Towards the site-selective modification of aminoglycoside antibiotics and their biological evaluation

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Chapter 4
Toward the site-selective modification of aminoglycoside antibiotics

Part of this chapter will be submitted for publication: Bianca S., et al. manuscript in preparation
4.1 Introduction

During the past century, important discoveries have been made in the field of antibiotics. As already mentioned in chapter 1, the first antibiotic, penicillin, was discovered in 1928 by Sir Alexander Fleming, and from that moment the so-called **golden era** of antibiotics started. Years later, in 1943, the first aminoglycoside antibiotic (AGA), streptomycin, was isolated from *Streptomyces griseus*, discovered by the Nobel Prize laureate in Medicine or Physiology, Selman Waksman. Streptomycin was used as the first effective antibiotic against tuberculosis caused by *Mycobacterium tuberculosis*. Aminoglycoside antibiotics form a class of antibacterial compounds based on amino sugars. Their mechanism of action consists of binding to the small ribosomal subunit of bacteria (30S) and they inhibit the bacterial protein synthesis. AGAs are mainly used to treat infections caused by aerobic gram-negative bacilli (for example *E. coli*), as well as some gram-positive staphylococci (for example *S. aureus*), streptococci, mycobacteria (example *M. tuberculosis*).

Before describing the mode of action of the aminoglycosides in detail, it is important to explain how protein synthesis takes place in cells. The biosynthesis of proteins in bacterial cells starts with the transcription of a gene on the DNA to form the mRNA. The amino acid sequence of the protein is encoded within this mRNA and in the subsequent step the mRNA is translated by the ribosome to form the polypeptide.

The translation of the mRNA takes place in the ribosome, which contains two subunits, a large subunit (50S) and a small one (30S) (Figure 1). The mRNA binds to the ribosome binding site (RBS). The codon that initiates the translation of mRNA is generally formed by the AUG sequence (A=adenine, U=uracil, G=guanine) which encodes for formyl-methionine in bacteria. The anticodon, corresponding to the complementary nucleobase sequence, is carried by the aminoacyl-tRNA (aa-tRNA). The tRNA functionalized with a specific amino acid residue will move from the acceptor site (A-site) to the peptidic site (P-site). In general, the codon-anticodon pairing will lead to the formation of a specific amino acid sequence (Figure 1a and 1b). The next incoming aa-tRNA carry also the elongation factor (EF-Tu) in complex with a GTP molecule. This will allow the formation of peptide bonds (Figure 1c). The process will continue until the ribosome arrives at the stop codon and the desired amino acid sequence has been formed (Figure 1d). The tRNA is finally released.
More precisely, under normal conditions, the protein synthesis starts in a loop located in the rRNA of the 30S subunit. This loop presents two adenines (A1492 and A1493) that play an important role in the pairing process of the incoming aa-tRNA and the mRNA. In particular, by making hydrogen bonding they give a positive signal to the A-site when a specific aa-tRNA is the correct one to pair with the mRNA. When this happens the two adenines will point out of the loop and the formation of the amino acid sequence will follow, if not the two adenines will remain inside the loop and therefore no pairing will take place 5.

In the presence of aminoglycoside, as showed with paromomycin, this biosynthetic process is disrupted 6. The AGA binds on the 30S subunit of the ribosome. Upon the binding of AGA into the loop, the two adenines point out of the loop, thereby disrupting the feedback loop to the A-site. Consequently, any incoming aa-tRNA is considered the correct one to be paired with, irrespective of the anticodon. This will lead to a mis-formation of the amino acid sequence which will further end in an
incorrect polypeptide without function for the bacterial cell, and therefore this cannot be described as a protein. In the process of synthesizing the wrong polypeptide, the bacterial cell has consumed a lot of energy. Slowly, this process will lead to bacterial death.

Structurally, AGAs are characterized by the presence of an aminocyclitol ring, called 2-deoxystreptamine (2-DOS) (Figure 2). The 2-DOS is functionalized with different monosaccharides via glycosidic linkages. If these are formed between the hydroxy at C4 and C5 of the 2-DOS the AGA belongs to the 4,5-disubstituted series (like neomycin, paromomycin, ribostamycin). While if the glycosidic bonds are formed with the hydroxy at C4 and C6 the AGA belongs to the 4,6-disubstituted series (like amikacin, kanamycin, gentamicin, tobramycin). Not all aminoglycosides contain a deoxystreptamine core. Several AGA contain a streptamine core. Examples are spectinomycin and the earliest discovered aminoglycoside streptomycin (Figure 3).

![Figure 2. Commonly distributed aminocyclitol core (2-DOS) among the AGAs](image)

![Figure 3. Some structural examples of aminoglycoside antibiotics (continue to next page)](image)
As it is apparent from Figure 3, the aminoglycosides contain multiple amino groups and hydroxyl groups. These functional groups play an important role in the binding process to the rRNA. Hydrogen bonds are formed with the hydroxy groups. While the positively charged ammonium groups bind with the negatively charged loop in the 30S ribosomal subunit.

4.2 Antibiotic resistance in aminoglycosides

As already discussed in chapter 1, antibiotic resistance is a very alarming problem leading to an antibiotic gap that by 2050 can cause an increase of death all around the
world as a consequence of bacterial infections. Also, the aminoglycoside antibiotics efficacy is affected by the bacterial resistance. There are three different mechanisms with which bacteria can develop resistance toward this class. Two of these mechanisms act by modifying specific cellular components, while the third mechanism acts on the AGAs directly. This latter mechanism will be discussed in more detail as it is the most common resistance mechanism that different bacteria classes and/or strains display towards AGAs, and because this is something that chemists can try to overcome by synthesizing derivatives.

However, a quick description of the cellular resistance mechanisms will be presented here. The first mechanism takes place at the ribosomal level and it is caused by methylase enzymes that selectively methylate the adenine residues that play an important role in the codon-anticodon pairing. In this way, the accommodation of the aminoglycoside inside the binding loop is very much disrupted, and therefore this process will lead to the final ineffectiveness of the aminoglycoside. The second resistance mechanism is the mutation and/or overexpression of efflux pumps. The efflux pumps are essential to any bacterial cell, as these transporter proteins export everything out of the cell that is not necessary and thereby they maintain homeostasis. Under normal conditions, however, the pumps do not export large molecules (such as antibiotics) efficiently. Consequently, the drug accumulates inside the cell, finally leading to the effectiveness of the antibiotic. However, bacteria can overcome this by either over-expressing or mutating the efflux pumps. When this process takes place, the bacterial cell can remove the antibiotics more efficiently from the cytosol. Consequently, the accumulation of the antibiotic is circumvented and the desired biological effect will not be reached.

The third mechanism of resistance comes from enzymes that modify the antibiotic. This latter resistance mechanism is in our interest because being the most widespread towards aminoglycosides among the different classes of bacteria and, also because of the resistance happening directly on the aminoglycosides drug. Blocking the enzymatic modification of the antibiotic should overcome resistance, and we used this consideration for our aimed approach. The enzymes responsible for counteracting the activity of AGA antibiotics are the Aminoglycoside Modifying Enzymes (AME). These enzymes are transferases that transfer a specific group onto a functional group of aminoglycosides (Figure 4). In particular, there are three major AME: phosphotransferase enzymes (APH), nucleotidyltransferase enzymes (ANT), and acetyltransferase enzymes (AAC). Among the transferase enzymes, our focus is
on the APH enzyme family which can be further divided in subclasses as reported below (Figure 5) \(^{12}\).

![Figure 4. Site-specific modification(s) from AME in 4,6-disubstituted AGAs (kanamycin) and 4,5-disubstituted AGAs (neomycin, paromomycin)](image)

![Figure 5. Phosphorylating enzyme distribution (adapted from literature) \(^{12}\)](image)

All the three different enzymes indicated previously: ANT, APH and AAC present different isozyme forms. These isoforms often have different regiospecificities in the substrate modification \(^{12}\). In bacterial strains expressing an AME, the aminoglycosides get inactivated once they get into the cell. This will preclude the interaction with the ribosome and consequently will contribute to the loss of their antibacterial activity. Since the discovery of these enzymes, a lot of effort has been put into chemically diversifying existing aminoglycosides to get new drugs immune to these inactivation mechanisms \(^{13}\). Several mechanistic studies have been performed to gain understanding of the different modifying enzymes. For example, Chen-
Goodspeed et al. proposed the mechanism of interaction for ATP and kanamycin A in the binding pocket of ANT(4’). By taking into account the interaction of ANT(4’) and kanamycin A, as reported in Figure 6A, it is observed that the Glu-145 acts as a base to activate the 4’-hydroxyl of the antibiotic. Subsequently, the nucleophilic attack at the α-phosphoryl group of the nucleotide triphosphate takes place. According to the authors, the attack is further facilitated by the proximity of the positively charged Lys-149 to the nucleotide group.

![Diagram of ATP and Kanamycin A](image)

Figure 6. A) Interactions of the active site of ANT(4’) with ATP and kanamycin A (adapted from literature) [15], B) key interactions of the NPL of APH(3’)-IIIa with ATP and kanamycin A (adapted from literature) [16].

Also, the mechanism of APH(3’) has been studied (Figure 6B). The amino acid residues Ser27 and Met26 of the nucleotide positioning loop (NPL) [16] play an important role in the binding of ATP and in the phosphate transfer. After the binding of Mg-ATP, the β-phosphate nonbridge oxygens interact with the Mg²⁺ ion and with Ser27 through a hydrogen bond formation. The Mg²⁺ ion also links with the γ-phosphate of ATP. Upon the binding of the aminoglycoside, the NPL conformation changes in such a way that the nonbridge oxygens of the β- and γ-phosphate start to eclipse and the enhanced repulsion of the two phosphates will lead to the weakening of the γ-PO bond. Next, Asp190 in the binding pocket of APH(3’) will act as a base for...
the deprotonation of the 3’-OH of the aminoglycoside to favor the phosphorylation 16,17.

