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SYNTHESIS OF 4'-O-GLYCOPYRANOSYL PAROMOMYCIN AMINOGLYCOSIDE ANTIBIOTICS: INFLUENCE OF THE GLYCOSIDE ON ANTIRIBOSOMAL, ANTIBACTERIAL ACTIVITY AND UTILIZATION OF ELECTROSPRAY IONIZATION MASS SPECTROMETRY TO EVALUATE REACTIVITY OF GLYCOSYL PHOSPHATE

by

WEIWEI CHEN

THESIS

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

2014

MAJOR: CHEMISTRY

Approved by:

____________________________________________________________________

Advisor Date
ACKNOWLEDGEMENTS

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>Ac</td>
<td>Acetyl</td>
</tr>
<tr>
<td>AGA</td>
<td>Aminoglycoside</td>
</tr>
<tr>
<td>AW-MS</td>
<td>Acidic washed molecular seives</td>
</tr>
<tr>
<td>Bn</td>
<td>Benzyl</td>
</tr>
<tr>
<td>BnBr</td>
<td>Benzyl bromide</td>
</tr>
<tr>
<td>Bu</td>
<td>Butyl</td>
</tr>
<tr>
<td>C</td>
<td>Concentration</td>
</tr>
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<td>Catalytic</td>
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<td>Calculated</td>
</tr>
<tr>
<td>CID</td>
<td>Collision induced dissociation</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
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<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPSO</td>
<td>Diphenyl sulfoxide</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>ESI-HRMS</td>
<td>Electrospray ionization high resolution mass spectroscopy</td>
</tr>
<tr>
<td>Et</td>
<td>Ethyl</td>
</tr>
<tr>
<td>exp</td>
<td>Exponential</td>
</tr>
<tr>
<td>G</td>
<td>Guanine</td>
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</tbody>
</table>
gt  gauche-trans
HPLC  High pressure liquid chromotography
Hz  Hertz
$k$  Rate constant
Me  Methyl
mmol  Milimole
MS  Molecular sieves
NIS  $N$-Iodosuccinimide
NMR  Nuclear Magnetic Resonance
$p$  para
PG  Protecting group
Ph  Phenyl
ppm  Parts per million
Py  Pyridine
quant.  Quantitatifs
RDS  Rate determining step
rf energy  Radio frequency energy
RNA  Ribonucleic acid
RRV  Relative reactivity value
RSV  Relative stability value
rt  Room temperature
SY$_{50}$  rf excitation energy used to achieve 50% fragmentation in CID
$T$  Temperature
<table>
<thead>
<tr>
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<tr>
<td>TBAI</td>
<td>Tetrabutyl ammonium iodine</td>
</tr>
<tr>
<td>Tf</td>
<td>Trifluoromethanesulfonyl</td>
</tr>
<tr>
<td>TfO₂</td>
<td>Triflic anhydride</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TfOH</td>
<td>Trifluoromethanesulfonic acid</td>
</tr>
<tr>
<td>tG</td>
<td><em>trans-gauche</em></td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
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<tr>
<td>TTBP</td>
<td>2,4,6-Tri-tert-butylpyrimidine</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VL</td>
<td>Visceral Leishmaniasis</td>
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PART I: SYNTHESIS OF 4’-O-GLYCOPYRANOSYL PAROMOMYCIN AMINOGLYCOSIDE ANTIBIOTICS: INFLUENCE OF THE GLYCOSIDE ON ANTIRIBOSOMAL, ANTIBACTERIAL ACTIVITY

CHAPTER 1

INTRODUCTION

1.1 An Overview of Aminoglycoside Antibiotics (AGAs)

Since the discovery of the first naturally produced antibiotic Penicillin, various types of antibiotics have emerged, either from natural sources or synthetic routes. Generally, antibiotics can be classified into four types according to their acting mechanism: cell wall synthesis inhibitors; protein synthesis inhibitors; oligonucleotides replication and repair inhibitors; and folate coenzyme biosynthesis inhibitors. Among them, aminoglycosides antibiotics (AGAs) are an old family of antibiotics, which target protein synthesis. They have been developed for around 70 years since the introduction of streptomycin in 1944. Early AGAs like neomycin, paromomycin (Figure 1) and gentamicin were used to treat Gram-negative and most Gram-positive bacterial infections effectively. The widely adopted resistance of bacteria towards AGAs drove the birth of a second generation of AGAs, the semisynthetic derivatives netilmicin (1976) etc. However, the toxicity limitations of those AGAs eventually led to their replacement by other broad spectrum antibiotics with fewer side effects, fluoroquinolones for example.
1.2 AGAs Mechanism of Action

AGAs bind to bacterial 16S rRNA helix 44. The binding site is part of the aminoacyl-tRNA acceptor site, which is commonly called A site (Figure 2). Even though AGAs all effectively inhibit elongation step in protein translation, their actions are mechanistically different. They affect protein synthesis either by inducing miscoding or by inhibiting translocation of the tRNA-mRNA complex. Paromomycin and apramycin represent two distinct mechanisms of action. Apramycin acts primarily by blocking the translocation of ribosome along mRNA while only limited codon misreading is observed. Paromomycin, however, destacks A1492 and A1493 and causes significant miscoding.

In the absence of paromomycin, energy is required to flip out A1492 and A1493 so they can contact with tRNA. However, paromomycin ring I forms so tight hydrogen bond with the phosphate backbone of A1493 that it locks the
structure in the flipped-out form. As a result, the energetic cost of both cognate
and non-cognate tRNA binding is reduced. An undifferentiated affinity of the A-
site for cognate and non-cognate tRNA is resulted and codon misreading takes
place (Figure 2).\textsuperscript{5}

![Figure 2: Paromomycin interacting with bacterial 30S subunit. [This figure
has been reproduced from “Perez-Fernandez, D.; Shcherbakov, D.; Matt, T.;

1.3 Paromomycin Interactions With 30S Ribosomal Subunit

Paromomycin is an efficient antibiotic against Gram-negative and many
Gram-positive bacteria. It is not used as an antibiotic anymore due to its toxicity,
but nevertheless paromomycin was licensed in India as an effective, well
tolerated treatment for Visceral Leishmaniasis (VL) in 2007.\textsuperscript{6} Understanding the
interactions between paromomycin with the decoding A site of bacterial ribosome is of great significance as it provides the basis for rational drug design. A crystallographic structure gives insight into how paromomycin docks into the major groove of Helix44 (Figure 2).  

Paromomycin ring IV and ring II contact the backbone of both sides of H44. Additionally, ring II also tightly binds to bases (Figure 2). Ring III only weakly binds to the rRNA. The binding model of ring I is very interesting and the most important one. It behaves like a nucleotide base, stacking against G1491 and hydrogen-bonding with A1408. The base at position 1408 of 16S rRNA is critical to determine the aminoglycosides selectivity. Drug-susceptible bacterial ribosomes are characterized by an adenine at position 1408, while drug resistant eukaryotic cytoplasmic ribosomes is conferred by a guanine at the homologous position. This is one of the most important reasons why we are interested in modifying ring I of paromomycin.

1.4 Selectivity and Ototoxicity

The selectivity of aminoglycoside derives from the fact that the decoding sites of rRNA small ribosomal subunits of bacteria and humans have minor sequence differences, as illustrated in Figure 3. The decoding A site of wild type bacterial 16S rRNA is shown in Figure 3A as shadow region. Figure 3B illustrates the homologous 18S rRNA sequence in human cytosolic ribosomes. Figure 3C is
homologous 12S rRNA sequence in human mitoribosomes. Figure 4D shows homologous 12S rRNA sequence in human mitoribosomes with a single mutation of A1555G, which confers to hyper-susceptibility to AGAs induced ototoxicity.³

Aminoglycosides are able to bind more strongly to the Prokaryotic ribosome than the Eukaryotic ribosome. On the other hand, the sequence difference at the decoding site is so minimal that competing binding of aminoglycosides to human ribosome is expected. Therefore, the toxicity and selectivity are intertwined together making it very challenging to modify AGAs to meet clinical requirements.¹¹

As aminoglycosides began to be used widely in clinics after 1950, the adverse effects became obvious. Among them, nephrotoxicity and ototoxicity are the major obstacles limiting the use of this type of antibiotics. Nephrotoxicity is resulted from accumulation of AGAs in the renal cortex tissue.¹¹,¹² Clinically people are using a large dose strategy to minimize this side effect. When a large dose of AGAs is applied within the clinical meaningful range, kidneys can only absorb limited amount of AGAs.¹³,¹⁴ In contrast, ototoxicity of aminoglycosides is mostly irreversible. The hearing loss results from degeneration of hair cells and neurons in the cochlea.¹¹ AGAs induced deafness is also affected by genetic factors. For example, individuals with a single mutation A1555G in the A-site of the mitoribosomal small subunit are hyper-susceptible to ototoxicity.¹⁵
1.5 Renaissance of Aminoglycosides

Due to the toxicity of aminoglycosides, widely adopted bacterial resistance and the emergence of other broad spectrum antibiotics with less side effects (fluoroquinolones etc.), interest in improving the pharmacological profile of AGAs declined in the late 1970s. However, all other common antibiotics have been encountered increasing resistance from bacteria. At the same time, limited biological targets in bacterial metabolism make it difficult to find new types of antibiotics. Therefore, there is a revived interest in modifying aminoglycosides.

Previously researchers were trapped by the lack of efficient genetic tools to study the interaction between eukaryotic rRNA with aminoglycosides. This hurdle has been overcome with the pioneering work of Böttger et. al. by engineering the rRNA decoding site of eukaryotic ribosome in bacteria. They replaced the
bacterial ribosomal A site with its eukaryotic counterpart, resulting in bacterial hybrid ribosomes with a fully functional eukaryotic rRNA decoding site (Figure 3E-3G). This technique allows the fast screening of aminoglycosides derivatives \textit{in vitro} to evaluate their potential ototoxicity. It is also one of the key techniques in this collaborative project with the Böttger group. Genetic analysis identified mitoribosome as the target in AGAs induced ototoxicity. This proposal is further supported by the finding that antibacterial activity of 4-monosubstituted 2-deoxystreptamine apramycin 2 can be separated from aminoglycosides ototoxicity. Recent published data demonstrated that modifications of paromomycin at the 4’ position reduce anti mitoribosomal activity but retain its antibiotic activity.

1.6 Recent Efforts to Improve AGAs Pharmaceutical Profile

Various modifications of paromomycin at C(4’), C(6’), C(5”), C(6’”) positions had been screened for activity and selectivity. Work done by the Vasella and Böttger groups revealed that 4’-O-alkyl, and 4’,6’-O-alkylidene paromomycin derivatives retain strong antibacterial activity comparable with paromomycin against \textit{Staphylococcus aureus} and of \textit{E. coli}. They showed minimal affinity to the hybrid human mitochondrial ribosomes carrying the A1555G or C1494U alleles, which are hyper-susceptible to AGA-induced hearing loss. 4’-O-(3-phenylpropyl) paromomycin ether 3, the 4’,6’-O-benzylidene paromomycin acetal
4, and the 4',6'-O-(3-phenypropyldene) paromomycin acetal 5 are shown here in Figure 4 to exemplify these two series.  

\[ \text{Figure 4. Potential paromomycin derivatives} \]

Crystallographic studies identify the same binding patterns between the bacterial decoding site and these derivatives to paromomycin itself. The chemically introduced 4'-O-substituents of paromomycin derivatives extend out of the binding pocket and extrude to approach the flipped out base A1492 (Figure 5B). An overlay of the X-ray crystal structures of apramycin 2 and 4',6'-O-modified paromomycin 4 (Figure 5C) display the common binding mode between the two. Most importantly, the 4',6'-modified paromomycin derivative can discriminate bacterial ribosome from hybrid mitoribosome A1555G which confers hyper-susceptibility to ototoxicity. These results identify the C4' position as a promising site to increase the selectivity of paromomycin derivatives via the manipulation of drug-target interaction.

The above studies lead to the hypothesis that the substituent at the 4’ position possibly exerts its influence over binding either by interacting with the zone of organized water into which it projects, or through the stabilization of the flipped out conformations. Therefore, the goal of this project is to synthesize series of 4’-O-glycosylated derivatives of paromomycin mimicking the aminoglucosyl ring of apramycin in order to improve selectivity and diminish toxicity.
CHAPTER 2

4’-O-GLYCOSYL PAROMOMYCIN SYNTHESIS AND BIOLOGICAL RESULTS

2.1 Results and Discussion

2.1.1 4’-O-Glycosyl Paromomycin Synthesis

2.1.1.1 Synthesis of 4’-O-Glycosyl Paromomycin Intermediate

Pentaazido-paromomycin 8 was prepared from paromomycin monosulfate 7 by diazo transfer from imidazole-1-sulfonyl azide hydrochloride (Stick’s reagent, 6) in the presence of an excess amount of K₂CO₃ and a catalytic amount of CuSO₄ (Scheme 2).²¹ Stick’s reagent can be conveniently prepared on a large scale from cheap starting materials. It is shelf-stable and easily recrystallizes from ethyl acetate under acidic conditions (Scheme 1).²¹

\[
\text{NaN}_3 \xrightarrow{i) \text{SO}_2\text{Cl}_2, \text{MeCN}} \xrightarrow{ii) \text{imidazole}} \xrightarrow{iii) \text{HCl in EtOH}} \text{N}_3\text{SO}-\text{N} \xrightarrow{\text{HCl}} \text{N}_3\text{SO}-\text{N} \xrightarrow{\text{HCl}} \text{6}
\]

Scheme 1. Stick’s reagent synthesis

As a cheap replacement of triflyl azide, Stick’s reagent also showed higher efficiency in this diazotransfer reaction to paromomycin: the yield increased from 49%¹⁰ to 63%. Regioselective protection of the 4’-OH and 6’-OH groups with benzylidene acetal gave 9. The remaining free hydroxyl groups were O-benzylated with benzyl bromide (BnBr) in the presence of sodium hydride (NaH) in THF to yield 10. Regioselective reductive cleavage of the benzylidene acetal of
with NaCNBH$_3$ in the presence of 2 M HCl$^{22,23}$ left the 4’ hydroxyl group of compound 11 open for further modification (Scheme 2).$^{10}$

Scheme 2. Synthesis of a selectively protected paromomycin acceptor

2.1.1.2 Glycosylation Reactions

After the intermediate 11 was obtained, it was glycosylated with a series of known monosaccharyl donors (Scheme 3).$^{24-26}$
Thioglycosides donors (12-15) were preactivated with DPSO/Tf$_2$O (DPSO: diphenyl sulfoxide)$^{27}$ at -72 °C in the presence of 2,4,6-tri-tert-butylpyrimidine (TTBP)$^{28}$ as a mild, hindered, non-nucleophilic base. Subsequent addition of paromomycin acceptor provided the glycosides as α- and β- mixtures. As it is evident from the results, except for the formation of mannopyranoside 17, all glycopyranoside formations were characterized by a α-selectivity, which is consistent with the previous reports (Table 1).$^{29-31}$ The attention was focused on the study of the antibiotic profile of both α and β-isomers. The α and β-isomers were separated by regular liquid chromatography first, and then by reverse phase
high pressure liquid chromatography. The yields and anomeric ratios of each glycosylation are indicated in Table 1.

2.1.1.3 Deprotection

Extensive exploration of the direct hydrogenolysis of benzyl ethers and azide groups with Pd(OH)$_2$/C (20% wt.) in various solvents was made. It identified methanol/water solvent system with a certain amount of acetic acid as the best solvent system. However, the one step deprotection was not reproducible and suffered low yield. Therefore, a stepwise sequential deprotection of azide groups using the Staudinger reaction followed by the deprotection of benzyl ether groups on hydrogenolysis was adopted (Scheme 4). In a small scale deprotection of these paromomycin derivatives, the amine groups would undergo condensation with the formaldehyde impurity within methanol solvent to form imine, which could be reduced by hydrogenolysis to methylamine compounds, as identified by high resolution mass spectrometry. The yields of the Staudinger reaction and the hydrogenolysis reaction are also included in Table 1.
Scheme 4. Deprotection

<table>
<thead>
<tr>
<th>Entry</th>
<th>Donor</th>
<th>Glycosylation yield, ratio</th>
<th>Staudinger reaction yield</th>
<th>Hydrogenolysis yield</th>
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<tr>
<td>1</td>
<td>12</td>
<td>16, 42%, α:β = 3.2:1</td>
<td>20α, 64%</td>
<td>24α, 39%</td>
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<tr>
<td>2</td>
<td></td>
<td></td>
<td>20β, 55%</td>
<td>24β, 87%</td>
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<tr>
<td>3</td>
<td>13</td>
<td>17, 51%, α:β = 1:3.6</td>
<td>21α, 91%</td>
<td>25α, 33%</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td>21β, 66%</td>
<td>25β, 25%</td>
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<td>5</td>
<td>14</td>
<td>18, 60%, α:β = 6.4:1</td>
<td>22α, 81%</td>
<td>26α, 62%</td>
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<td>6</td>
<td></td>
<td></td>
<td>22β, 78%</td>
<td>26β, 39%</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>19, 50%, α:β = 4.5:1</td>
<td>23α, 64%</td>
<td>27α, 64%</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td>23β, 48%</td>
<td>27β, 29%</td>
</tr>
</tbody>
</table>

Table 1. Synthesis of 4′‐O‐glycosyl paromomycin derivatives.

