]

most common form of aminoglycoside resistance, and therefore derivatives may exhibit greater antibacterial potency against resistant bacteria.
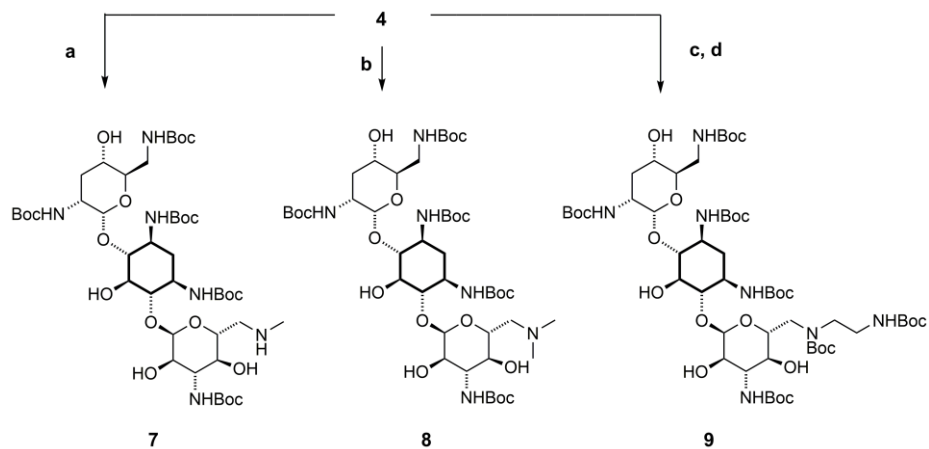
Synthesis

The parent aminoglycosides were converted into three key intermediates using known procedures.[22, 23] The synthetic approach for the conversion of the parent aminoglycosides into these intermediates is illustrated using tobramycin (**1a**) as an example (Scheme 4.1). First, all amines were globally *tert*-butyloxycarbonyl (Boc)-protected using di-*tert*-butyl dicarbonate. The single primary alcohol of (Boc)$_5$tobramycin (**3**) was then selectively converted to a sterically demanding sulfonate by treatment with 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl) in pyridine. Reflux in methanolic ammonia afforded 6''-deoxy-6''-amino(Boc)$_5$tobramycin (**5**). Alternatively, the TPS derivative could be converted to 6''-deoxy-6''-azido(Boc)$_5$tobramycin (**6**) by treating it with sodium azide.

6''-Deoxy-6''-triisopropylbenzylsulfonyl(Boc)$_5$tobramycin (**4**) can also undergo substitution reactions with a variety of other nucleophiles (Scheme 4.2). Reflux in ethanolic methylamine yielded 6''-deoxy-6''-methylamino(Boc)$_5$tobramycin (**7**). Reflux with dimethylamine in tetrahydrofuran (THF) and dimethylformamide (DMF) mixture gave 6''-deoxy-6''-dimethylamino(Boc)$_5$tobramycin (**8**). 6''-Deoxy-6''-(2-(aminoethyl)amino)-(Boc)$_7$tobramycin (**9**) was obtained by heating with ethylene diamine in methanol, followed by Boc protection using di-*tert*-butyl dicarbonate to facilitate purification of this intermediate.
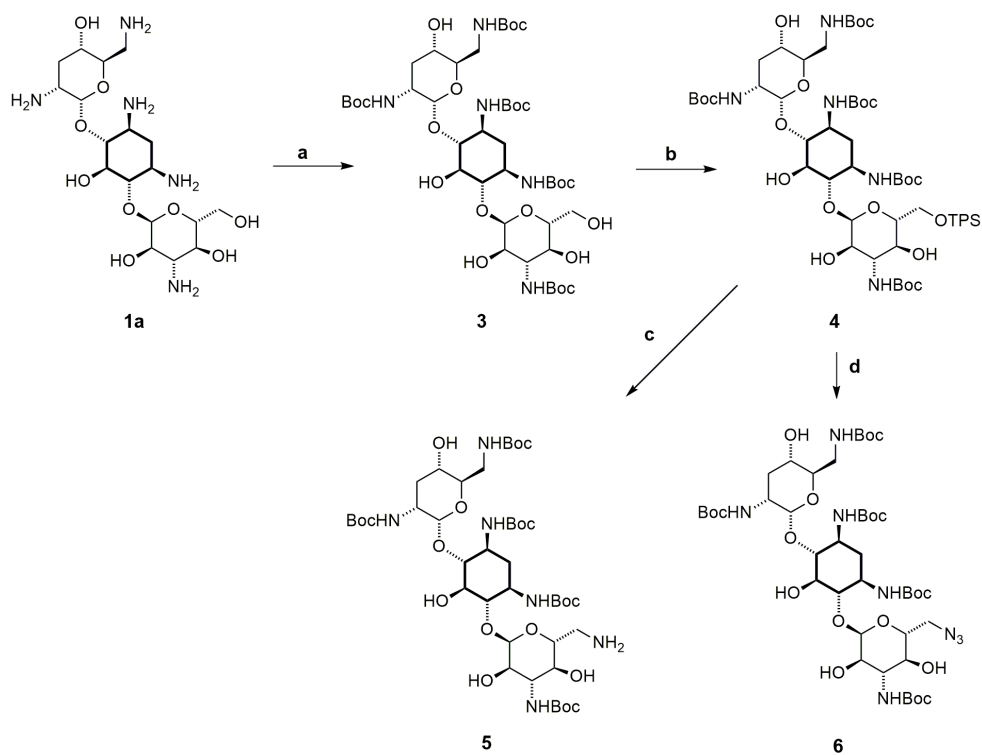
Scheme 4.1: Synthesis of key intermediates **4**, **5**, and **6**. *Reagents and conditions:* a) Boc₂O, Et₃N, H₂O, DMF, 55 °C; b) TPSCl, pyridine, RT; c) NH₃, MeOH, 80 °C; d) NaN₃, DMF, 55 °C.

Scheme 4.2: Substitution reactions of 6"-deoxy-6"-triisopropylbenzylsulfonyl(Boc)₅tobramycin (**4**). *Reagents and conditions:* a) Methylamine, EtOH, 80 °C; b) Dimethylamine, THF, DMF, 80 °C; c) Ethylene diamine, MeOH, 80 °C; d) Boc₂O, Et₃N, H₂O, DMF, 55 °C.
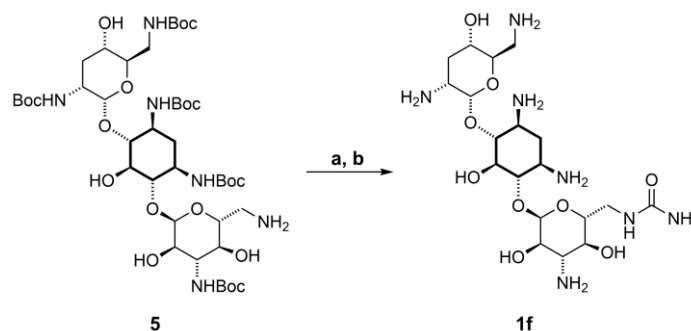
Scheme 4.3: Synthesis 6"-deoxy-6"-ureidotobramycin (**1f**). *Reagents and conditions:* a) 2, 4-Dimethoxybenzyl isocyanate, pyridine, RT; b) TFA, TIPS, CH$_2$Cl$_2$, RT.
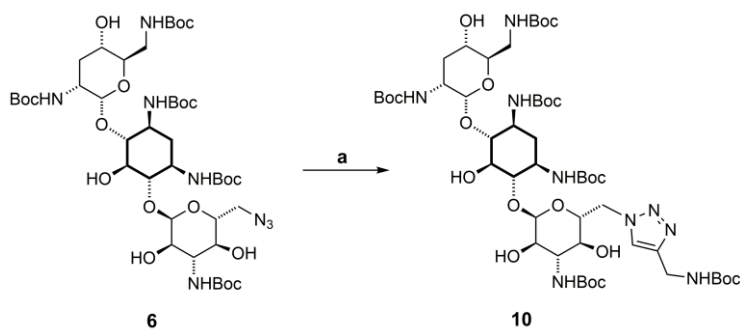
Scheme 4.4: Synthesis 6"-deoxy-6"-(4-(aminomethyl)-1H-1,2,3-triazol-1-yl)-(Boc)$_6$tobramycin (**10**). *Reagents and conditions:* a) Propargyl (Boc)amine, CuSO$_4$·5 H$_2$O, sodium ascorbate, THF, *t*-BuOH, H$_2$O, RT.

The free amine of 6''-deoxy-6''-amino(Boc)$_5$tobramycin (**5**) was used nucleophilically to react with 2,4-dimethoxybenzyl isocyanate in the presence of pyridine to give a 2,4-dimethoxybenzyl (DMB) protected urea. The DMB and Boc protecting groups were concurrently removed using a one to one mixture of trifluoroacetic acid (TFA) and dichloromethane with a tri-*iso*-propyl silane (TIPS) cation scavenger. HPLC purification, afforded the analytically pure 6''-deoxy-6''-ureidotobramycin (**1f**) (Scheme 4.3).

6''-Deoxy-6''-azido(Boc)$_5$tobramycin (**6**) was used in a cycloaddition reaction with propargyl (Boc)amine catalyzed by copper sulfate in the presence of a sodium ascorbate reductant to give 6''-deoxy-6''-(4-(aminomethyl)-1H-1,2,3-triazol-1-yl)-

(Boc)$_6$tobramycin (**10**) (Scheme 4.4). The intermediates **5**, **7**, **8**, **9**, and **10** were all be

deprotected using the aforementioned acidic conditions and HPLC purified to yield the

tobramycin analogs **1b**, **1c**, **1d**, **1e**, and **1g**. All the amikacin derivatives were made using

the same reagents as the tobramycin analogs.

Affinity for the bacterial 16S A-site RNA construct

To determine the affinity of all derivatives to the bacterial 16S A-site, we used a

modified version of a FRET-based assay that was previously developed in our lab.[24] This

modified version has been previously used to measure A-site affinities of modified

aminoglycosides.[22] In this assay, a coumarin–aminoglycoside conjugate binds to a 16S

A-site construct terminally labelled with Dy-547. Coumarin acts as a FRET donor to the

Dy-547 acceptor. The affinity of unlabeled ligands for the A-site can be measured in a

competition experiment, where the compound of interest is titrated in and displaces the

coumarin–aminoglycoside, resulting in a decreased emission of the FRET acceptor, Dy-

547. Different coumarin–aminoglycoside conjugates can be used to cover distinct affinity

ranges of A-site ligands. Amikacin has a much lower affinity to the A-site as compared to

tobramycin, so initial titrations on amikacin analogs were performed with a coumarin–

kanamycin derivative, the lowest affinity aminoglycoside conjugate (Table 4.1).

Tobramycin derivatives and higher affinity amikacin analogs were titrated against a

coumarin–neomycin derivative (Table 4.2). In all cases, binding curves were generated

by plotting the fractional fluorescence saturation of the FRET acceptor against the

concentration of the molecule of interest. Representative curves of kanamycin-coumarin

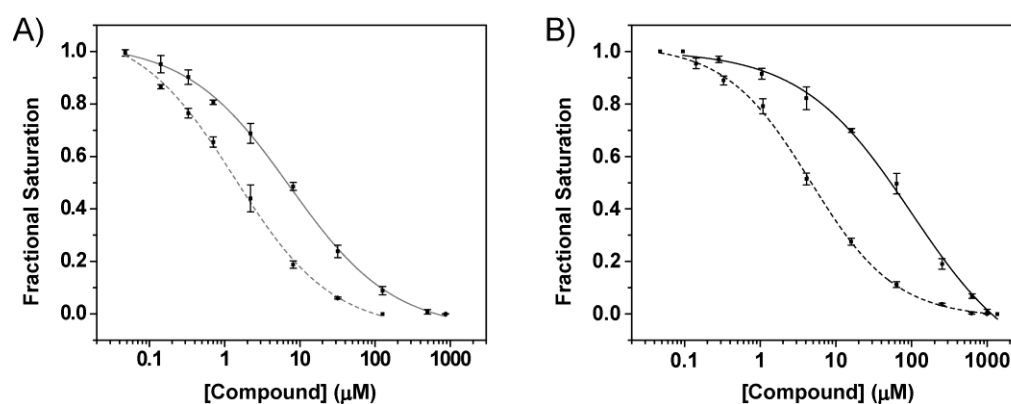and neomycin-coumarin are shown in Figure 4.3.

Figure 4.3: Representative displacement curves of A) Kanamycin-Coumarin by **2a** (grey solid) and **2c** (grey dashed), with IC$_{50}$ values of 6.7 ± 0.7 and 1.5 ± 0.2, respectively. B) Neomycin-Coumarin by **1a** (black solid) and **1b** (black dashed) with IC$_{50}$ values of 53.0 ± 6.0 and 4.7 ± 0.4, respectively.

All amikacin derivatives showed improved A-site binding with the exception of 6''-deoxy-6''-ureidoamikacin (**2f**), which had a much lower affinity than any other aminoglycoside tested. All amikacin analogs with modifications containing a single amine moiety: **2b-e** and **2g** showed similar binding to each other and were also comparable to tobramycin (**1a**). 6''-Deoxy-6''-(2-(aminoethyl)amino)amikacin (**2e**) showed binding superior to any of the other amikacin derivatives.

Table 4.1: IC$_{50}$ Values for Competing Off Kanamycin-Coumarin[a]	
Compound	IC$_{50}$ (μM)
Tobramycin (**1a**)	1.5 ± 0.2
Amikacin (**2a**)	6.7 ± 0.7
6''-Deoxy-6''-aminoamikacin (**2b**)	2.1 ± 0.2
6''-Deoxy-6''-methylaminoamikacin (**2c**)	1.5 ± 0.2
6''-Deoxy-6''-dimethylaminoamikacin (**2d**)	2.2 ± 0.2
6''-Deoxy-6''-(2-(aminoethyl)amino)amikacin (**2e**)	1.7 ± 0.03
6''-Deoxy-6''-ureidoamikacin (**2f**)	50.7 ± 5.5
6''-Deoxy-6''-(4-(aminomethyl)-1H-1,2,3-triazol-1-yl)amikacin (**2g**)	2.2 ± 0.1
[a] Conditions: A-site RNA (1 μM), kanamycin-coumarin (0.53 μM), cacodylate buffer pH 7.0 (20 mM), NaCl (100 mM), EDTA (0.5 mM)	

Table 4.2: IC$_{50}$ Values for Competing Off Neomycin-Coumarin[a]

Compound	IC$_{50}$ (μM)
Tobramycin (**1a**)	53.0 ± 6.0
6''-Deoxy-6''-aminotobramycin (**1b**)	4.7 ± 0.4
6''-Deoxy-6''-methylaminotobramycin (**1c**)	7.4 ± 0.6
6''-Deoxy-6''-dimethylaminotobramycin (**1d**)	6.8 ± 0.8
6''-Deoxy-6''-(2-(aminoethyl)amino)tobramycin (**1e**)	5.3 ± 0.5
6''-Deoxy-6''-ureidotobramycin (**1f**)	30.0 ± 4.0
6''-Deoxy-6''-(4-(aminomethyl)-1H-1,2,3-triazol-1-yl)tobramycin (**1g**)	9.8 ± 1.0
Amikacin (**2a**)	>100
6''-Deoxy-6''-aminoamikacin (**2b**)	46.7 ± 1.5
6''-Deoxy-6''-methylaminoamikacin (**2c**)	45.7 ± 5.8
6''-Deoxy-6''-dimethylaminoamikacin (**2d**)	46.4 ± 5.4
6''-Deoxy-6''-(2-(aminoethyl)amino)amikacin (**2e**)	20.2 ± 2.6
6''-Deoxy-6''-ureidoamikacin (**2f**)	>100
6''-Deoxy-6''-(4-(aminomethyl)-1H-1,2,3-triazol-1-yl)amikacin (**2g**)	47.6 ± 2.6

[a] Conditions: A-site RNA (1 μM), neomycin-coumarin (0.53 μM), cacodylate buffer pH 7.0 (20 mM), NaCl (100 mM), EDTA (0.5 mM)

All tobramycin analogs showed improved binding over tobramycin (**1a**). Like the amikacin derivatives, the urea modification resulted in the weakest binders. This urea tobramycin analog (**1f**) was the only one that was not superior to all of the amikacin derivatives. In contrast to the amikacin analogs, the tobramycin modifications showed substantial variability in their affinity for the A-site. 6''-Deoxy-6''-aminotobramycin (**1b**) and 6''-deoxy-6''-(2-(aminoethyl)amino)tobramycin (**1e**) showed the highest affinities of all derivatives tested. The methylamino (**1c**) and dimethylamino (**1d**) modified derivatives were the next best binders. 6''-Deoxy-6''-(4-(aminomethyl)-1H-

1,2,3-triazol-1-yl)tobramycin (**1g**) was worse than these, but still significantly better than the urea modified analog (**1f**).

Antibacterial activities

To assess the relative antibacterial activities of the synthetic derivatives, minimum inhibitory concentration (MIC) values of both the modified and parent antibiotics were determined against an array of bacterial strains (Tables 4.3 and 4.4). Multiple gram positive and gram negative strains were chosen to establish a broad spectrum representation of antibacterial activity. The compounds were first tested against the antibacterial susceptible control *E. coli* strain ATCC25922. No derivatives showed improvement against this strain and only one compound, 6''-Deoxy-6''-aminoamikacin (**2b**), even showed equal activity to its parent aminoglycoside with an MIC value of 6.25 – 12.5 μg/mL.

The aminoglycosides were tested against three *P. aeruginosa* strains, P4, PA01, and ATCC27853. Tobramycin (**1a**) shows much better activity than amikacin (**2a**) against these *P. aeruginosa* strains. Unfortunately, only one tobramycin derivative, 6''-Deoxy-6''-(4-(aminomethyl)-1H-1,2,3-triazol-1-yl)tobramycin (**1g**), showed even equal activity to tobramycin (**1a**) against any of these strains. Both had MIC values of 0.39 μg/mL against PA01. However, the amikacin derivatives, 6''-deoxy-6''-aminoamikacin (**2b**), 6''-deoxy-6''-methylaminoamikacin (**2c**), and 6''-deoxy-6''-(2-(aminoethyl)amino)amikacin (**2e**) showed improved activity. 6''-Deoxy-6''-(2-(aminoethyl)amino)amikacin (**2e**) showed superior activity against all three strains including a four-fold improvement to 6.25 μg/mL against P4. 6''-Deoxy-6''-

Table 4.3: Inhibitory activities of tobramycin (**1a**) and derivatives against a panel of bacterial strains (μg ml^{-1})[a]

Bacterial Strain	1a	1b	1c	1d	1e	1f	1g
E. coli (ATCC25922)	3.125	25-50	≥50	≥50	6.25-12.5	6.25	6.25
P. aeruginosa (P4)	0.78	25	>50	>50	3.125-6.25	3.125-6.25	1.56
P. aeruginosa (PA01)	0.39	12.5	50	50	1.56	0.78	*0.39*
P. aeruginosa (ATCC27853)	0.39	12.5-50	50	>50	3.125	0.78-1.56	0.78
K. pneumoniae (ATCC700603)	6.25	12.5	25	25-50	*6.25*	12.5	12.5
K. pneumoniae (GNR1100)	>50	>50	>50	>50	>50	>50	>50
MRSA (TCH1516)	0.78-1.56	6.25-12.5	12.5-25	25	6.25	3.125-6.25	6.25
MRSA (ATCC33591)	3.125	6.25	12.5-25	25	3.125-6.25	*3.125*	**0.78-1.56**
MRSA (Sanger 252)	>50	>50	>50	>50	>50	>50	>50

[a] Minimum inhibitory concentration (MIC) values [mg mL^{-1}]. MIC value equal to tobramycin (*italics*); MIC value lower than tobramycin (**bold**).

aminoamikacin (**2b**) was equal to amikacin (**2a**) with MIC values of 1.56 – 3.125 μg/mL against PA01, but was slightly improved against ATCC27853 with an MIC value of 1.56 – 3.125 μg/mL compared to a parent MIC value of 3.125 μg/mL. It also showed a four-fold improvement against P4.

The aminoglycosides were also tested against two *K. pneumoniae* strains, ATCC700603 and the highly drug resistant, *K. pneumoniae* carbapenemase producer GNR1100. Amikacin (**2a**) shows better activity than tobramycin (**1a**) against these strains. Again, the tobramycin derivatives were disappointing with only 6''-deoxy-6''-(2-(aminoethyl)amino)tobramycin (**1e**) showing even equal activity to the parent. Both had

Table 4.4: Inhibitory activities of amikacin (2a) and derivatives against a panel of bacterial strains (µg ml⁻¹)[a]							
Bacterial Strain	**2a**	**2b**	**2c**	**2d**	**2e**	**2f**	**2g**
E. coli (ATCC25922)	6.25 - 12.5	*6.25 - 12.5*	12.5 - 25	25 - 50	12.5	50	12.5
P. aeruginosa (P4)	25	**6.25**	**12.5**	>50	**6.25**	>50	25 - 50
P. aeruginosa (PA01)	1.56 - 3.125	*1.56 - 3.125*	3.125	12.5 - 25	**1.56**	25 - 50	*1.56 - 3.125*
P. aeruginosa (ATCC27853)	3.125	**1.56 - 3.125**	3.125 - 6.25	25	**1.56**	25 - 50	*3.125*
K. pneumoniae (ATCC700603)	0.781	0.781 - 1.56	1.56	3.125	*0.781*	6.25 - 12.5	*0.781*
K. pneumoniae (GNR1100)	50	*50*	>50	>50	**12.5 - 25**	>50	**12.5 - 25**
MRSA (TCH1516)	6.25 - 12.5	12.5 - 25	50	>50	12.5 - 25	>50	12.5 - 25
MRSA (ATCC33591)	25	**12.5 - 25**	*25*	>50	**12.5**	>50	**12.5**
MRSA (Sanger 252)	12.5 - 25	>50	>50	>50	25 - 50	>50	25 - 50

[a] Minimum inhibitory concentration (MIC) values [mg mL⁻¹]. MIC value equal to amikacin (*italics*); MIC value lower than amikacin (**bold**).

MIC values of 6.25 µg/mL against ATCC700603. 6''-Deoxy-6''-(2-(aminoethyl)amino)amikacin (**2e**) and 6''-deoxy-6''-(4-(aminomethyl)-1H-1,2,3-triazol-1-yl)amikacin (**2g**) showed equal activity to amikacin (**2a**) against ATCC700603 with MIC values of 0.781 µg/mL. Interestingly, they both improved from 50 µg/mL to 12.5 – 25 µg/mL against GNR1100 also.

To test efficacy against gram positive bacteria, the aminoglycosides were tested against MRSA strains TCH1516, ATCC33591, and Sanger 252. No amikacin or tobramycin derivatives showed any improvements or even equal activity to their parents against TCH1516 or Sanger 252. There were several compounds that showed improved

activity against ATCC33591, however. 6''-Deoxy-6''-(4-(aminomethyl)-1H-1,2,3-triazol-1-yl)tobramycin (**1g**) improved to an MIC value of 0.78 – 1.56 µg/mL from a parent value of 3.125 µg/mL. Several amikacin derivatives showed increased potency compared to the parent MIC value of 25 µg/mL for amikacin (**2a**). These included 6''-deoxy-6''-aminoamikacin (**2b**), with a slight improvement to 12.5 – 25 µg/mL and 6''-deoxy-6''-(4-(aminomethyl)-1H-1,2,3-triazol-1-yl)amikacin (**2g**) and 6''-deoxy-6''-(2-(aminoethyl)amino)amikacin (**2e**), which both had more significant improvements to 12.5 µg/mL.

4.3 Discussion

Tobramycin and amikacin analogs modified at the 6'' position were synthesized to evaluate their A-site affinities and antibacterial activity compared to their parent compounds. All tobramycin analogs showed superior affinity for the A-site as compared to tobramycin (**1a**). There was more variation in A-site affinity among the tobramycin analogs compared to the amikacin derivatives. The tightest binders were 6''-deoxy-6''-aminotobramycin (**1b**) and 6''-deoxy-6''-(2-(aminoethyl)amino)tobramycin (**1e**) and the worst tobramycin analog was 6''-deoxy-6''-ureidotobramycin (**1f**). The general trend among the tobramycin analogs suggests that binders with smaller steric bulk or with greater overall potential charge show higher affinity.