Our attention was on the aminoglycoside phosphotransferase enzymes (APH). We focused on the APHs because one of the isoenzymes, the APH(3’), can selectively phosphorylate the C3’-OH of the gluco-configured ring of the aminoglycoside antibiotics, thus causing loss of their antibacterial activity. From our experience and as explained in chapter 2, the C3’-OH of the gluco-configured ring can undergo to regioselective oxidation. In this way we aimed to further prepare AGA derivatives exclusively modified at C3’ with the intention of circumventing the phosphorylation caused by APH(3’). Among the members of this class, the most studied is APH(3’)-IIIa 18. This specific enzyme does not only phosphorylate the 3’-hydroxyl, but it can also modify the 5’'-hydroxy group of 4,5 disubstituted AGAs, as observed for lividomycin A, which does not possess the 3’-hydroxy 19,20. A study by the Wrights group showed that neomycin B, which contains hydroxy groups at both the 3’ and the 5’’, is phosphorylated at both positions with phosphorylation at 5’’-OH being favored. Interestingly, phosphorylation at 3’-OH is favored for butirosin A (Figure 7). However, HPLC analysis revealed that butirosin A also undergoes a second phosphorylation reaction. Wright’s group also stated that the second phosphorylation takes place only after the monophosphorylated derivatives have dissociated from the APH(3’)-IIIa enzyme 19.

![Figure 7. Structures of neomycin B and butirosin A](image)

4.3 Towards modified aminoglycoside antibiotics

In the last years, a new aminoglycoside has been approved for medical use, plazomicin, which was accepted by the FDA in 2018 to be used in the USA for the treatment of complicated urinary tract infection (cUTI) 21. This suggests the necessity
to work on this class of antibiotics as they are widely used in hospitals, mainly in topical use for the treatment of skin and ocular infections, since prolonged exposure to AGAs can cause ototoxicity and nephrotoxicity. Next, despite these toxicity issues, this class of antibiotics is used for the treatment of tuberculosis. They fall in the third line of defence option for treating TB patients mainly due to toxicity effects upon a long time of treatment, which can easily take place in TB patients as the treatment can require several months. However, despite the introduction of new AGA into clinical (e.g. plazomicin), the continuous bacterial resistance requires efforts in the preparation of derivatives that can overcome the resistance mechanisms that otherwise will limit their use.

Considering the modification caused by the AMEs, we focused on the preparation of regioselective modified aminoglycosides by applying a novel catalytic approach. In particular, our interest focused on the modification caused by APH(3') enzymes which phosphorylate the hydroxy group at C3' of the gluco-configured ring. Before going into details, it is important to present some of the modified aminoglycosides that have been already synthesized to overcome the action of AMEs.

Among the different approaches to avoid resistance mechanisms, one of the most inspiring starting points of our approach was the work published by Mobashery et al. in 1999. Based on the fact that APH(3') catalyzes the phosphorylation at the C3' position of aminoglycosides, Mobashery’s group designed and prepared a kanamycin A analog (A) that is in equilibrium with its hydrated form (B). If B undergoes phosphorylation, the resulting product (C) spontaneously eliminates phosphate (Figure 8). The 3'-keto-kanamycin (A) was obtained in 11 steps with an overall yield of 9%.26
More recently, Crich’s group prepared several aminoglycoside derivatives and studied them both for their antibacterial activity but also for their toxicity. For example, they prepared 4’-O-glycopyranosyl paromomycin derivatives. Even though these compounds have a decreased activity compared to the parent compound paromomycin, they are promising starting points for the development of less toxic derivatives \(^{27}\). Also, they prepared derivatives with a modification at the 4’ position of paromomycin by the introduction of an ethylthio group or, even better, a propyl group as a replacement for the hydroxy group. This modification led to both a better activity against bacterial pathogens and to an increase of the ribosomal selectivity for bacterial ribosomes versus mitochondrial eukaryotic ribosomes, which is an indication for reduced toxicity of these derivatives \(^{28}\). They also investigated modifications at the 2’ position, showing that alkylation of this position of 4,5-disubstituted aminoglycoside antibiotics could overcome the resistance caused by the acetyltransferases and also led to an enhanced selectivity for the procaryotic ribosome versus the eukaryotic ones \(^{29}\).

Nevertheless, we focused on the APH(3’) enzymes due to the opportunity to apply a site-selective modification method already developed in the group and discussed in chapter 2. The modification method we aimed for is the regioselective C3’ oxidation. The resulting keto-aminoglycoside derivative can be modified further by using the ketone function as a powerful handle to selectively react without interfering with the other functional groups of the molecule (Scheme 1). To make this work, protection of the amino groups present in the molecules is necessary as already explained in chapter 2.
As a first goal, we planned to invert the stereochemistry of the hydroxyl group at C3’ as this appeared to be a valuable approach. Based on the X-ray structure of the APH(3’)-IIIa in complex with kanamycin and ATP (Figure 9), it was hypothesized that the 3’-OH group will probably not be phosphorylated anymore upon stereochemical inversion. The distance between the ATP and the 3’ hydroxy group would increase going from equatorial to axial.

Figure 9. Cartoon representation of kanamycin (cyan) and ATP (green) binding the APH(3’)-IIIa enzyme. The 2-DOS moiety is highlighted in orange. A label identifies the 3’ position in the gluco-configured ring (PDB code: 1L8T)

4.4 Results and discussion
4.4.1 Toward the complete protection of the amino groups in aminoglycosides

To begin our synthetic modifications, the complete protection of the amino groups was necessary as it was hypothesized that free amines would coordinate to the catalyst, thereby inhibiting the oxidation reaction. Protection was performed at room temperature with the selected protecting group reagent and a base (K₂CO₃ or Et₃N) in a water/THF (1:1) mixture or in DMSO. This approach was successful with Cbz and Boc protecting groups as already shown in Jäger’s Ph.D. thesis. With the hope to increase the lipophilicity of the protected aminoglycosides and thereby improve the
Solubility of protected AGAs in organic solvents, we also attempted to protect AGAs with the protecting groups prepared in chapter 2 and 3 using these conditions. However, our attempts didn’t perform as desired, probably due to mainly two aspects. The first relates to the steric clash between the carbamate protecting group and the aminoglycosides that need to be protected at the amino groups, especially when considering the reactions that need to take place with the amine of the aminoglycoside and the bulky protecting groups. The second aspect is the difference in polarity of the AGA substrates and the protecting group reagents. Initially, the aminoglycoside was dissolved in water and the protecting group in THF. However, upon mixing the two solutions, a cloudy suspension was obtained probably due to the precipitation of one of the two reagents in the solvent mixture. Indeed, the protecting groups weren’t soluble in water. Neither did the aminoglycoside appear to dissolve in THF. Also in the case of DMSO, both kanamycin and the protecting groups seemed to not properly being dissolved in the reaction flask, leading to a cloudy suspension. As consequence, partial protection of the AGA was observed (UPLC-MS) for most of the selected protecting group reagents. Approximately two to three amino groups out of the six in neomycin and out of the four in kanamycin were protected in these reactions. In one attempt, four out of six amino groups of neomycin seemed to be protected, but purification was troublesome due to the presence of partially unprotected material. We increased the temperature to 50 °C but this did neither increase the solubility of the reagents and products nor improve the conversion. A selection of the UPLC-MS spectra used to monitor the reactions is reported in the experimental section of this chapter to show the complexity in monitoring and analyzing the reaction mixture. Analysis by TLC showed that a very complex and tailing mixture had formed, which was too difficult to purify.

Confronted with these difficulties, it was decided to go back to the classical protecting groups (Cbz and Boc) already used in the previous work reported in Manuel Jäger’s Ph.D. thesis and depicted in Scheme 2. This approach leads, however, to poorly soluble compounds, especially the Boc-protected form. Therefore, the Cbz protecting group was used for neomycin and paromomycin, whereas the Boc protection was used for kanamycin. Kanamycin was also protected with Cbz and despite full protection was obtained, a very insoluble material was obtained, as already mentioned in Jäger’s thesis.
Scheme 2. Procedure for the protection of paromomycin 1 and kanamycin 3

The protected AGAs were subjected to purification. (Cbz)-neomycin and (Cbz)-paromomycin were purified by silica gel chromatography. Cbz protected paromomycin was obtained in a low yield after purification, and therefore, it was decided to check the purity of the starting material by means of qNMR analysis. It turned out that commercial paromomycin with a reported purity of 95% (HPLC based) had in fact a purity of 64% based on qNMR. Therefore, the yield of the protection initially of 55% was adjusted accordingly after the purity check to 86%. Boc-protected kanamycin was purified by adding water to the reaction mixture and collecting the formed precipitate by filtration through a glass-sintered filter. Drying under vacuum overnight provided the product in 92% yield with 84% purity (based on qNMR), which probably contained residual carbonate and/or sulfate salts as a result of the not possible purification by means of silica gel column chromatography.