2.1.2 Antiribosomal Activity
2.1.2.1 Anti Bacterial Ribosomal Activity

2.1.2.1.1 Anti Wild Type Bacterial Ribosomal Decoding A Site Activity

To access the interaction between the synthesized paromomycin derivatives and bacteria 16S rRNA A site, cell-free translation assays were conducted. Compound antiribosomal activity in the translation inhibiting assay is defined as the concentration to inhibit the *in vitro* translation reaction by 50% (inhibitory concentration, IC$_{50}$). IC$_{50}$ values of all compounds are in µg/ml. Paromomycin 1, apramycin 2, and the 4'-paromomycin derivatives 3 and 5 were also tested as comparators to the 4'-O-glycosyl paromomycin derivatives synthesized. As shown in Table 2, with the exceptions of 4'-O-4-amino-α-d-glucopyranosyl paromomycin 27a, all the paromomycin derivatives synthesized 24-27 showed significant increase of IC$_{50}$ toward wild type bacterial ribosome, compared to paromomycin itself. If compare the IC$_{50}$ values of the corresponding α, β-glycosyl paromomycin carefully, it is clear that both anomers of 4'-O-α-d-glucopyranosyl paromomycin 24 and 4'-O-α-manno pyranosyl paromomycin 25 showed similar activity to inhibit cell-free translation, while a significant IC$_{50}$ difference of two times or more existing between the α, β anomers of other glycosyl paromomycin derivatives. A close scrutiny of the data between anomers revealed something even more interesting. While the α anomers of 4'-O-α-d-galactopyranosyl paromomycin 26 and 4'-O-4-amino-α-d-glucopyranosyl paromomycin 27 showed much stronger anti wild-type ribosome activity than the corresponding β anomers.
<table>
<thead>
<tr>
<th>Cmpd</th>
<th>Wild Type</th>
<th>G1491C</th>
<th>G1491A</th>
<th>A1408G</th>
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</thead>
<tbody>
<tr>
<td>1</td>
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<td>10.42±2.86</td>
<td>0.57±0.09</td>
<td>0.26±0.04</td>
</tr>
<tr>
<td>2</td>
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<td>43.01±11.31</td>
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</table>

**Table 2. Compound interaction with polymorphic residues in the drug binding pocket (IC_{50}, µg/ml)**
a) All measurements were made in triplicate for compounds 1, 2, 3, 5, 24-27.

4’-O-4-Amino-ß-D-glucopyranosyl paromomycin 27 has greater inhibiting activity than other paromomycin derivatives 24-26, which also have a 6-carbon monosaccharyl substituent at 4’ position. This may be resulting from the
increased electrostatic attraction between the positively charged paromomycin derivatives and negatively charged ribosome\textsuperscript{33-35} caused by the extra amino group. To be more specific, as mentioned before in the crystal structure of paromomycin interacting with bacterial A site, additional hydrogen bonds link the hydroxyl groups at positions 3’ and 4’ to the phosphate groups of the two adenine bases 1492 and 1493, helping to stabilize the position of ring I (Figure 6). The positively charged amine group in 4-amino-\textalpha-D-glucopyranoside probably increased the affinity of the paromomycin derivative to the ribosome via interacting with the negatively charged phosphate groups in A1492 and A1493 nearby.\textsuperscript{9} As shown in Figure 6, the 4’-OH group forms a hydrogen bond with the phosphate backbone of A1493, suggesting if the hydrogen bond is diminished, a weaker binding would be resulted. But 4’-deoxyparomomycin only showed minimal loss of inhibiting activity.\textsuperscript{10,36} It seems that the hydrogen bonding between the 4’-OH and the phosphate backbone does not affect considerably the binding affinity. However, in this study, most of the 4’-glycosyl paromomycin derivatives suffered significant loss of binding activity.
This phenomena probably can be explained by the weakened CH-π stacking between paromomycin ring I and G1491 at the bottom of the binding pocket.\textsuperscript{37,38} Ring I fits into the binding pocket well mostly by stacking interactions with G1491 and hydrogen bonds with A1408.\textsuperscript{9} Weakened CH-π interaction may be caused by three factors. The first factor is the reduced electron density in C4’-H bond. Alkylation of O4’, would lower the electron density at this position, thus reduce the electron density at C4’. The electron density would be further reduced from an acetal linkage (compound 5) or a glycosidic bond (compounds 24-27) at this position. Another factor is the increased steric hindrance resulting from the 4’-O substituent. It also may explain the different activities between α, β anomers. The third factor is to which degree the 4’-O pyranoside disturb the solvent shell surrounding the binding site. X-ray crystallographic studies of aparamycin 2, 4’,6’-O-benzylidene paromomycin acetal 3 revealed a common binding pattern at the
bacterial A site as shown in figure 5. The aminoglucosyl residue of apramycin and the 4'-substituent of compounds 3 both extend into the solvent shell between A1492 and G1491. If the 4'-glycosyl paromomycin derivatives adopt the same binding pattern, to which extent the 4'-substituent fits well into this kind of hydrophilic environment, which is composed of organized water molecules, it would affect the stability of the binding pattern as a whole. Studies showed that mannopyranosides, glucopyranosides have less affinity to water molecules compared to galactopyranosides. Therefore, they can fit better into the organized water and disrupt the hydrophilic environment less than galactopyranosides. As a result, the galactosyl derivatives 26α,β showed least inhibiting activity compared to other pyranosyl derivatives. Mannosyl derivatives 24α,β and glucosyl derivatives 25α,β had similar anti-ribosomal activity to each other as it can be explained by their similar affinity to water.

2.1.2.1.2 Anti Mutant Bacterial Ribosomal Decoding A Site Activity

Structurally, in the binding pocket two slightly different bases—residues 1408 and 1491 determine the selectivity of AGAs. Residue 1408 is an adenine in bacterial binding site and in mitochondrial ribosomal binding site. It is a guanine in cytosolic ribosomal binding site. Residue 1491 is a guanine in bacterial binding site, but an adenine in cytosolic ribosomal binding pocket, and a cytosine in mitochondrial ribosomal binding pocket. Other than that, the bases within the
binding pockets are the same (Figure 3). So it is important to understand how a single allelic residue change could subtly affect the binding of AGAs. Therefore, the 4’-glycosyl paromomycin derivatives were assayed for their ability to inhibit three ribosomes with single mutation as shown in table 2. G1491C stands for the 1491G at the bacterial decoding A site was replaced by a cytosine found in human mitochondrial rRNA. G1491A stands for the 1491G at the bacterial decoding A site was replaced by an adenine found in human cytosolic rRNA. A1408G stands for the 1408A at the bacterial decoding A site was replaced by a guanine found in human cytosolic rRNA. 3 Data in table 2 illustrate that all of the compounds tested showed significant loss of inhibiting ability against the three mutant ribosomes compared to the wild type ribosome. This loss implies a disruption of the binding mode as a result of a single base change at the binding pocket. More importantly, it seems the G1491C mutant ribosome endures greater loss of binding affinity to paromomycin derivatives than G1491A. One possible explanation is that adenine has similar structure to guanine, which has two conjugated π rings. 1491 base is at the bottom of the binding pocket, providing CH-π interaction to stabilize paromomycin ring I inside the bacterial A site. Both, adenine and guanine have big π system to support the strong CH-π stacking, while on the other side, cytosine only has one aromatic ring. Another possible explanation is that in the wild type ribosome, there is a strong base pair between C1409 and G1491 to
make a tighter binding pocket, but in G1491C and G1491A mutants, this base pair bonding has been diminished. A1491 is still able to form a pseudobase interaction with C1409 by N6, N1 of adenine donating hydrogen bond to N3 and the carbonyl group of cytosine, though not as strong as G1491. But C1491 is not able to form this kind of pseudobase interaction. Therefore, a looser binding pocket in G1491C mutant has been formed, leading to weaker affinity to paromomycin derivatives.

All of the compounds experienced loss of activity towards the A1408G mutant ribosome. In wild type ribosome, ring I of paromomycin forms a pseudo base-pair interaction with adenine 1408. N6 of adenine donates a hydrogen to ring oxygen of ring I. The 6’–OH donates a hydrogen to the N1 of adenine (Figure 6). In A1408G mutant, an alternative similar pseudobase interaction with paromomycin is possible according to the modeling study. Nevertheless, the lower anti A1408G ribosomal activity was observed across paramomycin, apramycin and all the paramomycin derivatives shown in table 2 suggesting that such an alternative pseudobase pair is weaker than the original one. If the 6’-OH group forms inter-residue hydrogen bonds with the 4’-O-glycosyl ring, it can reduce the availability for 6’-OH to form a hydrogen bond with 1408 adenine. That is another possible reason for the lower activity of paromomycin derivatives against wild type bacterial decoding A site. In the case of A1408G mutant, such guanine-ring I pseudobase interaction is even weakened in the binding with 4’-O-
glycosyl paromomycin.

When comparing the anti ribosomal activity of α, β anomers, it is clear that with the exception of 4'-O-4-amino-α-D-glucopyranosyl paromomycin 27, compounds 24-26 all showed a similar pattern that β anomers inhibit the translation function of those mutant ribosomes much less than the corresponding α anomers. α, β isomers of 4'-O-4-amino-α-D-glucopyranosyl paromomycin 27 have similar anti mutant ribosome activity to each other.

2.1.2.2 Anti Hybrid Ribosomal Activity

As mentioned before, ototoxicity is the major hurdle for paromomycin derivatives to overcome. Recent advances showed a strong correlation between the aminoglycoside-induced ototoxicity and aminoglycoside-induced dysfunction of the mitochondrial ribosome. There are two groups of people hyper-susceptible to AGA-induced ototoxicity—one with A1555G mutation in their mitochondrial ribosome decoding site, another with C1494U mutation.9,44,45 In order to access the probability of the synthesized paromomycin derivatives to induce deafness, their activity against hybrid wild type mitochondrial ribosome and mutant A1555G ribosome was tested (Table 3). To create the hybrid ribosome, complete eukaryotic decoding A site was inserted into the bacterial ribosome.16

In a general way as shown in Table 3, the eukaryotic ribosomes, whether it is wild type mitochondrial ribosome, A1555G mutant ribosome or cytosolic
ribosome, all endure less translation function loss than the bacterial decoding ribosome. 4',6'-O-phenylpropylidene paromomycin acetal 5 stands out as the most selective paromomycin derivative to avoid ototoxicity as demonstrates its big IC$_{50}$ value towards both wild type mitochondrial ribosome and A1555G mutant ribosome. While paromomycin itself can inhibit the translation of A1555G mutant ribosome much stronger than the wild type mitochondrial ribosome, that is not the case for all of its 4'-O modified derivatives. That is especially obvious for 24α, 25α, 26α, 27α, which showed much less activity against A1555G mutant than wild type mitochondrial ribosome. It suggests that the 4'-O-glycosyl paromomycin provides a promising modification pattern to reduce the hearing loss within the group hyper-susceptible to the AGA-induced ototoxicity.

In wild type mitochondrial ribosome A site, there are two non-Watson-Crick base pairs at the bottom of the binding pocket: 1493C•1556C, 1494C•1555A. In the mutant mitochondrial ribosome A site, one of the non-Watson-Crick base pairs has been replaced with a strong Watson-Crick base pair of 1494CΞ1555G. This results in a more rigid binding pocket within the mutant ribosome. Presumably, 4'-O-substituents fit less into the mutant decoding site, thus affect the translation function less. Noteworthy, 4'-O-4-amino-d-glucopyranosyl paromomycin has stronger activity against wild type bacterial decoding A site, but it also shows stronger activity against both mitochondrial wild type ribosome and also A1555G mutant. It seems the introduction of an extra
amine group provides more hydrogen bond to the ribosomes, but it binds unselectively. 4-amino-α-D-glucopyranosyl paromomycin does not show much differentiation between the various ribosomes tested.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Bacterial</th>
<th>Eukaryotic A site</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild Type</td>
<td>Mitochondrial</td>
</tr>
<tr>
<td>1</td>
<td>0.03±0.02</td>
<td>50.54±13.04</td>
</tr>
<tr>
<td>2</td>
<td>0.06±0.04</td>
<td>58.03±18.80</td>
</tr>
<tr>
<td>3</td>
<td>0.20±0.07</td>
<td>49.85±15.39</td>
</tr>
<tr>
<td>5</td>
<td>0.10±0.03</td>
<td>330.74±115.30</td>
</tr>
<tr>
<td>24α</td>
<td>0.32±0.05</td>
<td>1.86±0.36</td>
</tr>
<tr>
<td>24β</td>
<td>0.37±0.06</td>
<td>59.12±6.57</td>
</tr>
<tr>
<td>25α</td>
<td>0.29±0.03</td>
<td>2.19±0.07</td>
</tr>
<tr>
<td>25β</td>
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<td>54.60±8.3</td>
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<tr>
<td>26α</td>
<td>0.55±0.08</td>
<td>2.23±0.33</td>
</tr>
<tr>
<td>26β</td>
<td>1.52±0.30</td>
<td>141.35±3.08</td>
</tr>
<tr>
<td>27α</td>
<td>0.12±0.04</td>
<td>0.34±0.01</td>
</tr>
<tr>
<td>27β</td>
<td>0.29±0.07</td>
<td>7.62±3.30</td>
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</tbody>
</table>

Table 3. Compound interaction with hybrid ribosomes (IC<sub>50</sub>, µg/ml)<sup>a</sup>

<sup>a</sup> All measurements were made in triplicate for compounds 1, 2, 3, 5, 36-39

In table 4, the selectivity of drugs toward eukaryotic ribosomes against bacterial ribosome is measured as a ratio between their absolute IC<sub>50</sub> values. The relative activity values can better reflect the selectivity of those compounds.
The bigger the relative values are, the more selective they are. When $\alpha$, $\beta$ anomers are compared, it is clear that $\beta$ anomers of compounds 24-27 inhibit the function of both, mitochondrial wild type ribosome and mutant A1555G ribosome much less than their corresponding $\alpha$ anomers as evident in Table 4. It seems that the equatorial orientation of the glycosidic bond at the 4'-glycosyl ring would increase the selectivity of paromomycin derivatives towards bacterial decoding A site. This kind of equatorially oriented modification at 4’ position should bring less ototoxicity.

The cytosolic 14 hybrid ribosome (Cyt 14) cell-free translation was also tested in the presence of paromomycin derivatives. The ribosome carries the A site of human cytosolic rRNA, incorporating both of the G1491A and A1408G single point mutations. The test stands for general toxicity, because cytosolic ribosomes are widely spread in the cytosol and are responsible for translating most proteins of the cells. Aside from that, AGAs does not need to go through the two-layered membrane of mitochondria to bind cytosolic ribosomes. All the compounds tested showed less antiribosomal activity towards the Cyt 14 hybrid than towards the bacterial wild type. Noteworthy, 4’-O-4-amino-D-glucopyranosyl paromomycin 27 even exhibited little selectivity towards Cyt14 hybrid ribosome. This further proves the previous hypothesis that 4-amino-D-glucopyranosyl paromomycin binds to the ribosome binding pocket unselectively, and are not able to differentiate the subtle structural differences between ribosomes.
### Table 4. Compound selectivity toward eukaryotic A site over bacterial A site

<table>
<thead>
<tr>
<th>Compound</th>
<th>Bacterial Wild</th>
<th>Mit13/Sms</th>
<th>A1555G/Sms</th>
<th>Cyt14/Sms</th>
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<tr>
<td>1</td>
<td>0.03±0.02</td>
<td>1684.67</td>
<td>194.33</td>
<td>346.33</td>
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<td>967.17</td>
<td>434.33</td>
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<td>0.20±0.07</td>
<td>249.25</td>
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<td>5</td>
<td>0.10±0.03</td>
<td>3307.40</td>
<td>1268.80</td>
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<td>24α</td>
<td>0.32±0.05</td>
<td>5.81</td>
<td>43.69</td>
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<tr>
<td>24β</td>
<td>0.37±0.06</td>
<td>159.78</td>
<td>226.05</td>
<td>317.65</td>
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<td>0.29±0.03</td>
<td>7.55</td>
<td>62.24</td>
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<td>25β</td>
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<tr>
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<td>26.28</td>
<td>59.93</td>
<td>73.90</td>
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</table>

**2.1.2.3 Antibacterial Activity**

All the paromomycin derivatives along with paromomycin 1 and apramycin 2 were accessed for their activity against clinical isolates of *Staphylococcus aureus* and *Escherichia coli* (Table 5). Their antibacterial activity was evaluated by the
determination of Minimal Inhibitory Concentration (MIC, µg/ml). The results of these studies are indicated in the table 5 below.