All amikacin analogs showed improved A-site binding with the exception of 6''-deoxy-6''-ureidoamikacin (**2f**), which had by far the lowest A-site affinity of any compound tested. It is the only modification made without a basic functionality, which likely contributed to its lack of affinity. The amikacin analogs with one additional basic

functional group showed similar IC_{50} values including the bulky 6''-deoxy-6''-(4-(aminomethyl)-1H-1,2,3-triazol-1-yl)amikacin (**2g**). 6''-Deoxy-6''-(2-(aminoethyl)amino)amikacin (**2e**) has two additional basic amines as compared to amikacin (**2a**) and it was the highest affinity amikacin analog. In contrast to the tobramycin analogs, amikacin analogs appear to exhibit affinities based solely on electrostatic effects with no apparent steric preference among the analogs tested.

When analyzing MIC values, it is important to remember that aminoglycoside affinities to the A-site do not correlate with antibacterial potency.[25] Interestingly, all but one derivative, 6''-deoxy-6''-aminoamikacin (**2b**), showed inferior antibacterial activity against the control *E. coli* strain ATCC25922. This suggests that improvements in activity against resistant strains is at least partially due to overcoming bacterial resistance mechanisms.

The tobramycin analogs generally showed disappointing antibacterial activity. The most successful analog was 6''-deoxy-6''-(4-(aminomethyl)-1H-1,2,3-triazol-1-yl)tobramycin (**1g**). It showed better activity than tobramycin (**1a**) against a MRSA strain and equal activity against one *P. aeruginosa* strain. In most other cases its MIC values were two fold worse. This was also one of the more successful modifications among the amikacin analogs. 6''-Deoxy-6''-(4-(aminomethyl)-1H-1,2,3-triazol-1-yl)amikacin (**2g**) showed equal or better activity than amikacin (**2a**) against five out of nine strains tested against and in all other cases its MIC was within one serial dilution. It is intriguing that this modification was so efficacious since it was the most structurally significant alteration made.

6''-Deoxy-6''-aminoamikacin (**2b**) was also promising with equal or improved activity against six out of nine strains, including all three *P. aeruginosa* strains. It is interesting to note that antibacterial activity was reduced across the entire panel for 6''-deoxy-6''-methylaminoamikacin (**2c**) and even more so for 6''-deoxy-6''-dimethylaminoamikacin (**2d**). This trend was also present in the tobramycin derivatives. This suggests hydrogen bonding may be playing a role in the increased activity of 6''-deoxy-6''-aminoamikacin (**2b**).

The most successful derivative made, however, was 6''-deoxy-6''-(2-(aminoethyl)amino)- amikacin (**2e**). This compound showed increased activity against five strains and equal activity against one. It was universally better against the *P. aeruginosa* strains and it showed equal or better activity against both *K. pneumoniae* strains including an improvement against GNR1100. This makes the broad spectrum improvement of some of the amikacin derivatives particularly fascinating given that amikacin itself is a semi-synthetic aminoglycoside with an amino 2-hydroxybutyryl (AHB) side chain, which lowers its susceptibility to aminoglycoside-modifying enzymes.[26] It is possible that the AHB and 6'' modifications operate synergistically to further decrease its affinity for these enzymes. This is a hypothesis that we have previously posited when we observed increased antibacterial activity in an analog with a guanidinium group in this position.[22]

4.4 Conclusions

A series of 6'' modified tobramycin and amikacin analogs were synthesized. In all cases the derivatives showed improved A-site affinity compared with their parent

antibiotics when tested in an in vitro FRET-based assay with the exception of 6''-deoxy-6''-ureidoamikacin (**2f**), which showed greatly decreased binding. Tobramycin analogs generally showed disappointing antibacterial activity. In contrast, several amikacin analogs exhibited promising antibacterial potency. Most notably 6''-deoxy-6''-(2-(aminoethyl)amino)amikacin (**2e**) showed greater potency than amikacin (**2a**) against the majority of strains that were tested in MIC assays.

4.5 Experimental Section

Materials

Unless otherwise specified, materials purchased from commercial suppliers were used without further purification. Tobramycin (**1a**) and amikacin (**2a**) were obtained from Sigma–Aldrich as their free bases. Propargyl (Boc)amine was synthesized according to an established procedure.[27] Anhydrous NH_3 was purchased from Airgas. All other anhydrous solvents and reagents, and ion exchange resins were purchased from Sigma–Aldrich. NMR solvents were purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

The Dy-547-labeled A-site construct was purchased from Thermo Scientific and purified by gel electrophoresis. Kanamycin–coumarin and neomycin–courmarin conjugates were synthesized and purified according to established procedures.[24] Chemicals for preparing buffer solutions (enzyme grade) were purchased from Fisher Biotech. Autoclaved water was used in all fluorescence titrations.

Mueller–Hinton broth used for sensitivity testing was obtained from Hardy Diagnostics (Santa Maria, CA, USA). Polystyrene 96-well microplates for MIC testing

were purchased from Corning Inc. (Corning, NY, USA). Bacterial strains for sensitivity testing included five strains from the American Type Culture Collection (Manassas, VA, USA): hospital-associated MRSA strain 33591 rendered resistant to rifampicin by serial passage, USA300 MRSA strain TCH1516 (BAA-1717), *K. pneumoniae* strain 700603, *P. aeruginosa* strain 27853, and *E. coli* strain 25922. *P. aeruginosa* strain PA01 was used as a general antibiotic-sensitive *P. aeruginosa* strain.[28] USA200 MRSA strain Sanger 252 was obtained from the Network on Antimicrobial Resistance in *S. aureus* (NARSA) program supported under NIAID/NIH contract number HHSN272200700055C. Other Gram-negative strains used were clinical isolates obtained from a tertiary academic hospital in the New York metropolitan area; these were: *K. pneumoniae* strain GNR1100 (respiratory isolate) and *P. aeruginosa* strain P4 (sputum isolate).

Instrumentation

NMR spectra were recorded on Varian Mercury 300 and 400 MHz, Varian VX 500 MHz, and Jeol ECA 500 MHz spectrometers. Mass spectra (MS) were recorded at the University of California, San Diego Chemistry and Biochemistry Mass Spectrometry Facility, utilizing an Agilent 6230 HR-ESI-TOF mass spectrometer. Reverse-phase HPLC (Vydac C18 column) purification and analysis were carried out using an Agilent 1200 series instrument. Products were lyophilized utilizing a Labconco FreeZone 2.5 freeze drier. Steady-state fluorescence experiments were carried out in a microfluorescence cell with a path length of 1.0 cm (Hellma GmH & Co KG, Mullenheim, Germany) on a Jobin Yvon Horiba FluoroMax-3 luminescence spectrometer. A background spectrum (buffer) was subtracted from each sample. A

VersaMax plate reader (Molecular Devices, Mountain View, CA, USA) set at 600 nm wavelength was used for MIC assays.

Synthesis

(Boc)$_5$tobramycin (**3**), 6''-deoxy-6''-triisopropylbenzylsulfonyl(Boc)$_5$tobramycin (**4**), 6''-deoxy-6''-amino(Boc)$_5$tobramycin (**5**), (Boc)$_4$amikacin (**11**), 6''-deoxy-6''-triisopropylbenzylsulfonyl(Boc)$_4$amikacin (**12**), and 6''-deoxy-6''-amino(Boc)$_4$amikacin (**13**) were previously synthesized.[22, 23]

Tobramycin Derivatives

6''-Deoxy-6''-azido-(Boc)$_5$tobramycin (6). DMF (8.3 mL) was added to 6''-deoxy-6''-triisopropylbenzylsulfonyl-(Boc)$_5$tobramycin (**4**)[22] (818 mg, 0.663 mmol). Sodium azide (344 mg, 5.30 mmol) was added. The yellow solution was heated to 65 °C and stirred for 1 day. The solvent was removed under reduced pressure and the resulting solid was dissolved in DCM and washed with water. The organic layers were dried with sodium sulfate and the solvent was removed under reduced pressure. The product was isolated by flash chromatography (3.5, 4, and 5% methanol in DCM). Product: White solid (469 mg, 0.472 mmol, 71% yield). ^{1}H NMR (400 MHz, CD$_3$OD): δ 5.10 (brs, 1H), 5.09 (brs, 1H), 4.21 − 4.10 (m, 1H), 3.81 − 3.27 (m, 15H), 2.19 − 1.95 (m, 2H), 1.65 (q, J = 12 Hz, 1H), 1.60 − 1.20 (m, 46H); HR-ESI-MS calculated for C$_{43}$ H$_{76}$ N$_8$ O$_{18}$ Na [M+Na]$^+$ 1015.5159, found 1015.5161.

6''-Deoxy-6''-aminotobramycin (1b). DCM (3.0 mL) and TIPS (180 μL) were added to 6''-Deoxy-6''-amino-(Boc)₅tobramycin (**5**) (180 mg, 0.182 mmol). TFA (3.0 mL) was added. The yellow solution was stirred for 2.5 hours. The solvent was removed under reduced pressure. The remaining white solid was dissolved in water and purified by reverse phase HPLC [0 – 1.5% ACN in water (0.1% TFA) over 7 mins, flow rate is 3 mL / min, eluted after 5.6 min], then lyophilized and desalted. Product: White solid (65.1 mg, 0.140 mmol, 75% yield). ^{1}H NMR (500 MHz, CD₃OD): δ 5.14 (d, J = 4.0 Hz, 1H), 5.02 (d, J = 4 Hz, 1H), 3.85 (m, 1H), 3.62 – 3.58 (m, 2H), 3.52 – 3.46 (m, 2H), 3.30 (t, J = 9.5 Hz, 1H), 3.22 (t, J = 5 Hz, 1H), 3.19 (t, J = 10 Hz, 1H), 3.00 – 2.92 (m, 4H), 2.89 – 2.82 (m, 2H), 2.74 (td, J_1 = 13.5 Hz, J_2 = 7 Hz, 2H), 2.00 (dt, J_1= 12 Hz, J_2 = 4.5 Hz, 1H), 1.92 (dt, J_1= 13 Hz, J_2 = 4.5 Hz, 1H), 1.59 (q, J = 12 Hz, 1H), 1.20 (q, J = 12.5 Hz, 1H); ^{13}C NMR (125 MHz, D₂O): δ 100.74, 100.24, 88.51, 87.20, 75.00, 74.01, 72.46, 72.38, 71.35, 66.80, 54.71, 51.27, 50.00, 49.70, 42.11, 41.72, 36.06, 35.45; HR-ESI-MS calculated for $C_{18} H_{39} N_6 O_8$ [M+H]$^+$ 467.2828, found 467.2828.

6''-Deoxy-6''-methylamino-(Boc)₅tobramycin (7). A solution of 33% methylamine in ethanol (10 mL) was added to 6''-deoxy-6''-triisopropylbenzylsulfonyl-(Boc)₅tobramycin (**4**) [21] (0.143 g, 0.116 mmol) in a pressure tube. The vessel was capped and heated to 80 °C overnight. The vessel was cooled to 0 °C and opened, and let warm to room temperature. The solvent was removed under reduced pressure. The remaining residue was redissolved in methanol (5 mL) and DOWEX® Monosphere® 550A ion exchange resin (OH⁻ form) was added. The reaction was stirred for 12 hours at rt and filtered. The solvent was removed under reduced pressure. The product was isolated by

flash chromatography (7% methanol, then 12% methanol and 0.5% TEA in DCM).

Product: White solid (0.100 mg, mmol, 88% yield). ^{1}H-NMR (300 MHz, CD3OD): δ

5.16 (brs, 2H), 4.33 – 4.22 (m, 1H), 3.71 (t, J=10.2 Hz, 1H), 3.66 – 3.33 (m, 11H), 3.21

(t, J = 9.8 Hz, 1H) 3.14– 3.07 (m, 1H), 2.86 (p, J = 6.9 Hz, 1H), 2.77 (s, 3H), 2.02 (brs,

2H), 1.65 (q, J = 12.4 Hz, 1H), 1.55 – 1.35 (m, 46H). HR-ESI-MS calculated for $C_{44}H_{81}$

N_6O_{18} [M+H]$^+$ 981.5602, found 981.5598.

6''-Deoxy-6''-methylaminotobramycin (1c). DCM (1.7 mL) and TIPS (0.10 mL) were

added to 6''-Deoxy-6''-methylamino-(Boc)$_5$tobramycin (**7**) (100 mg, 0.101 mmol). TFA

(1.7 mL) was added. The yellow solution was stirred for 2.5 hours. The solvent was

removed under reduced pressure. The remaining white solid was dissolved in water and

purified by reverse phase HPLC [0 – 0.1% ACN in water (0.1% TFA) over 10 mins, flow

rate is 3 mL / min, eluted after 5.3 min], then lyophilized and desalted. Product: White

solid (21.5 mg, 0.045 mmol, 44% yield). ^{1}H NMR (500 MHz, D$_2$O): δ 5.13 (d, J = 4.0

Hz, 1H), 5.03 (d, J = 4.0 Hz, 1H), 4.00 (m, 1H), 3.64 – 3.58 (m, 2H), 3.55 – 3.46 (m, 2H),

3.31 (t, J = 9.5 Hz, 1H), 3.24 (t, J = 9.5 Hz, 1H), 3.18 (t, J = 9.5 Hz, 1H) , 3.03 – 2.93 (m,

4H), 2.92 – 2.82 (m, 2H), 2.81 – 2.72 (m, 2H), 2.40 (s, 3H), 2.00 (dt, J_1= 11.5 Hz, J_2 =

4.5 Hz, 1H), 1.92 (dt, J_1= 13 Hz, J_2 = 4.5 Hz, 1H), 1.59 (q, J = 11.5 Hz, 1H), 1.20 (q, J =

13 Hz, 1H); ^{13}C NMR (125 MHz, D$_2$O): δ 100.57, 88.31, 87.65, 75.04, 72.38, 71.96,

70.30, 66.78, 54.68, 51.70, 51.28, 49.99, 49.86, 49.71, 42.13, 36.08, 35.51, 35.26; HR-

ESI-MS calculated for $C_{19}H_{41}N_6O_8$ [M+H]$^+$ 481.2980, found 481.2983.

6''-Deoxy-6''-dimethylamino-(Boc)₅tobramycin (8). Anhydrous DMF (4 mL) was added to 6''-deoxy-6''-triisopropylbenzylsulfonyl-(Boc)₅tobrmamycin (**4**) [22] (320 mg, 0.26 mmol) in a pressure tube. A 2 M solution of dimethylamine in THF (4 mL) was added. The vessel was capped and heated to 80 °C overnight. The vessel was cooled to 0 °C and opened. The solvent was removed under reduced pressure. The remaining residue was redissolved in methanol (6.2 mL) and DOWEX® Monosphere® 550A ion exchange resin (⁻OH form) was added. The reaction was stirred for 12 hours at rt and filtered. The solvent was removed under reduced pressure. Product: White solid (235 mg, 0.246 mmol, 91% yield). ^{1}H NMR (400 MHz, CD₃OD): δ 5.28 (br s, 1H), 5.05 (br s, 1H), 4.10 – 3.96 (m, 1H), 3.77 – 3.28 (m, 11H), 3.16 – 3.07 (m, 1H), 2.65 – 2. 56 (m, 1H), 2.53 – 2.41 (m, 1H), 2.32 (s, 6H), 2.46 (t, J = 7.6 Hz, 1H), 2.17 – 1.90 (m, 2H), 1.74 – 1.22 (m, 47H); HR-ESI-MS calculated for $C_{45} H_{83} N_6 O_{18}$ [M+H]$^+$ 995.5758, found 995.5756.

6''-Deoxy-6''-dimethylaminotobramycin (1d). DCM (3.3 mL) and TIPS (199 μL) were added to 6''-Deoxy-6''-dimethylamino-(Boc)₅tobramycin (**8**) (198 mg, 0.200 mmol). TFA (3.3 mL) was added. The yellow solution was stirred for 2.5 hours. The solvent was removed under reduced pressure. The remaining white solid was dissolved in water and purified by reverse phase HPLC [0 – 1.5% ACN in water (0.1% TFA) over 7 mins, flow rate is 3 mL / min) eluted after 5.3 min], then lyophilized and desalted. Product: White solid (72.8 mg, 0.147 mmol, 74% yield) ^{1}H NMR (500 MHz, D₂O): δ 5.10 (d, J = 3.5 Hz, 1H), 5.05 (d, J = 4 Hz, 1H), 4.02 – 3.96 (m, 1H), 3.59 – 3.52 (m, 1H), 3.51 (dd, J_1 = 10 Hz, J_2 = 4 Hz, 1H), 3.31 (t, J = 9.5 Hz, 1H), 3.28 (t, J = 9.5 Hz, 1H), 3.14 (t, J = 9.5 Hz, 1H), 3.06 – 2.94 (m, 3H), 2.94 – 2.84 (m, 2H), 2.76 (dd, J_1 = 14 Hz, J_2 = 7.5 Hz, 1H),

2.68 (dd, J_1 = 16 Hz, J_2 = 2 Hz, 1H), 2.56 (dd, J_1 = 13.5 Hz, J_2 = 8.5 Hz, 1H), 2.05 (dt, J_1 = 12 Hz, J_2 = 4.5 Hz, 1H), 1.97 (dt, J_1 = 13.5 Hz, J_2 = 4.5 Hz, 1H), 1.62 (q, J = 12 Hz, 1H); 1.24 (q, J = 12.5 Hz, 1H); ^{13}C NMR (125 MHz, D$_2$O): δ 101.02, 100.46, 88.67, 88.08, 75.24, 74.30, 72.67, 72.49, 70.11, 66.84, 60.87, 54.70, 51.14, 50.29, 49.89, 45.65, 42.22, 36.21, 35.35; HR-ESI-MS calculated for $C_{20}H_{43}N_6O_8$ [M+H]$^+$ 495.3139, found 495.3139.

6''-Deoxy-6''-(2-(aminoethyl)amino)-(Boc)$_5$tobramycin (9). Anhydrous methanol (15 mL) was added to 6''-deoxy-6''-triisopropylbenzylsulfonyl-(Boc)$_5$tobrmaycin (**4**) [22] (0.170 g, 0.138 mmol) in a pressure tube. Ethylene diamine (0.4 mL, 6 mmol) was added. The vessel was capped and heated to 80 °C for 2 days. The vessel was cooled to 0 °C and opened. The solvent was removed under reduced pressure. The remaining residue was suspended in toluene and the solvent was removed under reduced pressure. This was repeated three more times. DCM (17.5 mL) and methanol (1 mL) were added to the remaining pale yellow solid. TEA (0.66 mL, 4.8 mmol) and di-tert-butyl dicarbonate (2.62 g, 12 mmol) were added. The orange solution was stirred overnight. The solvent was removed under reduced pressure. The product was isolated by automated flash chromatography (15 - 80% ethyl acetate in hexanes over 18 mins) eluted after 13 min. Product: Light yellow solid (96.6 mg, 0.080 mmol, 58% yield). ^{1}H NMR (500 MHz, CD$_3$OD): δ 5.09 (br s, 1H), 5.01 (d, J = 3.6 Hz, 1H), 4.19 – 4.11 (m, 1H), 3.99 (dd, J_1 = 9.0 Hz, J_2 = 3.8 Hz, 1H), 3.84 (t, J = 5.4 Hz, 1H), 3.77 – 3.60 (m, 5H), 3.51 – 3.32 (m, 6H), 3.25 – 3.08 (m, 4H), 3.00 – 2.63 (m, 6H), 2.16 – 2.07 (m, 1H), 1.99 – 1.91 (m, 1H),

1.81 – 1.72 (m, 1H), 1.63 – 1.55 (m, 1H), 1.51 – 1.25 (m, 63H); HR-ESI-MS calculated

for $C_{55} H_{99} N_7 O_{22}$ Na $[M+Na]^+$ 1232.6735, found 1232.6733.

6''-Deoxy-6''-(2-(aminoethyl)amino)tobramycin (1e). DCM (1.68 mL) and TIPS (120

μL) were added to 6''-Deoxy-6''-(2-(aminoethyl)amino)-(Boc)₅tobramycin (**9**) (96.6 mg,

0.16 mmol). TFA (1.68 mL) was added. The yellow solution was stirred for 2.5 hours.

The solvent was removed under reduced pressure. The remaining white solid was

dissolved in water and purified by reverse phase HPLC [0 – 0.8% ACN in water (0.1%

TFA) over 8 mins, flow rate is 3 mL / min), eluted after 5.0 min], then lyophilized and

desalted. Product: Light yellow solid (16.1 mg, 0.032 mmol, 40% yield). ^{1}H NMR (500

MHz, D_2O): δ 5.47 (d, J = 3.2 Hz, 1H), 5.17 (d, J = 3.6 Hz, 1H), 4.38 – 4.34 (m, 1H),

4.24 (dd, J_1 = 9.2 Hz, J_2 = 4.2 Hz, 1H), 4.11 – 3.98 (m, 2H), 3.88 – 3.73 (m, 4H), 3.65

(dd, J_1 = 10.2 Hz, J_2 = 3.8 Hz, 1H), 3.60 – 3.34 (m, 12H), 3.21 (q, J = 6.8 Hz, 1H), 3.14

(t, J = 7.2 Hz, 2H), 2.21 – 2.11 (m, 2H), 1.97 – 1.88 (m, 1H), 1.77 (q, J = 12.6 Hz, 1H);

^{13}C NMR (125 MHz, D_2O): δ 173.08, 99.05, 96.86, 78.40, 73.53, 71.97, 71.80, 70.69,

70.49, 69.84, 69.21, 68.77, 67.90, 55.24, 50.97, 49.69, 48.60, 46.07, 41.69, 40.21, 40.12,

38.98, 36.16, 33.15; HR-ESI-MS calculated for $C_{20} H_{44} N_7 O_8$ $[M+H]^+$ 510.3046, found

510.3243.

6''-Deoxy-6''-ureidotobramycin (1f). Anhydrous pyridine (1.78 mL) was added to to

6''-deoxy-6''-amino-(Boc)₅tobramycin (**5**) (200 mg, 0.207 mmol). Dimethoxybenzyl

isocyanate (59 μL, 0.197 mmol) was added. The yellow solution was wrapped in tinfoil

and stirred overnight. The solvent was removed under reduced pressure. The product was

isolated by flash chromatography (6% methanol in DCM). The pale yellow solid product

was carried on without further purification. DCM (2.24 mL) and TIPS (134 µL) were added to the crude product. TFA (2.24 mL) was added. The yellow solution was stirred for 3.5 hours. The solvent was removed under reduced pressure. The remaining white solid was dissolved in water and purified by reverse phase HPLC [0 – 1% ACN in water (0.1% TFA) over 7 min, eluted after 5.3 min], then lyophilized and desalted. Product: White solid (53 mg, 0.104 mmol, 53%). ^{1}H NMR (500 MHz, D$_2$O): δ 5.13 (d, J = 3.5 Hz, 1H), 4.98 (d, J = 4.0 Hz, 1H), 3.94 – 3.87 (m, 1H), 3.61 – 3.52 (m, 2H), 3.51 – 3.44 (m, 2H), 3.40 (dd, J$_1$ = 14 Hz, J$_2$ = 3 Hz, 1H), 3.31 – 3.22 (m, 2H), 3.19 (t, J = 10 Hz, 1H), 3.13 (t, J = 10 Hz, 1H), 3.00 – 2.88 (m, 3H), 2.88 – 2.79 (m, 2H), 2.72 (dd, J$_1$ = 14 Hz, J$_2$ = 7.5 Hz, 1H), 1.99 (dd, J$_1$ = 12 Hz, J$_2$ = 4.5 Hz, 1H), 1.89 (dd, J$_1$ = 13 Hz, J$_2$ = 4 Hz, 1H), 1.57 (q, J = 12 Hz, 1H), 1.17 (q, J = 12.5 Hz, 1H); ^{13}C NMR (125 MHz, D$_2$O): δ 162.21, 100.71, 100.10, 88.49, 87.07, 74.83, 74.03, 72.35, 71.46, 71.17, 88.82, 54.48, 51.18, 50.01, 49.71, 42.11, 40.93, 35.97, 35.44; HR-ESI-MS calculated for C$_{19}$ H$_{39}$ N$_7$ O$_9$ Na [M+Na]$^+$ 532.2704, found 532.2701.

6''-Deoxy-6''-(4-(aminomethyl)-1H-1,2,3-triazol-1-yl)-(Boc)$_6$tobramycin (10). A 3:1:1 of THF: tert-butanol:water solution (2.94 mL) was added to 6''-Deoxy-6''-azido-(Boc)$_5$tobramycin (**6**) (86.6 mg, 0.148 mmol) and *N*-Boc-propargylamine (27.2mg, 0.174 mmol). The solution was degassed by bubbling through argon for 25 minutes. A 1 M aqueous sodium ascorbate solution (94 µL) was added. Then a 7.5% weight / volume aqueous copper sulfate pentahydrate solution (38 µL) was added. The solution turned brown and then yellow overnight. The solvent was removed under reduced pressure. The product was isolated by flash chromatography (5% methanol in DCM). Product: Light

yellow solid (80.7 mg, 0.090 mmol, 81% yield). ^{1}H NMR (400 MHz, CD$_3$OD): δ 7.92 (s, 1H), 5.23 (s, 2H), 4.76 – 4.22 (m, 5H), 3.82 – 3.22 (m, 12H), 3.00 (t, J = 9.6Hz, 1H), 2.01 (brs, 2H), 2.68 (q, J = 12Hz, 1H), 1.60 – 1.20 (m, 55H); HR-ESI-MS calculated for C$_{51}$ H$_{89}$ N$_9$ O$_{20}$ Na [M+Na]$^+$ 1170.6116, found 1170.6112.