4.4.2 Regioselective oxidation of amino-protected aminoglycoside antibiotics

In order to obtain the site-selective modified AGAs, we applied the palladium-catalyzed regioselective oxidation. This approach leads to the 3'-keto aminoglycoside derivatives. The keto-handle allows a selective modification at C3' without interference with the other functional groups. As reported in Scheme 3, the 3'-keto
paromomycin 5 and 3'-keto kanamycin 6 are formed. Contrary to what has been observed in mono-saccharides \(^3\), full oxidation was not obtained in the case of the AGAs. The main hurdle, especially in the oxidation of kanamycin, was the low solubility in DMSO, the solvent of choice for the oxidation reaction. The poor solubility might very well have led to reduced reaction rates, which may explain the incomplete oxidation of kanamycin. Extending the reaction time did not improve the conversion, presumably because the catalyst decomposed over time either by air exposure or due to the possible presence of residual carbonate and/or sulfate salts. This reasoning does not explain the partial oxidation of paromomycin and neomycin, as these dissolved well under the reaction conditions. We hypothesize that one cause, in this case, could be that the conformation of the aminoglycoside in solution might have led to inefficient chelation of the catalyst with the 3'-OH and 4'-OH of paromomycin/neomycin. This would affect the efficiency of the palladium complex formation and thus lead to a lower conversion. Indeed, while for kanamycin increasing the reaction temperature neither changed the final recovery nor the solubility, for paromomycin an increase in the temperature gave higher conversion. This enforces our hypothesis.

Scheme 3. General procedure for the regioselective oxidation of paromomycin and kanamycin

Initially, the oxidation reactions were carried out in DMSO. Later, also oxidation in methanol and trifluoroethanol \(^3\) was carried out for the Cbz-protected paromomycin
and neomycin. This still did not lead to full conversion but the removal of the solvent was much easier than in the case of DMSO. For Boc-protected kanamycin, the solvent of choice was DMSO mainly due to the bad solubility of the substrate in methanol or trifluoroethanol. However, even in DMSO, as previously mentioned, the solubility was not great. In all cases, incomplete conversion to the desired products was observed. Also attempts to reach full conversion by adding either extra equivalents of 1,4-benzoquinone or a higher loading of the catalyst, did not lead to full conversion. However, double oxidation was observed for (Boc)-kanamycin. The amount of double oxidized kanamycin varied per batch and with the reaction time. The oxidation of kanamycin was extensively monitored by UPLC-MS. However, the differences could not accurately be determined making it difficult to determine the reason behind it. A logical explanation could be that due to the low solubility of (Boc)-kanamycin and a slightly better solubility of its keto-form, the latter will be more easily to react further in solution. Indeed a complexation can be expected also from the catalyst with the 4''-OH and the primary alcohol in 6'', despite the 3'-OH/4'-OH trans-diol being more favorable in coordinating the catalyst. In this way, the already mono-oxidized kanamycin is more accessible and thus leads to the formation of the double oxidation. We were forced to combine the best reaction conditions to avoid double oxidation. We stopped the reaction as soon as a decent conversion to the desired mono-oxidized product was observed.

4.4.3 Reduction of 3'-keto-(Cbz)-paromomycin

In order to support and prove our hypothesis as described in Figure 9, we aimed to perform a straightforward stereochemical inversion of the C3' position by reducing the ketone with NaBH₄. As already shown in chapter 2 and in prior work from our group, the hydride will attack the ketone from the top face of the 3'-keto glycosides, due to less steric hindrance compared to the bottom face. In this way, the hydroxy group in C3' is inverted from equatorial to axial. With this approach, we aimed to block phosphorylation by APH(3') enzymes in ring I. The reduction was performed in ethanol and NaBH₄ was added as the reducing reagent. In the case of paromomycin we were able to separate the epi-paromomycin from the unreacted starting material. Whereas the same reduction could not be applied on kanamycin seen the troublesome purification after oxidation. The reaction was generally performed overnight at room temperature, and after this time full conversion was observed. The reaction was quenched by the addition of amberlite resin R-120 in its H⁺ form, followed by stirring for 30 min. After this time, the suspension was filtered
and the solvent was then removed by rotary evaporation. A column chromatography purification afforded the 3’-epi-(Cbz)-paromomycin 7 (Figure 10). Hydrogenolysis conditions were used for the removal of Cbz to get the final paromomycin derivative in its acetate salt form in 26% yield adjusted based on the purity determined by qNMR (20%), as reported in the experimental section.

![Figure 10. 3’-epi-(Cbz)-paromomycin](image_url)

**4.4.4 Indium-mediated allylation and propargylation of 3’-keto aminoglycosides**

While carrying out this research, a publication from the Crich’s group appeared showing that a propyl group on the 4’ position of paromomycin did not only overcome the resistance caused by ANT(4’), which was the initial goal of their project, but also led to susceptibility in those bacterial strains containing the genes encoding for the expression of APH(3’). A possible reason for this could be that the propyl chain, upon binding of this derivative to the APH(3’) enzymes, disrupts the binding of ATP.

Based on Crich’s observations, we also desired to investigate if our modification at C3’ could also interfere with the action of the ANT(4’) enzyme on the nearby C4’ position. In this sense, very preliminary assays on some bacterial strains presenting the genes expressing for ANT(4’) will be shown in chapter 5. Therefore, based on this hypothesis, our approach started from the possible modification of a ketone by applying the indium-mediated allylation or propargylation, as our group described for 3’-keto glucosides. In this way, we could look at the difference in activity of the prepared derivatives when an extra substituent is placed on the C3’ position. The indium-mediated reactions were performed in water: THF mixtures, generally a 1:1 ratio was used. As a reagent, 1.5 eq of either allyl-bromide or propargyl-bromide was used and 1.5 eq of indium powder was also added (Figure 11). The reactions were monitored by UPLC-MS and they were stopped upon completion. Next, the
suspension was filtered over celite to remove as much of the formed indium salts as possible, and subsequently the solvent was removed under reduced pressure.

Figure 11. General reaction scheme for the indium-mediated allylation and propargylation of 3'-keto-aminoglycosides

The 3’-allyl-(Cbz)-paromomycin 8 was obtained in 74% yield after purification. Some mixed fractions were recovered too. Therefore a higher recovery of the desired product was not achieved. The mixed fractions contained co-eluted benzoquinone and catalyst that were present in the starting material, 3’-keto-(Cbz)-paromomycin. In any case, for paromomycin, a much better solubility than the one observed for kanamycin, led to a shorter reaction time to achieve complete consumption of the starting ketone. Indeed the poor solubility of the Boc protected keto-kanamycin caused very slow reaction time and the reaction mixture was very turbid. This impacted the yields of the allyl and propargyl kanamycin derivatives which were drastically lower than the yield of allyl paromomycin.

A possible explanation of why the low solubility of the starting material might affect the reaction time comes from an organoindium chemistry review 34. As reported in the review, the indium-mediated allylation proceeds via the following intermediates (Scheme 4). The allylindium (III) dibromide (allyl-InBr₂) and the diallylindium bromide (allyl≡InBr) are formed in both aqueous medium and organic solvents (such as THF) 34. These are considered the most active allylindium species among the others.
reported in the review, although the diallylindium bromide can decompose in water leading to the formation of the allyl(μ-hydroxido)indium bromide species and propene as hypothesized from NMR and X-ray crystallographic studies.

These observations might explain why, especially in kanamycin, the reaction sometimes proceeded quite slow. Indeed, in the already cloudy mixture where the substrate was not very soluble, the degradation of one of the reactive allyl indium species with the formation of a less reactive and bulkier species might lead to slower conversion to the desired product. However, after allylation/propargylation, the solubility of the kanamycin derivatives in the organic solvents (CH2Cl2 and MeOH) used for purification improved slightly, but the solubility was still not optimal. In particular, 3′-allyl-(Boc)-kanamycin 8 was isolated in 10-28% yield, 3′,4′′-di-allyl-(Boc)-kanamycin 10 and 3′-propargyl-(Boc)-kanamycin 11 were isolated in 10% yield and 3′-propyl-(Boc)-kanamycin 12 was isolated in 6% yield after hydrogenation of the terminal alkene of 9 (Figure 12).

NMR analysis of the isolated 3′-allyl aminoglycoside and 3′-propargyl aminoglycoside revealed that the carbon chain was at the equatorial position while the hydroxy group was axial (based on NOESY NMR). Also in this case a top face attack is favored for the same reason as described in the previous paragraph. For compound 10, the second allylation was confirmed to be in C4′ after TOCSY NMR analysis.

Deprotection of the Boc kanamycins (9-12) in TFA: CH2Cl2: 0.5:1 followed to give full conversion of the final products in their TFA salt form with yields 30%-66% and purities 47%-66% as reported in the experimental section. While hydrogenolysis conditions were used for the removal of Cbz from the allyl-paromomycin 8 with also
reduction of the double bond to single bond to get the final paromomycin derivative in its acetate salt form in 22% yield adjusted based on the purity determined by qNMR (10%), as reported in the experimental section.

**Figure 12.** Isolated compounds (structure, yield and purity) after the indium-mediated allylation and propargylation

In some cases other modifications were attempted starting from compound 9, as described in the next paragraphs, involving for instance alkene cross-metathesis.
4.4.5 Cross-metathesis with terminal 3'-allyl-(Boc)-kanamycin

Starting from the allyl kanamycin derivative we investigated the application of ruthenium-catalyzed cross-metathesis reactions with the aim to prepare compound 13 after Boc removal (Scheme 5). In this derivative, the modification at C3' might not only overcome the resistance caused by APH(3') but also it might act as an electrophilic trap. In this latter case, the kanamycin derivative 13 could react with a nucleophilic residue, in the binding site of APH enzymes (APH(3')-Ia and APH(3')-IIa – Figure 13), thereby covalently blocking the substrate binding site of the enzyme. In particular, for APH(3')-IIa in Figure 13B, this is an attractive approach as the active site of this enzyme contains a suitably positioned cysteine residue that may attack the Michael acceptor. The distance between the 3'-OH group of kanamycin and the thiol of this cysteine residue is approximately 4.7 Å, a distance that is expected to be reduced with the installation of our desired modification and hence this cysteine may react with the Michael acceptor to make a covalent bond.