Aside from AG 039 and AG 042, all the other strains are less resistant to the paromomycin derivatives than paromomycin with the exception of 4’-O-4-amino-α-D-glucopyranosyl paromomycin. It is consistent with the result obtained from anti wild type bacterial decoding A site activity. Generally, α anomers can inhibit the growth of the strains better than β anomers. It is also consistent with the data from anti wild type bacterial decoding A site activity. The consistency in these results further proved that the cell-free translation assay is a fast and reliable model to evaluate the activity of AGAs to inhibit bacteria growth. Strains AG 039 and AG042 achieve their resistance toward AGAs through phosphorylation at 3’ or 4’ position at AGAs.\textsuperscript{46,47} 4’-modified paromomycin derivatives block the possible phosphorylation at 4’ position. As a result, there is a broadly weaker resistance of the two strains to paromomycin derivatives, especially in the case of compounds 3, 5, 24α and 27α.

<table>
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<td>\textit{Staphylococcus aureus}</td>
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Table 5. Minimal inhibitory concentrations (MIC, µg/ml) of clinical isolates

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2.2 Conclusion

A series of 4’-O-glycopyranosyl paromomycin have been synthesized. The α and β anomers of these analogs were tested individually for their ability to inhibit cell-free translation from both, bacterial ribosomes and hybrid bacterial ribosomes with eukaryotic decoding A site. All paromomycin derivatives synthesized (compounds 24-27) suffered significant loss of anti wild type bacterial ribosome activity, compared to their parental paromomycin. All the paromomycin analogues synthesized showed less ability to inhibit the translation of both mutant bacterial ribosomes and hybrid bacterial ribosomes than the
original wild type bacterial ribosome. In the study of anti hybrid ribosome activity assays, it is clear that 4’-O-glycosyl paromomycin derivatives with equatorially oriented glycosidic bonds at the 4’-O-glycosyl ring has more selectivity toward mitochondrial hybrid ribosomes than their corresponding derivatives with axially oriented glycosidic bonds. As AGAs induced ototoxicity is believed to be associated with the anti mitoribosome activity, it implies future modification at 4’-O position can rely on introducing equatorially positioned substituents to improve selectivity of the antibiotics.

Among all the paromomycin derivatives tested in this study, phenylpropyl paromomycin ether 3, 4’,6’-O-phenylpropylidene paromomycin acetal 5 are the most selective drugs as detailed in Table 4. Even though 4’-O-4-amino-α-D-glucopyranosyl paromomycin 27 has comparable activity as parental paromomycin, its selectivity has been greatly compromised as evident in the small values of Mit13/Sms, A1555G/Sms, Cyt14/Sms (Table 4).

The crystal structures of 4’-glycosyl paromomycin derivatives interacting with bacterial A site is not known yet. The lack of X-ray structures of the interaction of paromomycin with the mitochondrial A site limits the development of paromomycin derivatives with small binding affinity to the mitochondrial ribosome. Ultimately, an ideal AGA candidate should display a strong inhibiting activity towards the wild type bacterial ribosome translation while leaving the mitochondrial ribosome undisturbed. To that end, crystallographic and modeling
studies would be of a crucial need to make further advancements in the field.
PART II: UTILIZATION OF ELECTROSPRAY IONIZATION MASS SPECTROMETRY TO EVALUATE REACTIVITY OF GLYCOSYL PHOSPHATE

CHAPTER 3

INTRODUCTION

3.1 One-Pot Oligosaccharide Synthesis

3.1.1 Challenges of Oligosaccharide Synthesis

Oligosaccharides are involved in many biological processes, ranging from inflammation to immune response to viral infections. They are frequently found ligated to cell membrane proteins and control the information flow between different cells by triggering cell-cell recognition. If the automated synthesis of oligonucleotides and peptides is routine, efficient automated synthesis of complex oligosaccharides is still in expansion in the carbohydrate field. The difficulty in synthesizing oligosaccharides partly lies in the lack of general and selective glycosylation methods. As a result, the chemical synthesis of oligosaccharides frequently appears as a difficult and labor-intensive task with respect to the control of the stereoselectivity of the glycosidic bond formation as well as the multiple protection and deprotection manipulation steps.

3.1.2 Introduction to One-Pot Oligosaccharide Synthesis

Different approaches to oligosaccharide synthesis have been developed,
for instance solid-phase based synthesis, enzyme assisted synthesis and one-pot sequential synthesis.\textsuperscript{54} The traditional solid-phase based synthesis requires complicated on-resin group protection and deprotection. On the other hand, the enzymatic synthesis could be used as an alternative, but the low availability and the high cost of the catalysts and substrates limit the usage of this technique.\textsuperscript{53}

Therefore, the one-pot sequential glycosylation is an advantageous method for oligosaccharide synthesis. The efficiency of this approach is based on the tuning of glycoside donors by varying protecting groups and the nature of the leaving group. The most reactive donor is placed at the non-reducing end while the least reactive one is placed at the reducing end. The one-pot synthesis therefore can be achieved by sequentially adding less active donors into the reaction system as shown in Scheme 5.\textsuperscript{49,50,54}

\textbf{Scheme 5: Strategy for one-pot synthesis of linear oligosaccharides}\textsuperscript{a}

a) X represents the leaving group\textsuperscript{50}

\textbf{3.1.3 Reactivity-Based One-Pot Synthesis of Oligosaccharides}

One pot synthesis allows to carry multiple sequential glycosylations to be conducted without the need for purification after each step. However, this method
is limited by the lack of precise reactivity values of building blocks\textsuperscript{49}. In 1999, Wong et. al\textsuperscript{50} reported a general procedure to quantitatively measure the relative reactivity values (RRVs) of glycosyl donors using HPLC. In his study, Wong et al. used a direct competition assay as shown in Scheme 6.\textsuperscript{50} Two different glycosyl donors A1 and A2 were used in large excess in presence of a limited amount of electrophile (E\textsuperscript{+}), to give the activated glycosyl intermediates B1 and B2. Secondly, B1 and B2 collapse into the transient oxocarbenium ions C1 and C2, which are finally trapped by the acceptors to form the desired products. The formation of the glycosyl oxocarbenium ion is assumed to be the rate-determining step of the reaction. As a result, a pseudo-first order reaction was observed. The RRVs were obtained from the relative ratio of the observed rate constants (k_{obs}), which were calculated by monitoring the disappearance of glycosyl donors A1 and A2 by HPLC (Equation 1). [A\textsubscript{t}] stands for donor concentration at time t; [A\textsubscript{0}] stands for donor concentration at the beginning.\textsuperscript{50}

\begin{equation}
[A_t] = [A_0] - \frac{k_{obs}}{k_1 + k_2} [A_0] t
\end{equation}

\textbf{Scheme 6: Competition experiment}\textsuperscript{49}
A library of the RRVs of fifty common glycosyl donors has been determined according to this protocol. In order to prove the applicability of this database, Wong et al. did a series of oligosaccharide synthesis based on the RRVs of glycosyl donors. The building blocks were added sequentially in order of decreasing RRVs\(^{50}\). During the years followed, Wong’s group was able to synthesize many oligosaccharides based on this RRV library\(^{52,55-57}\).

3.2 Methods Using Electrospray Ionization Mass Spectrometry (ESI-MS) to Determine the relative stability of glycosides

3.2.1 Cone Voltage Fragmentation

Electrospray ionization (ESI) uses electrical energy to transfer ions from solution into gaseous phase, and then ions are processed by the analyser\(^{58}\). When the sampling cone voltage is set at a low potential, for example 40V, the molecular ion reaches the mass analyzer intact. However, at higher voltage, an in-source collision-induced dissociation (CID) will take place. The sprayed charged species will accelerate between the sampling cone and the skimmer (Figure 7). During this process, the ions collide with the residual gas molecules. As a result, the kinetic energy is converted into internal energy to break the
bonds in the molecular ions. The kinetic energy got from the increased cone voltage is not that significant. The cone voltage fragmentation is only enough to break weak bonds within molecules ions. In this project, the fragmentation of glycosyl phosphate donors in regular ESI-MS were studied since the phosphate group is a good leaving group.

Figure 7. Schematic illustration of in-source fragmentation

The purpose of this study is to establish a fast and accurate method to access the reactivity of glycosyl phosphate donors as a function of stereochemistry and protecting groups with the use of regular ESI-MS, considering that most organic laboratories have easy access to regular ESI-MS. An initial attempt to use ESI-MS cone fragmentation as a useful means to access the influence of protecting groups on the stability of the sialyl oxocarbenium ions, had been made by the Crich group. From the values of onset cone voltage as shown in Figure 8, it was concluded that the oxazolidinone and the carbonate protecting groups are strongly electron-withdrawing, and thus prevent the positive
charge to build up at the anomeric center. Therefore, higher cone voltages are required to fragment compounds 31-32 rather than compounds 28-30.\textsuperscript{60}

![Images of chemical structures]

**Figure 8: Cone voltage fragmentation of sialyl phosphates\textsuperscript{60}**

### 3.2.2 Collision Induced Dissociation Mass Spectrometry (CID-MS)

CID-MS uses inert gas molecules to collide with accelerated molecular ions. Unlike the cone voltage fragmentation, CID-MS relies on a collision cell positioned between two mass analyzers to provide high potential to accelerate and then collide the ions (Figure 9). Compared to cone voltage fragmentation, the in-cell collision can provide more kinetic energy available to convert to internal energy. Therefore, the CID-MS can break stronger bonds than the cone voltage fragmentation. CID process is a sequence of two steps. The first step of this s (10^{-14} \text{ s to } 10^{-16} \text{ s}), and involves a collision between the molecular ions and an inert gas. During this step, part of the kinetic energy of the ions is transformed into internal energy, bringing the ions into an excited state. The activated ions then undergo an unimolecular decomposition in the subsequent step.\textsuperscript{61} With the
use of CID, Sandler et al. accessed the stability of glycosyl oxocarbenium ions and found that the pyranosides with trans configuration between the diacetyloxy groups at C1 and C2 is more active.\textsuperscript{62} They attributed it to the involvement of neighboring group participation.\textsuperscript{62}

![Figure 9. Schematic illustration of CID mass spectrometer](image-url)
CHAPTER 4

UTILIZATION OF CONE VOLTAGE FRAGMENTATION AND CID TO ACCESS REACTIVITY OF GLYCOSYL PHOSPHATES

4.1 Cone Voltage Fragmentation

4.1.1 Method for Quantitative Measurement

In ESI-MS, the ion signal is proportional to the analyte concentration. Theoretically, the ion signal is linear from a concentration of the analyte ranging from pmol/L to µmol/L. Nevertheless, to make an accurate quantitative analysis, an internal standard is necessary to minimize the variations of sample preparation and sensitivity of mass spectrometer\textsuperscript{63}. The aim of this study is to quantitatively determine the relative reactivity values of several glycosyl phosphate donors. With the use of NIS/TfOH activating system, a series of glycosyl phosphates were synthesized from their corresponding thioglycosides\textsuperscript{60} (see experimental part). The internal standard used here is the (2,3,4,6-tetra-O-(p-methylbenzyl)-α-D-glucosyl) dibutylphosphate \textbf{33}. Compared to glycosyl phosphate donors \textbf{34} and \textbf{35}, the internal standard \textbf{33} has an extra mass of 56 amu. Therefore, it is easy to differentiate from a mixture of the internal standard and the interested glycosyl phosphate donor, the peak of the internal standard phosphate based on the mass spectrum obtained.

All the samples were prepared using the same amount of internal standard and of the interested glycosyl phosphate and were dissolved in a sodium acetate
methanolic solution ([NaOAc] = 0.18 mM). At the standard cone voltage of 40 V, both molecular ions hit the mass detector without losing phosphate groups (Figure 8A). As the cone voltage was increased gradually, both glycosyl phosphates began to fragment more as shown in Figure 8B. The fragment ions observed either resulted from the loss of dibutyl phosphoric acid from the parental molecular ion or from the loss of both dibutyl phosphoric acid and a the benzyl group from parental molecular ion.

The relative abundance of the fragmentation ion with respect to the parental molecular ion in the spectrum is a good reflection of the stability of glycosyl phosphates. The in-source fragmentation study of glycosyl phosphates was carried by increasing the cone voltage gradually from 40V until 2% fragmentation of the internal standard was observed. The relative stability value (RSV) was calculated as following: $RSV = \frac{R_M}{R_S}$, where $R_M$ is the relative abundance of fragmentation ion with respect to the corresponding parental interested glycosyl phosphate ion, and $R_S$ is the relative abundance of fragmentation ion with respect to the corresponding parental internal standard ion. An example of the mass spectra obtained from the fragmentation of glycosyl phosphates is shown in Figure 10; the fragmentation spectrum represented here is from (2,3,4,6-tetra-O-benzyl-α-D-mannosyl) dibutylphosphate. In Figure 8B, P1 is the internal standard parental ion; P2 is (2,3,4,6-tetra-O-benzyl-α-D-mannosyl) dibutylphosphate parental ion; D1 is daughter ion of internal standard and D2 is
daughter ion of (2,3,4,6-Tetra-O-benzyl-α-D-mannosyl) dibutylphosphate.

![Mass Spectrum](image)

**Figure 10**: Mass Spectrum of (2,3,4,6-Tetra-O-benzyl-α-D-mannosyl) dibutylphosphate under different cone voltages.

### 4.1.2 Results and Discussion

A series of α, β anomers of mannosyl phosphates and glucosyl phosphates have been synthesized and tested quantitatively to determine their stability as shown in Table 5. Mannosyl phosphates and glucosyl phosphates were either fully protected with benzyl protecting groups or selectively protected with the 4,6-O-benzylidene acetal group. Based on the formula of RSV
calculation, it is obvious that as the RSV decreases, the stability of the glycosyl phosphate increases. During different fragmentation experiments, the cone voltage for internal standard achieving 2% fragmentation varies in 150 V-155 V region. This variation demonstrated the importance of using internal standard to ensure the accuracy and the reproducibility of these quantitative experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sample structure</th>
<th>CV</th>
<th>Rs average</th>
<th>R_M average</th>
<th>RSV average</th>
</tr>
</thead>
<tbody>
<tr>
<td>36α</td>
<td>![Image]</td>
<td>151</td>
<td>2.30±0.11</td>
<td>105.24±1.51</td>
<td>45.84±1.91</td>
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<td>2.43±0.01</td>
<td>84.47±0.33</td>
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<td>12.75±1.49</td>
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<td>2.40±0.10</td>
<td>7.54±0.19</td>
<td>3.14±0.12</td>
</tr>
</tbody>
</table>

Table 6. Relative stability values from cone voltage fragmentation^b^

a) CV stands for the cone voltage when internal standard glycosyl phosphate reaches approximately 2% fragmentation.
b) Each fragmentation experiment was duplicated for three times.
The α anomers of glucosyl phosphates are more stable than their corresponding β anomers regardless of the protecting group used as shown in compounds 35α and 37α. In contrast, the α anomers of mannosyl phosphates are less stable than their corresponding β anomers irrespective of the protecting group used as shown compounds 34α and 36α. Generally, it is accepted that α glycosyl phosphates are more stable than their β counterparts because of the anomeric effect. While studies from glucosyl phosphates support this assumption, the results from mannosyl phosphate studies contradict it. One possible explanation advances that when the dibutyl phosphate group at C1 is syn to the proton at C2 (compounds 34α, 35β, 36α, 37β), the observed fragmentation undergoes a one-step pericyclic syn-elimination (McLafferty rearrangement) (Scheme 7, path a) rather than going through the oxocarbenium ion formation as shown in pathway b (Scheme 7, path b). Scheme 7 uses mannosyl phosphate donor 36α as an example to illustrate the two alternative pathways that take place during the fragmentation of all the glycosyl phosphates studied here. Because the glycosyl oxocarbenium ion builds up a significant positive charge on the anomeric carbon, path b needs higher threshold energy to reach the transient oxocarbenium ion specie rather than path a. While path a is suitable for compounds 34α, 35β, 36α, 37β because of the favorable syn orientation of the dibutyl phosphate group at C1 with respect to the hydrogen atom at C2, such a
mechanism is not a possible for compounds $34\beta$, $35\alpha$, $36\beta$, $37\alpha$ because of the anti periplanar orientation of the C1-O1 and C2-H2 bonds. As a result, compounds $34\beta$, $35\alpha$, $36\beta$, $37\alpha$ have to go through the unfavorable path b.

The McLafferty rearrangement is a common reaction observed during mass spectrometric fragmentation. In solution, the glycosylation proceeds through the formation of an oxocarbenium ion intermediate as outlined in path b (Scheme 7). Therefore, the RSVs of glycosyl phosphates carrying the dibutyl phosphate group at C1 trans to the hydrogen atom at C2 can be correlated to the reactivity of the glycosyl phosphates observed in glycosylation reactions since the fragmentation of compounds $34\beta$, $35\beta$, $36\alpha$ and $37\alpha$ follow the common formation of the oxocarbenium ion intermediate.