6''-Deoxy-6''-(4-(aminomethyl)-1H-1,2,3-triazol-1-yl)tobramycin (1g). DCM (1.8 mL) and TIPS (90 μL) were added to 6''-Deoxy-6''-(4-(aminomethyl)-1H-1,2,3-triazol-1-yl)-(Boc)$_6$tobramycin (**10**) (62 mg, 0.054 mmol). TFA (1.8 mL) was added. The yellow solution was stirred for 2.5 hours. The solvent was removed under reduced pressure. The remaining white solid was dissolved in water and purified by reverse phase HPLC [0 – 0.1% ACN in water (0.1% TFA) over 6.5 mins, eluted after 5.6 min], then lyophilized and desalted. Product: White solid (27.5 mg, 0.050 mmol, 82% yield). ^{1}H NMR (500 MHz, D$_2$O): δ 7.85 (s, 1H), 5.06 (d, J = 2.5 Hz, 1H), 4.98 (d, J = 3.0 Hz, 1H), 4.62 (d, J = 3 Hz, 1H), 4.26 – 4.22 (m, 1H), 3.82 (s, 2H), 3.58 – 3.52 (m, 1H), 3.51 – 3.44 (m, 1H), 3.35 (dd, J$_2$ = 3 Hz, J$_1$ = 10 Hz, 1H), 3.21 (t, J = 9.5 Hz, 1H), 3.14 (t, J = 9.5 Hz, 1H), 3.01 – 2.87 (m, 4H), 2.83 – 2.74 (m, 2H), 2.74 – 2.65 (m, 1H), 2.04 – 1.97 (m, 1H), 1.91 – 1.83 (m, 1H), 1.56 (q, J = 11.5 Hz, 1H), 1.14 (q, J = 12.5 Hz, 1H); ^{13}C NMR (125 MHz, D$_2$O): δ 148.86, 125.17, 100.51, 100.10, 88.05, 86.93, 74.77, 74.09, 72.16, 70.93, 70.73, 66.81, 54.68, 51.29, 51.16, 49.96, 49.66, 42.15, 36.26, 36.03, 35.53; HR-ESI-MS calculated for C$_{21}$ H$_{41}$ N$_9$ O$_8$ Na [M+Na]$^+$ 570.2970, found 570.2971.

Amikacin Derivatives
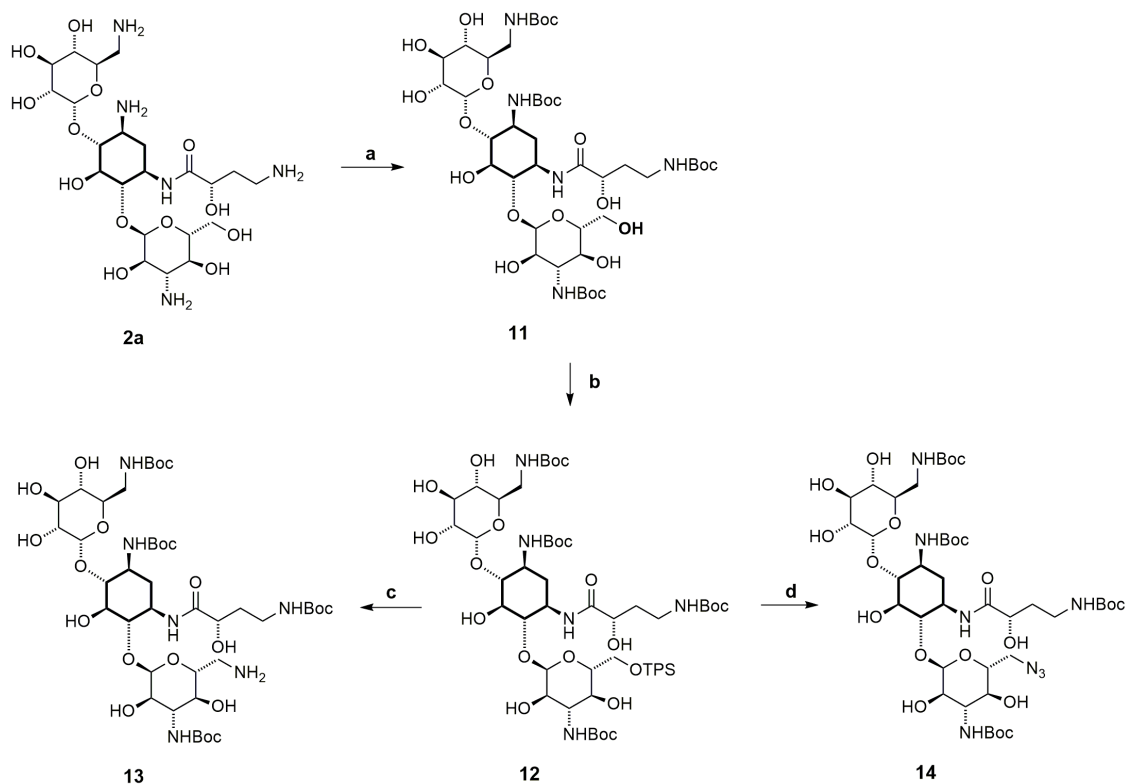
Scheme 4.5: Synthesis of key intermediates **12**, **13**, and **14**. *Reagents and conditions:* a) Boc$_2$O, Et$_3$N, H$_2$O, DMF, 55 °C; b) TPSCl, pyridine, RT; c) NH$_3$, MeOH, 80 °C; d) NaN$_3$, DMF, 55 °C

6''-Deoxy-6''-azido-(Boc)$_4$amikacin (14). DMF (48.6 mL) was added to 6''-deoxy-6''-triisopropylbenzylsulfonyl-(Boc)$_4$amikacin (**12**) [22] (2.5 g, 2 mmol). Sodium azide (520 mg, 8 mmol) was added. The yellow solution was heated to 55 °C and stirred for 2 days. The solvent was removed under reduced pressure and the resulting solid was dissolved in DCM and washed with water. The organic layers were dried with sodium sulfate and the solvent was removed under reduced pressure. The product was isolated by automated flash chromatography (0 - 20% methanol in DCM over 25 mins) eluted after 16 min. Product: White solid (1.7 g, 1.7 mmol, 85% yield). ^{1}H NMR (500 MHz, CD$_3$OD): δ 5.09 (d, J = 2 Hz, 1H), 5.01 (d, J = 3.5 Hz, 1H), 4.13 − 4.08 (m, 1H), 4.01 − 3.93 (m, 1H), 3.68 − 3.57 (m, 4H), 3.51 − 3.34 (m, 6H), 3.28 − 3.10 (m, 8 H), 2.06 − 2.00 (m, 2H), 1.94 −

1.86 (m, 1H), 1.81 − 1.77 (m, 1H), 1.47 − 1.37 (m, 36H); HR-ESI-MS calculated for

$C_{50}H_{87}N_9O_{22}Na$ [M+Na]$^+$ 1188.5858, found 1188.5859.
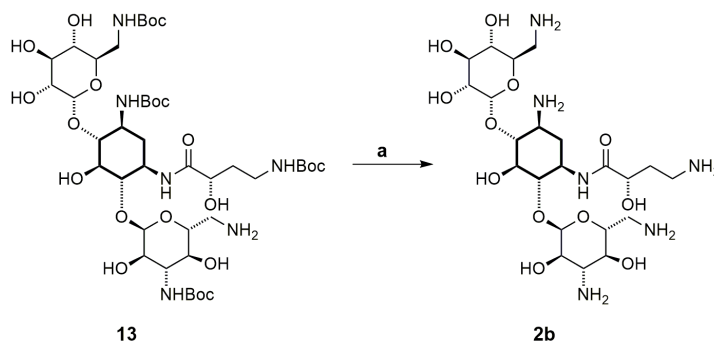
Scheme 4.6: Synthesis of 6"-deoxy-6"-aminoamikacin (**2b**). Reagents and conditions: a) TFA, TIPS, CH_2Cl_2, RT

6''-Deoxy-6''-aminoamikacin (2b). DCM (3.9 mL) and TIPS (0.23 mL) were added to

6''-deoxy-6''-amino-(Boc)$_4$amikacin (**13**) (160 mg, 0.16 mmol). TFA (3.9 mL) was

added. The yellow solution was stirred for 2.5 hours. The solvent was removed under

reduced pressure. The remaining white solid was dissolved in water and purified by

reverse phase HPLC [0 − 0.1% ACN in water (0.1% TFA) over 10 mins, flow rate is 2

mL / min] eluted after 7.8 min, then lyophilized and desalted. Product: White solid (82

mg, 0.14 mmol, 86% yield). ^{1}H NMR (400 MHz, CD$_3$OD): δ 5.28 (d, J = 4.0 Hz, 1H),

5.05 (d, J = 3.6 Hz, 1H), 4.15 (dd, J$_1$ = 9.2 Hz, J$_2$ = 3.6 Hz, 1H), 4.01 − 3.89 (m, 1H), 3.77

− 3.66 (m, 3H), 3.57 (dd, J$_1$ = 9.6 Hz, J$_2$ = 4.0 Hz, 1H), 3.36 − 3.27 (m, 3H), 3.16 (t, J =

9.6 Hz, 2H), 2.98 − 2.84 (m, 5H), 2.76 − 2.67 (m, 3H), 1.95 − 1.82 (m, 2H), 1.74 − 1.64

(m, 1H), 1.39 (q, J = 12.4 Hz, 1H); ^{13}C NMR (125 MHz, D$_2$O): δ 174.89, 97.92, 96.19,

85.10, 77.96, 72.58, 71.14, 71.10, 70.27, 70.09, 69.80, 69.12, 68.89, 67.99, 52.12, 47.80,

46.69, 39.60, 39.37, 35.41, 34.45, 32.36; HR-ESI-MS calculated for $C_{22}H_{44}N_6O_{12}Na$ $[M+Na]^+$ 607.2909, found 607.2914.
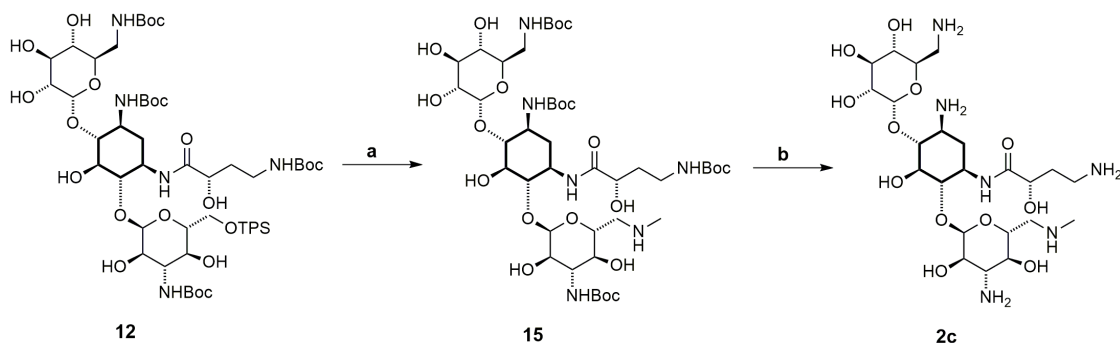
Scheme 4.7: Synthesis of 6"-Deoxy-6"-methylaminoamikacin (**2c**). Reagents and conditions: a) Methylamine, EtOH, 80 °C; b) TFA, TIPS, CH_2Cl_2, RT.

6''-Deoxy-6''-methylamino-(Boc)₄amikacin (15). A solution of 33% methylamine in ethanol (22 mL) was added to 6''-deoxy-6''-triisopropylbenzylsulfonyl-(Boc)₄amikacin (**12**) [22] (320 mg, 0.26 mmol) in a pressure tube. The vessel was capped and heated to 80 °C overnight. The vessel was cooled to 0 °C and opened. The solvent was removed under reduced pressure. The remaining residue was redissolved in methanol (15 mL) and DOWEX® Monosphere® 550A ion exchange resin (OH⁻ form) was added. The reaction was stirred for 12 hours at rt and filtered. The solvent was removed under reduced pressure. The product was isolated by flash chromatography (10% methanol, 1% TEA in DCM). Product: White solid (230 mg, 0.23 mmol, 90% yield). ^1H NMR (400 MHz, CD₃OD): δ 5.08 (br s, 1H), 5.00 (s, 1H), 4.14 – 4.06 (m, 1H), 3.99 – 3.93 (m, 1H), 3.84 – 3.75 (m, 1H), 3.75 – 3.39 (m, 5H), 3.20 – 3.05 (m, 3H), 2.99 – 2.52 (m, 6H), 2.40 – 2.30 (m, 3H), 2.25 (s, 3H), 1.95 – 1.87 (m, 2H), 1.77 – 1.65 (m, 1H), 1.41 (br s, 37H); HR-ESI-MS calculated for $C_{43}H_{78}N_6O_{20}Na$ $[M+Na]^+$ 1021.5163, found 1021.5165.

6''-Deoxy-6''-methylaminoamikacin (2c). DCM (2.1 mL) and TIPS (0.13 mL) were added to 6''-Deoxy-6''-methylamino-(Boc)$_4$amikacin (**15**) (85 mg, 0.085 mmol). TFA (2.1 mL) was added. The yellow solution was stirred for 2.5 hours. The solvent was removed under reduced pressure. The remaining white solid was dissolved in water and purified by reverse phase HPLC [0 – 0.1% ACN in water (0.1% TFA) over 10 mins, flow rate is 2 mL / min, eluted after 8.0 min], then lyophilized and desalted. Product: White solid (40 mg, 0.067 mmol, 79% yield). ^{1}H NMR (500 MHz, D$_2$O): δ 5.28 (d, J = 4.0 Hz, 1H), 5.03 (d, J = 3.5 Hz, 1H), 4.13 (dd, J$_1$ = 9.3 Hz, J$_2$ = 3.5 Hz, 1H), 4.03 – 3.92 (m, 2H), 3.72 – 3.65 (m, 4H), 3.57 (dd, J$_1$ = 9.8 Hz, J$_2$ = 4.0 Hz, 1H), 3.34 – 3.26 (m, 3H), 3.15 – 3.07 (m, 1H), 2.96 – 2.87 (m, 3H), 2.81 – 2.79 (m, 1H), 2.75 – 2.60 (m, 4H), 2.29 (s, 3H), 1.92 – 1.83 (m, 2H), 1.71 – 1.65 (m, 1H), 1.38 (q, J = 12.5 Hz, 1H); ^{13}C NMR (125 MHz, D$_2$O): δ 174.77, 98.06, 96.17, 85.15, 77.81, 72.84, 71.29, 71.10, 70.16, 69.85, 69.83, 69.19, 68.60, 68.09, 52.17, 49.72, 47.93, 46.95, 39.74, 35.51, 34.37, 33.26, 32.49; HR-ESI-MS calculated for C$_{23}$H$_{47}$N$_6$O$_{12}$ [M+H]$^+$ 599.3246, found 599.3245.
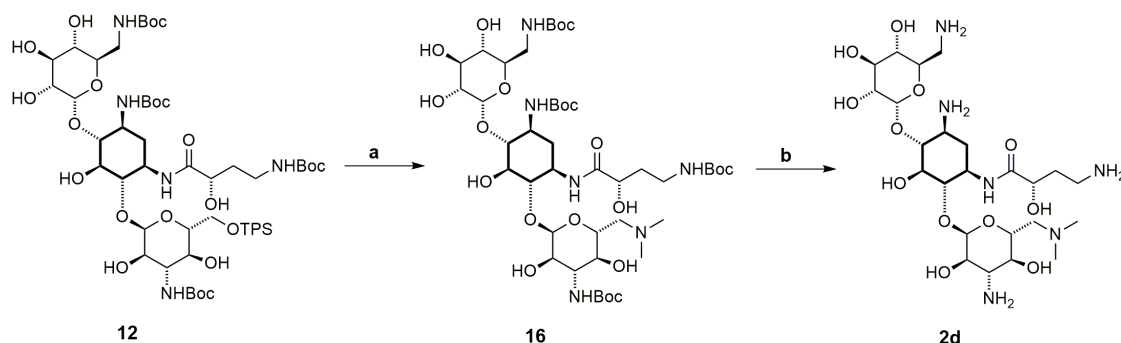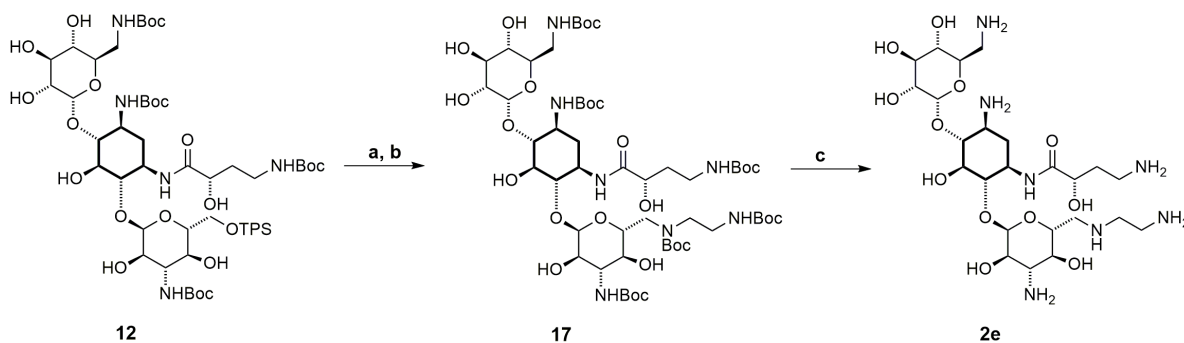

Scheme 4.8: Synthesis of 6''-deoxy-6''-dimethylaminoamikacin (**2d**). Reagents and conditions: a) Dimethylamine, THF, DMF, 80 °C; b) TFA, TIPS, CH$_2$Cl$_2$, RT.
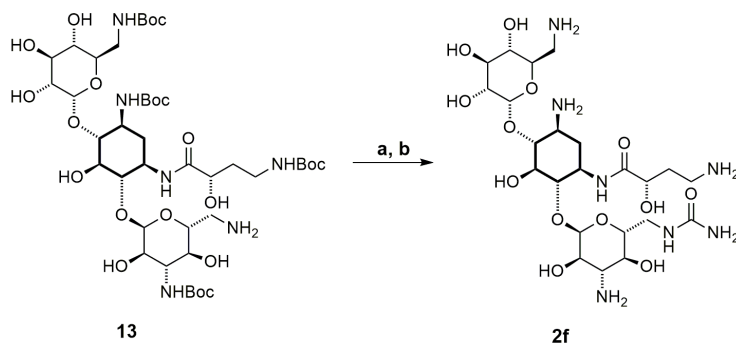
6''-Deoxy-6''-dimethylamino-(Boc)$_4$amikacin (16). Anhydrous DMF (3.1 mL) was added to 6''-deoxy-6''-triisopropylbenzylsulfonyl-(Boc)$_4$amikacin (**12**) [22] (320 mg, 0.26 mmol) in a pressure tube. A 2 M solution of dimethylamine in THF (3.1 mL) was added. The vessel was capped and heated to 80 °C overnight. The vessel was cooled to 0 °C and opened. The solvent was removed under reduced pressure. The remaining residue was redissolved in methanol (6.2 mL) and DOWEX® Monosphere® 550A ion exchange resin (OH⁻ form) was added. The reaction was stirred for 12 hours at rt and filtered. The solvent was removed under reduced pressure. The product was isolated by flash chromatography (10% methanol, 1% TEA in DCM). Product: White solid (160 mg, 0.16 mmol, 62% yield). ^{1}H NMR (400 MHz, CD$_3$OD): δ 5.08 (br s, 2H), 3.99 (dd, J$_1$ = 8.4 Hz, J$_2$ = 3.6 Hz, 1H), 3.84 – 3.78 (m, 1H), 3.72 – 3.56 (m, 3H), 3.52 – 3.34 (m, 3H), 3.24 – 3.16 (m, 2H), 3.07 – 2.82 (m, 9H), 2.69 (s, 6H), 2.46 (t, J = 7.6 Hz, 1H), 1.85 – 1.77 (m, 2H), 1.50 – 1.41 (m, 38H); HR-ESI-MS calculated for C$_{44}$H$_{80}$N$_6$O$_{20}$Na [M+Na]$^+$ 1035.5320, found 1035.5322.

6''-Deoxy-6''-dimethylaminoamikacin (2d). DCM (2.1 mL) and TIPS (0.13 mL) were added to 6''-Deoxy-6''-dimethylamino-(Boc)$_4$amikacin (**16**) (85 mg, 0.085 mmol). TFA (2.1 mL) was added. The yellow solution was stirred for 2.5 hours. The solvent was removed under reduced pressure. The remaining white solid was dissolved in water and purified by reverse phase HPLC [0 – 0.1% ACN in water (0.1% TFA) over 14 mins, flow rate is 2 mL / min, eluted after 7.9 min], then lyophilized and desalted. Product: White solid (46 mg, 0.076 mmol, 89% yield) ^{1}H NMR (300 MHz, D$_2$O): δ 5.26 (d, J = 3.9 Hz,

1H), 5.03 (d, J = 3.9 Hz, 1H), 4.12 (dd, J_1 = 9.3 Hz, J_2 = 3.8 Hz, 1H), 4.07 − 3.89 (m,

2H), 3.76 − 3.62 (m, 4H), 3.53 (dd, J_1 = 9.6 Hz, J_2 = 4.2 Hz, 1H), 3.34 − 3.20 (m, 3H),

3.07 − 2.84 (m, 4H), 2.76 − 2.43 (m, 5H), 2.21 (s, 6H), 1.95 − 1.79 (m, 2H), 1.74 − 1.64

(m, 1H), 1.35 (q, J = 12.6 Hz, 1H); ^{13}C NMR (125 MHz, D$_2$O): δ 174.13, 97.92, 95.68,

84.63, 77.32, 72.61, 70.89, 70.77, 70.01, 69.87, 69.39, 68.78, 67.68, 67.03, 58.21, 51.60,

47.49, 46.85, 39.35, 36.64, 35.03, 33.14, 32.85, 32.06; HR-ESI-MS calculated for

$C_{24}H_{49}N_6O_{12}$ [M+H]$^+$ 613.3403, found 613.3407.

Scheme 4.9: Synthesis of 6"-deoxy-6"-(2-(aminoethyl)amino)amikacin (**2e**). Reagents and conditions: a) Ethylene diamine, MeOH, 80 °C; a) Boc$_2$O, Et$_3$N, H$_2$O, DMF, 55 °C; c) TFA, TIPS, CH$_2$Cl$_2$, RT.

6''-Deoxy-6''-(2-(aminoethyl)amino)-(Boc)₆amikacin (17). Anhydrous methanol (75

mL) was added to 6''-deoxy-6''-triisopropylbenzylsulfonyl-(Boc)₄amikacin (**12**)^{22} (1.5

g, 1.2 mmol) in a pressure tube. Ethylene diamine (4 mL, 60 mmol) was added. . The

vessel was capped and heated to 80 °C for 2 days. The vessel was cooled to 0 °C and

opened. The solvent was removed under reduced pressure. The remaining residue was

suspended in toluene and the solvent was removed under reduced pressure. This was

repeated three more times. DCM (17.5 mL) and methanol (1 mL) were added to the

remaining pale yellow solid. TEA (0.66 mL, 4.8 mmol) and di-tert-butyl dicarbonate

(2.62 g, 12 mmol) were added. The orange solution was stirred overnight. The solvent was removed under reduced pressure. The product was isolated by automated flash chromatography (0 – 20% methanol in DCM over 30 mins) eluted after 13 min. Product: Light yellow solid (905 mg, 0.74 mmol, 61% yield). ^{1}H NMR (400 MHz, CD$_3$OD): δ 5.09 (br s, 1H), 5.01 (d, J = 3.6 Hz, 1H), 4.19 – 4.11 (m, 1H), 3.99 (dd, J$_1$ = 9.0 Hz, J$_2$ = 3.8 Hz, 1H), 3.84 (t, J = 5.4 Hz, 1H), 3.77 – 3.60 (m, 5H), 3.51 – 3.32 (m, 6H), 3.25 – 3.08 (m, 4H), 3.00 – 2.63 (m, 6H), 2.16 – 2.07 (m, 1H), 1.99 – 1.91 (m, 1H), 1.81 – 1.72 (m, 1H), 1.63 – 1.55 (m, 1H), 1.51 – 1.25 (m, 54H); HR-ESI-MS calculated for C$_{54}$H$_{97}$N$_7$O$_{24}$Na [M+Na]$^+$ 1250.6477, found 1250.6483.