Scheme 5. General reaction scheme for the synthesis of compound 13 (a) and (b) proposed reaction scheme of cysteine and kanamycin derivative obtained upon cross-metathesis of reaction on allyl-kanamycin
Figure 13. Interaction of kanamycin (cyan sticks) and cysteine residues (magenta lines) in the binding pocket in A) APH(3')-Ia (PDB: 4FEU) and B) APH(3')-IIa (PDB: 1ND4). Visualization on PyMol.

For our study, we used a chelating benzylidene-ether pre-catalyst (Hoveyda-type catalyst)\(^35\). We opted for the Grubbs-Hoveyda 2\(^{nd}\) generation catalyst containing an N-heterocyclic carbene (NHC). The reaction was performed in the presence of allyl kanamycin, ethyl acrylate, Grubbs-Hoveyda catalyst in 1,2-dichloroethane (DCE) as the non-coordinating solvent. The reaction was carried out in the dark under an inert atmosphere and stirred for 24 hours or upon completion. The product was isolated in 15% yield as the E isomer. Deprotection in TFA: CH\(_2\)Cl\(_2\) 0.5:1 followed to give full conversion of the final product \(13\) in its TFA salt form.

4.4.6 Epoxidation of terminal alkene from 3'-allyl-(Boc)-kanamycin

Starting from the allyl kanamycin derivative we aimed to prepare derivatives that could perform as an inhibitor of the APH(3') enzymes. In this case, we opted for epoxidation of the alkene in the presence of \(m\)CPBA. This approach would lead, after deprotection in TFA: CH\(_2\)Cl\(_2\) 0.5:1, to the formation of the epoxide derivative \(14\) (Scheme 6) that in presence of cysteine residues or other nucleophiles in the binding pocket could form a covalent bond. Consequently, epoxide \(14\) might be a covalent inhibitor of APH(3')s.

![Scheme 6. Cysteine residues and epoxide ring-opening](image)

\(^35\)This type of catalyst was chosen due to its known efficiency in carbene functionalization reactions.
We performed the reaction with 3'-allyl-(Boc)-\(\text{\textregistered}\)kanamycin as the starting material. \(m\)CPBA was used as the reagent and chloroform as the solvent.\(^{40}\) The reaction led to a complex mixture that was very difficult to purify. Very tailing spots showed, perhaps, the presence of two co-eluting products as this could be expected by the formation of two diastereomers. However, at this stage, an attempted purification did not lead to the removal of residual \(m\)CPBA. Therefore, it was decided to carry out the deprotection reaction on the crude mixture and do further analysis on the final product mixture in order to confirm if the desired product was indeed formed and to perhaps be able to remove \(m\)CPBA by decantation. The \(^1H\)-NMR spectrum of this crude product mixture clearly showed the presence of signals indicative of an epoxide (as reported in the experimental part of this chapter). However, the analysis remained still very complex and not a clean spectrum was obtained.

### 4.4.7 Wittig reaction of 3'-keto-(Boc)-\(\text{\textregistered}\)kanamycin

Starting from keto-kanamycin we also decided to investigate other chemical modifications. M. Duca in our group had already shown that a reaction between ethyl 2-(triphenyl-\(\lambda^5\)-phosphaneylidene)acetate and methyl-3-keto-glucose gave an \(E/Z\) mixture of the Wittig products (A) and the cyclized corresponding lactones (B and C) (Scheme 7).

![Scheme 7. Wittig reaction and possible products on \(\alpha\)-OMe-glucose](image)

The approach is attractive as with a single reaction we could potentially get 3 kanamycin derivatives (15, 16 and 17), for which it will be necessary to determine further whether there is selectivity in the formation of products 15, 16, or 17 (Figure 14). The three products would lead to a modification of C3' and therefore phosphorylation would be avoided. In addition, compound 16 would lead to block the 4'-OH group and therefore this modification may possibly overcome resistance both by APH(3') and by ANT(4'). Important to mention is that compound 17 could also be less likely the major product due to steric hindrance that could make its formation unfavorable.
The reaction was performed under the same conditions as used by M. Duca. A mixture containing the desired 3'-keto-(Boc)-kanamycin and traces of 3'-(Boc)-kanamycin was suspended in acetonitrile and 3 eq of the Wittig reagent were added. The reaction mixture was heated at 50 °C. After overnight stirring, an additional amount of Wittig reagent was added. Silica gel chromatography using CH₂Cl₂ and MeOH as the eluent was performed before deprotection. However, very tailing spots were observed on TLC. Separation via silica gel chromatography was very troublesome. It seemed that most of the remaining and unreacted (Boc)-kanamycin was removed but NMR analysis was inconclusive. It was nevertheless decided to deprotect the mixture in the presence of TFA: CH₂Cl₂:0.5:1 also in order to get a better interpretation of the NMR spectrum. From the observed triplets at 6.09 and 6.14 ppm which correspond to the CH proton of the modified segment, we concluded that the desired products had formed and this also suggests that only two products were formed, most likely being E/Z isomers. These protons also have an HSQC correlation with carbons at 113 ppm and 114 ppm confirming that the desired reaction had taken place. However, at this stage, the difficulty in analyzing the NMR spectra did not clarify which of the three products was formed. Next to this, it remained unclear whether also kanamycin was still present. Being certain that no kanamycin is present in the final product is important for the biological assays. The presence of kanamycin interferes with the results in determining the potential antibacterial activity of the prepared derivative together with its hypothesized inhibitory activity of APH(3') enzymes.

4.4.8 Epoxidation of 3'-keto-(Cbz)-neomycin

Another possible modification was applied on 3'-keto-neomycin. The approach came from a recent work reported by our group. The idea was to install a good electrophile, an epoxide (Scheme 8), which could react with nucleophiles in the enzyme binding pocket.
Initially, we attempted to prepare the epoxide from purified 3'-keto-(Cbz)-Neomycin via the Corey-Chaykovsky reaction with trimethylsulfoxonium iodide (1.15 eq), NaH (1.05 eq) in DMSO (2.5 M). However, UPLC-MS analysis showed that a product formed with a mass that corresponds to E1cB elimination of the gluco-configured ring (Figure 15).

Therefore we switched to the use of diazomethane as recently reported by our group. This approach showed full conversion to the desired product. A mixture of the axial and the equatorial epoxide was formed, as two peaks with the same mass and very similar retention time was observed in the chromatogram of the UPLC-MS. The obtained products were very difficult to separate hence they were deprotected as a mixture via Pd/C and Pd(OH)$_2$/C hydrogenolysis. However, upon deprotection a complex mixture of products was formed which could not be identified.
4.5 Conclusions

In conclusion, we have synthesized several aminoglycoside derivatives with inverted stereochemistry at C3’ from the keto-AGAs. Reduction with NaBH₄ gave the 3’-epi-paromomycin 7 and indium-mediated allylation/propargylation led to the formation of compounds 8-12. The intermediates bearing these aminoglycoside derivatives were not obtained in high yields, especially for the Boc-protected kanamycin derivatives. The low isolated yields are the result of the low solubility of protected AGAs in organic solvents, which complicates their purification. Nevertheless, the desired derivatives were obtained in sufficient quantity to perform the biological studies (see chapter 5). The purity of all the prepared compounds used to carry out the biological assays were determined by qNMR analysis (see experimental part of this chapter for further details).

Next, starting from the indium-mediated allylation we also investigated further modifications with the main goal to prepare potential APH(3’) inhibitors via covalent interaction with the cysteines residues and other nucleophiles present in the binding pocket, leading to the preparation of 13 and 14. Another attempted approach starting from 3’-keto-(Boc)-kanamycin aimed at the preparation of single and/or double modified kanamycins (15-17) upon reaction with a stabilized Wittig reagent as reported in paragraph 4.4.7. However, both this last attempt and the one leading to compound 14 led to complex mixtures. These last reactions would need further work as they seemed to be promising. However, better purification methods are required to separate the product mixtures to fully characterize the obtained derivatives and test their biological activity.