![Scheme 7 Fragmentation mechanism]
However, during the study of the influence of different protecting groups on the stability of glycosyl phosphates, it was found that glycosyl phosphate donors protected with 4, 6-O-benzylidene acetal groups are less stable than their corresponding tetra benzylated glycosyl phosphate donors with the only exception of 34β and 36β, which are similar to each other. Thus, the 4,6-O-benzylidene-2,3-O-benzyl-α-D-glucosyl phosphate donor 37α was much less stable than the (2,3,4,6-tetra-O-benzyl-α-D-glucosyl) dibutyl phosphate donor 35α. Yet, the 4,6-O-benzylidene-2,3-O-benzyl-β-D-mannosyl dibutyl phosphate 36β donor was found to have a similar stability than the (2,3,4,6-tetra-O-benzyl-β-D-mannosyl) dibutyl phosphate donor 34β. The results are contradictory to those usually observed in solution media where, for example, it was found that 4,6-O-benzylidene protected glucoside was hydrolyzed more slowly than the corresponding 4,6-di-O-benzyl glucoside. The explanation advanced for this solution phase observation was attributed to the benzylidene effect, which results from locking C5-C6 bond in its most deactivating trans-gauche (tg) conformation leading to a maximization of the electron-withdrawing ability of the C6-O6 bond. Therefore, it seems that the RSV cannot be used as a support to determine the reactivity of glycosyl phosphate donors in glycosylation reaction.

4.2 CID Fragmentation
4.2.1 Survival Yield

With the use of CID-MS, it is possible to measure the stability of glycosyl phosphate donors without worrying about side reactions as parental molecular ions fragment in the collision cell in the gaseous state. In this study, the CID fragmentation experiment was carried on a Bruker amaZon ETD ion trap mass spectrometer. These experiments were conducted in collaboration with Dr. Mary Rodger’s group. The parental ions were fragmented with rf excitation energy (radio frequency energy) increasing from zero voltage until all the parental ions are fragmented. The survival yield of parental ion was calculated at each rf excitation energy applied. Hence, the survival yield curve obtained for each glycosyl phosphate donor is represented in Figure 11. The rf excitation energy used to achieve 50% fragmentation of the parental ion is used to access the stability of the glycosyl phosphate. The rf excitation energy applied at 50% fragmentation was named as $SY_{50}$. As $SY_{50}$ increases, the glycosyl phosphate is more stable.
Figure 11 Survival-yield curves of energy-resolved CID of eight glycosyl phosphates. Data were obtained on a Bruker amaZon ETD quadrupole ion trap mass spectrometer. Four parameter nonlinear dynamic fits to the survival-yield curves were generated to determine the rf excitation energies at which 50% of the precursor ions dissociate to evaluate the relative stabilities of the glycosyl phosphates. A) comparison of six glycosyl phosphates B) comparison of α,β anomers of 4,6-O-benzylidene-2,3-O-benzyl-D-mannosyl phosphate.

4.2.2 Results and Discussion

From the comparison of SY_{50} values of each glycosyl phosphate tested (Table 7), the CID fragmentation experiment gave similar result as it from the cone voltage fragmentation experiment. In other words, the α anomers of glucosyl phosphate are more stable than their corresponding β anomers regardless the protecting group used as show compounds 35α and 37α. Similarly, the α anomers of mannosyl phosphate are less stable than their corresponding β anomers irrespective with the protecting groups used as show compounds 34α and 36α. Glycosyl phosphate donors protected with 4, 6-O-benzylidene acetal groups are also less stable than their corresponding tetra benzylated glycosyl
phosphate donors with the only exception of α-glucosyl phosphate. It is likely that these glycosyl phosphates undergo the same mechanism pathway during the fragmentation step in either CID-MS or cone voltage fragmentation experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sample structure</th>
<th>RSV average</th>
<th>SY_{50}/V</th>
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<tbody>
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<td>36α</td>
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<td>0.470±0.003</td>
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<td>0.471±0.003</td>
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<td>0.677±0.003</td>
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<tr>
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<td>0.780±0.004</td>
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<td>3.14±0.12</td>
<td>0.737±0.003</td>
</tr>
</tbody>
</table>

Table 7. Comparison of RSV from cone voltage fragmentation and SY_{50} from CID-MS fragmentation

4.3 Conclusion

Based on the results obtained from both, cone voltage and CID fragmentation experiments, it is likely that glycosyl phosphates with the C1-
dibutyl phosphate bond syn to the C2-H bond as for compounds \textit{34\alpha}, \textit{35\beta}, \textit{36\alpha} and \textit{37\beta}, dissociate through a McLafferty rearrangement. On the other hand, glycosyl phosphates with the C1-dibutyl phosphate bond anti to C2-H bond as for compounds \textit{34\beta}, \textit{35\alpha}, \textit{36\beta} and \textit{37\alpha} dissociate through an oxocarbenium ion, a common intermediate specie of the glycosylation reaction. However, based on the cone voltage fragmentation experiment, the 4,6-O-benzylidene protected glucosyl fragments more readily than the corresponding 4,6-di-O-benzyl glucosyl phosphate, which is contradictory to the well-known benzylidene effect. Therefore, the RSVs of compounds \textit{34\beta}, \textit{35\alpha}, \textit{36\beta} and \textit{37\alpha} cannot be used to determine the relative reactivity of the glycosyl phosphate donor. The different behavior of these glycosyl phosphate donors observed during ESI-MS fragmentation experiments, and not during the glycosylation reactions, is probably due to the fact that in ESI-MS, the glycosyl phosphate donors are in a gaseous phase and exist as sodiated, unsolvated ions. Evidently, either sodiation or the lack of solution changes the order of oxocarbenium ion stability from that seen in solution.
CHAPTER 5

EXPERIMENTAL SECTION

General. Glycosyl acceptor 11\textsuperscript{10} was prepared by the literature method. All reactions were conducted under an inert atmosphere of nitrogen or argon. ESI high resolution mass spectrometra were recorded with an electrospray source coupled to a time-of-flight mass analyzer. Optical rotation values were measured on an Autopol III automatic polarimeter. NMR data were acquired on either a Varian 500 MHz NMR machine or varian 600 MHz NMR machine for \textsuperscript{1}H and \textsuperscript{13}C spectra.

A. General Procedure for Glycosylation under DPSO/TTBP/Tf\textsubscript{2}O Conditions. Dichloromethane solution of glycosyl donor (0.47 mmol, 1.3 eq), DPSO (0.55 mmol, 1.5 eq), TTBP (1.05 mmol, 2.9 eq) and activated 3 Å molecular sieves in dichloromethane (3 mL) was stirred at room temperature for 3 h before it was cooled to -72 °C temperature. Tf\textsubscript{2}O (0.55 mmol, 1.5 eq) was added slowly to the solution. After 1 h at the same temperature a solution of acceptor 11 (0.36 mmol, 1 eq) in dichloromethane (1.5 mL) was added dropwise to the reaction mixture. The resulting solution was stirred at the same temperature for 2 h before the reaction was quenched by the addition of a saturated solution of NaHCO\textsubscript{3}. The reaction mixture was diluted with EtOAc and the organic phase was washed with a saturated solution of NaHCO\textsubscript{3} three times. The organic layer was dried over
Na$_2$SO$_4$, concentrated and purified by column chromatography over silica gel.

**B. General Procedure for the Reduction of Azides to Amines under Staudinger Reaction Conditions**$^{10}$. The substrate (0.06 mmol, 1 eq) in THF (5 mL) was treated at room temperature with 0.1M aqueous NaOH (0.3 mL, 0.03 mmol, 5 eq) and 1M trimethylphosphine in THF (0.6 mmol, 10 eq). The reaction mixture was stirred for 2 h at 50 °C, then cooled to room temperature. Before it was neutralized with 1 M AcOH solution to pH 7 before concentration. The resulting slurry was subjected to silica gel chromatography, eluting first with MeOH (100 mL), followed by 0.5% aqueous NH$_4$OH in MeOH (150 mL) to give the product.

**C. General Procedure for the Hydrogenolysis Reaction.** The substrate was dissolved in a mixture of methanol (1 mL), deionized water H$_2$O (2 mL), and glacial AcOH (0.1 mL). A catalytic amount of Pd(OH)$_2$ on carbon (20 wt. %) was added, and the reaction was stirred at room temperature under 1 atm of hydrogen (balloon) overnight. After completion, the reaction mixture was filtered over Celite® and the resulting filtrate was neutralized by the addition of Amberlite-IRA400 to pH 7 and filtered. The filtrate was concentrated to dryness and dissolved in 0.002 M aqueous AcOH solution (pH 4, 2 mL) before it was charged to a Sephadex column (CM Sephadex C-25). The Sephadex column was flushed sequentially with dionized water H$_2$O (50 mL), 0.5% aqueous
NH₄OH (20 mL), and 1.5% NH₄OH (40 mL). The fractions containing the hydrogenolysis product were combined and evaporated to give a sticky white solid, which was dissolved in 0.02 mM acetic acid (pH 5, 1 mL). The resulting solution was frozen by immersion in a dry ice acetone bath, and then the water was removed by liophilization to give the product as an acetate salt.

1,3,2′,2″,6″'-Pentaazido-6,3′,6′,2″′,5″′,3″″,4″″-hepta-O-benzyl-1,3,2′,2″′,6″″-pentadeamino paromomycin (8). A solution of potassium carbonate (24.2 g, 176 mmol) in 1:1 MeOH:H₂O (200 mL) was cooled on ice before paramomycin monosulfate (12.7 g, 20.7 mmol) was added slowly. Then Stick’s reagent (26 g, 124 mmol) was added portion-wise followed by a catalytic amount of copper sulfate. The reaction mixture was stirred at room temperature overnight during the course of which the color changed from blue to brown. After completion, the reaction mixture was filtered and the resulting filtrate concentrated under vacuum at room temperature. The remaining aqueous mixture was cooled on ice and acidified with 6 N hydrochloric acid to pH 3 and extracted with ethyl acetate three times. The extracts were concentrated under vacuum to afford the title product.
9.69 g (63%) in the form of a light yellow solid with spectral data identical to the literature values.⁸

\[ \text{4'-O-(4''',6'''-O-Benzylidene-2''',3'''-O-benzyl-\alpha-D-mannopyranosyl)-1,3,2',2''',6'''-pentaazido-6,3',6',2'',5'',3''',4'''-hepta-O-benzyl-1,3,2',2''',6'''-pentadeamino paromomycin (16\alpha)} \]

and

\[ \text{4'-O-(4''',6'''-O-Benzylidene-2''',3'''-O-benzyl-\beta-D-mannopyranosyl)-1,3,2',2''',6'''-pentaazido-6,3',6',2'',5'',3''',4'''-hepta-O-benzyl-1,3,2',2''',6'''-pentadeaminoparomomycin (16\beta).} \]

Compounds 16\alpha (76 mg), 16\beta (272 mg) were prepared from 11 (520 mg) by the general glycosylation procedure A. The glycosylation was conducted at -60 °C for 2 h and then gradually increased to room temperature before quenching by saturated aqueous NaHCO₃. The pure anomers were isolated in the form of white gums by gradient chromatography over silica gel (toluene:EtOAc 98:2 to 94:6) followed by preparative reverse phase-HPLC (Varian dynamax 250*21.4 mm C₁₈, acetonitrile:H₂O 50:50 to 100:0, 21.5 ml/min) in a α:β ratio of 1:3.6 with a total yield of 51%.

16\alpha: [\alpha]^{D}_{D} -27.3 (c 0.21, CHCl₃); ¹H NMR (600 MHz, CDCl₃) δ 7.50-7.00 (m, 50H, aromatic), 6.15 (d, J = 3.7 Hz, 1 H: H1'), 5.63 (d, J = 5.9 Hz, 1 H: H1''), 5.61 (s, 1 H: PhCH), 5.23 (s, 1H: H1''''), 4.30-4.94 (m, 16 H: PhCH₂), 4.86 (s, 1 H: H1'''), 4.21-4.27 (m, 4 H: H4'', H4'''', H3'', PhCH₂), 4.08-4.11 (m, 2 H: H6'''', PhCH₂), 4.05-4.10 (d, J = 9.9 Hz, 1 H: H5'), 3.97 (t, J = 9.2 Hz, 1 H: H3'), 3.90-3.94 (m, 3
H: H2”, H3””, H5), 3.84-3.87 (m, 1 H: H5””), 3.78 (t, J = 9.9 Hz, 1 H: H6””), 3.74-3.80 (m, 6 H: H5”, H6’, H6”, H5””, H3”), 3.66-3.70 (m, 2 H: H4’, H4), 3.62 (dd, J = 8.4 Hz, 12.9 Hz, 1 H: H6”), 3.55 (dd, J = 3.3 Hz, 10.3 Hz, 1 H: H5”), 3.39-3.50 (m, 2 H: H3, H1), 3.33 (s, 1 H: H2”), 3.22 (t, J = 9.5 Hz, 1 H: H6), 3.11 (s, 1 H: H4””), 2.93 (dd, J = 3.7 Hz, 10.3 Hz, 1 H: H2”), 2.90 (d, J = 4.0 Hz, 12.8 Hz, 1 H: H6”), 2.20-2.24 (dt, J = 4.8 Hz, 13.2 Hz, 1 H: H2_eq), 1.34 (m, 1 H: H2_ax). 

$^{13}$C NMR (125 MHz, CDCl$_3$) δ 106.24 (C1”), 101.40 (PhCH), 101.29 (C1””), 98.64 (C1”), 95.84 (C1”), 84.14 (C6), 82.28(C5), 82.05 (C4”), 81.96 (C2”), 79.94 (C3’), 78.99 (C4’”), 77.96 (C2””), 77.76 (C4”), 76.27 (C3””), 75.51 (C3”), 75.08 (C4), 74.31 (C5””), 72.89 (C3””), 71.48 (C4’”), 70.61 (C5’), 70.16 (C5”), 69.25 (C6’), 68.68 (C6’’), 65.32 (C5””), 62.86 (C2’), 60.45 (C1), 60.27 (C3), 57.29 (C2”), 51.07 (C6””), 32.12 (C2); PhCH$_2$ (9C: 74.88, 74.82, 74.11, 73.93, 73.36, 73.25, 73.16, 72.40, 71.73). HRESIMS calcd for C$_{99}$H$_{103}$N$_{15}$O$_{19}$Na [M+Na]$^+$ 1828.7452; found, 1828.7511.

16β: [a]$^{RT}_D$ +63.0 (c 0.33, CHCl$_3$); $^1$H NMR (600 MHz, CDCl$_3$) δ 7.70-7.00 (m, 50H, aromatic), 6.20 (d, J = 4.0 Hz, 1 H: H1’), 5.70 (d, J = 5.9 Hz, 1 H: H1”), 5.54 (s, 1 H: PhCH), 4.39-5.19 (m, 16 H: PhCH$_2$), 4.95 (s, 1 H: H1””), 4.45 (m, 1H: H1””), 4.32-4.35 (m, 2 H: H4”, PhCH$_2$ ), 4.27-4.29 (d, J = 12.1 Hz, PhCH$_2$), 4.26 (br s, 1 H, H3”), 4.17-4.20 (m, J = 9.9 Hz, 1 H: H5’), 4.03 - 4.14 (m, 3 H: H4’”, H6’”, H3’), 3.94-4.03 (m, 3 H: H4’, H2”, H5), 3.71 - 3.81 (m, 6 H: H5””, H2’”, H5”, H6’”).
H3”, H4, H5” ), 3.67-3.70 (d, J = 11.3 Hz, 1H: H6’), 3.58-3.63 (m, 2 H: H5”, H6”), 3.50-3.55 (dt, J = 3.8 Hz, 10.3 Hz, 1H: H3), 3.42-3.49 (m, 2 H: H6””, H1), 3.39 (dd, J = 0.7 Hz, 9.9 Hz, 1H: H3”), 3.36 (br s, 1H: H2”), 3.29-3.34 (t, J = 9.2 Hz, 1H: H6”), 3.24-3.28 (dd, J = 4.0 Hz, 10.3 Hz, 1H: H2’), 3.15 (br s, 1H: H4”), 3.07-3.11 (dt, J = 4.8 Hz, 9.6 Hz, 1H: H5””), 2.92-2.95 (dd, J = 4.4 Hz, 13.2 Hz, 1H: H6””), 2.26 (dt, J = 4.5 Hz, 8.6 Hz, 1H: H2_eq), 1.41 (m, 1H: H2_ax).

13C NMR (151 MHz, CDCl3) δ 106.17 (C1”), 101.71 (C1””), 101.33 (PhCH), 98.71 (C1”), 95.81 (C1’), 84.14 (C6), 82.35 (C4”), 82.10 (C5), 81.86 (C2”), 78.53 (C3”), 77.92 (C3””), 77.49 (C4”), 76.70 (C2””), 75.63 (C3”), 74.99 (C4), 73.74 (C5”), 73.48 (C3”), 71.46 (C4”), 70.66 (C5”), 70.03 (C5”), 68.61 (C6””), 68.49 (6”), 67.38 (C5””), 62.60 (C2’), 60.39 (C1), 60.31 (C3), 57.34 (C2”), 51.00 (C6”), 32.72 (C2); PhCH2 (9C: 75.10, 75.05, 74.98, 74.21, 73.30, 72.94, 72.42, 72.25, 71.76). HRESIMS calcd for C99H103N15O19Na [M+Na]⁺ 1828.7452; found, 1828.7444.