6''-Deoxy-6''-(2-(aminoethyl)amino)amikacin (2e). DCM (3.9 mL) and TIPS (0.23 mL) were added to 6''-Deoxy-6''-(2-(aminoethyl)amino)-(Boc)$_6$amikacin (**17**) (200 mg, 0.16 mmol). TFA (3.9 mL) was added. The yellow solution was stirred for 2.5 hours. The solvent was removed under reduced pressure. The remaining white solid was dissolved in water and purified by reverse phase HPLC [0 – 0.1% ACN in water (0.1% TFA) over 10 mins, flow rate is 2 mL / min, eluted after 7.5 min], then lyophilized and desalted. Product: White solid (75 mg, 0.12 mmol, 74% yield). ^{1}H NMR (400 MHz, D$_2$O): δ 5.47 (d, J = 3.2 Hz, 1H), 5.17 (d, J = 3.6 Hz, 1H), 4.38 – 4.34 (m, 1H), 4.24 (dd, J$_1$ = 9.2 Hz, J$_2$ = 4.2 Hz, 1H), 4.11 – 3.98 (m, 2H), 3.88 – 3.73 (m, 4H), 3.65 (dd, J$_1$ = 10.2 Hz, J$_2$ = 3.8 Hz, 1H), 3.60 – 3.34 (m, 12H), 3.21 (q, J = 6.8 Hz, 1H), 3.14 (t, J = 7.2 Hz, 2H), 2.21 – 2.11 (m, 2H), 1.97 – 1.88 (m, 1H), 1.77 (q, J = 12.6 Hz, 1H); ^{13}C NMR (125 MHz, D$_2$O): δ 174.68, 98.22, 96.10, 85.30, 77.62, 72.76, 71.16, 70.95, 69.89, 69.69,

69.04, 68.41, 67.94, 51.99, 48.87, 47.80, 46.83, 42.88, 40.86, 39.62, 37.85, 35.37, 34.28, 32.35; HR-ESI-MS calculated for $C_{24}H_{49}N_7O_{12}Na$ $[M+Na]^+$ 650.3331, found 650.3326.

Scheme 4.10: Synthesis 6"-deoxy-6"-ureidoamikacin (**2f**). *Reagents and conditions:* a) 2, 4-Dimethoxybenzyl isocyanate, pyridine, RT; b) TFA, TIPS, CH_2Cl_2, RT.

6''-Deoxy-6''-ureidoamikacin (2f). Anhydrous pyridine (1.78 mL) was added to to 6''-deoxy-6''-amino-(Boc)₄amikacin (**13**) (266 mg, 0.27 mmol). Dimethoxybenzyl isocyanate (82 μL, 0.27 mmol) was added. The yellow solution was wrapped in tinfoil and stirred overnight. The solvent was removed under reduced pressure. The product was isolated by automated flash chromatography (0 – 20% methanol in DCM over 11 mins) eluted after 7.5 min. The pale yellow solid product was carried on without further purification. DCM (6.4 mL) and TIPS (0.38 mL) were added to the crude product. TFA (6.4 mL) was added. The yellow solution was stirred for 3.5 hours. The solvent was removed under reduced pressure. The remaining white solid was dissolved in water and purified by reverse phase HPLC [0 – 1% ACN in water (0.1% TFA) over 8 mins, eluted after 5.1 min], then lyophilized and desalted. Product: White solid (95 mg, 0.15 mmol, 56%). ^{1}H NMR (500 MHz, D₂O): δ 5.28 (s, 1H), 4.98 (d, J = 3.5 Hz, 1H), 4.09 (dd, J_1 = 9.5 Hz, J_2 = 3.8 Hz, 1H), 4.02 – 3.89 (m, 2H), 3.79 – 3.60 (m, 4H), 3.55 (dd, J_1 = 9.4 Hz, J_2 = 4.2 Hz, 1H), 3.42 – 3.16 (m, 6H), 3.14 – 3.02 (m, 3H), 2.97 – 2.82 (m, 3H), 1.98 –

1.84 (m, 2H), 1.74 – 1.62 (m, 1H), 1.36 (q, J = 12.4 Hz, 1H); ^{13}C NMR (125 MHz, D$_2$O):

δ 173.42, 158.43, 96.03, 95.36, 79.63, 77.04, 71.45, 71.22, 69.71, 68.71, 68.68, 67.95,

67.37, 66.00, 57.18, 51.26, 46.59, 45.48, 42.47, 38.29, 34.97, 31.15, 26.42; HR-ESI-MS

calculated for C$_{23}$H$_{45}$N$_7$O$_{13}$Na [M+Na]$^+$ 650.2968, found 650.2974.
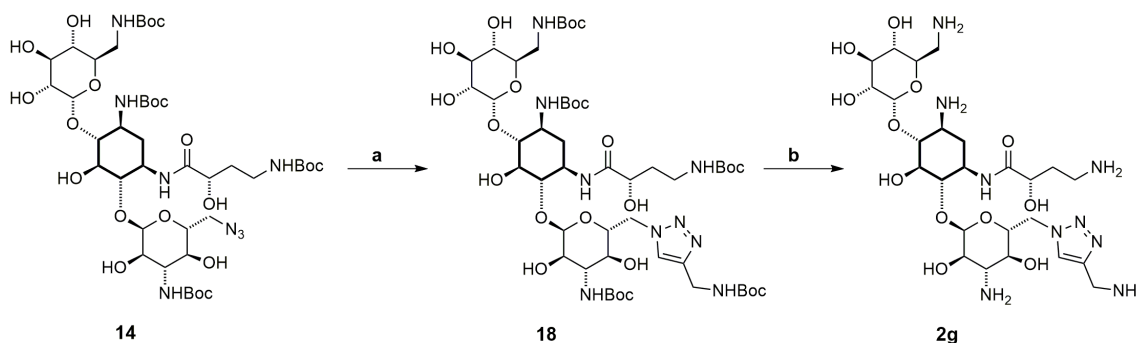
Scheme 4.11: Synthesis of 6"-deoxy-6"-(4-(aminomethyl)-1H-1,2,3-triazol-1-yl)amikacin (**2g**). *Reagents and conditions:* a) Propargyl (Boc)amine, CuSO$_4$ 5 H$_2$O, sodium ascorbate, THF, tBuOH, H$_2$O, RT; b) TFA, TIPS, CH$_2$Cl$_2$, RT.

6''-Deoxy-6''-(4-(aminomethyl)-1H-1,2,3-triazol-1-yl)-(Boc)$_5$amikacin (18). A 3 THF:

1 tert-butanol: 1 water solution (5.1 mL) was added to 6''-deoxy-6''-azido-

(Boc)$_4$amikacin (**14**) (150 mg, 0.148 mmol) and *N*-Boc-propargylamine (27 mg, 0.174

mmol). The solution was degassed by bubbling through argon for 25 minutes. A 7.5%

weight / volume aqueous copper sulfate pentahydrate solution (64 μL) was added. Then a

1 M aqueous sodium ascorbate solution (156 μL) was added. The solution turned from

light blue to orange. The solution was stirred overnight during which time it became a

yellow mixture. The solvent was removed under reduced pressure. The product was

isolated by automated flash chromatography (0 – 20% methanol in DCM over 11 mins)

eluted after 7.5 min. Product: Light yellow solid (120 mg, 0.103 mmol, 70% yield). ^{1}H

NMR (400 MHz, CD$_3$OD): δ 6.89 (s, 1H), 5.00 (d, J = 3.6 Hz, 1H), 4.97 (s, 1H), 4.67 –

4.39 (m, 6H), 4.34 – 4.24 (m, 1H), 3.67 – 3.43 (m, 6H), 3.19 – 2.79 (m, 8H), 2.68 (s, 1H), 2.01 – 1.85 (m, 2H), 1.75 – 0.97 (m, 56H); HR-ESI-MS calculated for $C_{42}H_{74}N_8O_{20}Na$ $[M+Na]^+$ 1033.4912, found 1033.4913.

6''-Deoxy-6''-(4-(aminomethyl)-1H-1,2,3-triazol-1-yl)amikacin (2g). DCM (2.45 mL) and TIPS (140 µL) were added to 6''-deoxy-6''-(4-(aminomethyl)-1H-1,2,3-triazol-1-yl)-(Boc)₅amikacin (**18**) (110 mg, 0.094 mmol). TFA (2.45 mL) was added. The yellow solution was stirred for 2.5 hours. The solvent was removed under reduced pressure. The remaining white solid was dissolved in water and purified by reverse phase HPLC [0 – 1.5% ACN in water (0.1% TFA) over 7 mins, eluted after 5.0 min], then lyophilized and desalted. Product: White solid (51 mg, 0.077 mmol, 82% yield). ^{1}H NMR (400 MHz, D₂O): δ 7.89 (s, 1H), 5.17 (d, J = 2.4 Hz, 1H), 5.02 (d, J = 4.0 Hz, 1H), 4.68 – 4.52 (m, 2H), 4.41 – 4.34 (m, 1H), 4.26 (t, J = 11.2 Hz, 1H), 4.15 (d, J = 8.8 Hz, 1H), 3.96 – 3.88 (m, 2H), 3.77 – 3.53 (m, 5H), 3.33 – 3.25 (m, 2H), 3.15 – 3.07 (m, 2H), 2.98 – 2.95 (m, 2H), 2.89 – 2.68 (m, 4H), 1.91 – 1.81 (m, 2H), 1.73 – 1.59 (m, 1H), 1.34 (q, J = 12.6 Hz, 1H); ^{13}C NMR (125 MHz, D₂O): δ 172.7, 147.18, 123.23, 99.32, 96.97, 86.43, 78.81, 78.06, 77.15, 74.63, 73.15, 72.03, 70.32, 69.92, 69.41, 69.05, 68.80, 53.10, 49.77, 48.52, 47.58, 40.47, 36.25, 34.93, 34.64; HR-ESI-MS calculated for $C_{25}H_{47}N_9O_{12}Na$ $[M+Na]^+$ 688.3236, found 688.3234.

Desalting

Aminoglycoside·TFA (up to 40 mg) was dissolved in autoclaved H₂O (0.6 mL) in a sterile eppendorf tube. Dowex Monosphere 550 A (100 mg) was added, and the

suspension wasshaken lightly on a Fisher Vortex Genie 2 overnight. The resin was

removed by centrifugal filtration and washed twice with autoclaved H_2O. The desalted

solutions were lyophilized, and the removal of TFA counterions was confirmed by ^{13}C

NMR spectroscopy.

A-site Binding Assay

All titrations were performed with working solutions of 1 μM Dy-547 labeled A-

site in 20 mM cacodylate buffer (pH = 7.0, 100 mM NaCl, 0.5 mM EDTA). The

solutions were heated to 75 °C for 5 min, cooled to room temperature over 2 h, cooled to

0 °C for 30 min, then allowed to warm back to room temperature. Kanamycin-courmarin

or neomycin-coumarin was added, to give a working concentration of 0.53 μM, just prior

to aminoglycoside titrations. Steady state fluorescence experiments were carried out at

ambient temperature (20 °C). Excitation and emission slit widths were 9 nm for

kanamycin-coumarin experiments and 7 nm for neomycin-coumarin. The system was

excited at 400 nm and changes in Dy-547 emission were monitored at 561 nm. Errors

were generated from three sets of measurements. IC_{50} values were calculated using

OriginPro 8.5 software by fitting a dose response curve (eq 1) to the fractional

fluorescence saturation (Fs) plotted against the log of antibiotic (A) concentration.

$F_s = F_0 + (F_\infty[A]^n)/([IC50]^n + [A]^n)$ (1)

F_s is the fluorescence intensity at each titration point. F_0 and F_∞ are the

fluorescence intensity in the absence of aminoglycoside or at saturation, respectively, and

n is the Hill coefficient or degree of cooperativity associated with binding. Representative

raw fluorescence data next to a normalized binding curve are previously pictured (Figure

2.12). The binding curves for the displacement of kanamycin-coumarin (Figure 4.4) and neomycin-coumarin (Figure 4.5) are pictured.
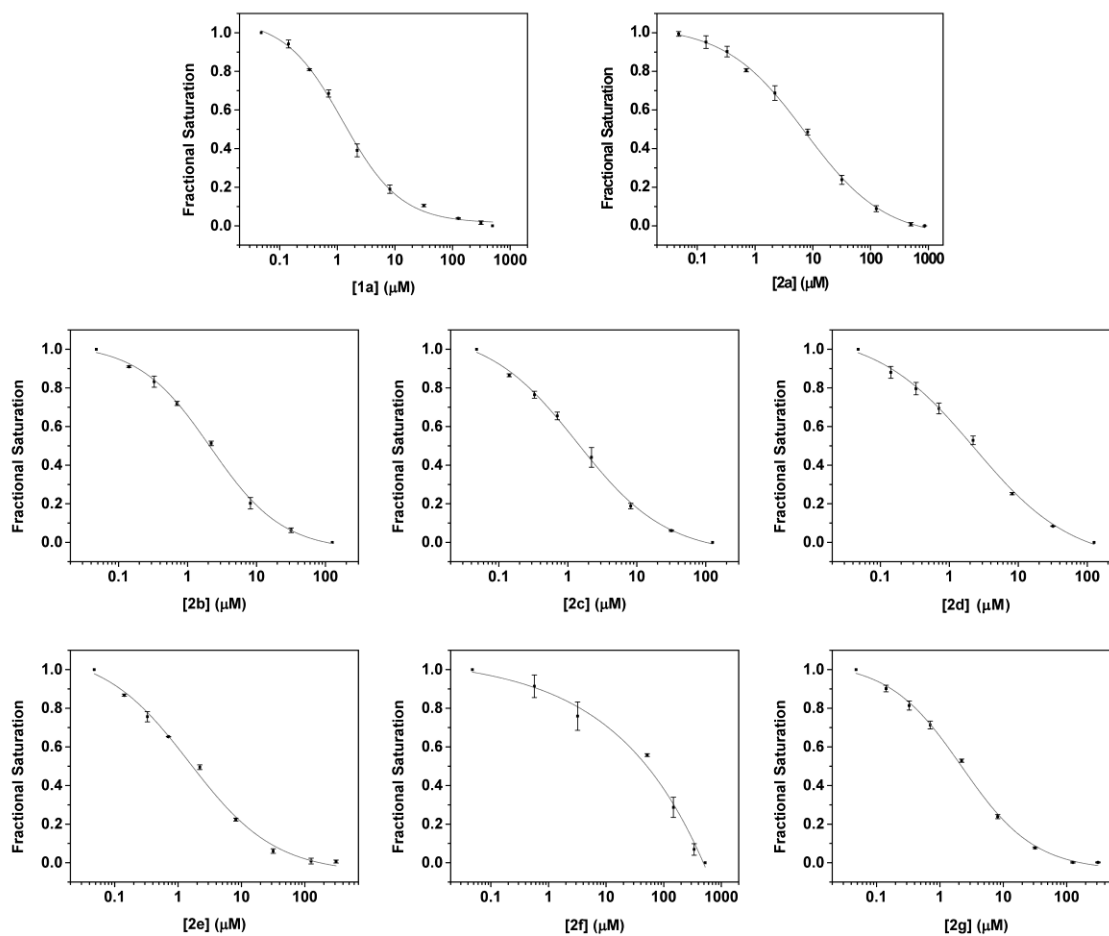

Figure 4.4: Kanamycin-Coumarin displacement curves for tobramycin (**1a**), amikacin (**2a**), 6''-deoxy-6''-aminoamikacin (**2b**), 6''-deoxy-6''-methylaminoamikacin (**2c**), 6''-deoxy-6''-dimethylaminoamikacin (**2d**), 6''-deoxy-6''-(2-(aminoethyl)amino)amikacin (**2e**), 6''-deoxy-6''-ureidoamikacin (**2f**), and 6''-deoxy-6''-(4-(aminomethyl)-1H-1,2,3-triazol-1-yl)amikacin (**2g**).
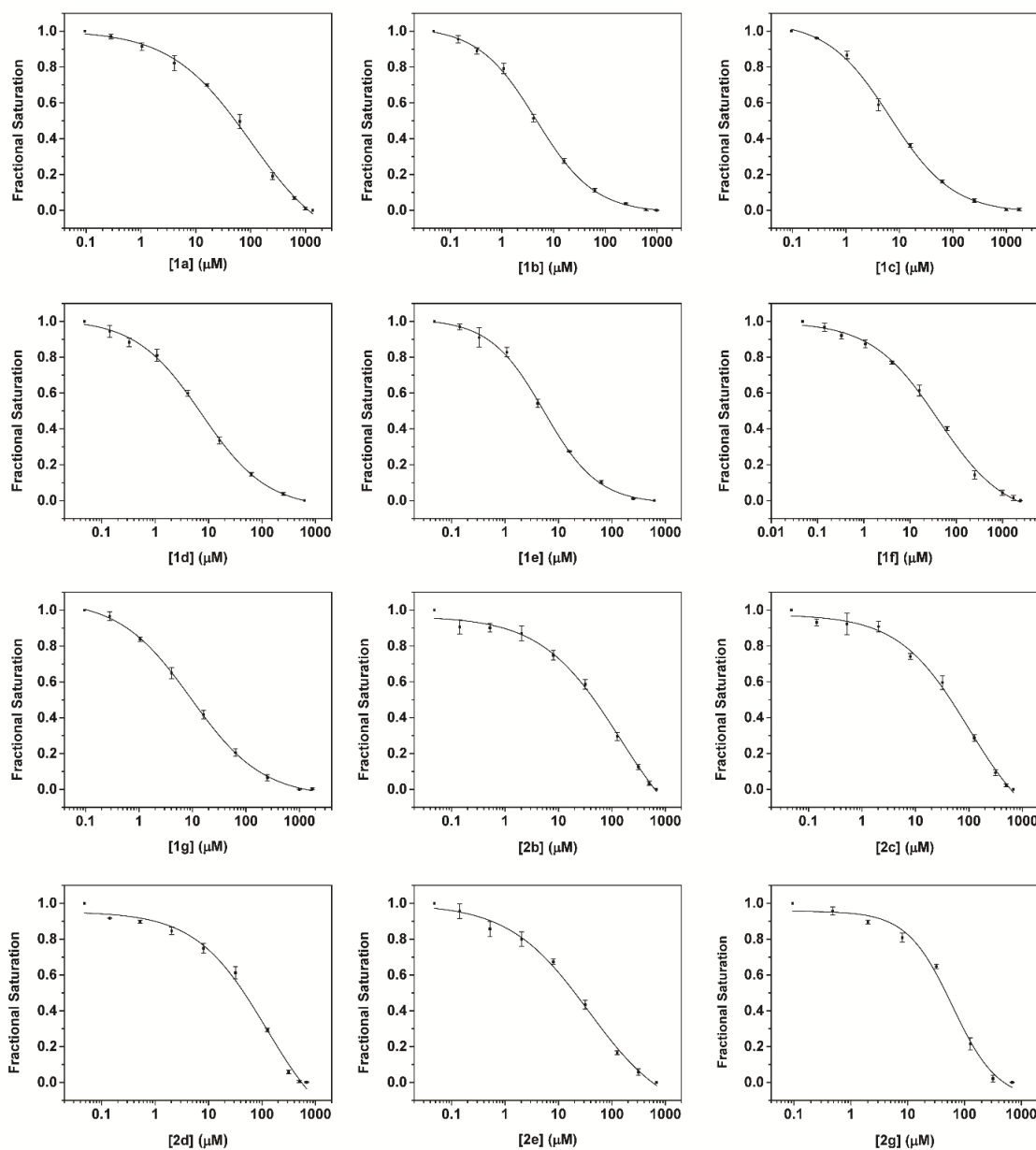
Figure 4.5: Neomycin-Coumarin displacement curves for tobramycin (**1a**), 6''-deoxy-6''-aminotobramycin (**1b**), 6''-deoxy-6''-methylaminotobramycin (**1c**), 6''-deoxy-6''-dimethylaminotobramycin (**1d**), 6''-deoxy-6''-(2-(aminoethyl)amino)tobramycin (**1e**), 6''-deoxy-6''-ureidotobramycin (**1f**), 6''-deoxy-6''-(4-(aminomethyl)-1H-1,2,3-triazol-1-yl)tobramycin (**1g**), 6''-deoxy-6''-aminoamikacin (**2b**), 6''-deoxy-6''-methylaminoamikacin (**2c**), 6''-Deoxy-6''-dimethylaminoamikacin (**2d**), 6''-deoxy-6''-(2-(aminoethyl)amino)amikacin (**2e**), and 6''-deoxy-6''-(4-(aminomethyl)-1H-1,2,3-triazol-1-yl)amikacin (**2g**).

Minimum inhibitory concentration (MIC) determinations

MIC values for aminoglycosides were determined using broth microdilution in accordance with Clinical Laboratory Standards Institute guidelines.[29] Bacterial strains of interest were grown on Mueller-Hinton agar. A colony was selected and grown in an overnight liquid culture of cation adjusted Mueller-Hinton broth (MHB) (10 mg/mL $CaCl_2$ and 10 mg/mL $MgCl_2$) at 37 °C. 100 μL of the overnight culture was added to 10 mL of fresh cation adjusted MHB in a 20 mL test tube. This was shaken at 37 °C to an optical density (OD) value of 0.4 – 0.6 at 600 nm to give a log phase culture. This usually took 1.5 – 3.5 hours, for the strains used in this study, and varied between bacterial species and strain. The log culture was poured into a falcon tube and pelleted by centrifugation at 4,000 rpm for 8 minutes. The cation adjusted Mueller-Hinton broth was decanted off. The bacteria pellet was suspended in 0.5 mL PBS. In a 10 mL test tube, the bacteria suspension was added step wise to 5 mL of PBS to give a final OD at 600 nm of 0.2 for *E. coli* and 0.4 for all other bacterial species used. Bacteria had to be diluted to to a final concentration of 5 x 10^5 cfu/mL just prior to addition to the 96-well test plate. *E. coli* strains were used directly. MRSA strains were diluted by addition of 0.5 mL of the OD = 0.4 solution to 9.5 mL of cation adjusted MHB. *K. pneumoniae* and *P. aeruginosa* strains were diluted by addition of 167 μL of OD = 0.4 solution to 9.83 mL of cation adjusted MHB. *A. baumannii* strains were diluted by addition of 833 μL of OD = 0.4 solution to 9.17 mL of cation adjusted MHB.

A 96-well compound dilution plate was prepared. The highest concentration well consisted of 90 μL of cation adjusted MHB and 10 μL of an aqueous stock solution at 5

mg/mL of compound. Serial dilutions in cation adjusted MHB were then made down the compound dilution plate.

Round bottom 96 well test plates were separately prepared. 80 µL of cation adjusted MHB was added to each well. 10 µL of the serial dilutions were transferred from the comound dilution plate using a multi-channel pipette. Each compound of interest was added to in two consecutive columns to give results in duplicate. In one column per test plate serial dilutions of a known antibiotic that the strain is sensitive to was added. For the strains used these were tobramycin for *P. aeruginosa*, tetracycline for *K. pneumoniae,* ciprofloxacin for *E. coli*, and vancomycin for MRSA. 10 mL of the 5 x 10^5 cfu/mL solution of bacteria were added to each well containing compound and also to three wells containing only media as a control. The plates were covered, parafilmed along

Table 4.5: Representative MIC assay raw data[a]									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**11**	**12**
A	0.051	0.049	0.048	0.048	**0.097**	0.052	0.051	0.055	0.059	0.044
B	0.045	0.045	0.045	0.045	**0.555**	**0.621**	0.045	0.048	0.055	0.059
C	0.044	0.044	0.044	0.044	**0.489**	**0.435**	0.046	**0.438**	0.053	0.043
D	**0.304**	0.043	**0.123**	0.048	**0.972**	**1.012**	**0.703**	**0.692**	0.056	0.044
E	**0.821**	**1.007**	**1.031**	**0.779**	**1.304**	**1.299**	**1.099**	**1.052**	0.079	**1.245**
F	**1.008**	**1.075**	**0.988**	**1.014**	**1.313**	**1.310**	**1.217**	**1.198**	0.075	**1.044**
G	**1.019**	**1.014**	**0.981**	**0.916**	**1.203**	**1.184**	**1.189**	**1.129**	0.070	**0.955**
H	**0.640**	**0.922**	**0.955**	**0.979**	**1.230**	**1.179**	**1.093**	**0.690**	**0.597**	0.044

[a] Optical density values at 600 nm. Compounds incubated with *E. coli* strain ATCC25922. Serial dilutions are from 50 µg/mL (row A) to 0.391 µg/mL (row H). Compound **2b** (column 1 and 2), **2e** (column 3 and 4), **2f** (column 5 and 6), **2c** (column 7 and 8). Ciprofloxacin positive control (column 11), bacteria and media only negative control (column 12 E-G), media only control (column 12 B-D). Blank columns 9 and 10 of the 96 well plate are omitted. Bacterial growth is highlighted in **bold**.

the sides, and shaken at 37 °C overnight.