As already mentioned, the key step leading to regioselective oxidation was not trivial. Incomplete conversion was obtained in all attempts and this hampered the purification of the keto products and, in the case of protected kanamycin, the keto product could not be separated from the unreacted starting material. Consequently, a mixture of protected keto-kanamycin and kanamycin was used in the follow-up modification reactions. Although the remaining protected kanamycin neither reacted, nor interfered with these follow up reactions, it did complicate the purification of products deriving from these reactions. This had an important impact on the final recovery of the desired modified kanamycins before their deprotection.
4.6 Experimental section

General remarks

All solvents used for extraction, filtration, and chromatography were of commercial grade and used without further purification. [(neocuproine)PdOAc](OTf)2 was prepared according to literature procedure.37 Benzoquinone was purified by recrystallization from ethanol. Celite (Celite® 545) was purchased from Merck. The palladium on carbon was supplied by Alfa Aesar (Palladium, 10% on carbon, Type 487, dry). Other reagents were purchased from Sigma-Aldrich, TCI, Fluorochem, and Acros and were used without further purification. Flash chromatography was performed manually with silica (SiliaFlash P60, 230-400 mesh, Silicycle) or performed with automated column chromatography using a Reveleris flash chromatography system purchased from Buchi. TLC was performed on Merck silica gel 60, 0.25 mm plates, and visualization was done by staining with anisaldehyde stain (a mixture of 135 mL EtOH, 5 mL H2SO4, 1.5 mL AcOH and 3.7 mL p-anisaldehyde) or ninhydrin stain (a mixture of 1.5 g ninhydrin, 100 mL n-butanol, and 3 mL AcOH). 1H-, 13C-NMR, APT-, HSQC, and TOCSY were recorded on a Bruker Avance NEO 600 (600, 150.9 MHz, respectively) at 25°C using MeOD-d4, DMSO-d6, or D2O as the solvent. Chemical shift values are reported in ppm with the solvent resonance as the internal standard (MeOD-d4: δ 3.31 for 1H, δ 49.00 for 13C; DMSO-d6: δ 2.50 for 1H, δ 39.52 for 13C; D2O: δ 4.79 for 1H). Data are reported as follows: chemical shifts (δ), multiplicity (s = singlet, d = doublet, dd = doublet of doublets, ddd = doublet of doublet of doublets, dddd = doublet of doublet of doublet of doublets, dt = doublet of triplets, ddt = doublet of doublet of triplets, dq = doublet of quartets, t= triplet, td = triplet of doublets, q = quartet, m = multiplet, bs = broad singlet), coupling constants J (Hz), and integration. High-resolution mass spectra (HRMS) were recorded on a Thermo Scientific LTQ Orbitrap XL. Residues of palladium were removed by using an MP-TMT resin purchased from Biotage. All UPLC-MS analysis for monitoring of reactions was performed using a Thermo Fischer Vanquish UPLC system in combination with Thermo Fischer LCQ Fleet Mass Spectrometer System. Separation of the components in the crude mixtures was achieved using an ACQUITY UPLC BEH C18 1.7 µm column, of the dimension 2.1 x 150 mm, using a gradient as shown in the following graph.
Graph 1. UPLC solvent gradient and MS settings used for monitoring the reaction progress

Solvent base (solvent A): water containing 0.1% of formic acid

Solvent B: acetonitrile containing 0.1% of formic acid

Quantitative NMR (qNMR) was used to determine the purity of the final compounds. The spectra were recorded by using dimethyl sulfone (DMS) TraceCERT purchased from Sigma as standard for quantitative NMR. The qNMR experiment was run either with DMS as an internal calibrant or as an external calibrant. For the internal calibration qNMR, the purity was determined by dissolving an accurately weighed amount of the sample (3.00-5.00 mg) and the internal calibrant (IC) dimethylsulfone in MeOD-d4 or D2O (0.6 mL). The experiment was recorded on a Bruker Avance NEO 600 with a Pulse Width of 90°, at 25 °C. Relaxation delay (d1) was adjusted at 60 s, acquisition time (AQ) of 4 s, number of scans (nt) of 32. The calculation of the purity (P%) was calculated as follow:

\[
P[\%] = \frac{n_{IC} \cdot Int_{IC} \cdot MW_{t} \cdot m_{t}}{n_{t} \cdot Int_{t} \cdot MW_{IC} \cdot m_{s}} \cdot P_{IC}
\]

where Int is the integral, MW is the molecular weight, m is the mass, n is the number of protons, P is the purity (in %), IC is the internal calibrant, s is the sample, and t is the target analyte/molecule. For a more detailed description see reference 38. The MW of the analyte was calculated as in its salt form, either trifluoroacetate (for the kanamycin derivatives) or in its acetate form (for the paromomycin derivatives). The purity of four compounds was determined by internal standard calibration, while for two compounds this was determined by external calibration due to the low amount
available. In any case, the used qNMR calibration is specified in the experimental part. External standard calibration was accomplished by measuring a series of dimethylsulfone (DMS) samples. The most concentrated sample (0.128 mol/L) was prepared by dissolving 14.5 mg DMS in 1.2 mL DMSO-d$_6$. From this solution, 0.6 mL was taken and diluted 2 times by adding another 0.6 mL DMSO- d$_6$. This procedure was repeated resulting in dilution series of 8 different concentrations with 0.001 mol/L as the lowest concentration. $^1$H NMR experiments were recorded at 25 °C on a Bruker Avance NEO 600 MHz instrument using 16 repetitions of a relaxation delay of 60 s, a 90° pulse, an acquisition time of 4.99 s. The integrated resonance lines were fitted using linear regression with a correlation coefficient of 0.999893. The unknown purities (P%) were calculated by dividing the concentrations as determined by qNMR by the concentrations as calculated by the weighted amount of analyte multiplied by 100%. The qNMR experiment was performed in order to correct the concentration used in the biological assay for the presence of NMR silent impurities (salts) and/or for the amount of water in the final amount (aminoglycosides are known for their high hygroscopicity). Based on these points we know that the relatively low purity obtained for the paromomycin derivatives other than caused by the high amount of water present in the sample is also because of a high amount of acetate salts. Both acetate and water were not removed by several freeze-drying cycles. Therefore the qNMR experiment was used to correct the sample purity. The reported yields of the final compounds were, therefore, reported by taking into consideration their qNMR-based purity.

**N-(Cbz)-Paromomycin (2)**

To a solution of paromomycin sulfate 1 (4 g, 5.6 mmol, 1 eq) in H$_2$O (29 mL), a solution of K$_2$CO$_3$ (2.7 g, 19.6 mmol, 3.5 eq) in H$_2$O (18 mL) was added. Then THF was added (30 mL) followed by dropwise addition of N-(benzyloxycarbonyloxy)-succinimide (12.6 g, 50.4 mmol, 9 eq) in THF (62 mL) over 30 min. A white suspension was formed which was stirred at rt overnight. After complete conversion (TLC in CH$_2$Cl$_2$: MeOH 95:5, detected with anisaldehyde stain), the reaction was quenched with glycine (3.8 g, 50.4 mmol, 9 eq) in a saturated solution
of NaHCO$_3$ (20 mL). The resulting mixture was stirred for 1 h at room temperature. THF was then removed in vacuo. Next, the mixture was basified with 1 M NaOH to pH 9. The resulting mixture was then extracted with EtOAc. The combined organic layers were dried over MgSO$_4$, filtered and concentrated in vacuo. The white solid was purified by flash column chromatography in CH$_2$Cl$_2$: MeOH (gradient 0%-10%). The desired product 2 was obtained as a white foam (4.1 g, 3.2 mmol, 86%) $^{38,39}$. The yield was adjusted based on the purity of the starting material (paromomycin sulfate) which was 64% (qNMR-based).

$^1$H NMR (400 MHz, Methanol-$d_4$) $\delta$ 7.45 – 7.22 (m, 25H), 5.28 – 4.94 (m, 12H), 4.82 (d, 1H), 3.98 (s, 1H), 3.91 (m, 3H), 3.81 (m, 3H), 3.78 – 3.69 (m, 3H), 3.69 – 3.54 (m, 5H), 3.49 (m 3H), 3.42 – 3.33 (m, 4H), 1.98 (bd, $J = 12.7$ Hz, 1H), 1.40 (m, 1H).

$^{13}$C NMR (151 MHz, Methanol-$d_4$) $\delta$ 174.9, 174.5, 159.2, 159.1, 159, 158.8, 158.7, 158.3, 158.2, 158.1, 129.6, 129.56, 129.5, 129.4, 129.3, 129.2, 129.1, 129.04, 129, 128.9, 128.89, 128.8, 128.6, 110.6, 100.5, 100.2, 100.16, 87.3, 83.7, 81.2, 79, 78.1, 75.8, 74.9, 74.5, 72.6, 71.6, 71.5, 70.3, 69.2, 67.9, 67.8, 67.7, 67.6, 67.4, 63.4, 63.1, 62.8, 57.8, 54.1, 52.3, 52.2, 49.9, 49.4, 49.3, 49.1, 49, 48.9, 48.7, 48.6, 43.5, 42.6, 35.4. HRMS (ESI+) calculated for C$_{63}$H$_{75}$O$_{24}$N$_{5}$Na ($[\text{M+Na}]^+$): 1308.4694, found: 1308.4714.

$N$-(Boc)-Kanamycin (4)

Kanamycin A sulfate 3 (5 g, 8.6 mmol, 1 eq) was suspended in H$_2$O (20 mL). Triethylamine (4.8 mL, 34.3 mmol, 4 eq) was added together with a solution of di-tert-butyldicarbonate (15 g, 68.7 mmol, 8 eq) in DMSO (60 mL). The reaction mixture was stirred overnight at rt. The reaction was monitored by UPLC-MS. Upon completion, the solid was filtered using a glass sintered funnel and the residue was washed once with H$_2$O. The resulting white solid was dried under vacuum to give the desired product 4 (7 g, 92%)$^{30,39}$.