4’-O-(2””,3””,4””,6””-Tetra-O-benzyl-α-D-glucopyranosyl)-1,3,2’, 2””,6””-pentaaazido-6,3’,6’,2”,5”,3””,4””-hepta-O-benzyl-1,3,2’,2””,6””-pentadeamino paromomycin (17α) and 4’-O-(2””,3””,4””,6””-Tetra-O-benzyl-β-D-glucopyranosyl)-1,3,2’, 2””,6””-pentaaazido-6,3’,6’,2”,5”,3””, 4””-hepta-O-benzyl-1,3,2’,2””,6””-pentadeamino paromomycin (17β). Compounds 17α (221 mg), 17β (69 mg) were prepared from 11 (500 mg) by the
general glycosylation procedure A, which was conducted at -60 °C for 1 h and then at -50 °C for 1 h before quenching with saturated aqueous NaHCO₃. The pure anomers were isolated in the form of white gums by chromatography over silica gel using a gradient eluate toluene:EtOAc 98:2 to 95:5, followed by preparative HPLC over silica gel (Agilent dynamax 250*21.4 mm SI, Hexanes: EtOAc 90:10, 21.5 ml/min) in a α:β ratio of 3.2:1 and a combined yield of 42%.

17α: [α]RT₀ +71.7 (c 4.20, CHCl₃); ¹H NMR (600 MHz, CDCl₃) δ 7.50-7.00 (m, 55H, aromatic), 6.11 (d, J = 4.03 Hz, 1 H: H1'), 5.64 (d, J = 5.5 Hz, 1 H: H1''), 5.44 (d, J = 2.94 Hz, 1 H: H1''''), 4.95 (m, 22 H: PhCH₂), 4.56 (s, 1 H, H1''''), 4.30 (s, 1 H: H4'''), 4.29 (s, 1 H: H3''), 4.22 (d, J = 9.9 Hz, 1 H: H5'), 4.12 (t, J = 9.35 Hz, 1 H: H3'''), 4.00-3.90 (m, 5 H: H2'', H3'', H6', H4', H5), 3.83 (d, J = 10.64, 1 H: H5'''), 3.81-3.75 (m, 4 H: H6', H5'', H5''', H3'''), 3.69 (t, J = 9.17 Hz, 1 H: H4), 3.66-3.60 (m, 2 H: H6'', H4'''), 3.55 - 3.60 (m, 2 H: H5'', H6'''), 3.40 - 3.51 (m, 4 H: H2''', H6''', H3, H1), 3.36 (s, 1 H: H2'''), 3.25 (t, J = 9.35 Hz, 1 H: H6), 3.16 (s, 1 H: H4''''), 3.11 (dd, J = 3.9 Hz, 9.8 Hz, 1 H: H2'), 2.92 (dd, J = 4.2 Hz, 12.8 Hz, 1 H: H6'''), 2.24 (dt, J = 4.5 Hz, 8.6 Hz, 1 H: H2eq), 1.31-1.41 (m, 1 H: H2ax). ¹³C NMR (151 MHz, CDCl₃) δ 106.27 (C1'), 98.57 (C1'''), 97.47 (C1''''), 95.99 (C1), 83.95 (C6), 82.17 (C2''), 81.91 (C4''), 81.89 (C3'''), 81.86 (C5), 79.80 (C3), 79.59 (C2'''), 77.61 (C4'''), 75.53 (C3''), 75.44 (C4), 74.26 (C4'), 73.67 (C5''), 71.49 (C5), 71.10 (C5'''), 70.88 (C3'''), 70.15 (C6'), 69.34 (C5''), 68.41(C6'''), 62.78
(C2'), 60.38 (C1), 59.90 (C3), 57.33 (C2''), 51.03 (C6'''), 32.38 (C2); PhCH₂ (11C: 75.31, 75.00, 74.90, 73.39, 73.23, 73.14, 73.07, 72.91, 72.85, 72.40, 71.74). HRESIMS calcd for C₁₀₆H₁₁₁N₁₅O₁₉Na [M+Na]⁺, 1920.8078; found, 1920.8175.

17β: [α]ⁿD +60.3 (c 0.80, CHCl₃); ¹H NMR (600 MHz, CDCl₃) δ 7.70-7.00 (m, 55H, aromatic), 6.09 (d, J = 4.03 Hz, 1 H: H1'), 5.62 (d, J = 5.50 Hz, 1 H: H1''), 5.13-4.22 (m, 22 H: PhCH₂), 4.82 (s, 1 H: H1'''), 4.38 (m, 1H: H1''''), 4.24 (s, 1 H: H4''), 4.20 (s, 1 H, H3''), 4.13 (m, J = 9.9 Hz, 1 H: H5'), 3.97-3.12 (m, 2 H: PhCH₂, 2 H', H5), 3.65 (t, J = 11.2 Hz, 1H: H4), 3.61 (t, J = 9.35 Hz, 1 H: H4'''), 3.49-3.34 (m, 3 H: H3, H2''', H1), 3.34 (s, 1 H: H2''), 2.86-2.92 (dd, J = 4.4 Hz, 13.2 Hz, 1 H: H6''), 2.18 (dt, J = 4.5 Hz, 8.6 Hz, 1 H: H2eq), 1.27-1.34 (m, 1 H: H₂ax). ¹³C NMR (600 MHz, CDCl₃) δ 105.99 (C1''), 102.87 (C1'''), 98.66 (C1'''), 95.84 (C1'), 84.71 (C3'''), 84.05 (C6), 82.61 (C2''), 82.23 (C4''), 81.97 (C2''), 81.70 (C5), 77.99 (C4'''), 77.50 (C3'), 77.21 (C4), 75.59 (C3'''), 75.17 (C5'''), 74.72 (C4), 74.09 (C5'''), 73.08 (C3'''), 71.71 (C5'), 69.86 (C6'''), 69.00 (C5'), 67.96 (C6'), 62.62 (C2'), 60.34 (C1), 60.19 (C3), 57.30 (C2'''), 50.89 (C6'''), 32.64 (C2); PhCH₂ (11C: 75.55, 75.10, 75.01, 74.90, 74.83, 73.23, 73.21, 72.90, 72.35, 71.41, 70.99). HRESIMS calcd for C₁₀₆H₁₁₁N₁₅O₁₉Na [M+Na]⁺, 1920.8078; found, 1920.8156.
4'-O-(2'''',3'''',4'''',6'''')-Tetra-O-benzyl-α-D-galactopyranosyl)-1,3,2',
2'''',6''''-pentaazido-6,3',6',2''',5'''',3'''',4''''-hepta-O-benzyl-1,3,2',2'''',6''''-pentadeamino paromomycin (18α) and 4'-O-(2'''',3'''',4'''',6'''')-Tetra-O-
benzyl-β-D-galactopyranosyl)-1,3,2',2'''',6''''-pentaazido-6,3',6',2''',5'''',3'''',4''''-hepta-O-benzyl-1,3,2',2'''',6''''-pentadeamino paromomycin (18β).

Compounds 18α (358 mg), 18β (56 mg) were prepared from 11 (500 mg) by the
general glycosylation procedure A, which was conducted at -72 °C for 2 h before
quenching with saturated aqueous NaHCO₃. The pure anomers were isolated as
white gums by gradient chromatography over silica gel (toluene:EtOAc 98:2 to
94:6). The two anomers were obtained as white gums with a α:β ratio of 6.4: 1
with a total yield of 60%.

18α: [α]RTD +82.97 (c 6.33, CHCl₃); ¹H NMR (600 MHz, CDCl₃) δ 7.50-7.00 (m, 55H, aromatic), 6.12 (d, J = 4.03 Hz, 1 H: H1'), 5.66 (d, J = 5.50 Hz, 1 H: H1''),
5.54 (d, J = 3.67 Hz, 1 H: H1''''), 4.91-4.34 (m, 21 H: PhCH₂), 4.88-4.91 (br s, 1 H, H1'''), 4.31-4.30 (m, 3 H: H4'', H3'', PhCH₂), 4.22-4.27 (dd, J = 3.3 Hz, 9.9 Hz, 1 H: H5''), 4.11 (t, J = 10.27 Hz, 1 H: H3''), 4.02-4.04 (dd, J = 3.45 Hz, 10.30 Hz, 1 H: H2'''), 4.01-3.98 (m, 2 H: H5''', H2''), 3.96-3.98 (br s, 1 H: H4'''), 3.85-3.96 (m, 4 H: H3'''', H5, H6', H4'), 3.76-3.84 (m, 4 H: H6', H5'', H3'''', H5''''), 3.73 (t, J = 9.35 Hz, 1 H: H4), 3.65 (dd, J = 8.44 Hz, 12.84Hz, 1 H: H6'''), 3.57-3.62 (dd, J = 3.11Hz, 10.27 Hz, 1 H: H5''), 3.50-3.55 (m, 2 H: H6'''), ) 3.41-3.50 (m, 2 H: H3,
H1), 3.36-3.41 (br s, 1 H: H2''), 3.27 (t, J = 9.35 Hz, 1 H: H6), 3.14-3.18 (s, 1 H: H4''), 3.09 (dd, J = 3.67 Hz, 10.27 Hz, 1 H: H2'), 2.95 (dd, J = 4.2 Hz, 13.02 Hz, 1 H: H6''), 2.24 (dt, J = 4.5 Hz, 8.6 Hz, 1 H: H2eq), 1.33-1.39 (m, 1 H: H2ax). ^13^C NMR (151 MHz, CDCl3) δ 120-144 (55C: aromatic), 106.27 (C1''), 98.63 (C1''), 98.22 (C1'''), 95.91 (C1'), 84.01 (C6), 82.24 (C2''), 81.96 (C4''), 81.94 (C3''), 79.94 (C3'), 78.87 (C5), 75.97 (C2'''),75.53 (C3''), 75.28 (C4), 75.09 (C4), 75.07 (C4'''), 74.28 (C5''''), 72.98 (C3'''), 71.57 (C4'''), 70.82 (C5''), 70.16 (C5'''), 70.14 (C5''''), 69.73 (C6'), 69.08 (C6''''), 62.92 (C2'), 60.42 (C1), 60.00 (C3), 57.38 (C2''), 51.05 (C6''), 32.46 (C2); PhCH2 (11C: 74.94, 74.72, 73.80, 73.38, 73.30, 73.28, 73.19, 72.93, 72.89, 72.43, 71.79). HRESIMS calcd for C106H111N15O19Na [M+Na]^+ , 1920.8078; found, 1920.8009.

18β: [α]^RT_D +42.37 (c 3.25, CHCl3); ^1^H NMR (600 MHz, CDCl3) δ 7.50-7.00 (m, 55H, aromatic), 6.12 (d, J = 3.7 Hz, 1 H: H1'), 5.65 (d, J = 5.9 Hz, 1 H: H1''), 5.14-4.23 (m, 22 H: PhCH2), 4.81 (s, 1 H: H1'''), 4.38 (d, J = 7.0 Hz, 1H: H1'''''), 4.20-4.27 (m, 3 H: H4'', H5', H3''), 4.03-4.07 (t, J = 9.2 Hz, 1 H: H3'), 3.99-4.02 (t, J = 9.2 Hz, 1 H: H4'), 3.90 - 3.98 (m, 4 H: H6', H2'', H5, H5''''), 3.82 (dd, J = 9.9, 7.7 Hz, 1 H: H2'''''), 3.66 - 3.79 (m, 5 H: H4, H5'', H3'''', H6', H5'''''), 3.52-3.58 (m, 3 H: H6'', H5'', H6'''''), 3.42-3.50 (m, 2 H: H3, H1), 3.40 (dd, J = 3.0 Hz, 9.9 Hz, 1 H: H3'''''), 3.33-3.37 (m, 2 H: H4'''', H6''''), 3.32 (br s, 1 H: H2'''), 3.24-3.27 (t, J = 9.5 Hz, 1 H: H6), 3.18-3.23 (dd, J = 4.8 Hz, 12.8 Hz, 1H: H2'), 3.10-3.15 (br s, 1H:
H4''), 2.93 (dd, J = 4.8 Hz, 12.8 Hz, 1 H: H6''), 2.24 (dt, J = 4.5 Hz, 8.6 Hz, 1 H: H2eq), 1.30-1.40 (m, 1 H: H2ax). $^{13}$C NMR (151 MHz, CDCl$_3$) δ 120-144 (55C: aromatic), 106.04 (C1''), 103.08 (C1''), 98.68 (C1'''), 95.78 (C1'), 84.06 (C6), 82.29 (C5), 82.26 (C3'''), 82.00 (C4'''), 81.76 (C2''), 79.95 (C2'''), 78.09 (C3'), 76.65 (C4'), 75.63 (C3''), 74.75 (C4), 74.10 (C5''), 73.14 (C4'''), 72.99 (C3'''), 71.07 (C5'), 69.92 (C5''), 68.13 (C6''), 68.06 (C6'), 62.74 (C2'), 60.20 (C1), 60.03 (C3), 57.34 (C2'''), 50.91 (C6'''), 32.65 (C2), PhCH$_2$ (11C: 75.35, 75.27, 75.01, 74.80, 73.37, 72.91, 72.69, 72.39, 71.76).

HRESIMS calcd for C$_{106}$H$_{111}$N$_{15}$O$_{19}$Na [M+Na]$^+$, 1920.8078; found, 1920.8101.

4'-O-(2''',3''',6'''-Tri-O-benzyl-4'''-azido-4'''-deoxy-α-D-glucopyranosyl)-1,3,2',2''',6''-pentaazido-6,3',6',2'',5'',3''',4'''-hepta-O-benzyl-1,3,2',2''',6'''-pentadeamino paromomycin (19α) and 4'-O-(2''',3''',6'''-Tri-O-benzyl-4'''-azido-4'''-deoxy-β-D-glucopyranosyl)-1,3,2',2''',6''-pentaazido-6,3',6',2'',5'',3''',4'''-hepta-O-benzyl-1,3,2',2''',6'''-pentadeamino paromomycin (19β). Compounds 19α (272 mg), 19β (61 mg) were prepared from 11 (500 mg) by the general glycosylation procedure A, conducted at -60 °C for 1 h and then -50 °C for 1 h before quenching by saturated aqueous NaHCO$_3$. The two anomers were isolated as white gums by gradient chromatography over silica gel (toluene:EtOAc 98:2 to 94:6) followed by preparative reverse phase HPLC ((Varian dynamax 250*21.4 mm C$_{18}$, acetonitrile:H$_2$O 70:30 to 100:0, 21.5
ml/min)) in a α:β ratio of 4.5:1 with a total yield of 50%.

19α: [α]D +93.21 (c 3.73, CHCl₃); ¹H NMR (600 MHz, CDCl₃) δ 7.50-7.00 (m, 50H, aromatic), 6.10 (d, J = 4.0 Hz, 1 H: H1'), 5.61 (d, J = 5.5 Hz, 1 H: H1'''), 5.41 (d, J = 3.7 Hz, 1 H: H1''''), 4.94-4.30 (m, 19 H: PhCH₂), 4.85 (s, 1 H, H1'''), 4.24-4.29 (m, 3 H: H4'', H3'', PhCH₂), 4.14-4.20 (d, J = 9.9 Hz, 1 H: H5'), 4.07-4.13 (t, J = 9.2 Hz, 1 H: H3'), 3.94-3.96 (t, J = 5.1 Hz, 1 H: H2''), 3.90-3.93 (m, 3 H: H4', H5, H6'), 3.82 (t, J = 9.4 Hz, 1 H: H3''''), 3.74-3.78 (m, 3 H: H5'', H3'''', H5''''), 3.73 (t, J = 8.4 Hz, 1 H: H6'), 3.67 (t, J = 9.5 Hz, 1 H: H4), 3.59-3.63 (m, 3 H: H6'', H4'''', H5'''''), 3.55 (dd, J = 3.3 Hz, 10.3 Hz, 1 H: H5'''), 3.39-3.49 (M, 5 H: H3, H6''''', H2'''', H6''''', H1), 3.32-3.36 (br s, 1 H: H2''''), 3.24 (t, J = 9.4 Hz, 1 H: H6), 3.11-3.15 (br s, 1 H: H4''''), 3.08 (dd, J = 10.1, 3.9 Hz, 1 H: H2'), 2.91 (dd, J = 13.0, 4.2 Hz, 1 H: H6''''), 2.24 (dt, J = 4.5 Hz, 8.6 Hz, 1 H: H2ax), 1.30-1.39 (m, 1 H: H2ax).