The 96-well plates were read at 600 nm using a plate reader. OD values at or below 0.065 were considered bacteria free. The first well that was cleared for a given compound is the MIC value. A representative table of raw data for the MIC assay showing amikacin derivatives tested against *E. coli* strain ATCC25922 is pictured below (Table 4.5). Each compound of interested was tested minimally in quadruplicate. A difference of one serial dilution for separate runs was commonly observed. In this case the MIC is reported as a range.

Parent Aminoglycoside Crystal Structures

Crystal structure representations (Figure 2) were made using PyMOL Molecular Graphics Systems, Version 1.4.1, Schrödinger, LLC. All structures were adapted from PDB files: tobramycin (1LC4), amikacin (2GSQ).

4.6 References

[1] Davies, J. *The Canadian journal of infectious diseases & medical microbiology* **2006**, *17*, 287.

[2] Norman, A.; Hansen, L. H.; Sorensen, S. J. *Philos T R Soc B* **2009**, *364*, 2275.

[3] Davies, J.; Davies, D. *Microbiol Mol Biol R* **2010**, *74*, 417.

[4] Rice, L. B. *J Infect Dis* **2008**, *197*, 1079

[5] Boucher, H. W.; Talbot, G. H.; Bradley, J. S.; Edwards, J. E.; Gilbert, D.; Rice, L. B.; Scheld, M.; Spellberg, B.; Bartlett, J. *Clin Infect Dis* **2009**, *48*, 1.

[6] Pendleton, J. N.; Gorman, S. P.; Gilmore, B. F. *Expert Rev Anti-Infe* **2013**, *11*, 297.

[7] Giske, C. G.; Monnet, D. L.; Cars, O.; Carmeli, Y.; Resistance, R.-A. A. *Antimicrob Agents Ch* **2008**, *52*, 813.

[8] Boucher, H. W.; Talbot, G. H.; Benjamin, D. K.; Bradley, J.; Guidos, R. J.; Jones, R. N.; Murray, B. E.; Bonomo, R. A.; Gilbert, D.; Amer, I. D. S. *Clin Infect Dis* **2013**, *56*, 1685.

[9] Spellberg, B.; Powers, J. H.; Brass, E. P.; Miller, L. G.; Edwards, J. E. *Clin Infect Dis* **2004**, *38*, 1279.

[10] Barbachyn, M. R.; Ford, C. W. *Angew Chem Int Edit* **2003**, *42*, 2010

[11] Carpenter, C. F.; Chambers, H. F. *Clin Infect Dis* **2004**, *38*, 994.

[12] Andries, K.; Verhasselt, P.; Guillemont, J.; Gohlmann, H. W. H.; Neefs, J. M.; Winkler, H.; Van Gestel, J.; Timmerman, P.; Zhu, M.; Lee, E.; Williams, P.; de Chaffoy, D.; Huitric, E.; Hoffner, S.; Cambau, E.; Truffot-Pernot, C.; Lounis, N.; Jarlier, V. *Science* **2005**, *307*, 223.

[13] Mullane, K. M.; Gorbach, S. *Expert Rev Anti-Infe* **2011**, *9*, 767.

[14] (a) Falagas, M. E.; Grammatikos, A. P.; Michalopoulos, A. *Expert Rev Anti-Infe* **2008**, *6*, 593; (b) Falagas, M. E.; Kasiakou, S. K. *Clinical Infectious Diseases* **2005**, *40*, 1333; (c) Hermann, T. *Cell Mol Life Sci* **2007**, *64*, 1841; (d) McCoy, L. S.; Roberts, K. D.; Nation, R. L.; Thompson, P. E.; Velkov, T.; Li, J.; Tor, Y. *Chembiochem* **2013**, *14*, 2083.

[15] Avent, M. L.; Rogers, B. A.; Cheng, A. C.; Paterson, D. L. *Intern Med J* **2011**, *41*, 441.

[16] Jackson, J.; Chen, C.; Buising, K. *Curr Opin Infect Dis* **2013**, *26*, 516.

[17] Moazed, D.; Noller, H. F. *Nature* **1987**, *327*, 389.

[18] Carter, A. P.; Clemons, W. M.; Brodersen, D. E.; Morgan-Warren, R. J.; Wimberly, B. T.; Ramakrishnan, V. *Nature* **2000**, *407*, 340.

[19] Ogle, J. M.; Brodersen, D. E.; Clemons, W. M.; Tarry, M. J.; Carter, A. P.; Ramakrishnan, V. *Science* **2001**, *292*, 897.

[20] Becker, B.; Cooper, M. A. *Acs Chem Biol* **2013**, *8*, 105.

[21] (a) Q. Vicens, E. Westhof, *Chem. Biol.* **2002**, 9, 747. (b) J. Kondo, B. François, R. J. M. Russell, J. B. Murray, E. Westhof, *Biochimie* **2006**, 88, 1027 –1031.

[22] R. J. Fair, M. E. Hensler, W. Thienphrapa, Q. N. Dam, V. Nizet, Y. Tor, *ChemMedChem* **2012**, 7, 1273.

[23] K. Michael, H. Wang, Y. Tor, *Bioorg. Med. Chem.* **1999,** *7,* 1361.

[24] a) Y. Xie, A. V. Dix, Y. Tor, *J. Am. Chem. Soc.* **2009**, 131, 17605. b) Y. Xie, A. V. Dix, Y. Tor, *Chem. Commun.* **2010**, 46, 5542.

[25] M. Kaul, C. M. Barbieri, D. S. Pilch, *J. Am. Chem. Soc.* **2006**, 128, 1261.

[26] H. Kawaguchi, T. Naito, S. Nakagawa, K. J. Fujisawa *J. Antibiot*. **1972**, 25, 695.

[27] T. T.Denton, X.D. Zhang, J.R. Cashman, *J. Med. Chem.* **2005**, *48*, 224.

[28] W. M. Huston, M. P. Jennings, A. G. McEwan, *Mol. Microbiol.* **2002**, 45, 1741.

[29] *Performance Standards for Antimicrobial Susceptibility Testing*, 19th Informational Supplement M100-S19, Clinical and Laboratory Standards Institute, Wayne, PA, USA, **2008**.

Chapter 4 is in full currently being prepared for submission: Fair, R. J.; McCoy, L. S.; Hensler, M. E.; Nizet, V.; Tor, Y. Singly Modified Amikacin and Tobramycin Derivatives Show Increased A-site Binding and Higher Potency against Resistant Bacteria. The dissertation author is the co-main author and researcher for this work.

Chapter 5

Synthesis and Biological Evaluation of Aromatic TAN-1057 Analogs

5.1 Introduction

A dramatic increase in the occurrence of dangerous, antibiotic resistant bacteria has been seen in clinics around the world for the past several decades. This increased resistance has been driven primarily by two factors; bacterial evolution of new resistance factors to the current arsenal of antibiotics and a concurrent decline in the development of new antibacterial agents. Bacterial evolution is largely a result of selective pressure caused by exposure to antibiotics and to other bacteria with already evolved resistance mechanisms capable of lateral gene transfer. Bacterial exposure to current antibiotics has been exacerbated by decades of wonton clinical prescription and irresponsible policies regarding their prophylactic use by the food industry. The decreased output of new antibiotic agents is due chiefly to a waning interest in antibiotic development by major pharmaceutical companies caused by lackluster profit margin due to a multitude of factors.

The majority of antibiotic development within the past forty years has been focused on the isolation or synthetic tailoring of new members of already existing antibiotic classes. This has served to prolong the utility of these classes. New members of already existing classes will often be subject to at least some of the resistance mechanisms manifest in older analogs, however. The development of totally novel

antibiotic classes is therefore also necessary. Unfortunately, there have been only five

totally novel antibiotic scaffolds approved since the 1960s. All five were developed in

response to the emergence to highly resistant gram positive bacteria and *Mycobacterium*

tuberculosis in the 1990s.

The vast majority of clinically approved antibiotics hamper a select few cellular

processes. The translation of mRNA by ribosomes into proteins is one of the most

successfully and commonly targeted processes. Structurally diverse antibiotics including

aminoglycosides, amphenicols, macrolides, tetracyclines, streptogramins, oxazolidinones,

and pleuromutilins all primarily target ribosomes. This historical success coupled with

the wealth of structural knowledge that has been elucidated about the ribosome make this

an attractive target for the development of new antibiotic scaffolds.

TAN-1057 (**1**) is dipeptide consisting of a dihydropyrimidinone core and a (S)-β-

homoarginine side chain (Figure 5.1). It was first isolated in 1993 by Takeda as an

epimeric mixture termed TAN-1057 A/B, hereafter referred to as TAN-1057, from soil

bacterium, *Flexibacter*.[1] It strongly inhibits both eukaryotic and prokaryotic translation

with greater inhibition of the former. The ribosomal binding site(s) of TAN-1057 is

currently unknown, though competitive binding experiments have suggested that the

binding site does not overlap with chloramphenicol, tetracycline, or erythromycin.[2] There

is some knowledge of which ribosomal processes it inhibits and its mechanism of action

appears to be complex. Binding of mRNA to the ribosome and tRNA binding to A- and

P-sites appear to be unaffected whereas initiation is slightly reduced. Elongation and the

peptidyl transferase reaction are strongly inhibited, and formation of the 50S ribosomal

subunit is also significantly inhibited.[2, 3] Experiments have suggested that active transport via dipeptide transporters are involved in its cellular uptake.[2] TAN-1057 shows good activity against gram positive bacteria, particularly *Staphylococci* including MRSA. It is also highly toxic in mouse models, however, which is unsurprising given its potent inhibition of eukaryotic translation. [1, 4]
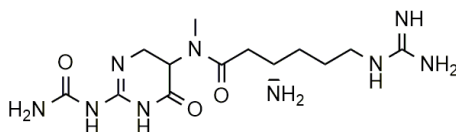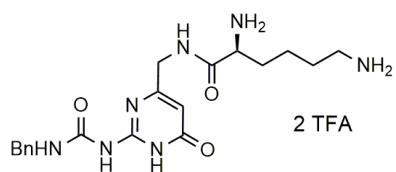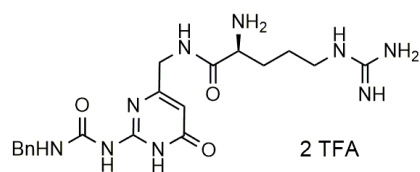

Figure 5.1: TAN-1057 A/B (**1**).

There are several total syntheses of TAN-1057,[5] including a diastereoselective synthesis of TAN-1057A,[6] but all of them are quite lengthy and none has greater than a 12% overall yield. Published analogs of TAN-1057 have largely focused on modifications to the (S)-β-homoarginine side chain.[6,7,8,9] Variations to the length of the side chain and to the terminal functional group were well tolerated and in some cases derivatives with improved activity and selectivity for prokaryotic translation were obtained. (S)-β-lysine and (S)-β-homolysine side chains were among those that were successful. Analogs with modifications the urea moiety have also been synthesized.[10] Most of these analogs showed significantly reduced antibiotic activity though some with appended nitrogen containing heterocycles retained activity.

TAN-1057 is unstable in aqueous solutions as the six membered ring is gradually hydrolyzed. This process can also be catalyzed by strong acid or base.[5b] In solution it also readily epimerizes at the C(6) position into TAN-1057 A/B, which is why most studies have been done on the epimeric mixture.[5a,b] It is known that TAN-1057A, which has an S
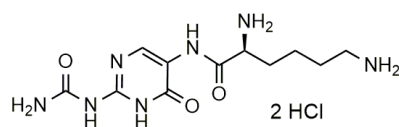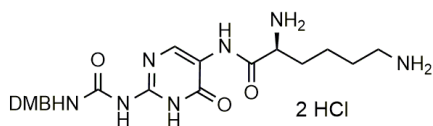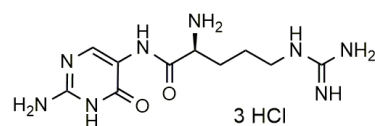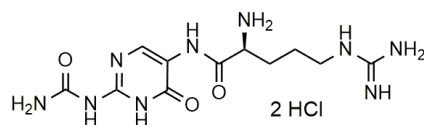
Lys-BnU-Cyc (**2**)

Arg-BnU-Cyc (**3**)
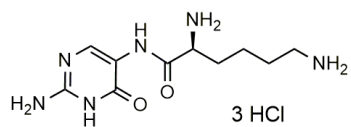
Lys-IsoC (**4**)

U-Lys-IsoC (**5**)

DMBU-Lys-IsoC (**6**)

Arg-IsoC (**7**)
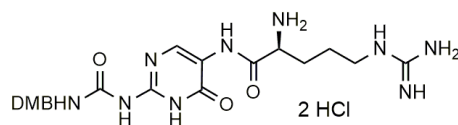
U-Arg-IsoC (**8**)

DMBU-Arg-IsoC (**9**)
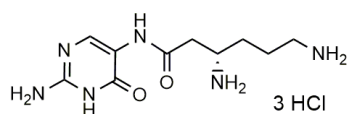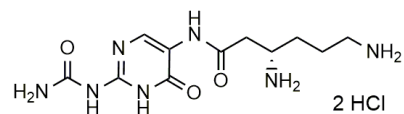
β-Lys-IsoC (**10**)
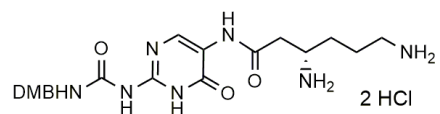
U-β-Lys-IsoC (**11**)

DMBU-β-Lys-IsoC (**12**)

Figure 5.2: Aromatic TAN-1057 Analogs.

configuration at the C(5) position has 2 to 4 fold more potent antibacterial activity, however.[1, 4]

The dihydropyrimidinone core is primarily responsible for the chemical instability and difficulty of synthesis of TAN-1057 and analogs. We rationalized that aromatic pyrimidine analogs would negate the issue of stability and would be much easier to synthesize. In the course of this project it is was found that Bayer had patented some aromatic analogs.[11] Most of them differed structurally from the analogs that we had envisioned and their synthesis was entirely different from what we had devised. Additionally, very little was detailed about the activity of these analogs, so we decided to continue with the project. Here we disclose the synthesis of a small, but diverse library of aromatic TAN-1057 analogs (Figure 5.2). Preliminary results from an in vitro Förster resonance energy transfer (FRET) based A-site binding assay, in vitro prokaryotic and eukaryotic translation assays, and antibacterial minimum inhibitory concentration (MIC) determinations of select molecules are detailed.

5.2 Results and Discussion

Design Strategy

We chose two different pyrimidinone cores in order to explore importance of the amino acid side chain positioning (Figure 5.3). C(6) substituted 2-ureido-pyrimidinones have been studied thoroughly as hydrogen bonding dimers.[12] It is likely that a hetero- and homo-dimers are possible for the TAN-1057 core as well (Figure 5.4). We have made analogs with side chains in the C(6) position and others with the side chain in the C(5) position by utilizing two different synthetic routes.
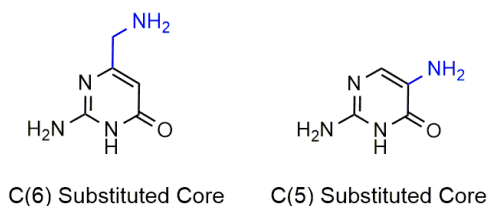
C(6) Substituted Core   C(5) Substituted Core

Figure 5.3: Pyrimidinone cores. Amino acid side chain attachment site highlighted in blue.

Most translation inhibiting antibiotics are highly cationic and/or have a large number of hydrogen bond donors and acceptors, which allows them to bind their rRNA targets. We hypothesized that TAN-1057 may bind an rRNA target as a dimer thereby forming a complex with a greater overall cationic character and an increased number of hydrogen bonding functional groups. To explore this possibility and to probe the importance of the urea moiety we made derivatives with benzyl ureas and amines at the C(2) position, both of which would form weaker dimers.
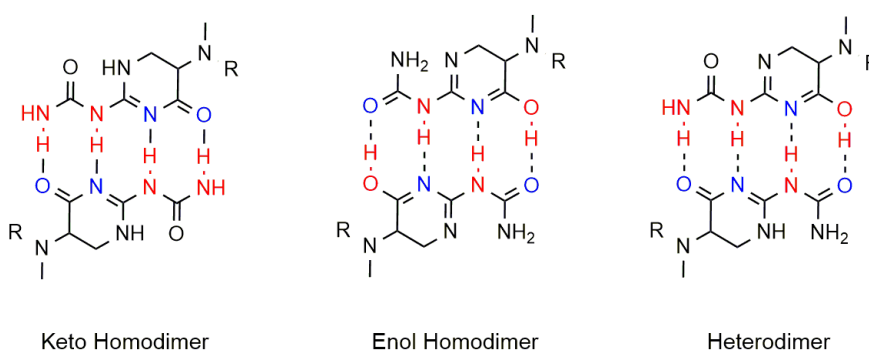

Keto Homodimer   Enol Homodimer   Heterodimer

Figure 5.4: 2-Ureido-pyrimidinone homodimers and heterodimer. Hydrogen bond donors in red, acceptors in blue.

We also wanted to examine the importance of the β positioning of the amine on the (S)-β-homoarginine side chain. Analogs haven't been made that altered this positioning, so we decided to explore inexpensive α-amino acids as surrogates. (S)-β-lysine was also investigated as a side chain. An analog with the dihydropyrimidinone

core and this side chain is known to exhibit similar antibacterial activity and improved

prokaryotic ribosomal selectivity to TAN-1057.[9]

Synthesis

The C(6) substituted pyrimidinones were made from ethyl 4-chloro-3-

oxobutanoate (**13**). The chloride was converted to a Boc-protected amine through a

known two step procedure (Scheme 5.1).[13] The chloride was first displaced through a

substitution reaction using sodium azide. The azide was subsequently converted to a

protected amine via a one-pot hydrogenolysis and protection with di-*tert*-butyl

dicarbonate. The heterocycle, **16**, is formed by heating the beta keto ester with guanidine

carbonate. The aryl amine was then reacted with electrophilic 4-

nitrophenylbenzylcarbonate to produce a benzyl protected urea (**17**).

Acidic deprotection of the Boc group was then effected using a one to one

solution of TFA and DCM with TIPS as a cation scavenger. The amino acid side chains

were then appended to the free primary amine using EDCI, TEA, catalytic DMAP, and

either lysine or arginine with Boc-protected amines. These were subsequently

deprotected under the same conditions as the previous Boc deprotection and HPLC

purified to give the analogs Lys-BnU-Cyc (**2**) (Scheme 5.2) and Arg-BnU-Cyc (**3**)
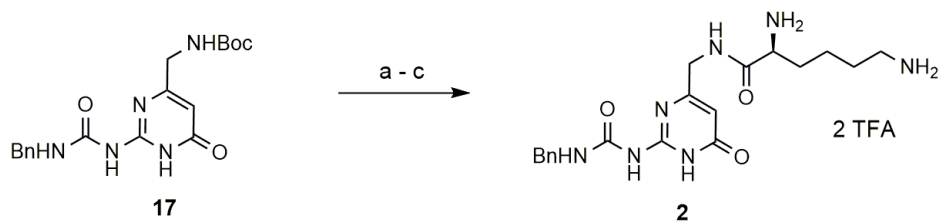
(Scheme 5.3).

Our original intent was to remove the benzyl protecting groups to give free urea

analogs as well. The benzyl protecting groups proved resistant to a number of

deprotection conditions, however, including a standard hydrogenolysis with 55 psi H_2

and a Pd(OH)$_2$ catalyst and a transfer hydrogenolysis with ammonium formate and Pd/C heated to 50 °C in a pressure tube.

The aryl amine, **16**, was instead reacted 2,4-dimethoxybenzylisocyante in pyridine to give a more labile DMB-protected urea. Subjecting this molecule to TFA / DCM with the TIPS scavenger led to removal of both the Boc and DMB protecting groups yielding the free urea, **18** (Scheme 5.4). Efforts became focused on the synthesis of analogs with C(5) amino acid side chains as they are more structurally analogous to TAN-1057. This core was never revisited after disappointing biological results obtained with C(5) derivatives led to the termination of this project.
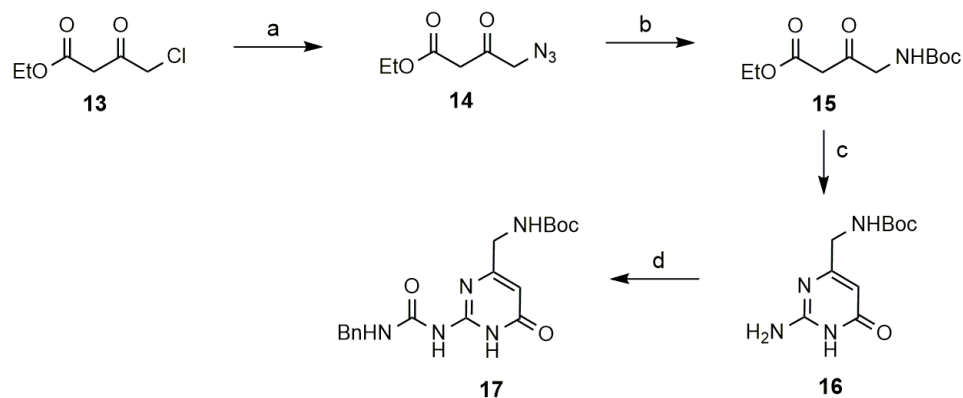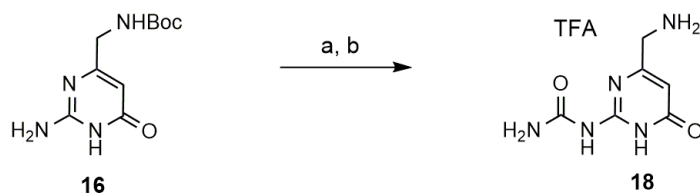

Scheme 5.1: BnU-Cyc (**17**) synthesis. (a) NaN$_3$, H$_2$O, Acetone. (b) Boc$_2$O, Pd/C, H$_2$, EtOAc. (c) Guanidine carbonate, EtOH. (d) 4-Nitrophenylbenzylcarbamate, TEA, DMF.

Scheme 5.2: Lys-BnU-Cyc (**2**) synthesis. (a) TFA, TIPS, DCM. (b) Boc-Lys(Boc)-OH DCHA, EDCI, TEA, DMAP, DMF. (c) TFA, TIPS, DCM.
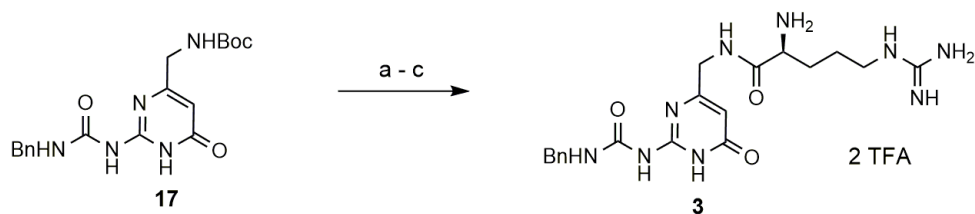
Scheme 5.3: Arg-BnU-Cyc (**3**) synthesis. (a) TFA, TIPS, DCM. (b) Boc-Arg-OH HCl, EDCI, TEA, DMAP, DMF. (c) TFA, TIPS, DCM.
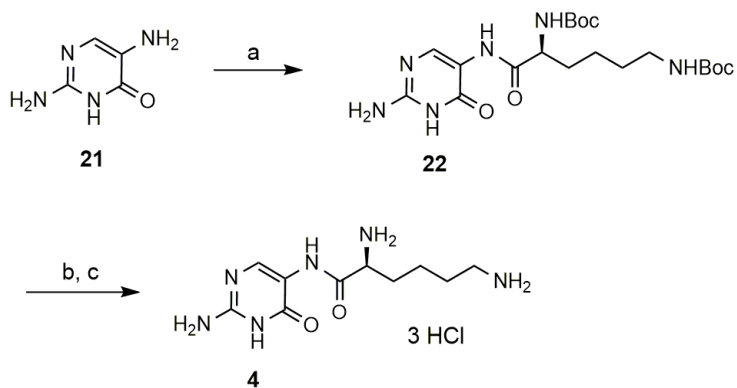

Scheme 5.4: UCyc (**18**) synthesis. (a) 2,4-Dimethoxybenzylisocyanate, Pyridine. (b) TFA, TIPS, DCM.