$^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 6.97 – 6.85 (m, 1H), 6.59 (d, $J = 7.1$ Hz, 1H), 6.51 (d, $J = 9.1$ Hz, 1H), 6.36 (t, $J = 5.0$ Hz, 1H), 5.44 – 5.35 (m, 1H), 5.26 (bs, 1H), 4.99 – 4.85 (m, 4H), 4.72 (d, $J = 6.4$ Hz, 1H), 4.24 (t, $J = 5.5$ Hz, 1H), 3.81 (dt, $J = 10.3, 3.2$ Hz, 1H), 3.57 (dd, $J = 8.6, 5.1$ Hz, 1H), 3.49 (t, $J = 4.6$ Hz, 3H), 3.36 (d, $J = 10.3$ Hz, 1H), 3.32 – 3.16 (m, 3H), 3.06 (dt, $J = 12.5, 6.1$ Hz, 1H), 1.85 – 1.75 (m, 1H), 1.44 – 1.32 (m, 37H). $^{13}$C NMR
(101 MHz, DMSO-d$_6$) $\delta$ 156.5, 156.3, 155.1, 101.2, 97.9, 84.1, 80.3, 77.99, 77.94, 77.4, 75.1, 73, 72.8, 72.2, 70.4, 70.2, 67.4, 60.3, 56, 50.2, 49.1, 41.5, 40.4, 34.9, 28.5, 28.3 $^{(*)}$ (12 CH$_3$ in tot). **HRMS (ESI$^+$) calculated for C$_{38}$H$_{69}$O$_{19}$N$_4$ ([M+H]$^+$): 885.4551, found: 885.4561

$N$-(Cbz)$_{-}$3'-keto-Paromomycin (5)

$N$-(Cbz)$_{-}$paromomycin 2 (1 g, 0.7 mmol, 1 eq) was dissolved in MeOH (8 mL). Benzoquinone (92 mg, 0.9 mmol, 1.1 eq) was added. Upon dissolution, [(neocuproine)PdOAc$_2$(OTf)$_2$ (20.4 mg, 0.02 mmol, 2.5 mol%) was added and the dark orange solution was stirred overnight at rt. An additional 5 mol% of catalyst was added (total 61.1 mg, 0.06 mmol, 7.5 mol%) and the mixture was heated to 45 °C. After 5 d, the reaction was stopped although incomplete conversion was observed (CH$_2$Cl$_2$: MeOH 9:1, stained with anisaldehyde). The mixture was concentrated *in vacuo*. The resulting orange foam was purified by manual column chromatography (CH$_2$Cl$_2$: MeOH gradient 0%-10%). The desired product was obtained as a light orange foam (871 mg, 0.68 mmol, 87%) containing traces of catalyst and benzoquinone. The real yield was calculated as in the following equation:

$$m_3 = m_{tot} \frac{i_3}{N_3} * MW_3 + \frac{i_{BQ}}{N_{BQ}} + \frac{i_{catalyst}}{N_{catalyst}}$$

$$m_3 = m_{tot} \frac{i_3}{N_3} * MW_3 + \frac{i_{BQ}}{N_{BQ}} * MW_{BQ} + \frac{i_{catalyst}}{N_{catalyst}} * MW_{catalyst}$$

$m$ is the mass, $i$ represents the integrals [$i_3$ is one of the integral referring to the compound 3 ($N$-(Cbz)$_{-}$3'-keto-Paromomycin); $i_{BQ}$ refers to the benzoquinone; $i_{catalyst}$ refers to one of the integral of the catalyst, $N$ is the number of nuclei represented by the peak, $MW$ stands for molecular weight. The real mass of the obtained product (3) is 644.5 mg (0.5 mmol, 65%). The reaction was also performed on a smaller scale. The reagents were used in the following amounts: $N$-(Cbz)$_{-}$Paromomycin (500 mg 0.39
mmol, 1 eq), MeOH (3.9 mL), benzoquinone (46.2 mg, 0.43 mmol, 1.1 eq),
[(neocuproine)PdOAc]2(OTf)2 (30.5 mg, 0.03 mmol, 7.5 mol%). In this case, due to a
smaller scale, purification leads to a cleaner isolated product. The recovered yield for
the 500 mg scale is 48% (240 mg, 0.19 mmol). NMR spectra attached to this file are
reported for both 1g scale and 500 mg scale reactions. The 1g scale product was then
used to perform the further steps as more material was aimed to obtain in order to be
able to perform the biological assays and since a further purification would follow
prior to the deprotection step.

\[ ^1\text{H NMR (400 MHz, Methanol-}d_4 \text{)} \delta 7.32 (m, 25H), 5.08 (m, 12H), 4.69 (d, } J = 4.4 \text{ Hz, 1H), 4.33 (d, } J = 9.8 \text{ Hz, 1H), 4.05 – 3.80 (m, 8H), 3.79 – 3.69 (m, 2H), 3.67 – 3.56 (m, 3H), 3.50 (bs, 3H), 3.44 – 3.34 (m, 4H), 2.03 – 1.91 (m, 1H), 1.50 – 1.35 (m, 1H). ^{13}\text{C NMR (101 MHz, Methanol-}d_4 \text{)} \delta 205, 159, 158, 151, 147.8, 138.3, 129.7, 129.5, 129.4, 129.2, 129, 128.9, 128.84, 128.81, 128.7, 116.8, 102.4, 96.4, 86, 73.5, 71.6, 69.2, 67.7, 67.6, 61.8, 54, 42.6, 42.6, 26.3. \text{HRMS (ESI+) calculated for C}_{63}\text{H}_{74}\text{O}_{24}\text{N}_{5} ([M+H]^{+}): 1284.4718, found: 1284.4755.} \]

\[ N-(\text{Boc})-3'\text{-keto-Kanamycin (6)} \]

\[ \text{(Boc)}-\text{kanamycin 4 (1eq) was dissolved in DMSO. Benzoquinone (3 eq) was added. Upon dissolution, [(neocuproine)PdOAc]}_2(\text{OTf})_2 (2.5 \text{ mol%}) \text{ was added and the dark brown solution was stirred at rt. The reaction was monitored through UPLC-MS and quenched when at least 50% of the starting material was converted to the desired product. The reaction was quenched by adding five volumes of water compared to the DMSO used. The precipitate was collected by filtration over a sintered-glass filter and washed with water and dried under vacuum to give the desired mono-oxidized product. Different batches of the starting material required different reaction times to reach at least the desired 50% conversion. However, occasionally this led to the formation of an undesired di-oxidized product as shown in one of the UPLC-MS traces below. This was observed independently of the reaction scale. Full conversion to one or both products was observed only once and in all other attempts, it was decided to stop the reaction in order to avoid the undesired over-oxidation. The crude was used without any further purification. The} \]

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The second oxidation site was determined by TOCSY-NMR after indium-mediated allylation of the crude oxidation mixture, and subsequent separation of the mono- and di-allylated Boc protected Kanamycin.

The batches used to run the indium-mediated alkylation come from several attempts as described in the procedures for the synthesis of derivatives 9, 10, and 11 in this supporting information.

**N-(Cbz)₃-3’-epi-Paromomycin (7)**

\[
N-(
\text{Cbz})₃-3’-\text{keto-Paromomycin 5 (295 mg, 0.2 mmol, 1 eq) was dissolved in MeOH (8.2 mL). Sodium borohydride (43.5 mg, 1.2 mmol, 5 eq) was added. The reaction mixture was stirred overnight at rt. Full conversion was observed (TLC CH₂Cl₂:MeOH 9:1, stained with anisaldehyde) and the mixture was subsequently stirred for one hour with 50 mg of amberlite-IR120 resin (H⁺ form), filtered and concentrated in vacuo. The resulting orange foam was purified by flash column chromatography (CH₂Cl₂:MeOH gradient 0%-10%) to give the desired product 7 as a light orange foam in 103.5 mg. Yield was calculated by taking into account the real amount of 3’-keto-paromomycin used (218.3 mg) as being of 0.08 mmol, 47%).
\]
\(^1\text{H NMR}\) (600 MHz, Methanol-\textit{d}_4) \(\delta\) 7.59 – 7.07 (m, 25H), 5.28 – 4.94 (m, 13H), 4.78 (s, 1H), 3.98 (t, 1H), 3.93 (dt, \(J = 6.6, 3.2\) Hz, 3H), 3.90 – 3.85 (m, 2H), 3.85 – 3.80 (m, 3H), 3.69 – 3.63 (m, 2H), 3.62 – 3.52 (m, 4H), 3.51 – 3.48 (m, 1H), 3.48 – 3.36 (m, 4H), 2.07 – 1.95 (m, 1H), 1.45 (s, 1H).

\(^{13}\text{C NMR}\) (151 MHz, Methanol-\textit{d}_4) \(\delta\) 159.2, 159, 158.6, 158.5, 138.3, 138.1, 137.8, 129.7, 129.65, 129.53, 129.52, 129.4, 129.1, 129, 128.97, 128.93, 128.9, 128.8, 128.7, 100.5, 99.96, 99.93, 86.2, 83.6, 78.2, 78.17, 75.8, 75.3, 74.5, 72.1, 72, 71.5, 69.5, 69.2, 68.2, 67.9, 67.6, 67.5, 62.6, 54.1, 53.4, 49.9, 48.6, 42.6, 30.7, 21. HRMS (ESI+) calculated for C\(_{63}\)H\(_{75}\)O\(_{24}\)N\(_5\)Na ([M+Na\(^+\)]: 1308.4694, found: 1308.4710

\(N\)-(Cbz)-3'-allyl-Paromomycin (8)

To a solution of 3'-keto-paromomycin 5 (weighed 530 mg, 0.4 mmol, 1eq) in THF:H\(_2\)O (14 mL, 1:1 v/v), indium powder (61.6 mg, 0.5 mmol, 1.3 eq) and allyl bromide (54 µL, 0.6 mmol, 1.5 eq) were added. The reaction mixture was stirred at rt and monitored by TLC (CH\(_2\)Cl\(_2\):MeOH 5%) and stained with \(p\)-anisaldehyde. After two days, full conversion to a less polar product was observed. The mixture was filtered over filter paper to remove part of the indium salts and concentrated \textit{in vacuo}. Flash column chromatography in CH\(_2\)Cl\(_2\):MeOH (gradient 2%–15%) gave the desired product 8 as a dark orange amorphous solid in 297 mg. Yield was calculated by taking into account the real amount of 3'-keto-paromomycin used (392 mg) as being of 0.22 mmol, 74%.