¹³C NMR (151 MHz, CDCl₃) δ 120-144 (55C: aromatic), 106.30 (C1''), 98.57 (C1'''), 97.26 (C1'''''), 95.96 (C1), 81.89 (C5), 79.76 (C3'''), 79.69 (C3'), 79.42 (C2'''), 77.21 (C6), 76.99 (C2''), 76.78 (C4''), 75.53 (C3'''), 75.36 (C4), 74.25 (C4''), 72.39 (C3'''), 71.52 (C4'''), 70.84 (C5'), 70.19 (C4''''), 70.14 (C5''), 69.20 (C6'), 68.51 (C6'''), 62.66 (C2'), 61.60 (C4''''), 60.38 (C1), 60.03 (C3), 57.32 (C2'''), 51.04 (C6'''), 32.45 (C2'); PhCH₂ (10C: 75.44, 75.01, 74.61, 73.39, 73.21, 73.17, 73.11, 72.93, 72.75, 71.75). HRESIMS calcd for C₉₉H₁₀₄N₁₈O₁₈Na [M+Na]+, 1855.7674; found, 1855.7692.
$19\beta$: $[\alpha]_{D}^{RT} +78.7$ (c 1.87, CHCl$_3$); $^1$H NMR (600 MHz, CDCl$_3$) δ 7.50-7.00 (m, 50H, aromatic), 6.12 (d, $J = 4.0$ Hz, 1 H: H1'), 5.63 (d, $J = 5.9$ Hz, 1 H: H1''), 5.09-4.29 (m, 21 H: PhCH$_2$), 4.80 (s, 1 H: H1''), 4.32 (d, $J = 7.7$ Hz, 1H: H1'''), 4.24-4.26 (m, 2 H: H4'', PhCH$_2$), 4.20 (m, H3''), 4.18 (d, $J = 9.5$ Hz, J 1 H: H5'), 4.01 (m, 2 H: H3', H4''), 3.88-3.96 (m, 3 H: H6', H2'', H5), 3.68-3.76 (m, 4 H: H5'', H3'', H5'', H6''), 3.65-3.67 (m, 2 H: H4, H6''), 3.60 (t, $J = 9.9$ Hz, 1 H: H4'''), 3.52-3.59 (m, 2 H: H6'', H5''), 3.48 (dd, $J = 4.4$ Hz, 11.0 Hz, 1 H: H6'''), 3.39-3.43 (m, 2 H: H1, H3), 3.37 (t, $J = 8.4$ Hz, 1 H: H2'''), 3.31 (br s, 1 H: H2''), 3.27 (t, $J = 9.2$ Hz, 1 H: H3''''), 3.24 (t, $J = 9.5$ Hz, 1 H: H6), 3.13 (dd, $J = 4.0$ Hz, 9.9 Hz), 3.11 (br s, 1 H: H4''), 3.02 (ddd, $J = 1.5$ Hz, 4.0 Hz, 6.2 Hz, 1 H: H5'''), 2.91 (dd, $J = 4.4$ Hz, 12.8 Hz, 1 H: H6'''), 2.24 (dt, $J = 4.5$ Hz, 8.6 Hz, 1 H: H2$_{eq}$), 1.29-1.36 (m, 1 H: H2$_{ax}$). $^{13}$C NMR (151 MHz, CDCl$_3$) δ 120-144 (50C: aromatic), 106.04 (C1''), 102.71 (C1''), 98.66 (C1'''), 95.89 (C1), 84.05 (C6), 82.77 (C3'''), 82.26 (C2'', C2''''), 81.99 (C4''), 81.73 (C5), 77.79 (C3'), 77.02 (C4'), 76.77 (C3''), 74.82 (C4), 74.11 (C5'''), 74.07 (C5'''), 72.91 (C3'''), 71.43 (C4'''), 70.90 (C5'), 69.89 (C5'''), 69.16 (C6'''), 67.88 (C6'), 62.64 (C2'), 62.08 (C4'''), 60.35 (C1), 60.19 (C3), 57.31 (C2''''), 50.92 (C6'''), 32.64 (C2), PhCH$_2$ (10C: 76.77, , 75.19, 75.03, 74.87, 73.31, 73.24, 73.21, 72.91, 72.37, 71.73). HRESIMS calcd for C$_{99}$H$_{104}$N$_{18}$O$_{18}$Na $[M+Na]^+$, 1855.7674; found, 1855.7615.

4'-O-(4''',6'''-O-Benzylidene-2'''',3''''-O-benzyl-α-D-mannopyranosyl)-6,
3′,6′,2′′,5′′,3′′′,4′′′-hepta-O-benzyl-1,3,2′,2′′′,6′′′-pentadeamino paromomycin (20α). Compound 20α (17 mg, 91%) was obtained in the form of a white gum from 16α (20 mg) by Staudinger reaction general procedure B after silica gel chromatography (NH₄OH: MeOH 0: 100 to 1:99). [α]ᵣᵣᵣᵣ₊10.2 (c 0.93, MeOH); HRESIMS calcd for C₉₉H₁₁₄N₅O₁₉[M+H]⁺, 1676.8108; found, 1676.8175. This compound was taken forward to the next step without further characterization.

4′-O-(4′′′,6′′′-O-Benzylidene-2′′′,3′′′-O-benzyl-β-D-mannopyranosyl)-6,3′,6′,2′′,5′′,3′′′,4′′′-hepta-O-benzyl-1,3,2′,2′′′,6′′′-pentadeamino paromomycin (20β). Compound 20β (68 mg, 66%) was obtained in the form of a white gum under the Staudinger reaction general procedure B of 110 mg 16β (110 mg) after silica gel chromatography (NH₄OH: MeOH 0: 100 to 1:99). [α]ᵣᵣᵣᵣ₋₅.₅ (c 1.75, MeOH); HR ESI MS calcd for C₉₉H₁₁₄N₅O₁₉[M+H]⁺, 1676.8108; found, 1676.8066. This compound was taken forward to the next step without further characterization.

4′-O-(2′′′,3′′′,4′′′,6′′′-Tetra-O-benzyl-α-D-glucopyranosyl)-6,3′,6′,2′′,5′′,3′′′,4′′′-hepta-O-benzyl-1,3,2′,2′′′,6′′′-pentadeamino paromomycin (21α). Compound 21α (35 mg, 64%) was obtained as a white gum under the Staudinger reaction general procedure B of 17α (60 mg) after silica gel chromatography (NH₄OH: MeOH 0: 100 to 1:99). [α]ᵣᵣᵣᵣ₊₂₇.₅₀ (c 2.00,
MeOH); HRESIMS calcd for C_{106}H_{122}N_{5}O_{19} [M+H]^+, 1768.8734; found, 1768.8747. This compound was taken forward to the next step without further characterization.

4'-O-(2'''',3'''',4'''',6''''-Tetra-O-benzyl-β-D-glucopyranosyl)-6,3',6',2'',5'',3'''',4''''-hepta-O-benzyl-1,3,2',2'',6''''-pentadeamino paromomycin (21β). Compound 21β (17 mg, 55%) was obtained as a white gum under the Staudinger reaction general procedure B of 17β (33 mg) after silica gel chromatography (NH₄OH: MeOH 0: 100 to 1:99). [α]_{D}^{RT} -2.1 (c 0.52, MeOH); HRESIMS calcd for C_{106}H_{122}N_{5}O_{19} [M+H]^+, 1768.8734; found, 1768.8700. This compound was taken forward to the next step without further characterization.

4'-O-(2'''',3'''',4'''',6''''-Tetra-O-benzyl-α-D-galactopyranosyl)-6,3',6',2'',5'',3'''',4''''-hepta-O-benzyl-1,3,2',2'',6''''-pentadeamino paromomycin (21α). Compound 21α (83 mg, 81%) was obtained as a white gum under the Staudinger reaction general procedure C of 110 mg 17α (110 mg) after silica gel chromatography (NH₄OH: MeOH 0: 100 to 1:99). [α]_{D}^{RT} +42.7 (c 6.67, MeOH); HRESIMS calcd for C_{106}H_{122}N_{5}O_{19} [M+H]^+, 1768.8734; found, 1768.8707. This compound was taken forward to the next step without further characterization.

4'-O-(2'''',3'''',4'''',6''''-Tetra-O-benzyl-β-D-galactopyranosyl)-6,3',6',2'',5'',3'''',4''''-hepta-O-benzyl-1,3,2',2'',6'''-
**pentadeaminoparomomycin (22β).** Compound 22β (24 mg, 78%) was obtained as a white gum under the Staudinger reaction general procedure B of 18β (33 mg) after silica gel chromatography (NH₄OH: MeOH 0: 100 to 1:99). [α]_{D}^{RT} -5.2 (c 0.56, MeOH); HRESIMS calcd for C_{106}H_{122}N_{5}O_{19} [M+H]^+, 1768.8734; found, 1768.8732. This compound was taken forward to the next step without further characterization.

4′-O-(2′′′,3′′′,6′′′-Tri-O-benzyl-4′′′′-amino-4′′′′-deoxy-α-D-glucopyranosyl)-6,3′,6′,2′′,5′′,3′′′,4′′′-hepta-O-benzyl-1,3,2′,2′′′,6′′′-pentadeamino paromomycin (23α). Compound 23α (23 mg, 64%) was obtained as a white gum under the Staudinger reaction general procedure B of 40 mg 19α (40 mg) after silica gel chromatography (NH₄OH: MeOH 0: 100 to 1:99). [α]_{D}^{RT} +23.0 (c 0.80, MeOH); HRESIMS calcd for C_{99}H_{117}N_{6}O_{18} [M+H]^+, 1677.8424; found, 1677.8425. This compound was taken forward to the next step without further characterization.

4′-O-(2′′′′,3′′′′,6′′′′-Tri-O-benzyl-4′′′′-amino-4′′′′-deoxy-β-D-glucopyranosyl)-6,3′,6′,2′′,5′′,3′′′,4′′′-hepta-O-benzyl-1,3,2′,2′′′,6′′′-pentadeamino paromomycin (23β). Compound 23β (18 mg, 48%) was obtained as a white gum under the Staudinger reaction general procedure B of 19β (28 mg) after silica gel chromatography (NH₄OH: MeOH 0: 100 to 1:99). [α]_{D}^{RT} +5.9 (c 1.20, CHCl₃); HRESIMS calcd for C_{99}H_{117}N_{6}O_{18} [M+H]^+, 1677.8424;
found, 1677.8474. This compound was taken forward to the next step without further characterization.

4'-O-α-D-Mannopyranosyl paramomycin (24α). Compound 24α (4 mg, 33%) was obtained in the form of a white solid by hydrogenolysis general procedure C of 20α (17 mg) after Sephadex chromatography. [α]^{RT}_D +55.0 (c 0.18, H₂O), \(^1\)H NMR (600 MHz, CDCl₃) \(\delta\) 5.59 (d, \(J = 4.1\) Hz, 1 H: H1'), 5.21 (br s, 1 H: H1''), 5.12 (s, 1 H: H2''), 4.15 (s, 1 H: H5''), 4.06 (s, 1 H: H4''), 4.04 (s, 1 H: H4''), 3.92 (t, \(J = 8.8\) Hz, 1 H: H3''), 3.88 (s, 1 H: H2'''), 3.63-3.79 (m, 7 H: H6''', H5'', H5', H6', H5, H6', H4''', H4), 3.54-3.63 (m, 4 H: H3''', H6''', H5'', H4''), 3.43-3.52 (m, 3 H: H5''', H4''', H6), 3.41 (s, 1 H: H2'''), 3.17-3.28 (m, 3 H: H6'''', H6''', H2'), 3.09-3.15 (m, 2 H: H1, H3), 2.17 (m, 1 H: H2eq), 1.50 (m, 1 H: H2ax). \(^{13}\)C NMR (151 MHz, CDCl₃) \(\delta\) 109.98 (C1''), 101.49 (C1'''), 95.64 (C1'), 95.27 (C1'''), 84.63 (C5), 81.01 (C4''), 79.02 (C4), 75.54 (C4'), 74.93 (C3''), 73.82 (C5'''), 73.27 (C6), 72.79 (C2''), 72.48 (C5'), 70.19 (C3'''), 70.16 (C5'''), 69.98 (C2''''), 69.41 (C3'), 67.62 (C3''''), 67.20 (C4'''), 66.36 (C4'''), 60.80 (C6''''), 60.03 (C6'), 59.94 (C5'''), 53.84 (C2'), 50.78 (C2'''), 50.00 (C1), 48.80 (C3), 40.29 (C6''''), 30.02 (C2), 22.95 (CH₃). HRESIMS calcd for C₂₉H₅₆N₅O₁₉[M+H]⁺, 778.3570; found, 778.3600.

4'-O-β-D-Mannopyranosyl paramomycin (24β). Compound 24β (11 mg, 33%) was obtained in the form of a white solid by hydrogenolysis general procedure C of 20β (68 mg) after Sephadex chromatography. [α]^{RT}_D +31.9 (c 0.54, H₂O), \(^1\)H
NMR (600 MHz, CDCl$_3$) δ 5.57 (d, $J = 4.0$ Hz, 1 H: H1''), 5.20 (br s, 1 H: H1'''), 5.11 (s, 1 H: H1''''), 4.57 (s, 1 H: H1'''''), 4.35 (t, $J = 5.5$ Hz, 1 H: H3'''), 4.20 (s, 1 H: H2''), 4.12 (s, 1 H: H5''), 4.04 (s, 1 H: H3''''), 4.02 (s, 1 H: H4''), 3.87 (m, 2 H: H3'', H2''''), 3.72-3.78 (m, 3 H: H6'', H5', H5''), 3.57-3.70 (m, 7 H: H6', H6', H5, H4'', H4, H5'', H4'), 3.52-3.55 (dd, $J = 6.6$ Hz, 12.1 Hz, 1 H: H6''''), 3.43-3.47 (m, 4 H: H6'', H5'', H6'', H2'), 3.07-3.11 (m, 2 H: H1, H3), 2.15 (m, 1 H: H2$_{eq}$), 1.49 (m, 1 H: H2$_{ax}$). $^{13}$C NMR (151 MHz, CDCl$_3$) δ 109.87 (C1''), 99.96 (C1'''), 95.61 (C1'), 95.35 (C1''), 84.64 (C5), 81.07 (C4''), 79.22 (C4), 77.79 (C4''), 76.39 (C5''''), 74.98 (C3''), 73.28 (C2''), 72.86 (C3'''), 73.63 (C6), 72.01 (C5'), 70.38 (C2'''), 70.15 (C5'''), 67.99 (C3), 67.62 (C3''), 67.19 (C4''), 66.52 (C4'''), 60.84 (C6''''), 59.91 (C5'''), 59.56 (C6'), 53.61 (C2'), 50.76 (C2'''), 50.01 (C1), 48.83 (C3), 40.29 (C6'''), 30.01 (C2), 23.00 (CH$_3$). HRESIMS calcd for C$_{29}$H$_{56}$N$_5$O$_{19}$ [M+H]$^+$, 778.3570; found, 778.3561.

4'-O-α-D-Glucopyranosyl paramomycin (25α). Compound 25α (29 mg, 39%) was obtained as a white solid by hydrogenolysis general producedure C of 21α (120 mg) after Sephadex chromatography. [α]$^\text{RTD}_{D}$ +73.2 (c 0.50, H$_2$O); $^1$H NMR (600 MHz, CDCl$_3$) δ 5.59 (d, $J = 4.0$ Hz, 1 H: H1''), 5.21 (d, $J = 3.0$ Hz, 1H: H1'''''), 5.18 (br s, 1 H: H1'''), 5.11 (s, 1 H: H1'''''), 4.34 (t, $J = 5.1$ Hz, 1 H: H3''''), 4.20 (s, 1 H: H2'''), 4.12 (s, 1 H: H5''''), 4.00-4.05 (m, 3 H: H3'''', H3', H4'''), 3.79 (t, $J = 8.8$ Hz, 1 H: H5'), 3.63-3.76 (m, 7 H: H5'', H4, H5, H6'''', H6', H6', H4'''), 3.55-3.60 (m, 3
H: H5”, H6”", H4’), 3.46-3.51 (m, 3 H: H3””, H5””, H6), 3.41-3.44 (dd, J = 3.7 Hz, 9.9 Hz, 1 H: H2””), 3.40 (s, 1 H: H2”), 3.28 (dd, J = 4.0 Hz, 9.9 Hz, 1 H: H2’), 3.22-3.26 (m, 3 H: H4”’, H6”’, H3), 3.16 (dd, J = 4.0 Hz, 14.0 Hz, 1 H: H2’), 3.22-3.27 (m, 3 H: H3, H4””, H6”), 3.16-3.19 (dd, J = 4.0 Hz, 14.0 Hz, 1 H: H6”), 3.13 (dt, J = 3.6 Hz, 10.1 Hz, 1 H: H1), 2.23 (m, 1 H: H2_eq), 1.60 (m, 1 H: H2_ax). 13C NMR (151 MHz, CDCl3) δ 109.98 (C1”), 99.44 (C1””), 95.26 (C1’, C1”), 84.64 (C5), 81.07 (C4”), 79.22 (C4), 77.79 (C4’), 76.39 (C5””), 74.98 (C3”), 73.28 (C2”), 72.86 (C3””), 73.63 (C6), 72.01 (C5”), 70.38 (C2””), 70.15 (C5””), 67.99 (C3’), 67.62 (C3””), 67.19 (C4”), 66.52 (C4””), 60.37 (C6’), 59.93 (C6””, C5”), 53.29 (C2’), 50.74 (C2””), 49.78 (C1), 48.70 (C3), 40.29 (C6””), 28.85 (C2), 23.05 (CH3).