The synthesis of the C(5) side chain analogs started with the conversion of isocytosine (**19**) to 5-aminoisocytosine (**21**) through an already published procedure (Scheme 5.5).[14] This was accomplished by first nitrating at the C(5) position using a classic electrophilic aromatic substitution with potassium nitrate and sulfuric acid. The nitro group was then reduced via hydrogenation in water and ethanol. Solvents more commonly used in hydrogenation reactions were tried, but the yields were lower than for the already published conditions.

This was then coupled to Boc-protected lysine at the more nucleophilic C(5) amine using HATU and DIPEA (Scheme 5.6). The regioselectivity of this reaction was verified by determining the crystal structure of the product (Figure 5.5). This was then Boc-deprotected, using the same acidic conditions that were previously discussed, and subsequently HPLC purified to yield a free aryl amine analog. All of the C(5) analogs were converted from trifluoroacetate salts to chloride salts for biological studies. Chloride
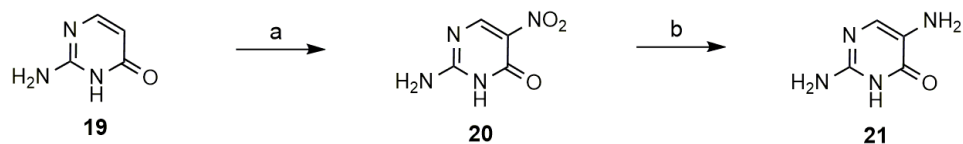
Scheme 5.5: 5-aminoisocytosine (**21**) synthesis. (a) KNO$_3$, H$_2$SO$_4$. (b) H$_2$, Pd/C, H$_2$O, EtOH.

Scheme 5.6: Lys-IsoC (**4**) synthesis. (a) Boc-Lys(Boc)-OH, HATU, DIPEA, DMF. (b) TFA, TIPS, DCM. (c) Amberlite IRA-900 (Cl$^-$), H$_2$O.
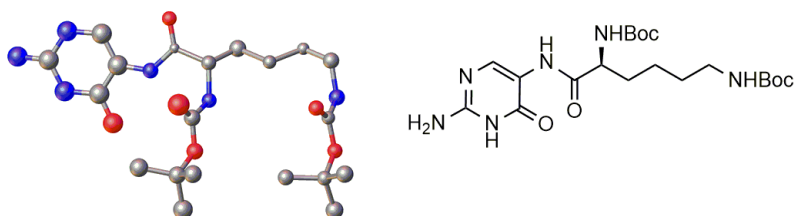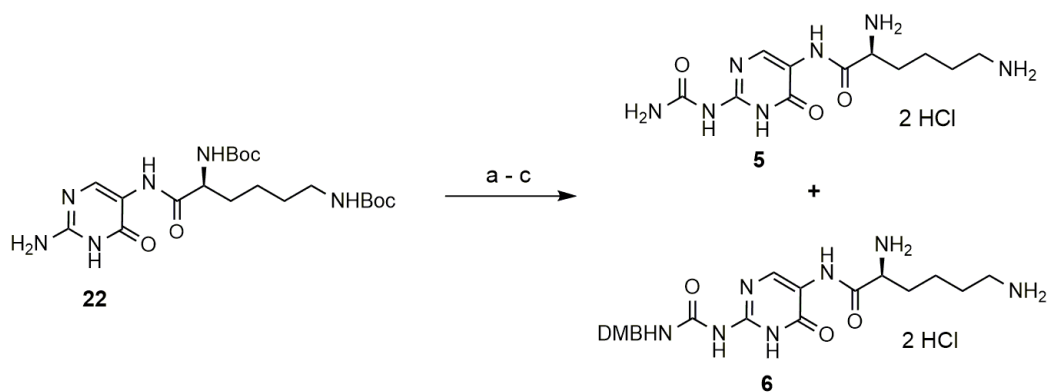

**Figure 5.5:** Crystal Structure of (Boc)Lys-IsoC (**22**).

Scheme 5.7: U-Lys-IsoC (**5**) and DMBU-Lys-IsoC (**6**) synthesis. (a) 2, 4-Dimethoxybenzylisocyanate, Pyridine. (b) TFA, TIPS, DCM. (c) Amberlite IRA-900 (Cl$^-$), H$_2$O.

salts were chosen because all previous studies on TAN-1057 analogs were done using this counterion.

(Boc)Lys-IsoC (**22**) was also reacted at the C(2) amine with 2,4-dimethoxybenzylisocyanate in pyridine to give DMB-protected urea (Scheme 5.7). Exposure to the acidic deprotection conditions led to full Boc deprotection, but only partial removal of the urea protecting group. This served our purposes because it yielded both the free urea (**5**) and DMB-protected urea (**6**) derivatives after HPLC purification.

Starting from 5-aminoisocytosine (**21**) the same synthetic scheme was followed to produce analogs with arginine side chains (Schemes 5.8 and 5.9). To make β-lysine derivatives 5-aminoisocytosine (**21**) was coupled with Fmoc-β-Lys(Boc)-OH. The same synthetic scheme was followed with the exception that the Fmoc protecting group was removed from the beta amine with piperidine for all derivatives prior to Boc deprotection (Schemes 5.10 and 5.11).
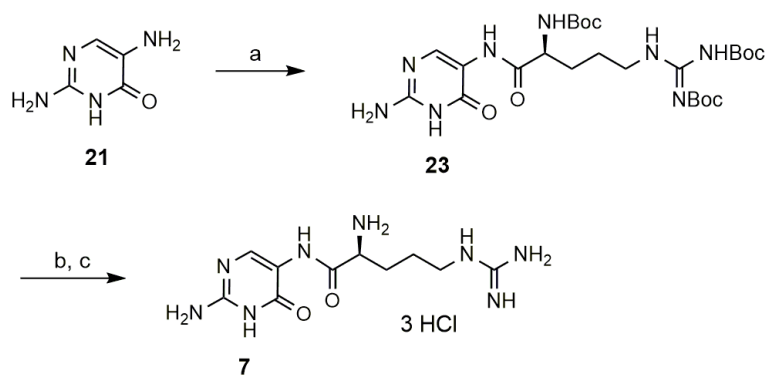
Scheme 5.8: Arg-IsoC (**7**) synthesis. (a) Boc-Arg(Boc)$_2$-OH, HATU, DIPEA, DMF. (b) TFA, TIPS, DCM. (c) Amberlite IRA-900 (Cl-), H$_2$O.
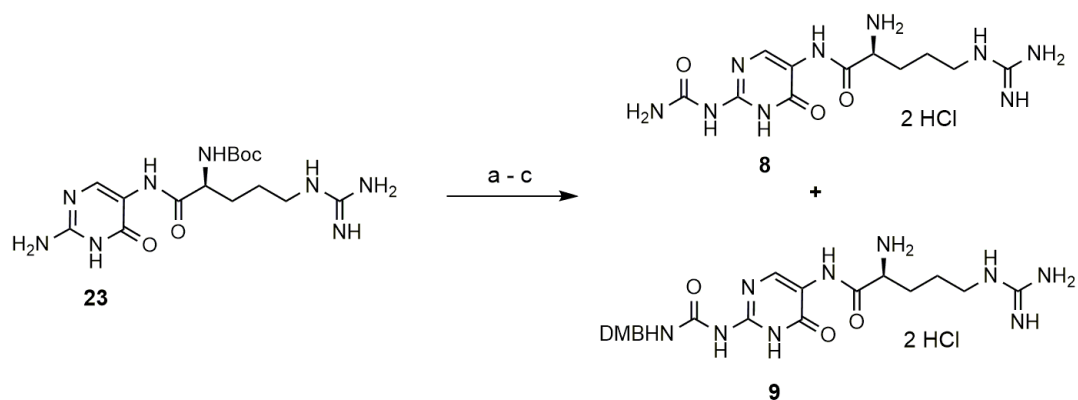
Scheme 5.9: U-Arg-IsoC (**8**) and DMBU-Arg-IsoC (**9**) synthesis. (a) 2, 4-Dimethoxybenzylisocyanate, Pyridine. (b) TFA, TIPS, DCM. (c) Amberlite IRA-900 (Cl⁻), H$_2$O.
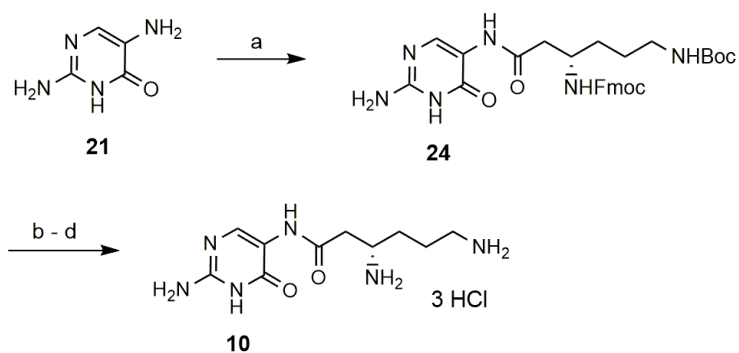
Scheme 5.10: β-Lys-IsoC (**10**) synthesis. (a) Fmoc-β-Lys(Boc)-OH, HATU, DIPEA, DMF. (b) Piperidine, DMF. (c) TFA, TIPS, DCM. (d) Amberlite IRA-900 (Cl⁻), H$_2$O.
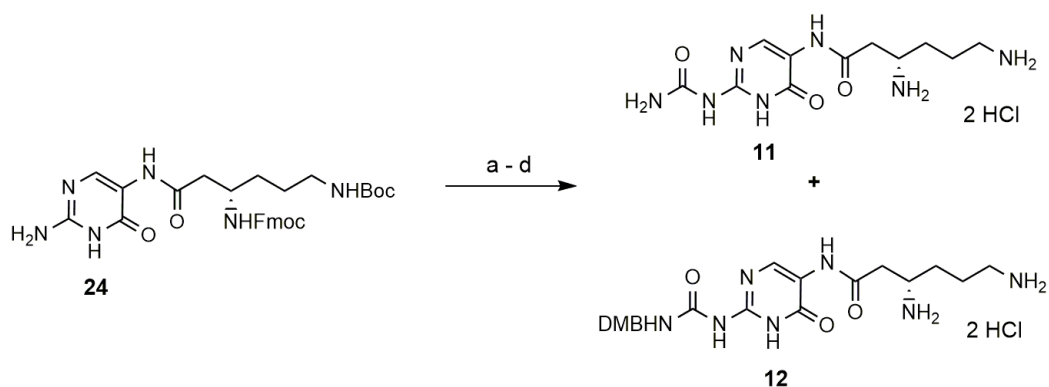
Scheme 5.11: U-β-Lys-IsoC (**11**) and DMBU-β-Lys-IsoC (**12**) synthesis. (a) 2, 4-Dimethoxybenzylisocyanate, Pyridine. (b) Piperidine, DMF. (c) TFA, TIPS, DCM. (d) Amberlite IRA-900 (Cl⁻), H$_2$O.

Affinity for the bacterial 16S A-site RNA construct

As previously discussed the ribosomal target(s) of TAN-1057 have not been conclusively identified. Most polycationic aminoglycosides bind to the A-site rRNA as their primary target. The similar overall positive charge that dimerized TAN-1057 has to most aminoglycosides prompted us to test the affinity of our analogs for this site. To determine the affinity of all derivatives to the bacterial 16S A-site, we used a version of a FRET-based assay that has been previously used in our lab to determine affinities of aminoglycosides for the A-site.[15] In this assay, a coumarin–aminoglycoside conjugate placeholder binds to a Dy-547-labeled 16S A-site construct. Coumarin acts as a FRET donor to its matched Dy-547 acceptor. The affinity of unlabeled ligands for the A-site can be measured in a competition experiment, where the compound of interest is titrated in and displaces the coumarin–aminoglycoside placeholder, resulting in a decreased sensitized acceptor emission. Binding curves can be created and from them IC_{50} values can be determined to give relative affinities.

Titrations were performed with a coumarin–kanamycin derivative, our lowest affinity placeholder aminoglycoside conjugate. This assay was performed using LysIsoC (**4**) and ArgIsoC (**7**). The displacement of kanamycin-coumarin was found to be linear with both analogs rather than a true sigmoidal binding curve. This led us to believe that displacement may be caused by other interactions with the RNA construct rather than actual binding of the A-site binding pocket. It was later observed in another RNA based assay that DMBU-LysIsoC (**6**) causes precipitation of RNA, which supports the possibility that the kanamycin-courmarin displacement may be an artifact of the assay.

In vitro translation inhibition

   The in vitro eukaryotic or prokaryotic translation inhibition of several analogs

was determined. The inhibition of eukaryotic translation was poor for the α-amino acid

side chain analog, U-Lys-IsoC (**5**) which was determined to not exhibit any measurable

inhibition of eukaryotic translation with an IC_{50} > 600 μM. Unfortunately, prokaryotic

inhibition was also poor with U-Arg-IsoC (**8**) IC_{50} ≈ 425 μM. U-β-Lys-IsoC (**11**) was

chosen as a representative β-amino acid side chain analog to assess the relative

prokaryotic translation inhibition of these analogs. It showed significant improvement

with an IC_{50} ≈ 150 μM. By comparison TAN-1057 and the aminoglycoside, kanamycin,

show IC_{50} = 0.3 μM and IC_{50} = 0.41 μM respectively, so this activity was still far below

what we were hoping to see for these analogs.[9,16]

Antibacterial activities

   TAN-1057 has been shown to have potent antibacterial activity against gram

positive bacteria.[4] To test the antibacterial activity of our analogs MIC values were

determined against MRSA strain TCH1516 and methicillin sensitive *S. aureus* (MSSA)

strain UAMS1. The MIC values were determined for Lys-IsoC (**4**), U-Lys-IsoC (**5**),

DMBU-Lys-IsoC (**6**), Arg-IsoC (**7**), U-Arg-IsoC (**8**), and DMBU-Arg-IsoC (**9**). They

were found to all be > 100 μg/mL meaning that both of these strains are highly resistant

to all of the analogs. U-β-Lys-IsoC (**11**) was tested against two MRSA strains,

ATCC33591 and Sanger 252. There was no significant activity for this derivative against

either strain MIC > 50 μg/mL.

5.3 Conclusions

A series of aromatic TAN-1057 analogs with varying C(2) functional groups, side chain positioning, proximal amine positioning, and terminal functional groups were synthesized. The analogs were obtained via two different synthetic routes based on the C(5) or C(6) positioning of their side chains. Inconclusive, but unpromising results were obtained from an in vitro FRET-based A-site affinity assay. Conclusively poor results were observed for in vitro translation assays and antibacterial activity determinations. The dubious performance of these analogs led to the termination of this project.

The reasons for the poor performance of these analogs have not been conclusively identified. There could of course be issues of uptake or efflux, but the lack of efficacy in the in vitro translation assays strongly suggests that there is a more fundamental lack of interaction with intracellular target(s). U-β-Lys-IsoC (**11**) differs only in the pi bond between C(5) and C(6) from TAN-1057 analogs that are highly effective translation inhibitors.[9]

The aromatic nature of the core changes several properties of these molecules. The orientation of the side chain will be changed, but since both epimers of TAN-1057 exhibit potent antibacterial properties this is unlikely to be the major cause of lost activity. These analogs are able to pi stack whereas TAN-1057 is not. This could lead to a number of interactions that unproductive towards antibacterial activity. Another possibility is the difference in reactivity of the cores. TAN-1057 has an electrophilic core that is capable of undergoing ring opening reactions. These analogs lack that reactivity. It

is possible that this reactive core acts as a suicide substrate or that TAN-1057 is in fact a prodrug that is converted to a ring-opened active drug either intra- or extracellularly.

5.4 Experimental Section

Materials

Unless otherwise specified, materials purchased from commercial suppliers were used without further purification. Boc-Lys(Boc)-OH was purchased as a DCHA salt, but in the synthesis of Boc-LysIsoC (**22**) it was converted to the acid by dissolving in DCM and extracting with cool 1 M HCl. 4-nitrophenylbenzylcarbamate was synthesized using an established procedure.[17] All other anhydrous solvents and reagents, and ion exchange resins were purchased from Sigma–Aldrich. NMR solvents were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). The Dy-547-labeled A-site construct was purchased from Thermo Scientific and purified by gel electrophoresis. Kanamycin–coumarin and neomycin–courmarin conjugates were synthesized and purified according to established procedures.[18] Chemicals for preparing buffer solutions (enzyme grade) were purchased from Fisher Biotech. Autoclaved water was used in all fluorescence titrations. Kits for the in vitro translation assays were purchased from Promega. Mueller–Hinton broth used for sensitivity testing was obtained from Hardy Diagnostics (Santa Maria, CA, USA). Polystyrene 96-well microplates for MIC testing were purchased from Corning Inc. (Corning, NY, USA). S. aureus strains TCH1516 (USA300 strain of community-associated MRSA) and hospital-associated MRSA strain 33591 rendered resistant to rifampicin by serial passage were obtained from American Type Culture Collection, Chantilly, VA. *S. aureus* strain UAMS1 (methicillin-sensitive *S.*

aureus) was kindly provided by Dr. Greg Somerville (University of Nebraska, Lincoln, NE; strain was originally obtained from Dr. Mark Smeltzer, University of Arkansas for Medical Sciences, Little Rock, AR). USA200 MRSA strain Sanger 252 was obtained from the Network on Antimicrobial Resistance in Staphylococcus aureus (NARSA) program supported under NIAID/NIH contract number HHSN272200700055C.

Instrumentation

NMR spectra were recorded on Varian Mercury 300 and 400 MHz, Varian VX 500 MHz, and Jeol ECA 500 MHz spectrometers. Mass spectra (MS) were recorded at the University of California, San Diego Chemistry and Biochemistry Mass Spectrometry Facility, utilizing an Agilent 6230 HR-ESI-TOF mass spectrometer. Automated flash chromatography purification was carried out using a Teledyne Isco CombiFlash® R_F 200 with RediSep® R_F normal phase silica. Reverse-phase HPLC (Vydac C18 column) purification and analysis were carried out using an Agilent 1200 series instrument. Products were lyophilized utilizing a Labconco FreeZone 2.5 freeze drier. Steady-state fluorescence experiments were carried out in a microfluorescence cell with a path length of 1.0 cm (Hellma GmH & Co KG, Mullenheim, Germany) on a Jobin Yvon Horiba FluoroMax-3 luminescence spectrometer. A background spectrum (buffer) was subtracted from each sample. Chemiluminescence in the translation assays was measured with a SPECTRAmax® GEMINI XS plate reader (Molecular Devices, Mountain View, CA, USA). A VersaMax plate reader (Molecular Devices, Mountain View, CA, USA) set at 600 nm wavelength was used for MIC assays.

Synthesis

C(6) side chain analogs

Ethyl 4-azido-3-oxobutanoate (**14**) and ethyl 4-((Boc)amino)-3-oxobutanoate (**15**) have been previously synthesized.[13]

(Boc)Cyc (16). Absolute ethanol (14.5 mL) was added to ethyl 4-((Boc)amino)-3-oxobutanoate (**15**) (350 mg, 1.44 mmol). Guanidine carbonate (130 mg, 1.45 mmol) was added. The reaction was refluxed under inert atmosphere for 1.5 days. The orange solution was cooled to rt, then the solvent was removed under reduced pressure. The product was isolated by flash chromatography (14% methanol in DCM). Product: Yellow solid (130 mg, 0.54 mmol, 38% yield). ^{1}H NMR (400 MHz, D_6-DMSO): δ 10.67 (br s, 1H), 7.24 (t, J = 5.6 Hz, 1H), 6.53 (br s, 2H), 5.35 (s, 1H), 3.72 (d, J = 6.4 Hz, 2H), 1.39 (s, 9H); ESI-MS calculated for $C_{10}H_{17}N_4O_3$ [M+H]$^+$ 241.13, found 240.90.

BnU-(Boc)Cyc (17). Dry pyridine (30 mL) was added to (Boc)Cyc (**16**) (400 mg, 1.67 mmol). 4-Nitrophenylbenzylcarbamate (1.0 g, 3.67 mmol) was added. The orange solution was stirred at 50 °C for 2 days. The solvent was removed under reduced pressure. The product was isolated by flash chromatography (4% methanol, 1% pyridine in DCM). Product: Yellow solid (395 mg, 1.07 mmol, 64% yield). ^{1}H NMR (400 MHz, D_6-DMSO): δ 11.58 (br s, 1H), 9.91 (br s, 1H), 7.94 (br s, 1H), 7.38 – 7.24 (m, 5H), 5.73 (s, 1H), 4.37 (d, J = 6.0 Hz, 2H), 3.86 (d, J = 5.6 Hz, 2H), 3.34 (s, 1H), 1.39 (s, 9H); ESI-MS calculated for $C_{18}H_{25}N_5O_4$ [M+H]$^+$ 374.18, found 374.07.

Lys-BnU-Cyc 2 TFA (2). DCM (6.1 mL) was added to BnU-(Boc)Cyc (**17**) (136 mg, 0.37 mmol). TIPS (0.36 mL) then TFA (6.1 mL) were added. The reaction was stirred for 2.5 hours. The solvent was removed under reduced pressure. DMF (2.4 mL) was added to

the crude solid. Boc-Lys(Boc)-OH DCHA (290 mg, 0.55 mmol) then TEA (76 µL, 0.55 mmol) were added. DMAP (5 mg, 0.037 mmol), then EDCI (105 mg, 0.55 mmol) were added. The mixture was stirred overnight. The solvent was removed under reduced pressure.). DCM (6.1 mL) was added to the crude solid. TIPS (0.36 mL) then TFA (6.1 mL) were added. The reaction was stirred for 2.5 hours. The solvent was removed under reduced pressure. The remaining yellow solid was dissolved in water (5% ACN) and purified by reverse phase HPLC (5 – 50% ACN (0.1% TFA) over 15 mins) eluted after 11.5 mins, then lyophilized. Product: White solid (144 mg, 0.23 mmol, 63% yield). ^{1}H NMR (400 MHz, D_2O): δ 7.41 – 7.30 (m, 5H), 5.99 (s, 1H), 4.43 (s, 2H), 4.26 (d, J = 17.2 Hz, 1H), 4.20 (d, J = 16.8 Hz, 1H), 4.03 (t, J = 5.6 Hz, 1H), 2.93 (t, J = 7.6 Hz, 2H), 1.95 – 1.88 (m, 2H), 1.70 – 1.63 (m, 2H), 1.44 – 1.36 (m, 2H); ESI-MS calculated for $C_{19}H_{28}N_7O_3$ $[M+H]^+$ 402.2, found 402.2.

Arg-BnU-Cyc 2 TFA (3). DCM (6.1 mL) was added to BnU-(Boc)Cyc (**17**) (136 mg, 0.37 mmol). TIPS (0.36 mL) then TFA (6.1 mL) were added. The reaction was stirred for 2.5 hours. The solvent was removed under reduced pressure. DMF (1.4 mL) was added to the crude solid. Boc-Arg-OH HCl (171 mg, 0.55 mmol) then TEA (0.13 mL, 0.92 mmol) were added. DMAP (5 mg, 0.037 mmol), then EDCI (105 mg, 0.55 mmol) were added. The mixture was stirred overnight. The solvent was removed under reduced pressure. DCM (6.1 mL) was added to the crude solid. TIPS (0.36 mL) then TFA (6.1 mL) were added. The reaction was stirred for 2.5 hours. The solvent was removed under reduced pressure. The remaining yellow solid was dissolved in water (5% ACN) and purified by reverse phase HPLC (5 – 50% ACN (0.1% TFA) over 15 mins) eluted after 11.8 mins,

then lyophilized. Product: White solid (164 mg, 0.25 mmol, 68% yield). ^{1}H NMR (400

MHz, D_2O): δ 7.40 – 7.27 (m, 5H), 6.01 (s, 1H), 4.41 (s, 2H), 4.31 (d, J = 17.2 Hz, 1H),

4.15 (d, J = 16.8 Hz, 1H), 4.04 (t, J = 6.2 Hz, 1H), 3.13 (t, J = 7.0 Hz, 2H), 1.91 – 1.87

(m, 2H), 1.61 – 1.54 (m, 2H); ESI-MS calculated for $C_{19}H_{28}N_9O_3$ [M+H]$^+$ 430.5, found

430.2.