\(^1\text{H NMR}\) (600 MHz, Methanol-\textit{d}_4) \(\delta\) 7.42 – 7.20 (m, 25H), 5.91 – 5.74 (m, 1H), 5.30 – 4.91 (m, 15H), 4.09 – 3.77 (m, 8H), 3.77 – 3.51 (m, 7H), 3.52 – 3.43 (m, 3H), 3.43 – 3.33 (m, 3H), 2.44 (dd, \(J = 13.7, 8.4\) Hz, 1H), 2.31 – 2.21 (m, 1H), 2.05 – 1.95 (m, 1H), 1.49 – 1.38 (m, 1H). \(^{13}\text{C NMR}\) (151 MHz, Methanol-\textit{d}_4) \(\delta\) 164.3, 159.2, 159, 141, 138.3, 138.1, 134.2, 129.7, 129.6, 129.5, 129.4, 129.1, 129, 128.9, 128.8, 128.7, 127.7, 127.6, 119.9, 119.6, 101.2, 100.7, 100.5, 86.4, 83.6, 78, 76, 75.1, 74.4, 72.2, 71.5, 68.3, 67.8, 67.6, 67.5, 64.3, 63.2, 62.8, 54.1, 53.5, 42.6, 39.1, 30.7, 30, 29.5, 24.2. HRMS (ESI+) calculated for C\(_{66}\)H\(_{79}\)O\(_{24}\)N\(_5\)Na ([M+Na\(^+\)]: 1348.5007, found: 1348.5022
This first batch of reaction containing crude product (total weight of SM: 4g) was used to run the indium-mediated allylation leading to compound 9. The estimated conversion to the desired N-(Boc)-3’-keto-Kanamycin 6 was 50%. Peak with a retention time of 16.07 as showed in the Base Peak MS corresponds to the unreacted N-(Boc)-Kanamycin 4 (mass value of 782.51, with the loss of 1 Boc). Peak with a retention time of 16.44 as showed in the Base Peak MS corresponds to the N-(Boc)-3’-keto-Kanamycin 6 (mass value of 780.52, with the loss of 1 Boc group). This observation leads to setup the indium-mediated allylation reaction when considering the amount of the starting material 6 to be around 2g.
To a solution of \( N\text{-}(Boc)\text{-3'}\text{-keto-kanamycin} \) containing unreacted \( N\text{-}(Boc)\text{-kanamycin} \) in THF:MeOH (60 mL, 1:1 v/v), indium powder (390 mg, 3.4 mmol, 1.5 eq) and allyl bromide (0.3 mL, 3.4 mmol, 1.5 eq) were added. The suspension changed color from dark orange to dark green. The reaction mixture was stirred at rt and monitored both by TLC (CH\(_2\)Cl\(_2\): MeOH 5%; staining with ninhydrin), and by UPLC-MS. After five days a more apolar product was observed. The mixture was filtered over filter paper to remove part of the indium salts and concentrated in vacuo. A hardly soluble material was obtained. The presence of a small amount of unreacted SM was observed. Silica gel column chromatography in CH\(_2\)Cl\(_2\): MeOH (from 2% to 15%) followed. The desired monoallylated product (9) was obtained as an orange solid (210 mg, 0.23 mmol, 10%) (210 mg, 0.23 mmol, 5% over two steps). The recovery was low due to the presence of mixed fractions and still a low solubility of the kanamycin derivative. This second batch crude of reaction (total weight 1.9 g) was used to run another indium-mediated allylation leading to compounds 9 and 10. The estimated conversion to the desired \( N\text{-}(Boc)\text{-3'}\text{-keto-Kanamycin} \) was not easy to calculate due to a poor retention time. The indium-mediated allylation was therefore set up considering the whole 1.9 g as being the SM. Excess of reagents are therefore being used, however no side reactions are expected. A closer look to the peak showed an apparently almost full conversion to the desired keto-derivative (mass value of 780.70, with the loss of 1 Boc) together with the formation of the double oxidized kanamycin derivative as by-product (mass value of 778.75, with the loss of 1 Boc). The final yield is determined by considering a higher amount of starting material, due to the presence of traces of unreacted \( N\text{-}(Boc)\text{-Kanamycin} \). Therefore, the final yields could be higher than the one reported.
To a solution of \(\text{(Boc)}^4\)-3'-keto-kanamycin containing some \(\text{(Boc)}^4\)-3',4''-di-keto-kanamycin (1.9 g, 2.2 mmol, 1 eq) in THF:H\text{2}O (60 mL, 1:1 v/v), indium powder (329 mg, 2.9 mmol, 1.3 eq) and allyl bromide (0.3 mL, 3.3 mmol, 1.5 eq) were added. The suspension changed color from dark orange to dark green. The reaction mixture was stirred at rt and monitored both by TLC (\text{CHCl}_3: \text{MeOH} 5\%; staining with ninhydrin), and by UPLC-MS. After three days full conversion to a more apolar product was observed. The mixture was filtered over filter paper to remove part of the indium salts and concentrated \textit{in vacuo}. Silica gel column chromatography in \text{CHCl}_3: \text{MeOH} (from 2\% to 15\%) followed. The desired monoallylated product (9) was obtained as a grey solid (290 mg, 0.31 mmol, 28\%) and the di-allylated side-product (10) was obtained as brown solid (112 mg, 0.12 mmol, 10\%). In the TOCSY-NMR of the diallylated product, a spin system correlation between the protons at C5'' and C6'' but not at C4'' was observed, strongly suggesting the presence of a second allyl substituent at C4''. The recovery was low due to a lot of mixed fractions and to the relatively poor solubility of the kanamycin derivatives.

\textit{N-}(\text{Boc})^4\text{-3'\text{-allyl-kanamycin}}: ^1\text{H NMR} (600 MHz, Methanol-\text{d}_4) \delta 5.80 \text{ (ddt, } J = 17.6, 10.0, 7.7 \text{ Hz, 1H), 5.20 – 5.05 \text{ (m, 4H), 4.02 \text{ (m, 1H), 3.90 \text{ (d, } J = 10.1 \text{ Hz, 1H), 3.80 \text{ (dd,}
$J = 11.7, 2.4 \text{ Hz, 1H)}, 3.72 - 3.63 \text{ (m, 3H)}, 3.56 - 3.47 \text{ (m, 5H)}, 3.43 - 3.37 \text{ (m, 3H)}, 3.34 \text{ (s, 1H)}, 3.26 \text{ (d, } J = 10.1 \text{ Hz, 1H)}, 2.48 \text{ (d, } J = 7.7 \text{ Hz, 2H)}, 2.10 \text{ (m, 1H)}, 1.45 \text{ (m, 37H)}.$

$^{13}$C NMR (151 MHz, Methanol-$d_4$) $\delta$ 159.5, 158, 157.7, 134.7, 119.5, 103.2, 99.9, 85.6, 80.6, 80.4, 80.1, 76.8, 76.7, 74.5, 72.2, 69.9, 69.2, 69, 67.8, 62.4, 54.5, 50.8, 42, 40.4, 38.3, 28.9-28.8 (9 CH$_3$).

HRMS (ESI+) calculated for C$_{41}$H$_{72}$O$_{19}$N$_4$Na$^+$: 947.4683, found: 947.4694.

$N$-(Boc)$\delta$-3',4''-diallyl-Kanamycin: $^1$H NMR (600 MHz, Methanol-$d_4$) $\delta$ 5.92 - 5.74 (m, 2H), 5.13 (m, 6H), 4.21 (dd, $J = 7.0, 2.6$ Hz, 1H), 3.90 - 3.84 (m, 2H), 3.83 - 3.74 (m, 2H), 3.74 - 3.62 (m, 3H), 3.60 - 3.46 (m, 5H), 3.38 (m, 1H), 3.27 (d, $J = 10.1$ Hz, 1H), 2.57 - 2.42 (m, 3H), 2.23 - 2.13 (m, 2H), 2.10 - 2.04 (m, 1H), 1.51 - 1.39 (m, 37H). $^{13}$C NMR (151 MHz, Methanol-$d_4$) $\delta$ 159.4, 158.6, 157.9, 157.7, 134.5, 133.6, 119.6, 119.1, 102.9, 99.9, 85.3, 82.6, 76.7, 76.6, 76.4, 73.4, 73.3, 70.1, 68.9, 67.6, 62.2, 61.7, 55.9, 54.7, 50.7, 41.9, 40.8, 38.3, 29.5-28.7 (12 CH$_3$). HRMS (ESI+) calculated for C$_{44}$H$_{76}$O$_{19}$N$_4$Na$^+$: 987.4996, found: 987.5007.

$N$-(Boc)$\delta$-3'-propargyl-Kanamycin (11)

A crude reaction mixture of the oxidation reaction (total weight 147 mg) was used to run the indium-mediated propargylation leading to compound 11. The estimated conversion to the desired $N$-(Boc)$\delta$-3'-keto-Kanamycin 6 was hard to determine due to a very poor retention time and an improper separation of peaks. However, from the below-reported picture peak with a retention time of 0.98 min as shown in the Base Peak MS corresponds to a small amount of the unreacted $N$-(Boc)$\delta$-Kanamycin 4 and to the desired $N$-(Boc)$\delta$-3'-keto-Kanamycin 6. A peak with a retention time of 1.93 min shows a similar outcome with the presence of also the double oxidized kanamycin derivative. In this case, due to complexity in determining the ratio, the indium-mediated propargylation reaction was set up considering 147 mg as the amount of only the starting material 6. An excess of reagents could be safely used since no reaction with the other functional groups is expected. Therefore, the final yield calculated based on 147 mg of SM could be higher than the one reported.
To a solution of 3'-keto-(Boc)-kanamycin 6 (147 mg, 0.2 mmol, 1eq) in THF:H₂O (4 mL, 1:1 v/v), indium powder (28.7 mg, 0.3 mmol, 1.5 eq) and propargyl bromide (24 µL, 0.3 mmol, 1.5 eq) were added. The reaction mixture was stirred at rt and monitored both by TLC (CH₂Cl₂: MeOH 5%) and stained with ninhydrin and by UPLC-MS. After two days full conversion to a more apolar product was observed. The mixture was filtered over filter paper to remove part of the indium salts and concentrated in vacuo. Flash column chromatography in CH₂Cl₂:MeOH (gradient 0%-10%) followed. The desired product was obtained as a light grey solid (16 mg, 0.017 mmol, 10%).