HRESIMS calcd for C29H56N5O19 [M+H]+, 778.3570; found, 778.3594.

4’-O-β-D-Glucopyranosyl paramomycin (25β). Compound 25β (9 mg, 87%) was obtained in the form of a white solid by hydrogenolysis general produceure C of 21β (17 mg) after Sephadex chromatography. [α]DRT +30.0 (c 0.45, H2O); 1H NMR (600 MHz, CDCl3) δ 5.59 (d, J = 3.7 Hz, 1 H: H1’), 5.19 (d, J = 2.9 Hz, 1 H: H1”), 5.09 (s, 1H: H1””), 4.34 (t, J = 6.6 Hz, 1 H: H3”), 4.31 (d, J = 8.1 Hz, 1 H: H1””), 4.19 (br s, 1 H: H2”), 4.12 (t, J = 4.4 Hz, 1 H: H5””), 4.03 (t, J = 3.3 Hz, 1 H: H3””), 4.01(m, 1 H: H4”), 3.82 (t, J = 10.6 Hz, 1 H: H3’), 3.77 (m, 1 H: H5””), 3.72-3.74 (m, 2 H: H6””, H5”), 3.68 (dd, J = 4.1 Hz, 12.4 Hz, 1 H: H6”), 3.62-3.65 (m, 2 H: H5, H4””), 3.59 (dd, J = 4.8 Hz, 12.5 Hz, 1 H: H5”), 3.52-3.57 (m, 4 H: H6’, H4’, H6’, H6), 3.43 (t, J = 10.3 Hz, 1 H: H4), 3.37 (s, 1 H: H2””), 3.31 (t, J = 9.1 Hz,
1 H: H3”), 3.27 (dd, J = 2.4 Hz, 5.8 Hz, 1 H: H5”), 3.16-3.26 (m, 4 H: H6”, H4”, H2’, H6”), 3.11 (t, J = 9.2 Hz, 1 H: H2”), 2.99-3.07 (m, 2 H: H1, H3), 2.10 (dt, J = 4.0 Hz, 12.5 Hz, 1 H: H2eq), 1.44 (m, 1 H: H2ax).

13C NMR (151 MHz, CDCl3) δ 109.80 (C1”), 102.44 (C1’”), 95.80 (C1’), 95.44 (C1’”), 84.67 (C5), 81.03 (C4”), 79.94 (C6), 77.73 (C4’), 75.90 (C5’), 75.38 (C3’”), 74.99 (C3”), 73.25 (C2’”), 73.08 (C2”), 73.04 (C4), 72.14 (C5’”), 70.19 (C5’”), 69.33 (C4’”), 68.21 (C3’), 67.71 (C3’”), 67.23 (C4’”), 60.46 (C5’), 59.96 (C6’), 59.93 (C6’”), 53.84 (C2’), 50.82 (C2’”), 50.12 (C1), 48.93 (C3), 40.30 (C6’”), 30.55 (C2), 23.12 (CH3).

HRESIMS calcd for C29H56N5O19 [M+H]+, 778.3570; found, 778.3537.

4’-O-α-D-Galactopyranosyl paramomycin (26α). Compound 26α (36 mg, 62%) was obtained as a white solid by hydrogenolysis general producedure C of 22α (100 mg) after Sephadex chromatography. [α]D +9.2 (c 0.45, H2O); 1H NMR (600 MHz, CDCl3) δ 5.59 (s, 1 H: H1’), 5.20 (s, 1H: H1’”), 5.16 (s, 1 H: H1”), 5.09 (s, 1 H: H1’”), 4.32 (br s, 1 H: H3”), 4.19 (s, 1 H: H2”), 4.10 (s, 1 H: H5’”), 3.98-4.05 (m, 3 H: H3’”, H3’, H4’”), 3.80 (t, J = 10.3 Hz, 1 H: H4), 3.73-3.79 (m, 3 H: H5’, H3’”, H5’”), 3.65-3.72 (m, 5 H: H5”, H6’”, H5, H2’”, H6’”), 3.60-3.64 (m, 2 H: H4’”, H4””), 3.51-3.59 (m, 4 H: H6’, H6’, H5”, H4’”), 3.48 (t, J = 9.5 Hz, 1 H: H6), 3.38 (s, 1 H: H2’”), 3.29-3.34 (m, 2 H: H3, H2’), 3.22 (m, 1 H: H6”), 3.11-3.18 (m, 2 H: H6””, H1), 2.24-2.28 (m, 1 H: H2eq), 1.63 (m, 1 H: H2ax). 13C NMR (151 MHz, CDCl3) δ 110.01 (C1”), 99.66 (C1’”), 95.30 (C1’’”), 95.11 (C1’), 84.19 (C5), 81.22 (C4’”), 77.43 (C4), 75.38 (C4’), 75.13 (C3’”), 73.31 (C2”), 73.20 (C5’),
72.21 (C6), 71.89 (C5''), 70.16 (C5''), 69.06 (C3'''), 68.83 (C3), 68.22 (C2'''), 67.56 (C3'''), 67.19 (C4'''), 61.15 (C6'), 60.03 (C5''), 59.97 (C6'''), 53.17 (C2'), 50.78 (C2''), 49.69 (C1), 48.68 (C3), 40.32 (C6''), 28.27 (C2), 22.59 (CH3). HRESIMS calcd for C29H56N5O19 [M+H]⁺, 778.3570; found, 778.3577.

4'-O-β-D-Galactopyranosyl paramomycin (26β). Compound 26β (5.5 mg, 39%) was obtained in the form of a white solid by hydrogenolysis general procedure C of 22β (24 mg) after Sephadex chromatography. [α]RT D +38.7 (c 0.37, H2O); ¹H NMR (600 MHz, CDCl3) δ 5.65 (s, 1 H: H1'), 5.23 (s, 1 H: H1''), 5.15 (s, 1 H: H1'''), 4.38 (s, 1 H: H3''), 4.31 (s, 1 H: H1'''), 4.23 (s, 1 H: H2''), 4.17 (s, 1 H: H5'''), 4.05-4.10 (m, 2 H: H3'', H4''), 3.95 (t, J = 10.3 Hz, 1 H: H3'), 3.86 (t, J = 9.5 Hz, 1 H: H4), 3.69-3.84 (m, 6 H: H6''', H5'', H4''', H5'', H5, H6'''), 3.68 (s, 1 H: H4'''), 3.56-3.67 (m, 4 H: H5'', H6', H5, H4', H6'), 3.50-3.57 (m, 3 H: H5', H3'''', H6), 3.45 (s, 1 H: H2'''), 3.32-3.40 (m, 3 H: H2'''', H3, H2'), 3.27-3.30 (br s, 1 H: H6'''), 3.18-3.25 (m, 2 H: H1, H6'''), 2.34 (m, 1 H: H2eq), 1.68 (m, 1 H: H2ax). ¹³C NMR (151 MHz, CDCl3) δ 109.97 (C1''), 102.82 (C1'''), 95.31 (C1'''), 95.18 (C1'), 84.24 (C5), 81.21 (C4''), 77.27 (C4), 77.19 (C4'), 75.39 (C5'), 75.06 (C3''), 73.33 (C2''), 72.94 (C5'''), 72.39 (C6), 72.22 (C3'''), 68.22 (C2'''), 70.11 (C5'''), 68.41 (C4'''), 67.58 (C3'), 67.55 (C3'''), 67.19 (C4'''), 61.06 (C6'), 60.03 (C5''), 59.39 (C6''''), 53.22 (C2'), 50.73 (C2'''), 49.63 (C1), 48.66 (C3), 40.31 (C6'''), 28.19 (C2), 21.83 (CH3). HRESIMS calcd for C29H56N5O19 [M+H]⁺, 778.3570; found, 778.3588.
4′-O-(4′′′′-Amino-4′′′′-deoxy-α-D-glucopyranosyl) paramomycin (27α).

Compound 27α (20 mg, 64%) was obtained as a white solid by hydrogenolysis general procedure C of 23α (54 mg) after Sephadex chromatography. [α]_{D}^{20} +53.8 (c 0.40, H_{2}O); \textsuperscript{1}H NMR (600 MHz, CDCl\textsubscript{3}) δ 5.63 (s, 1 H: H1'), 5.32 (s, 1 H: H1''), 5.19 (s, 1 H: H1'''), 5.11 (s, 1 H: H1''''), 4.34 (t, J = 5.5 Hz, 1 H: H3'''), 4.21 (s, 1 H: H2'''), 4.12 (br s, 1 H: H5'''), 4.01-4.07 (m, 3 H: H3'''', H3, H4''), 3.79-3.84 (m, 2 H: H5'', H4), 3.68-3.77 (m, 5 H: H5'', H4, H6''', H6, H3'''), 3.57-3.66 (m, 6 H: H5'', H6, H6'', H4', H4''), 3.49-3.54 (m, 2 H: H2'''', H6), 3.41 (s, 1 H: H2''), 3.30-3.39 (m, 2 H: H3, H2'), 3.24 (dd, J = 6.6 Hz, 13.9 Hz, 1 H: H6'''), 3.15-3.20 (m, 2 H: H1, H6'''), 3.07 (t, J = 10.6 Hz, 1 H: H4'''), 3.13 (dt, J = 3.6 Hz, 10.1 Hz, 1 H: H1), 2.29 (m, 1 H: H2_{eq}), 1.66 (m, 1 H: H2_{ax}). \textsuperscript{13}C NMR (151 MHz, CDCl\textsubscript{3}) δ 110.03 (C1'''), 99.66 (C1'''), 95.29 (C1'''), 95.25 (C1'), 84.24 (C5), 81.20 (C4''), 77.18 (C4), 75.15 (C3''), 75.13 (C4'), 73.31 (C2'''), 72.68 (C5'), 72.20 (C2''''), 71.22 (C6), 70.10 (C5'''), 69.03 (C5'''), 68.93 (C3'''), 68.81 (C3'), 67.53 (C3''), 67.18 (C4'''), 60.24 (C6'), 59.94 (C5'), 59.89 (C6'''), 53.31 (C2'), 52.08 (C4'''), 50.73 (C2''), 49.63 (C1), 48.61 (C3), 40.30 (C6'''), 28.15 (C2), 21.74 (CH\textsubscript{3}).

HRESIMS calcd for C\textsubscript{29}H\textsubscript{57}N\textsubscript{6}O\textsubscript{18} [M+H]\textsuperscript{+}, 777.3729; found, 777.3718.

4′-O-(4′′′′-Amino-4′′′′-deoxy-β-D-glucopyranosyl) paramomycin (27β).

Compound 27β (5 mg, 29%) was obtained as a white solid by hydrogenolysis general procedure C of 23β (22 mg) after Sephadex chromatography. [α]_{D}^{20} +66.86 (c 0.17, H_{2}O); \textsuperscript{1}H NMR (600 MHz, CDCl\textsubscript{3}) δ 5.66 (s, 1 H: H1'), 5.25 (s, 1
H: H1”), 5.16 (s, 1H: H1”), 4.42-4.47 (m, 2H: H1””, H3”), 4.24 (s, 1H: H2”), 4.17 (s, 1H: H5””), 4.07-4.10 (m, 2H: H3””, H4”), 3.94 (br s, 1H: H3’), 3.88 (br s, 1H: H4’), 3.73-3.84 (m, 7H: H5, H5”, H6’, H6’’, H6”), 3.69 (s, 1H: H4’’), 3.59-3.66 (m, 3H: H5’’, H4’, H5’”), 3.54-3.58 (m, 2H: H3’’, H6), 3.46 (s, 1H: H2’’), 3.40 (br s, 1H: H3) 3.35 (br s, 1H: H2’), 2.33 (m, 1H: H2eq), 1.70 (m, 1H: H2ax). ^13C NMR (151 MHz, CDCl3) δ 110.01 (C1”), 102.45 (C1””), 95.42 (C1””), 95.35 (C1’), 84.27 (C5), 81.20 (C4”), 77.50 (C4), 77.36 (C4’), 75.26 (C3”), 73.44 (C2””), 73.42 (C2”), 72.98 (C5”), 72.28 (C5’”), 72.22 (C3’”), 71.74 (C6), 70.19 (C5”), 67.59 (C3’), 67.56 (C3”), 67.28 (C4”), 60.32 (C6’), 60.03 (C5”), 59.37 (C6””), 53.38 (C2’), 52.18 (C4””), 50.84 (C2”), 49.70 (C1), 48.75 (C3), 40.40 (C6””), 28.22 (C2), 21.47 (CH3). HRESIMS calcd for C29H57N6O18 [M+H]^+ 777.3729; found, 777.3704.

(2,3,4,6-Tetra-O-(p-methyl benzyl)-α-D-glucopyranosyl) dibutyl phosphate (33).

(2,3,4,6-Tetra-O-benzylmethyl-β-D-glucopyranosyl) thioglycoside (270 mg, 1 eq), dibutyl phosphate (233 ul, 3 eq) and Acid Washed Molecular Sieves 3 Å (AW-MS
300) was stirred in anhydrous DCM at room temperature for 1.5 h and then cooled to 0 °C. 185 mg NIS was added (2.1 eq) followed by a catalytic amount of TfOH. The solution was turned to red color from brown color. It was stirred at 0 °C for 1.5 h before quenched with Hunig’s base. The molecular sieves were filtered out. The obtained filtrate was washed with sat. Na$_2$S$_2$O$_3$ three times. The organic layer was then condensed and subject to silica gel chromatography (Hexanes: EtOAc=3:1, R$_f$=0.27). 250 mg white solid product (yield: 81%) was obtained. $[$α$]^{RT}_D$ -25.3 (c 0.29, CHCl$_3$); $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.10-7.40 (m, 20 H, aromatic), 5.91 (dd, $J$ = 3.4 Hz, 7.0 Hz, 1 H: H1), 4.40-4.92 (m, 8 H: PhCH$_2$), 3.97-4.07 (m, 4 H: PO$_2$(OCH$_2$CH$_2$CH$_2$CH$_3$)$_2$), 3.94 (d, $J$ = 9.5 Hz, 1 H: H5), 3.91(t, $J$ = 9.5 Hz, 1 H: H3), 3.71-3.73 (dd, $J$ = 3.7 Hz, 11.0 Hz, 1 H: H6), 3.66-3.70 (t, $J$ = 9.8 Hz, 1 H: H4), 3.58-3.61 (m, 2 H: H2, H6'), 2.34 (m, 12 H: OBnCH$_3$), 1.55-1.66 (m, 4 H: PO$_2$(OCH$_2$CH$_2$CH$_2$CH$_3$)$_2$), 1.27-1.39 (m, 4 H: PO$_2$(OCH$_2$CH$_2$CH$_2$CH$_3$)$_2$), 0.90 (t, $J$ = 7.3 Hz, 3 H: PO$_2$(OCH$_2$CH$_2$CH$_2$CH$_3$)), 0.87 (t, $J$ = 7.6 Hz, 3 H: PO$_2$(OCH$_2$CH$_2$CH$_2$CH$_3$)); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 95.22 (C1), 81.07 (C3), 77.80 (C2), 77.27 (C4), 72.72 (C5), 67.67 (C6), 67.79 (PO$_2$(OCH$_2$CH$_2$CH$_2$CH$_3$)), 67.42 (PO$_2$(OCH$_2$CH$_2$CH$_2$CH$_3$)), 32.18 (PO$_2$(OCH$_2$CH$_2$CH$_2$CH$_3$)), 32.14 (PO$_2$(OCH$_2$CH$_2$CH$_2$CH$_3$)), 21.17 (OBnCH$_3$), 18.63 (PO$_2$(OCH$_2$CH$_2$CH$_2$CH$_3$)), 18.57 (PO$_2$(OCH$_2$CH$_2$CH$_2$CH$_3$)), 13.60 (PO$_2$(OCH$_2$CH$_2$CH$_2$CH$_3$)$_2$); PhCH$_2$ (4 C: 75.49, 75.01, 73.33, 72.36 ); HR ESI MS calcd for C$_{46}$H$_{61}$O$_9$NaP [M+Na]$^+$, 811.3951; found, 811.3979.
(2,3,4,6-Tetra-O-benzyl-α-D-mannopyranosyl) dibutyl phosphate (34α).