U-Cyc TFA (18). Dry pyridine (4.1 mL) was added to (Boc)Cyc (**16**) (150 mg, 0.63

mmol). 2, 4-Dimethoxybenzyl isocyanate (0.28 mL, 0.94 mmol) was added. The yellow

solution was stirred at 55 °C overnight. The solvent was removed under reduced pressure.

The product was isolated by automated flash chromatography (0 - 20% methanol in DCM

over 11 mins) eluted after 5 min. DCM (15.6 mL) was added to the yellow solid. TIPS

(0.86 mL) then TFA (15.6 mL) were added. The reaction was stirred for 2 hours. The

solvent was removed under reduced pressure. The product was isolated by flash

chromatography (20% MeOH, 1% TEA in DCM). Product: Pale yellow solid (85 mg,

0.29 mmol, 46% yield). ^{1}H NMR (400 MHz, D_6-DMSO): δ 12.08 (br s, 1H), 8.99 (br s,

3H), 7.32 (br s, 2H), 6.00 (s, 1H), 3.80 (s, 2H); ESI-MS calculated for $C_6H_{10}N_5O_2$

[M+H]$^+$ 184.08, found 184.09.

C(5) side chain analogs

5-nitroisocytosine (**20**) and 5-aminoisocytosine (**21**) have been previously synthesized.[14]

(Boc)Lys-IsoC (22). DMF (2 mL) was added to Boc-Lys(Boc)-OH (258 mg, 0.71

mmol). DIPEA (0.41 mL, 2.37 mmol) then HATU (302 mg, 0.79 mmol) were added. The

yellow solution was stirred for 5 min. 5-Aminoisocytosine (**21**) (100 mg, 0.79 mmol)

dissolved in DMF (3.5 mL) was added to the reaction. The red solution was stirred

overnight. The solvent was removed under reduced pressure. Water was added to the remaining red oil and the solution was extracted with ethyl acetate. The organics were dried over sodium sulfate. The solvent was removed under reduced pressure. The product was isolated by automated flash chromatography (0 - 20% methanol in DCM over 10 mins) eluted after 5 min. Product: Yellow solid (223 mg, 0.49 mmol, 69% yield). ^{1}H NMR (400 MHz, D_6-DMSO): δ 11.29 (br s, 1H), 8.72 (s, 1H), 8.21 (s, 1H), 7.22 (br s, 1H), 6.77 (s, 1H), 6.47 (br s, 2H), 3.99 (br s, 1H), 2.91 – 2.80 (m, 2H), 1.63 – 1.19 (m, 24H); ESI-MS calculated for $C_{20}H_{34}N_6O_6Na$ [M+Na]$^+$ 477.24, found 477.11.

Lys-IsoC 3 HCl (4). DCM (8.1 mL) was added to (Boc)Lys-IsoC (**22**) (150 mg, 0.33 mmol). TIPS (0.45 mL) then TFA (8.1 mL) were added. The reaction was stirred for 2 hours. The solvent was removed under reduced pressure. The remaining yellow solid was dissolved in water (5% ACN) and purified by reverse phase HPLC (5 – 50% ACN (0.1% TFA) over 15 mins) eluted after 5.3 mins, then lyophilized. The product was converted to a chloride salt using the general ion-exchange procedure. Product: White solid (95 mg, 0.26 mmol, 79% yield). ^{1}H NMR (500 MHz, D_6-DMSO): δ 10.07 (s, 1H), 8.40 (s, 3H), 8.29 (s, 1H), 8.13 (br s, 2H), 7.98 (br s, 3H), 4.17 – 4.13 (m, 1H), 2.76 – 2.72 (m, 2H), 1.77 – 1.73 (m, 2H), 1.57 – 1.53 (m, 2H), 1.40 – 1.35 (m, 2H); ESI-MS calculated for $C_{10}H_{19}N_8O_2$ [M+H]$^+$ 255.2, found 255.2.

U-Lys-IsoC 2 HCl (5) and DMBU-Lys-IsoC 2 HCl (6). Dry pyridine (3.2 mL) was added to (Boc)-LysIsoC (**22**) (223 mg, 0.49 mmol). 2, 4-Dimethoxybenzyl isocyanate (0.22 mL, 0.74 mmol) was added. The yellow solution was stirred overnight. The solvent was removed under reduced pressure. The product was isolated by automated flash

chromatography (0 - 20% methanol in DCM over 12.5 mins) eluted after 7 min. DCM

(1.5 mL) was added to the yellow solid. TIPS (0.1 mL) then TFA (1.5 mL) were added.

The reaction was stirred for 3.5 hours. The solvent was removed under reduced pressure.

The remaining yellow solid was dissolved in water (5% ACN) and purified by reverse

phase HPLC (5 – 50% ACN (0.1% TFA) over 15 mins) eluted after 5.4 mins (U-Lys-

IsoC) and 12.3 min (DMBU-Lys-IsoC), then lyophilized. The product was converted to a

chloride salt using the general ion-exchange procedure. Products: White solids (U-Lys-

IsoC: 23 mg, 0.06 mmol, 13% yield), (DMBU-Lys-IsoC: 59 mg, 0.11 mmol, 23% yield).

U-Lys-IsoC (**5**): ^{1}H NMR (500 MHz, D$_6$-DMSO): δ 9.94 (s, 1H), 8.49 (s, 1H), 8.36 (br s,

3H), 7.93 (br s, 3H), 7.23 (br s, 1H), 6.88 (br s, 1H), 5.51 (br s, 2H), 4.20 (br s, 1H), 2.77

– 2.73 (m, 2H), 1.78 – 1.74 (m, 2H), 1.58 – 1.53 (m, 2H), 1.39 – 1.36 (m, 2H); ESI-MS

calculated for C$_{11}$H$_{20}$N$_7$O$_3$ [M+H]$^+$ 298.2, found 298.2. DMBU-Lys-IsoC (**6**): ^{1}H NMR

(500 MHz, D$_6$-DMSO): δ 9.91 (s, 1H), 8.46 (s, 1H), 8.37 (br s, 3H), 7.95 (br s, 3H), 7.74

(br s, 1H), 7.12 (d, J = 8.0 Hz, 1H), 6.57 (s, 1H), 6.47 (d, J = 8.0 Hz, 1H), 4.22 -4.20 (m,

3H), 3.81 (s, 3H) 3.74 (s, 3H), 2.77 – 2.69 (m, 2H), 1.77 – 1.71 (m, 2H), 1.59 – 1.52 (m,

2H), 1.40 – 1.33 (m, 2H); ESI-MS calculated for C$_{20}$H$_{30}$N$_7$O$_5$ [M+H]$^+$ 448.2, found

448.3.

(Boc)Arg-IsoC (23). DMF (11.8 mL) was added to Boc-Arg(Boc)$_2$-OH (2 g, 4.21

mmol). DIPEA (2.43 mL, 14.05 mmol) then HATU (1.79 g, 4.68 mmol) were added. The

yellow solution was stirred for 5 min. 5-Aminoisocytosine (**21**) (592 mg, 4.68 mmol)

dissolved in DMF (20.7 mL) was added to the reaction. The purple solution was stirred

overnight. The solvent was removed under reduced pressure. Water was added to the

remaining red oil and the solution was extracted with ethyl acetate. The organics were dried over sodium sulfate. The solvent was removed under reduced pressure. The product was isolated by automated flash chromatography (0 - 20% methanol in DCM over 25 mins) eluted after 14 min. Product: Yellow solid (1.40 g, 2.40 mmol, 57% yield). ^{1}H NMR (400 MHz, D$_6$-DMSO): δ 9.16 (br s, 1H), 8.91 (s, 1H), 8.25 (s, 1H), 7.26 (d, J = 8.0 Hz, 1H), 6.83 (br s, 2H), 4.10 (br s, 1H), 3.77 (br s, 2H), 3.20 – 3.10 (m, 2H), 1.66 – 1.22 (m, 31H); ESI-MS calculated for C$_{25}$H$_{43}$N$_8$O$_8$ [M+H]$^+$ 583.3, found 583.5.

Arg-IsoC 3 HCl (7). DCM (6.3 mL) was added to (Boc)Arg-IsoC (**23**) (150 mg, 0.26 mmol). TIPS (0.35 mL) then TFA (6.3 mL) were added. The reaction was stirred for 2 hours. The solvent was removed under reduced pressure. The remaining yellow solid was dissolved in water (5% ACN) and purified by reverse phase HPLC (5 – 50% ACN (0.1% TFA) over 15 mins) eluted after 5.3 mins, then lyophilized. The product was converted to a chloride salt using the general ion-exchange procedure. Product: White solid (73 mg, 0.19 mmol, 72% yield). ^{1}H NMR (500 MHz, D$_6$-DMSO): δ 10.09 (s, 1H), 8.43 (s, 3H), 8.31 (s, 1H), 8.13 (br s, 2H), 7.83 (t, J = 6.0 Hz, 1H), 7.48 (br s, 2H), 7.02 (br s, 2H), 4.20 – 4.17 (m, 1H), 3.15 – 3.11 (m, 2H), 1.78 – 1.73 (m, 2H), 1.57 – 1.47 (m, 2H); ESI-MS calculated for C$_{10}$H$_{19}$N$_6$O$_2$ [M+H]$^+$ 283.2, found 283.3.

U-Arg-IsoC 2 HCl (8) and DMBU-Arg-IsoC 2 HCl (9). Dry pyridine (2.8 mL) was added to (Boc)-ArgIsoC (23) (250 mg, 0.43 mmol). 2, 4-Dimethoxybenzyl isocyanate (0.19 mL, 0.64 mmol) was added. The yellow solution was stirred overnight. The solvent was removed under reduced pressure. The product was isolated by automated flash chromatography (0 - 20% methanol in DCM over 10 mins) eluted after 4.5 min. DCM

(1.5 mL) was added to the yellow solid. TIPS (0.1 mL) then TFA (1.5 mL) were added. The reaction was stirred for 3.5 hours. The solvent was removed under reduced pressure. The remaining yellow solid was dissolved in water (5% ACN) and purified by reverse phase HPLC (5 – 50% ACN (0.1% TFA) over 15 mins) eluted after 6.5 mins (U-Arg-IsoC) and 12.6 min (DMBU-Arg-IsoC), then lyophilized. The product was converted to a chloride salt using the general ion-exchange procedure. Products: White solids (U-Arg-IsoC: 14 mg, 0.03 mmol, 8% yield), (DMBU-Arg-IsoC: 41 mg, 0.08 mmol, 17% yield). U-Arg-IsoC (**8**): ^{1}H NMR (500 MHz, D$_6$-DMSO): δ 9.99 (d, J = 15 Hz, 1H), 9.90 (s, 1H), 9.75 (s, 1H), 9.02 (s, 1H), 8.52 (d, J = 6.0 Hz, 1H), 8.46 (s, 1H), 8.26 (s, 2H) , 7.40 – 6.40 (m, 6H), 4.17 (br s, 1H), 3.30 – 3.26 (m, 2H), 1.81 – 1.68 (m, 2H), 1.58 – 1.48 (m, 2H); ESI-MS calculated for C$_{11}$H$_{19}$N$_9$O$_3$ [M+H]$^+$ 326.2, found 326.3. DMBU-Arg-IsoC (**9**): ^{1}H NMR (500 MHz, D$_6$-DMSO): δ 10.29 (br s, 0.5H), 9.96 (d, J = 2.5 Hz, 1H), 9.07 (br s, 0.5H), 8.55 (br s, 1H), 8.48 (s, 1H), 8.40 (br s, 3H), 7.78 (t, J = 6.0 Hz, 1H), 7.55 – 7.21 (m, 6H), 7.12 (d, J = 8.0 Hz, 1H), 6.57 (d, J = 2.5 Hz, 1H), 6.47 (dd, J$_1$ = 8.0 Hz, J$_2$ = 2.5 Hz, 1H), 4.23 – 4.20 (m, 4H), 3.84 (s, 3H), 3.74 (s, 3H), 3.14 – 3.10 (m, 1H), 1.77 – 1.73 (m, 2H), 1.61 – 1.46 (m, 2H); ESI-MS calculated for C$_{20}$H$_{30}$N$_9$O$_5$ [M+H]$^+$ 476.2, found 476.4.

Fmoc-β-Lys(Boc)-IsoC (24). DMF (0.75 mL) was added to Fmoc-β-Lys(Boc)-OH (125 mg, 0.27 mmol). DIPEA (0.15 mL, 0.89 mmol) then HATU (76 mg, 0.30 mmol) were added. The yellow solution was stirred for 5 min. 5-Aminoisocytosine (**21**) (38 mg, 0.30 mmol) dissolved in DMF (1.3 mL) was added to the reaction. The orange solution was stirred overnight. The solvent was removed under reduced pressure. Water was added to

the remaining red oil and the solution was extracted with ethyl acetate. The organics were dried over sodium sulfate. The solvent was removed under reduced pressure. The product was isolated by automated flash chromatography (0 - 20% methanol in DCM over 10 mins) eluted after 7 min. Product: Yellow solid (78 mg, 0.14 mmol, 51% yield). ^{1}H NMR (400 MHz, D$_6$-DMSO): δ 11.28 (br s, 1H), 8.80 (s, 1H), 8.16 (s, 1H), 7.88 (d, J = 7.2 Hz, 2H), 7.65 (d, J = 4.4 Hz, 2H), 7.40 (t, J = 7.4 Hz, 2H), 7.29 (t, J = 7.4 Hz, 2H), 7.19 (d, J = 8.4 Hz, 1H), 6.77 (s, 1H), 6.42 (br s, 2H), 4.26 – 4.17 (m, 2H), 3.80 (br s, 1H), 2.87 (d, J = 5.2 Hz, 2H), 2.45 – 2.40 (m, 2H), 1.37 – 1.16 (m, 13H); ESI-MS calculated for C$_{30}$H$_{37}$N$_6$O$_6$ [M+H]$^+$ 577.3, found 577.4.

β-Lys-IsoC 3 HCl (10). DMF (1.5 mL) was added to Fmoc-β-Lys(Boc)-IsoC (**24**) (20 mg, 0.035 mmol). Piperidine (0.13 mL) was added. The reaction was stirred for 45 min. The solvent was removed under reduced pressure. DCM (0.85 mL) was added to the crude yellow solid. TIPS (0.05 mL) then TFA (0.85 mL) were added. The reaction was stirred for 2 hours. The solvent was removed under reduced pressure. The remaining yellow solid was dissolved in water (5% ACN) and purified by reverse phase HPLC (5 – 50% ACN (0.1% TFA) over 15 mins) eluted after 5.3 mins, then lyophilized. The product was converted to a chloride salt using the general ion-exchange procedure. Product: White solid (8 mg, 0.022 mmol, 63% yield). ^{1}H NMR (400 MHz, D$_6$-DMSO): δ 9.70 (s, 1H), 8.26 (s, 1H), 8.10 (br s, 4H), 7.96 (br s, 6H), 3.51 – 3.42 (m, 1H), 2.83 – 2.68 (m, 4H), 1.69 – 1.53 (m, 4H); ESI-MS calculated for C$_{10}$H$_{18}$N$_6$O$_2$ [M+H]$^+$ 255.2, found 255.2.

U-β-Lys-IsoC 2 HCl (11) and DMBU-β-Lys-IsoC 2 HCl (12). Dry pyridine (0.84 mL) was added to Fmoc-β-Lys(Boc)-IsoC (**24**) (75 mg, 0.13 mmol). 2, 4-Dimethoxybenzyl isocyanate (57 µL, 0.20 mmol) was added. The yellow solution was stirred overnight. The solvent was removed under reduced pressure. The product was isolated by automated flash chromatography (0 - 20% methanol in DCM over 11 mins) eluted after 4 min. DMF (1.5 mL) was added to the yellow solid. Piperidine (0.13 mL) was added. The reaction was stirred for 45 min. The solvent was removed under reduced pressure. DCM (3.2 mL) was added to the yellow solid. TIPS (0.2 mL) then TFA (3.2 mL) were added. The reaction was stirred for 3.5 hours. The solvent was removed under reduced pressure. The remaining yellow solid was dissolved in water (5% ACN) and purified by reverse phase HPLC (5 – 50% ACN (0.1% TFA) over 15 mins) eluted after 5.4 mins (U-β-Lys-IsoC) and 12.3 min (DMBU-β-Lys-IsoC), then lyophilized. The product was converted to a chloride salt using the general ion-exchange procedure. Products: White solids (U-β-Lys-IsoC: 6 mg, 0.016 mmol, 12% yield), (DMBU-Lys-IsoC: 16 mg, 0.030 mmol, 23% yield). U-β-Lys-IsoC (**11**): ^{1}H NMR (400 MHz, D$_6$-DMSO): δ 10.05 (br s, 1H), 9.53 (s, 1H), 8.72 (br s, 2H), 8.44 (s, 1H), 8.20 – 7.87 (m, 4H), 7.15 (br s, 2H), 5.46 (br s, 2H), 3.29 – 3.14 (m, 2H), 3.04 (p, J = 7.2 Hz, 1H), 2.83 – 2.73 (m, 2H), 1.17 – 1.21 (m, 2H); ESI-MS calculated for C$_{11}$H$_{19}$N$_7$O$_3$ [M+H]$^+$ 298.2, found 298.3. DMBU-β-Lys-IsoC (**12**): ^{1}H NMR (400 MHz, D$_6$-DMSO): δ 9.99 (br s, 1H), 9.78 (br s, 1H), 9.61 (br s, 1H), 9.42 (br s, 1H), 8.42 (s, 1H), 8.13 – 7.82 (m, 6H), 7.51 (br s , 2H), 7.12 (d, J = 8.0 Hz, 1H), 6.57 (d, J = 2.8 Hz, 1H), 6.47 (dd, J$_1$ = 8.0 Hz, J$_2$ = 2.5 Hz, 1H), 4.21 (d, J = 6.0 Hz, 2H),

3.81 (s, 3H), 3.74 (s, 3H), 3.46 (br s, 1H), 2.87 – 2.68 (m, 4H), 1.70 – 1.53 (m, 4H); ESI-MS calculated for $C_{20}H_{29}N_7O_5$ [M+H]$^+$ 448.2, found 448.4.

General ion-exchange procedure

Amberlite IRA-900 (Cl$^-$ form) was prepared by washing with MeOH, followed by a saturated brine solution, and then water. Water (0.6 mL / 20 mg of compound) was added to the HPLC purified TFA salts. Amberlite IRA-900 (Cl$^-$ form) (100 mg / 20 mg of compound) was added. The reaction was gently shaken on a Fisher Vortex Genie 2 overnight. The reaction was filtered and the ion exchange beads were washed with water. The filtrate was lyophilized and the exchange of TFA counterions was confirmed by ^{13}C NMR.

A-site binding assay

All titrations were performed with working solutions of 1 μM Dy-547 labeled A-site in 20 mM cacodylate buffer (pH = 7.0, 100 mM NaCl, 0.5 mM EDTA). The solutions were heated to 75 °C for 5 min, cooled to room temperature over 2 h, cooled to 0 °C for 30 min, then allowed to warm back to room temperature. Kanamycin-courmarin or neomycin-coumarin was added, to give a working concentration of 0.53 μM, just prior to aminoglycoside titrations. Steady state fluorescence experiments were carried out at ambient temperature (20 °C). Excitation and emission slit widths were 9 nm for kanamycin-coumarin experiments and 7 nm for neomycin-coumarin. The system was excited at 400 nm and changes in Dy-547 emission were monitored at 561 nm. Errors were generated from three sets of measurements. IC50 values were calculated using

OriginPro 8.5 software by fitting a dose response curve (eq 1) to the fractional

fluorescence saturation (Fs) plotted against the log of antibiotic (A) concentration.

$F_s = F_0 + (F_\infty [A]^n)/([IC50]^n + [A]^n)$ (1)

F_s is the fluorescence intensity at each titration point. F_0 and F_∞ are the

fluorescence intensity in the absence of ligand or at saturation, respectively, and *n* is the

Hill coefficient or degree of cooperativity associated with binding.

Translation assays

Bacterial *in vitro* translation was quantified using a coupled transcription

translation assay (S30 T7 High-Yield Protein Expression System). A DNA plasmid (100

ng/uL) containing the renilla luciferase gene under control of a T7 phage RNA

polymerase promoter was used. For each reaction S30 premix plus (3.2 µL) and T7 S30

extract (2.88 µL) were premixed just before use. S30 premix plus contains amino acids,

rNTPs, tRNAs, an ATP-regenerating system, IPTG, and salts conducive to recombinant

protein expression. T7 S30 extract contains T7 RNA polymerase and all components

needed for translation. The experiment was done in strip tubes with compound or water

(1.2 µL), plasmid DNA (1.2 µL), and the premixed S30 extract mixture (6.08 µL) for a

total reaction volume of 8.48 µL. Reactions were incubated at 37°C for 30 min. in a

thermoclycler, and the reaction was cooled to 4°C when completed. Then 5 µL of each

reaction was added to a 96-well plate. Renilla luciferin substrate in buffer (25 µL) was

added and luminescence was immediately measured with a plate reader.

Eukaryotic *in vitro* translation was quantified using a coupled transcription

translation assay (TnT® SP6 Coupled Reticulocyte Lysate System). A DNA plasmid (100

ng/µL) containing the luciferase gene under control of a SP6 phage RNA polymerase promoter was used. For each reaction, rabbit reticulocyte (3.75 µL), TnT buffer (0.3 µL), amino acids (0.15 µL), SP6 polymerase (0.15 µL), and an RNase inhibitor (0.15 µL) were premixed just before use. The experiment was done in strip tubes with compound or water (1.5 µL), plasmid DNA (1.5 µL), and the premixed reticulocyte mixture (4.5 µL) for a total reaction volume of 7.5 µL. Reactions were incubated at 30°C for 30 min. in a thermocycler, and the reaction was cooled to 4°C upon completion. Then 5 µL of each reaction is added to a 96-well plate. Firefly luciferase substrate in buffer (25 µL) was added and luminescence was immediately measured in the same manner as above.

The half-maximum inhibitor concentration (IC_{50}) was determined from relative luminescence (% of the control) plotted against the log of compound concentration by fitting a dose-response curve using OriginPro 8 software.

MIC determinations

MIC values for aminoglycosides were determined using broth microdilution in accordance with Clinical Laboratory Standards Institute guidelines.[19]

5.5 References

[1] Y. Funabashi, S. Tsubotani, K. Koyama, N. Katayama, S. Harada, *Tetrahedron* **1993**, 49, 13228.

[2] N. Böddeker, G. Badahor, C. Gibbs, E. Mabery, J. Wolf, L. Xu, J. Watson, *RNA* **2002**, 8, 1120.

[3] W. S. Champney, J. Pelt, and C. L. Tober. *Curr. Microbiol.* **2001**, 43, 340.

[4] N. Katayama, S. Fukusumi, Y. Funabashi, T. Iwahi, H. Ono, *J. Antibiot.* **1993**, *46*, 606.

[5] (a) R. M., Williams, C. J. Yuan, *J. Am. Chem. Soc.* **1997**, 119, 11777. (b) V. V. Sokolov, S. I. Kozhushkov, S. Nikolskaya, V. N. Belov, M. Es-Sayed, A. de Meijere, *Eur. J. Org. Chem.* **1998**, 777. (c) L. Zhang, L. Xu, C. U. Kim, *Tet. Lett.* **2003**, 44, 5871.

[6] V. N. Belov, N. Brands, S. Raddatz, J. Kruger, S. Nikolskaya, V. Sokolov, A. de Meijere, *Tetrahedron*. **2004**, 60, 7579.

[7] N. Aguilar, J. Kruger, *Molecules*. **2002**, 7, 469.

[8] M. Brands, R. Endermann, R. Gahlmann, J. Kruger, S. Raddatz, J. Stoltefuβ, V. N. Belov, S. Nizamov, V. V. Sokolov, A. de Meijere, *J. Med. Chem.* **2002**, 45, 4246.

[9] M. Brands, R. Endermann, R. Gahlmann, J. Kruger, S. Raddatz, *Bioorg. Med. Chem. Lett.* **2003**, 13, 241.

[10] M. Brands, Y. G. Grande, R. Endermann, R. Gahlmann, J. Kruger, S. Raddatz, *Bioorg. Med. Chem. Lett.* **2003**, 13, 2641.

[11] M. Brands, R. Endermann, R. Gahlmann, S. Raddatz, June **2002**. Patent DE10061542 (A1).

[12] (a) M.M.L. Nieuwenhuizen, T.F.A. de Greef, R.L.J. van der Bruggen, J.M.J. Paulusse, W.P.J. Appel, M.M.J. Smulders, R.P. Sijbesma, E.W. Meijer, *Chem. Eur. J.* **2010**, 16, 1601. (b) T.F.A. de Greef, G.B.W.L. Ligthart, M. Lutz, A.L. Spek, E.W. Meijer, R.P. Sijbesma, *J. Am. Chem. Soc.* **2008**, 130, 5479. (c) H.M. Keizer, J.J. González, M. Segura, P. Prados, R.P. Sijbesma, E.W. Meijer and J. de Mendoza, *Chem. Eur. J.* **2005**, 11, 4602.

[13] J. Chen, X. G. Fu, L. Zhou, J. T. Zhang, X. L. Qi, X. P. Cao, *J. Org. Chem.* **2009**, 74, 4149.

[14] G. K. Mittapalli, Y. M. Osornio, M. A. Guerrero, K. R. Reddy, R. Krishnamurthy, A. Eschenmoser, *Angew. Chem. Int. Ed.* **2007**, 46, 2470.

[15] R. J. Fair, M. E. Hensler, W. Thienphrapa, Q. N. Dam, V. Nizet, Y. Tor, *ChemMedChem* **2012**, 7, 1273.

[16] L. S. McCoy, K. D. Roberts, R. L. Nation, P. E. Thompson, T. Velkov, J. Li, Y. Tor, *ChemBioChem* **2013**, 14, 2083.

[17] Q. Liu, N. Q. Luedtke, Y. Tor, *Tetrahedron Lett.* **2001**, 42, 1445.

[18] (a) Y. Xie, A. V. Dix, Y. Tor, *J. Am. Chem. Soc.* **2009**, 131, 17605. (b) Y. Xie, A. V. Dix, Y. Tor, *Chem. Commun.* **2010**, 46, 5542.

[19] *Performance Standards for Antimicrobial Susceptibility Testing*, 19th Informational Supplement M100-S19, Clinical and Laboratory Standards Institute, Wayne, PA, USA, **2008**.

Chapter 6

A Safe and Cost-Effective Synthesis of β^3-Lysine

6.1 Introduction

β^3-lysine is an amino acid that is prevalent in many structurally diverse bacterial natural products that possess a variety of biological activities. Selected examples are shown in Figure 6.1. Streptothricins are broad spectrum antibiotics isolated from actinomycetes that contain repeating L-β^3-lysine oligomer side chains up to seven units in length. Dozens of members of this class of molecules have been discovered to date, some as recently as 2012.[1] Tuberactinomycins, also isolated from actinomycetes, are cyclic peptide antibiotics with some isoforms having a monomeric β^3-lysine side chain. Capreomycin (**2**), a tuberactinomycin, is one of the leading treatments for MDR tuberculosis.[2] Tallysomycin A (**3**), a glycopeptide anticancer antibiotic produced by *Streptoalloteichus hindustanus*, also has a β^3-lysine monomer in its pendant side chain.[3] Myomycin A (**4**), an antibiotic pseudodisaccharide with a L-β^3-lysine dimer has been isolated from *Nocardia* species of bacteria.[4] Much smaller β^3-lysine derivatives have also been shown to have interesting biological properties. Bellenamine (**5**), an amide derivative of D-β^3-lysine, is a natural product of *Streptomyces nashvillensis*. While this small molecule only has very weak antibacterial activity, it is a potent inhibitor of the secondary spread of HIV.[5]
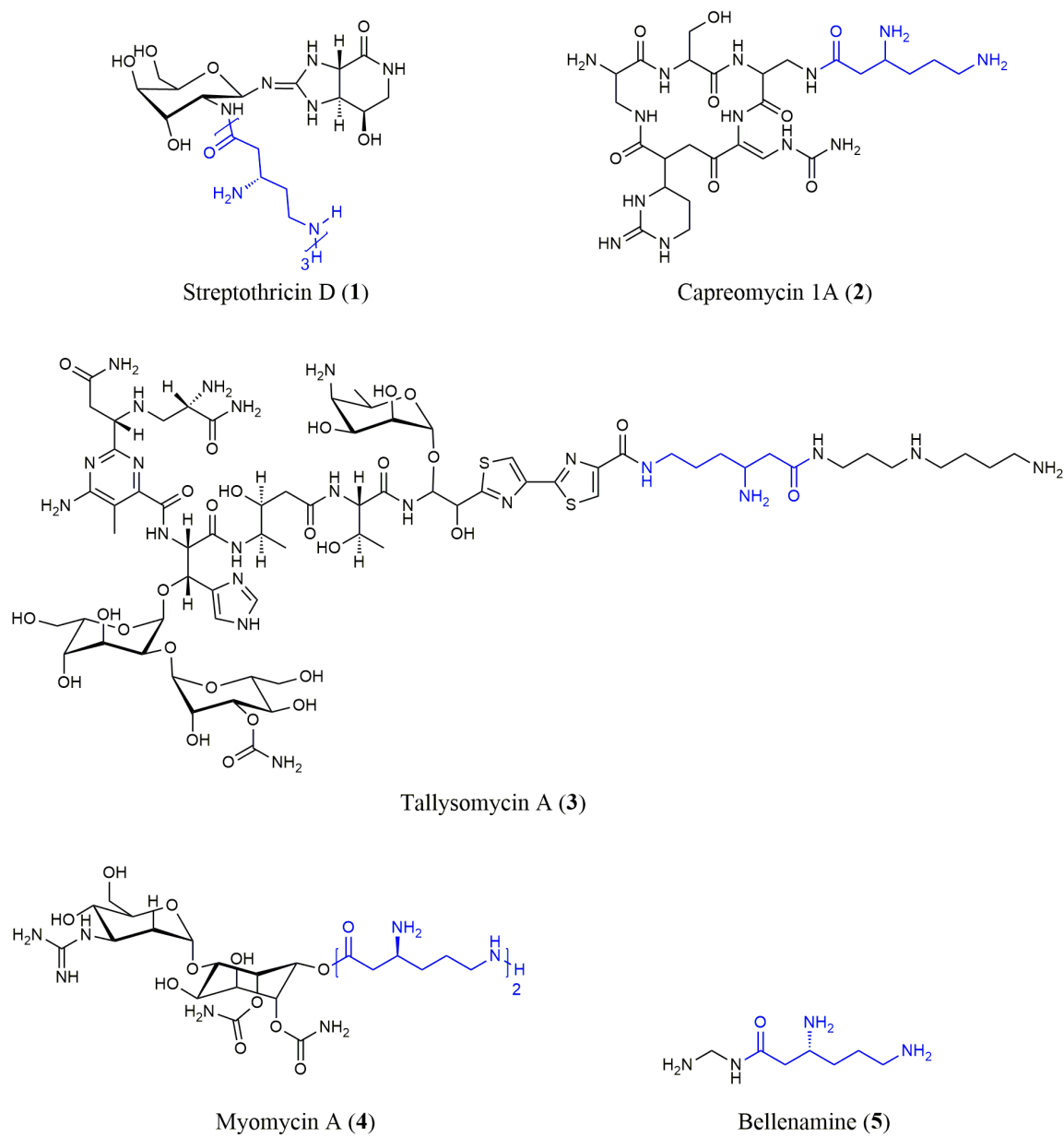
Streptothricin D (**1**)

Capreomycin 1A (**2**)

Tallysomycin A (**3**)

Myomycin A (**4**)

Bellenamine (**5**)

**Figure 6.1:** β^3-Lysine containing natural products.

It has even been suggested in *in vitro* studies that lysine analogs, including β^3-lysine, can themselves enhance bacterial growth inhibition when administered in combination with N^5-hydroxy-L-arginine.[6] β^3-lysine has also played a role in recent

biophysical research. One recent study, for example, has suggested that in gram negative bacteria elongation factor P is activated by a D-β^3-lysine post-translational modification.[7]

The prevalence of β^3-lysine in diverse bioactive natural products suggests that it is a privileged structure with biological properties worthy of further scientific investigation. There are many syntheses of β-amino acids, however the vast majority of them either don't apply or haven't been verifiably used for β^3-lysine.[8] Historically there were several ground up approaches for synthesizing racemic β^3-lysine.[9] These routes were largely abandoned with the advent of enantioselective methods. This is presumably due to the relative length of these routes and for the obvious advantage of isolating a product of a defined chirality. One notable example takes advantage of a nitrone cycloaddition specifically in the synthesis and isolation of both enantiomers of β^3-lysine (Scheme 6.1).[10] This method was notable in that it was the first synthetic route to give good optical yields of these products. It has a significant number of steps, however, and although yields are generally high there is a low yielding oxidation step. It also requires chiral resolution of nitrone intermediate (**9**), which can be taken forward to give both enantiomers of β^3-lysine. The current gold standard for β^3-homoamino acid synthesis is undoubtedly the Arndt-Eistert homologation based method. Though early variants of this method weren't high yielding for many of the more complex side chain containing amino acids, the current modifications give good yields for orthogonally protected β^3-homolysine (Scheme 6.2).[11] Although, it isn't detailed in this paper β^3-lysine can likely also be made in good yield via this method since it is actually β^3-homoornithine, a one
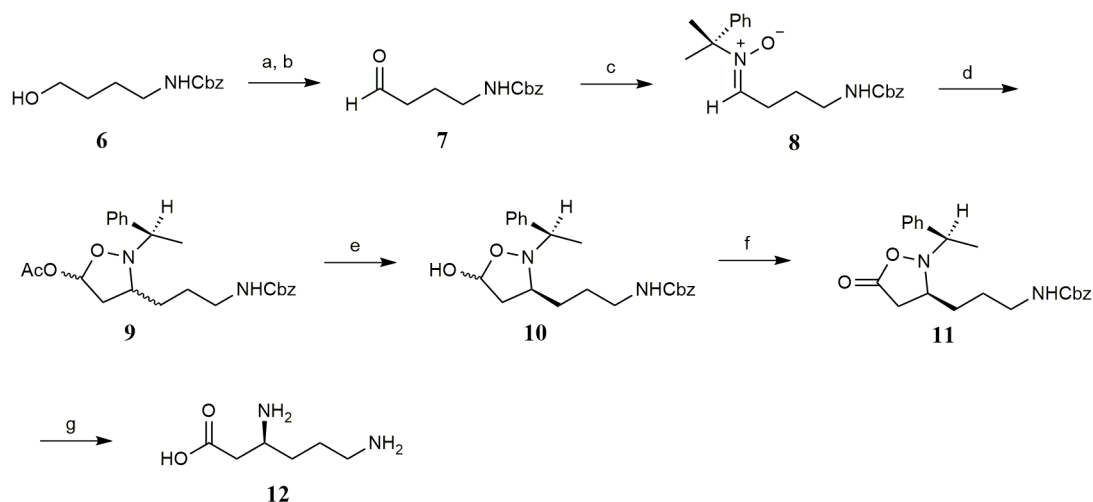
Scheme 6.1: Nitrone cycloaddition based synthesis of L-β^3-lysine (**12**). *Reagents and conditions:* a) Oxalyl chloride, DMSO, -60 °C; b) TEA; c) Hydroxylamine oxalate, TEA, DCM, 20 °C, (91% yield - 3 steps); d) Vinyl acetate, Reflux, 68% yield; e) K$_2$CO$_3$, MeOH, H$_2$O, 20 °C, >98% yield; f) CrO$_3$, DCM, pyridine, 0 °C, 40% yield; g) 20% Pd(OH)$_2$/C, H$_2$, EtOH, 20 °C to 70 °C, 98% yield
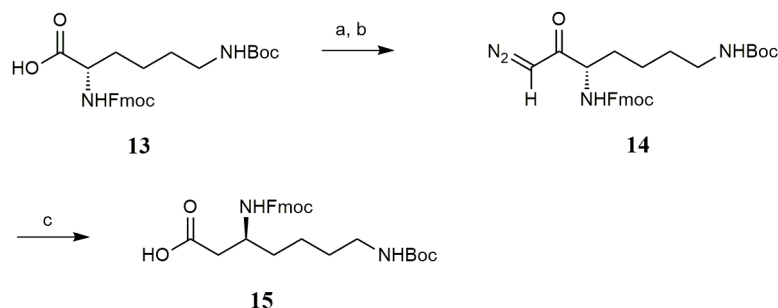
Scheme 6.2: Arndt-Eistert based synthesis of L-Fmoc-β^3-homolysine(Boc)-OH (**15**). *Reagents and conditions:* a) iBuOCOCl, NMM, THF, -20 °C to -5 °C; b) CH$_2$N$_2$, Et$_2$O, -5 °C, (88% yield - 2 steps); c) CF$_3$CO$_2$Ag, NMM, THF, H$_2$O, 0 °C to RT, 81% yield

carbon shorter variant of β^3-homolysine. The Arndt-Eistert method shows an impressive

brevity and economy in employing cheap, commercially available α-amino acids as

starting materials. A downside to this method, however, is that it requires the use of

diazomethane, a reagent that is both extremely dangerous and requires specialized

equipment to use.[12] As a consequence this route may be unattractive to some who want to

make gram scale quantities of these amino acids. It is also worth noting that there has

recently been an increase in research on lysine 2,3-aminomutase, a bacterial enzyme that

converts α-lysine into β^3-lysine, however at this time the enzyme is not known to be commercially available.[13] There are disadvantages associated with all of the present synthetic routes to β^3-lysine and so we devised a route that is complementary to the present methods.

6.2 Results and Discussion

Synthetic Strategy

The early racemic syntheses of β^3-lysine became irrelevant because of their length of synthesis and lack of chiral resolution.[9] However, upon examination of these routes it became evident to us that if updated with the vast advances in chemistry that have taken place since their publication they could potentially serve as inspiration for an effective and safe synthesis of both enantiomers of β^3-lysine. By searching current literature it was found that racemic 3,6-dibromohexanoic acid (**19**), an obvious intermediate for the synthesis of these amino acids, could be made in three steps from simple starting materials (Scheme 6.3).[14] From the 3,6-dibromohexanoic acid (**19**), 3, 6-diaminohexanoic acid (**21**) (racemic β^3-lysine) could be made in two more steps (Scheme 6.4). This could then theoretically be resolved into pure enantiomers.

Synthesis

The low cost starting material, 2,3-dihydrofuran (**16**), was first hydrated by stirring with aqueous HCl to give 2-hydroxytetrahydrofuran (**17**) using a known procedure.[14a] It was found that this intermediate could be made fairly pure simply by rotary evaporation under high vacuum as all major byproducts and the starting material are volatile under these conditions. The hemiacetal was then converted to racemic
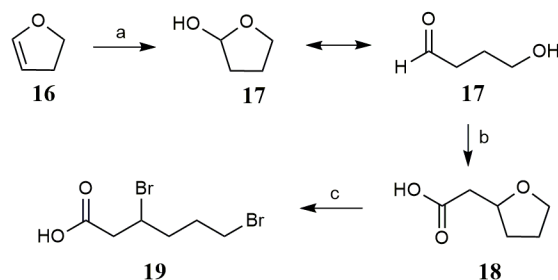
Scheme 6.3: Synthesis of 3, 6-dibromohexanoic acid (**19**). *Reagents and Conditions*: a) HCl, H$_2$O, RT; b) Malonic acid, piperidine, AcOH, DMSO, 100 °C; c) HBr, H$_2$O, 90 °C.
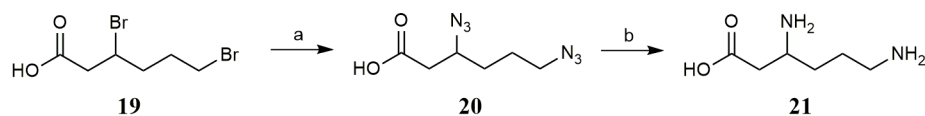
Scheme 6.4: Synthesis of β^3-lysine (**21**). *Reagents and Conditions*: a) NaN$_3$, DMF, 75 °C; b) Pd/C, H$_2$, THF.

tetrahydrofuran-2-ylacetic acid (**18**) via a recently devised modified Knoevenagel condensation/decarboxylation utilizing a piperidinium acetate catalyst.[14b] It is worth noting that these first two intermediates are commercially available, but they are very expensive considering how easily they are made. The tetrahydrofuran ring can then be opened and the resulting terminal alcohol substituted for a bromine by heating with aqueous HBr to yield 3,6-dibromohexanoic acid (**19**) also through a known reaction.[14c] A double substitution can then be effected by heating with sodium azide to give 3,6-diazidohexanoic acid (**20**). This reaction was tried at a variety of temperatures, but reacting at 75 °C led to the highest yield of 74%. A near quantitative hydrogenolysis can then afford free, racemic β^3-lysine (**21**). Several solvents were tried for the hydrogenolysis including acetic acid, ethanol, and ethyl acetate, but using THF led to the highest yield.

Chiral resolution of racemic β^3-lysine (**21**) has thus far been unsuccessful. Resolution on cellulose preparative thin layer chromatography (TLC) and reverse phase

silica preperative TLC with the chiral resolving agent, tartaric acid, as a mobile phase

additive was unsuccessful under a variety of conditions. Quantities of the tartaric acid

additive were varied from 0.5% by weight to 5% by weight. A water / acetonitrile mobile

phase was used. Both amines of racemic β^3-lysine (**21**) were *tert*-butyloxycarbonyl

(Boc)-protected then the acid was made into diastereomeric salts with both brucine and

quinine counterions. Preparative TLC on both cellulose and alumina stationary phases

was attempted on these salts. Water / acetonitrile mobile phases in some cases with a

pyridine additive were explored. Chiral recrystallization of these salts also proved so far

unsuccessful. β^3-lysine (**21**) was esterified via Fischer esterification in sulfuric acid and

methanol. Resolution by enantioselective hydrolysis using porcine liver esterase (PLE) in

a phosphate buffer (0.1 M, pH = 7.4) was attempted also without success. Chiral HPLC is

the last likely method of resolution that remains to be tried.

6.3 Conclusions

Racemic β^3-lysine (**21**) was synthesized in good yield from cost-effective starting

materials. The synthesis is safer and likely more scale-able than the current standard

synthesis of β^3-lysine. Chiral resolution, which isn't necessary for other modern methods,

has so far remained elusive, however. It will be necessary to establish a chiral resolution

procedure for this method to be useful.

6.4 Experimental Section

Materials

Unless otherwise specified, materials purchased from commercial suppliers were used without further purification. All reagents were obtained from Sigma-Aldrich. NMR solvents were purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

Instrumentation

All column chromatography was performed using the Teledyne Isco Combiflash® R_f 200 automated flash purification system with RediSep® R_f normal phase silica columns. NMR spectra were recorded on Varian Mercury 300 and 400 MHz, Varian VX 500 MHz, and Jeol ECA 500 MHz spectrometers. Mass spectra (MS) were recorded at the University of California, San Diego Chemistry and Biochemistry Mass Spectrometry Facility, utilizing an Agilent 6230 HR-ESI-TOF mass spectrometer.

Synthesis

Synthesis and characterization of 2-hydroxytetrahydrofuran (**17**), tetrahydrofuran-2-ylacetic acid (**18**), and 3,6-dibromohexanoic acid (**19**) have been previously reported.[14]

3,6-Diazidohexanoic acid (20). DMF (66.6 mL) was added to 3,6-dibromohexanoic acid (**19**) (1.5 g, 5.52 mmol). Sodium azide (3.59 g, 55.17 mmol) was added. The yellow solution was heated to 75 °C and stirred overnight. The solvent was removed under reduced pressure. The resulting solid was suspended in DCM and extracted with aqueous 1 M HCl. The combined aqueous layers were neutralized with a saturated sodium bicarbonate solution and disposed of. The organics were dried with sodium sulfate and the solvent was removed under reduced pressure. The product was isolated by flash chromatography (45% EtOAc in hexanes). Product: Yellow oil (809 mg, 4.08 mmol, 74%

yield). ^{1}H-NMR (400 MHz, CDCl$_3$): δ 4.63 – 4.58 (m, 1H), 3.54 – 3.47 (m, 2H), 2.57 (t, J = 8.2 Hz, 2H), 1.97 – 1.85 (m, 4H)

β^3-lysine (3,6-diaminohexanoic acid) (21). THF (12.9 mL) was added to 3,6-diazidohexanoic acid (**20**) (530 mg, 2.68 mmol). Pd/C (10%, 97 mg, 0.27 mmol) was added. The solvent was degassed by bubbling through H$_2$ and the mixture was stirred under atmospheric H$_2$ for 36 hours. The reaction was filtered through celite, washing with methanol. The solvent was removed under reduced pressure. The remaining gel was suspended in toluene and the solvent was again removed under reduced pressure to remove trace remaining solvent. No further purification was necessary. Product: Light yellow solid (385 mg, 2.64 mmol, 98% yield). ^{1}H-NMR (400 MHz, D$_2$O): δ 3.23 – 3.16 (m, 1H), 2.89 (t, J = 7.2 Hz, 2H), 2.36 (dd, J$_1$ = 15 Hz, J$_2$ = 5.8 Hz, 1H), 2.26 (dd, J$_1$ = 15 Hz, J$_2$ = 7.8 Hz, 1H), 1.69 – 1.43 (m, 4H)

6.5 References

[1] M. Gan, X. Zheng, Y. Liu, Y. Guan, C. Xiao. *Bioorg. Med. Chem. Lett.* **2012**, 22, 6151.

[2] (a) R. Akbergenov, D. Shcherbakov, T. Matt, S. Duscha, M. Meyer, D. N. Wilson, E. C. Böttger. *Antimicrob. Agents Chemother.* **2011**, 55, 4712; (b) R. E. Stanley, G. Blaha, R. L Grodzicki, M. D. Strickler, T. A. Steitz. *Nat. Struc. Mol. Bio.* **2010**, 17, 289; (c) M. G. Thomas, Y. A. Chan, S. G. Ozanick. *Antimicrob. Agents Chemother.* **2003**, 47, 2823.

[3] (a) M. L. Sznaidman, S. M. Hecht. *Org. Lett.* **2001**, 3, 2811; (b) L. Wang, M. Tao, E. Wendt-Pienkoski, U. Galm, J. M. Coughlin, B. Shen *J. Biol. Chem.* **2009**, 284, 8256.

[4] (a) W. J. McGahren, B. A. Hardy, G. 0. Morton, F. M. Lovell, N. A. Perkinson, R. T. Hargreaves, D. B. Borders, G. A. Elleetad *J. Org. Chem.* **1981**, 46, 792; (b) J. C. French, L. E. Anderson, R. H. Bunge, J. D. Howells. March **1974**. Patent US3795668 (A).

[5] (a) R. Ikeda, Y. Haraguchi, Y. Ikeda, S. Kondo, T. Takeuchi, H. Hoshino *Antiviral Res.* **1996**, 29, 163; (b) E. de Clerq *Med. Res. Rev.* **2000**, 20, 323.

[6] D. Perlman *J. Antibiotics* **1975**, 28, 997.

[7] T. J. Bullwinkle, S. B. Zou, A. Rajkovic, S. J. Hersch, S. Elgamal, N. Robinson, D. Smil, Y. Bolshan, W. Wiley Navarre, M. Ibba *J. Biol. Chem.* **2013**, 288, 4416.

[8] E. Jurasti: Enantioselective Synthesis of β-Amino Acids. Wiley-VCH; New York, New York: **1997**.

[9] (a) L. Birkofer, I. Hartwig, *Chem. Ber.* **1956**, 89, 1608; (b) F. F. Pelizzoni, *Annali di Chimica*, **1959**, 49, 1461.

[10] D. Keirs, D. Moffat, K. Overton, *J. Chem. Soc., Chem. Commun.* **1988**, 654.

[11] G. Guichard, S. Abele, D. Seebach, *Helv. Chim. Acta* **1998**, 81, 187.

[12] (a) USDOL (United States department of labor) Occupational Safety and Health Guideline for Diazomethane, **2000**; (b) T.J. van 't Erve, R.H. Rautiainen, L.W. Robertson, G. Luthe *Environment International* **2010**, 36, 835; (c) R. W. Barnhart, D. Dale, M. D. Ironside, S. M. Shaw, *Org. Process Res. Dev.* **2012**, 16, 1980.

[13] (a) C.-C. Hung, M.-C. Lai, *J. Microbiol., Immunol. Infec.* **2013**, 46, 1; (b) S. Müller, T. Hoffmann, H. Santos, S.H. Saum, E. Bremer, V. Müller, *Appl. Microbiol. Biotechnol.* **2011**, 91, 689.

[14] (a) K. Kojima, M. Kimura, S. Uedab, Y. Tamaru, *Tetrahedron* **2006**, 62, 7512; (b) V. Ragoussis, V. Theodorou, *Synthesis* **1993**, 84; (c) D. S. Kemp, M. D. Sidell, T. J. Shortridge, *J. Org. Chem.* **1979**, 44, 4473.