(2,3,4,6-Tetra-O-benzyl-α-D-mannopyranosyl) thioglycoside (50 mg, 1 eq), dibutyl phosphate (48 μl, 3 eq) and acid washed molecular sieves 3 Å (AW-MS 300) was stirred in anhydrous DCM at room temperature for 1 h and then cooled to -10 °C. 37 mg NIS was added (2.1 eq) followed by a catalytic amount of TfOH. The solution was turned to a red color from brown color. It was stirred at -10 °C for 3 h before quenched with Hunig’s base. The molecular sieves were filtered out. The obtained filtrate was washed with sat. Na2S2O3 three times. The organic layer was then condensed and subject to silica gel chromatography (Hexanes: EtOAc=3:1, Rf=0.3). 40 mg white solid was obtained (yield: 69%). It is a pure α anomer. \([\alpha]^{\text{RT}}_D +48.0 (c 0.20, \text{CH}_2\text{Cl}_2); \) 1H NMR (600 MHz, CDCl3) \(\delta 7.10-7.40 \) (m, 20 H, aromatic), 5.75 (d, \(J = 6.2 \) Hz, 1 H: H1), 4.49-4.90 (m, 8 H: PhCH2), 3.71 (t, \(J = 9.5 \) Hz, 1 H: H4), 3.89-4.03 (m, 6 H: PO2(OCCH2CH2CH3)2, H3, H5), 3.83 (br s, 1 H: H2), 3.78-3.81 (dd, \(J = 4.8 \) Hz, 11.0 Hz, 1 H: H6), 3.70 (d, \(J = 11.0 \) Hz, 1 H: H6'), 1.57 (m, 4 H: PO2(OCCH2CH2CH3)2), 1.33 (m, 4 H: PO2(OCCH2CH2CH3)2), 0.90 (t, \(J = 7.3 \) Hz, 3 H: PO2(OCCH2CH2CH3)), 0.80 (t, \(J = 7.0 \) Hz, 3 H: PO2(OCCH2CH2CH3)); 13C NMR (151 MHz, CDCl3) \(\delta 95.85 \).
(C1), 78.85 (C3), 74.58 (C2), 74.21 (C4), 73.82 (C5), 68.81 (C6), 67.74
(PO₂(OCH₂CH₂CH₂CH₃)), 67.64 (PO₂(OCH₂CH₂CH₂CH₃)), 32.20
(PO₂(OCH₂CH₂CH₂CH₃)), 32.13 (PO₂(OCH₂CH₂CH₂CH₃)), 18.60
(PO₂(OCH₂CH₂CH₂CH₃)), 18.51 (PO₂(OCH₂CH₂CH₂CH₃)), 13.57
(PO₂(OCH₂CH₂CH₂CH₃)), 13.55 (PO₂(OCH₂CH₂CH₂CH₃)); PhCH₂ (4 C: 75.20,
73.42, 72.72, 72.23); HR ESI MS calcd for C₄₂H₅₃O₉NaP [M+Na]⁺, 755.3325;
found, 755.3293.

(2,3,4,6-Tetra-O-benzyl-β-D-mannopyranosyl) dibutyl phosphate (34β).

A 1 ml anhydrous DCM solution of (2,3,4,6-Tetra-O-benzyl-α-D-mannopyranosyl)
thioglycoside (40 mg, 1 eq), DPSO (19 mg, 1.2 eq), TTBP (60 mg, 3 eq) and
activated 3 Å molecular sieves was stirred at R.T. for 3 hours before cooled down
to -78 °C. Tf₂O (16 ul, 1.2 eq) was added slowly to the solution to activate the
donor. After the solution was stirred for 30 min, a solution of dI H₂O (10 eq) in
THF was added. The resulting solution was stirred at the low temperature for 0.5
hours before quenched by Sat. NaHCO₃. It was then diluted by EtOAc and the
organic phase was washed with Sat. NaHCO₃ three times. The organic layer was
then dried over Na₂SO₄, concentrated and subjected to column chromatography.
(Hexanes: EtOAc=1:1, Rf=0.3). 31 mg product (yield: 90%) was obtained. It is a white sticky solid. 25 mg product (1 eq) was dissolved in anhydrous THF and stirred with activated 3 Å molecular sieves at room temperature for 1 h. 3.6 mg NaH (2.5 eq) was added. The solution was then cooled down to -78 °C. 31 ul (BuO)2PO2Cl (2.5 eq) was added slowly. The reaction was allowed to stir at -78 °C before being quenched by sat. NaHCO3. It was washed with brine and concentrated for column chromatography (Hexanes: EtOAc=4:1 Rf=0.2). The white sticky solid got is β anomer as expected. [α]RTD +18.6 (c 1.32, CHCl3); 1H NMR (600 MHz, CDCl3) δ 7.10-7.40 (m, 20 H, aromatic), 5.21 (d, J = 7.3 Hz, 1 H: H1), 4.48-4.92 (m, 8 H: PhCH2), 3.94-4.08 (m, 6 H: PO2(OCH2CH2CH2CH3)2, H2, H5), 3.73-3.77 (m, 2 H: H6', H6), 3.50-3.57 (m, 2 H: H3, H4), 1.59-1.64 (m, 4 H: PO2(OCH2CH2CH2CH3)2), 0.90 (t, J = 7.3 Hz, 3 H: PO2(OCH2CH2CH2CH3)), 0.80 (t, J = 7.3 Hz, 3 H: PO2(OCH2CH2CH2CH3)); 13C NMR (151 MHz, CDCl3) δ 96.79 (C1), 81.70 (C3), 76.17 (C5), 74.70 (C2), 74.09 (C4), 69.13 (C6), 68.10 (PO2(OCH2CH2CH2CH3)), 67.84 (PO2(OCH2CH2CH2CH3)), 32.22 (PO2(OCH2CH2CH2CH3)), 32.18 (PO2(OCH2CH2CH2CH3)), 18.63 (PO2(OCH2CH2CH2CH3)), 18.60 (PO2(OCH2CH2CH2CH3)), 13.62 (PO2(OCH2CH2CH2CH3)), 13.58 (PO2(OCH2CH2CH2CH3)); PhCH2 (4 C: 75.10, 74.34, 73.37, 71.96); HR ESI MS calcd for C42H53O9NaP [M+Na]+, 755.3325; found, 755.3289.
(2,3,4,6-Tetra-O-benzyl-α-D-glucopyranosyl) dibutyl phosphate (35α).

(2,3,4,6-Tetra-O-benzyl-β-D-glucopyranosyl) thioglycoside (100 mg, 1 eq), dibutyl phosphate (94 ul, 3 eq) and acid washed molecular sieves 3 Å (AW-MS 300) was stirred in anhydrous DCM at room temperature for 1 h and then cooled to 4 °C. 38 mg NIS was added (1.05 eq) followed by a catalytic amount of TfOH. The solution was turned to a red color from brown color. It was stirred at 4 °C for 10 min before quenched with Hunig’s base. The molecular sieves were filtered out. The obtained filtrate was washed with sat. Na2S2O3 three times. The organic layer was then condensed and subject to silica gel chromatography (Hexanes: EtOAc=3:1, Rf=0.26). 104 mg white solid was obtained (yield: 90%). It is a mixture of α, β anomers (α: β=1:1). It was then subjected to gradient Reverse Phase-HPLC purification (acetonitrile: H2O 80:20 to 90:10) to get the isolated anomers. [α]RTD +44.4 (c 0.068, CH2Cl2); 1H NMR (500 MHz, CDCl3) δ 7.10-7.40 (m, 20 H, aromatic), 5.91 (dd, J = 3.4 Hz, 7.3 Hz, 1 H: H1), 4.47-4.98 (m, 8 H: PhCH2), 3.98-4.07 (m, 5 H: PO2(OCH2CH2CH2CH3)2, H5), 3.98 (t, J = 9.1 Hz, 1 H: H3), 3.75-3.78 (dd, J = 3.7 Hz, 10.7 Hz, 1 H: H6), 3.73 (t, J = 9.5 Hz, 1 H: H4), 3.63-3.66 (m, 2 H: H2, H6′), 1.59 (m, 4 H: PO2(OCH2CH2CH2CH3)2), 1.33 (m, 4 H:
PO₂(OCH₂CH₂CH₂CH₃)₂, 0.90 (t, J = 7.3 Hz, 3 H: PO₂(OCH₂CH₂CH₂CH₃)), 0.80 (t, J = 7.6 Hz, 3 H: PO₂(OCH₂CH₂CH₂CH₃)); ¹³C NMR (125 MHz, CDCl₃) δ 95.09 (C1), 81.18 (C3), 79.35 (C2), 77.25 (C4), 72.84 (C5), 67.75 (C6), 68.10 (PO₂(OCH₂CH₂CH₂CH₃)), 67.42 (PO₂(OCH₂CH₂CH₂CH₃)), 32.18 (PO₂(OCH₂CH₂CH₂CH₃)), 32.14 (PO₂(OCH₂CH₂CH₂CH₃)), 18.62 (PO₂(OCH₂CH₂CH₂CH₃)), 18.55 (PO₂(OCH₂CH₂CH₂CH₃)), 13.59 (PO₂(OCH₂CH₂CH₂CH₃)), 13.57 (PO₂(OCH₂CH₂CH₂CH₃)); PhCH₂ (4 C: 75.64, 75.18, 73.51, 72.38); HR ESI MS calcd for C₄₂H₅₃O₉NaP [M+Na]+, 755.3325; found, 755.3290.

(2,3,4,6-Tetra-O-benzyl-β-D-glucopyranosyl) dibutyl phosphate (35β). [α]ᵣₑₒᵣₐ₁ = -10.4 (c 0.09, CHCl₃); ¹H NMR (600 MHz, CDCl₃) δ 7.10-7.40 (m, 20 H, aromatic), 5.16 (t, J = 7.3 Hz, 1 H: H1), 4.48-4.89 (m, 8 H: PhCH₂), 3.90-4.10 (m, 4 H: PO₂(OCH₂CH₂CH₂CH₃)₂), 3.73 (dd, J = 3.6 Hz, 11.0 Hz, 1 H: H6), 3.71 (t, J = 8.8 Hz, 1 H: H4), 3.69 (m, 1 H: H6'), 3.66 (t, J = 8.8 Hz, 1 H: H3), 3.55 (d, J = 9.5 Hz, 1 H: H5), 3.53 (t, J = 8.4 Hz, 1 H: H2),1.61 (quintet, J = 7.0 Hz, 2 H: PO₂(OCH₂CH₂CH₂CH₃)), 1.55 (quintet, J = 7.0 Hz, 2 H: PO₂(OCH₂CH₂CH₂CH₃)), 1.33-1.37 (sextet, J = 7.7 Hz, 2 H: PO₂(OCH₂CH₂CH₂CH₃)), 1.24-1.31 (sextet, J = 7.7 Hz, 2 H: PO₂(OCH₂CH₂CH₂CH₃)), 0.88 (t, J = 7.3 Hz, 3 H: PO₂(OCH₂CH₂CH₂CH₃)); ¹³C NMR (150 MHz, CDCl₃) δ 98.85 (C1), 84.35 (C3), 82.02 (C2), 77.24 (C4), 75.37...
(C5), 68.47 (C6), 67.70 (PO$_2$(OCH$_2$CH$_2$CH$_2$CH$_3$)), 67.66

(PO$_2$(OCH$_2$CH$_2$CH$_2$CH$_3$)), 32.16 (PO$_2$(OCH$_2$CH$_2$CH$_2$CH$_3$)), 32.12

(PO$_2$(OCH$_2$CH$_2$CH$_2$CH$_3$)), 18.59 (PO$_2$(OCH$_2$CH$_2$CH$_2$CH$_3$)), 18.51

(PO$_2$(OCH$_2$CH$_2$CH$_2$CH$_3$)), 13.55 (PO$_2$(OCH$_2$CH$_2$CH$_2$CH$_3$)), 13.50

(PO$_2$(OCH$_2$CH$_2$CH$_2$CH$_3$)); PhCH$_2$ (4 C: 75.64, 75.18, 74.98, 74.78); HR ESI MS calcd for C$_{42}$H$_{53}$O$_9$NaP [M+Na]$^+$, 755.3325; found, 755.3319.

**In-source Fragmentation of Glycosyl Phosphates:**

The mass spectrometric study was carried out using a Waters LCT Permiere Xe TOF mass spectrometer. The spectra were recorded in positive ion mode with a source temperature of 120 °C using a desolvation gas flow of 800 L/h. Samples were dissolved in an sodium acetate methanolic solution ([NaOAc]=0.18 mM). The sodium acetate methanolic solution was prepared by adding 750 µl aqueous NaOAc solution ([NaOAc]=12.20 mM) to 50 ml methanol. Ions were detected using the broadband detection mode covering a mass range from 20 to 1000 amu. For each sample, the compound was dissolved in ethyl acetate solvent first to make a 0.508 mM solution considering that the compounds doesn’t dissolve well in methanol solution directly. 2 µl of this kind of ethyl acetate solution of a interested glycosyl phosphate sample and 2 µl of this kind of ethyl acetate solution of internal standard phosphate sample was then added to 1 ml sodium acetate methanolic solution. It was then mixed thoroughly before injecting to the
mass spectrometer. A 2 µl methanol solution of the phosphate was infused into the ESI-MS source at room temperature. The in-source fragmentation study of the glycosyl phosphate was carried by increasing cone voltages starting from 40V with an incremental change in cone voltage till 2% fragmentation of standard phosphate was observed. Each fragmentation experiment was duplicated for three times.

**Data processing from the Mass Spectrum obtained In-source Fragmentation Experiment:**

Table 8 shows the direct reading of relative abundance of all the fragment ions and interested glycosyl phosphate parental ion to internal standard parental ion from the mass spectrum of cone voltage fragmentation experiment. CV stands for the cone voltage at which internal standard reached approximately 2% fragmentation. RA1 and RA2 are the relative abundance of daughter ions of interested glycosyl phosphate to internal standard parental ion. RA1 belongs to the daughter ion lost both phosphoric acid and one benzyl group in fragmentation. RA2 belongs to the daughter ion lost only phosphoric acid. RA3 is the relative abundance of daughter ion of internal standard to internal standard parental ion itself. RA4 is the relative abundance of interested glycosyl phosphate parental ion to internal standard parental ion.

Table 9 shows the processed data from Table 8. Rs is the relative abundance of daughter ion of internal standard to internal standard parental ion.
itself. It’s the same value as RA3. RM1 and RM2 are the relative abundance of daughter ions of interested glycosyl phosphate to interested glycosyl phosphate parental ion itself. $R_{M1}=RA1/RA4$; $R_{M2}=RA2/RA4$. RM is the total relative abundance of daughter ions of interested glycosyl phosphate to interested glycosyl phosphate itself. $R_{M}=R_{M1}+R_{M2}$. Relative stability value (RSV) is calculated as $RSV=R_{M}/Rs$. The data shown in Table 6 are the averages of 3 times duplicate experiments.
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Table 8. Relative abundance of all the fragment ions and interested glycosyl phosphate parental ion to internal standard parental ion.
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Table 9. Processed data in cone voltage fragmentation experiment
REFERENCES


ABSTRACT

SYNTHESIS OF 4’-O-GLYCOPYRANOSYL PAROMOMYCIN AMINOGLYCOSIDE ANTIBIOTICS: INFLUENCE OF THE GLYCOSIDE ON ANTIRIBOSOMAL, ANTIBACTERIAL ACTIVITY AND UTILIZATION OF ELECTROSPRAY IONIZATION MASS SPECTROMETRY TO EVALUATE REACTIVITY OF GLYCOSYL PHOSPHATE

by

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May 2014

Advisor: Dr. David Crich
Major: Chemistry
Degree: Master of Science

The data in this thesis is trying to address two problems. The first problem is paromomycin associated ototoxicity; the second problem is the lack of precise reactivity data of glycosyl donors in one-pot synthesis.

Paromomycin is out of the market as an antibiotic due to its ototoxicity. Previous work done by Vasella and Böttger's groups identified 4’-O position of paromomycin as a promising site of modification to reduce the side effect. A series of 4’-O-glycopyranosyl paromomycin has been synthesized and tested for their biological activity and selectivity. From the results of anti bacterial ribosomal activity, anti hybrid ribosomal activity and antibacterial activity, it was found that 4’-O-4-amino-α-D-glucopyranosyl paromomycin is the most active among the sixteen 4’-O-glycosyl paromomycin derivatives synthesized. However, 4’-O-4-
amino-α-D-glucopyranosyl paromomycin shows an inability to differentiate eukaryotic ribosomal A site from prokaryotic ribosomal A site. 4′-O-glycosyl paromomycin derivatives with equatorially oriented glycosidic bond at the 4′-O-glycosyl ring has more selectivity toward mitochondrial hybrid ribosomes than their corresponding derivatives with axially oriented glycosidic bonds. Therefore, it is promising to introduce an equatorially oriented substituent to the 4′-O position of paromomycin in the future drug design to reduce ototoxicity.

To evaluate the relative reactivity of glycosyl phosphate donors as a function of stereochemistry and protecting groups, electrospray ionization mass spectrometric fragmentation experiments were performed. Two methods to quantitatively access relative stability of glycosyl phosphates have been used: cone voltage fragmentation experiment and collision induced dissociation. Results from both methods showed that the α anomers of glucosyl phosphates are more stable than their corresponding β anomers regardless the protecting group. In the contrary, the α anomers of mannosyl phosphates are less stable than their corresponding β anomers irrespective with the protecting group. So it's likely glycosyl phosphates with the C1-dibutyl phosphate bond syn to the C2-H bond dissociate through a McLafferty rearrangement, but glycosyl phosphates with the C1-dibutyl phosphate bond anti to C2-H bond dissociate through an oxocarbenium ion. Cone voltage fragmentation experiment gave results contradicting the well-known benzylidene effect. Therefore, it seems the relative
stability of glycosyl phosphates cannot reflect their relative reactivity in glycosylation reaction.
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Publication