¹H NMR (600 MHz, Methanol-d₄) δ 5.20 – 5.03 (m, 2H), 4.01 (ddd, J = 9.7, 5.1, 2.3 Hz, 1H), 3.92 – 3.88 (m, 1H), 3.88 – 3.75 (m, 2H), 3.73 – 3.63 (m, 4H), 3.58 – 3.46 (m, 5H), 3.46 – 3.32 (m, 4H), 2.61 (t, J = 3.1 Hz, 1H), 2.37 – 2.30 (m, 1H), 2.10 (s, 1H), 1.48 – 1.42 (m, 37H). ¹³C NMR (151 MHz, MeOD) δ 144.4, 137.7, 127.5, 124.5, 122.6, 97.1, 89.5, 80.5, 76.7, 75.4, 72.1, 65.7, 62.4, 59.8, 58.3, 55.6, 42.8, 33.4, 30.8, 29, 28.9, 28.8, 24.2, 20.9, 18.8, 18.4, 15, 14.5.
In a three-neck flask, after 3 vacuum/nitrogen cycles, Pd/C (130 mg, 0.1 mmol, 0.3 eq) and Pd(OH)$_2$ (86 mg, 0.1 mmol, 0.3 eq) were added under inert atmosphere. A small amount of ethyl acetate was added. To this slurry, methanol (2 mL) was carefully added via syringe. (Boc)$_4$-$\alpha$-propyl-kanamycin 9 (377 mg, 0.4 mmol, 1 eq) was added as a solution in methanol (4 mL). The reaction was stirred and the flask was evacuated till the solvent started to boil, then the flask was carefully backfilled with nitrogen (x3). A balloon of hydrogen was attached and the reaction flask was evacuated until the solvent began to boil and then the reaction flask was backfilled with hydrogen (x3). After overnight no conversion was observed therefore the mixture was filtered over celite and the reaction was restarted and it was left to stir overnight. After this time the reaction was completed (as indicated by UPLC-MS). The hydrogen balloon was closed off and detached. The flask was filled with nitrogen. The reaction mixture was filtered over celite and washed with water. The water layer was freeze-dried overnight and column chromatography ($\text{CH}_2\text{Cl}_2$: MeOH gradient 0%-11%) was followed to afford the product 12 in very low yield as a dark orange solid film (22 mg, 0.024 mmol, 6%).

$^1$H NMR (600 MHz, Methanol-$d_4$) $\delta$ 5.14 (s, 1H), 5.06 (d, $J$ = 3.7 Hz, 1H), 4.55 (s, 1H), 4.02 (ddd, $J$ = 10.0, 5.0, 2.5 Hz, 1H), 3.93 – 3.86 (m, 1H), 3.80 (dd, $J$ = 11.7, 2.5 Hz, 1H), 3.72 – 3.63 (m, 3H), 3.58 – 3.46 (m, 5H), 3.45 – 3.33 (m, 4H), 2.14 – 2.07 (m, 1H), 1.45 (m, 3H), 1.29 (d, $J$ = 4.2 Hz, 2H), 0.94 (t, 3H), 0.92 – 0.84 (m, 2H).

$^{13}$C NMR (151 MHz, Methanol-$d_4$) $\delta$ 157.7 (4 C), 126.2 (4 C), 105.7, 101.6, 101.4, 86.4, 80.6, 80.4, 76.8, 76.7, 74.5, 72.2, 69.5, 69, 62.4, 48.6, 42.2, 36.5, 28.9-28.8 (12 CH$_3$), 18.4, 15, 13.3. HRMS (ESI+) calculated for C$_{41}$H$_{74}$O$_{19}$N$_4$Na ([M+Na]$^+$): 949.48395, found: 949.48394
A three-neck flask was purged with 3 vacuum/nitrogen cycles, after which Pd/C (25 mg, 0.02 mmol, 0.3 eq) and Pd(OH)\(_2\) (16.4 mg, 0.02 mmol, 0.3 eq) were added under inert atmosphere. A small amount of ethyl acetate was added. To this, methanol (1.2 mL) was carefully added via syringe. N-(Cbz)-3’-epi-paromomycin 7 (100 mg, 0.08 mmol, 1 eq) in methanol (2 mL) was added. Acetic acid (22 μL, 0.4 mmol, 5 eq) was added and the reaction was stirred and the air in the flask was evacuated till the solvent started to boil, then the flask was carefully backfilled with nitrogen (process repeated 3 times). A balloon of hydrogen was attached and the reaction flask was evacuated until the solvent began to boil and then backfilled with hydrogen (x3). The reaction mixture was stirred at rt. After overnight the reaction was completed (monitoring by UPLC-MS). The hydrogen balloon was closed off and detached. The flask was filled with nitrogen. The reaction mixture was filtered over the Whatman filter and the filter was washed with water. The resulting aqueous solution was stirred for one hour with MP-TMT resin and filtered. The water aqueous solution was freeze-dried overnight to get the final product as a white fluffy powder (96 mg, 0.16 mmol, 26%). The purity as determined by qNMR was 20% (qNMR experiment with internal standard).

\( ^1H \text{ NMR} (600 \text{ MHz}, D_2O) \delta 5.51 (d, J = 4.3 \text{ Hz}, 1H), 5.37 (d, J = 2.6 \text{ Hz}, 1H), 5.23 (d, J = 1.9 \text{ Hz}, 1H), 4.51 (dd, J = 6.5, 4.9 \text{ Hz}, 1H), 4.35 (dd, J = 5.0, 2.6 \text{ Hz}, 1H), 4.28 (d, J = 5.4 \text{ Hz}, 1H), 4.19 (m, 3H), 3.98 (ddd, J = 9.0, 6.0, 2.5 \text{ Hz}, 1H), 3.93 – 3.90 (m, 1H), 3.89 (t, J = 2.2 \text{ Hz}, 1H), 3.82 – 3.73 (m, 4H), 3.72 – 3.65 (m, 2H), 3.56 (dd, J = 10.4, 9.3 \text{ Hz}, 1H), 3.52 (tdt, J = 13.5, 6.8, 3.9 \text{ Hz}, 1H), 3.46 (s, 1H), 3.41 (dd, J = 13.6, 7.0 \text{ Hz}, 1H), 3.35 (dd, J = 13.6, 3.9 \text{ Hz}, 1H), 3.24 – 3.11 (m, 2H), 2.27 (dt, J = 12.7, 4.3 \text{ Hz}, 1H), 1.58 (q, J = 12.6 \text{ Hz}, 1H). \) 

\( ^{13}C \text{ NMR} (151 \text{ MHz}, D_2O) \delta 106.1, 93, 92.6, 81, 78, 76.5, 72.1, 70.6, 70, 67.2, 65.7, 65.1, 64.4, 64, 62.5, 57.3, 57.1, 48, 47.2, 47, 46, 37.2, 27.7. \) HRMS (ESI+) calculated for C\(_{23}\)H\(_{46}\)O\(_{14}\)N\(_5\) ([M+H]\(^{+}\)) : 616.3041, found: 616.3035.

For a clearer comparison the NMR of Paromomycin sulphate was recorded too (stacked NMR available in the NMR section): \( ^1H \text{ NMR} (600 \text{ MHz}, D_2O) \delta 5.78 (d, J = 4.0 \text{ Hz}, 1H), 5.39 (d, J = 2.4 \text{ Hz}, 1H), 5.29 (s, 1H), 4.56 – 4.51 (m, 1H), 4.44 – 4.39 (m,
1H), 4.32 (dd, J = 7.4, 4.0 Hz, 1H), 4.25 (t, J = 3.2 Hz, 1H), 4.21 (dt, J = 7.3, 3.8 Hz, 1H), 4.02 – 3.89 (m, 5H), 3.84 – 3.77 (m, 2H), 3.77 – 3.70 (m, 3H), 3.59 (d, J = 3.0 Hz, 1H), 3.54 – 3.45 (m, 2H), 3.44 – 3.38 (m, 2H), 3.38 – 3.32 (m, 2H), 2.46 (dt, J = 12.6, 4.3 Hz, 1H), 1.85 (q, J = 12.6 Hz, 1H). 13C NMR (151 MHz, D2O) δ 109.97, 95.99, 95.18, 84.25, 81.14, 77.95, 74.82, 73.67, 73.14, 72.35, 70.29, 69.27, 68.95, 67.64, 67.13, 60.29, 60.08, 53.92, 50.80, 49.87, 48.89, 40.35, 28.56.

3'-propyl-Paromomycin x5AcOH (SBI236)

In a three-neck flask, after 3 vacuum/nitrogen cycles, Pd/C (48 mg, 0.05 mmol, 0.3 eq) and Pd(OH)2 (31.8 mg, 0.05 mmol, 0.3 eq) were added under inert atmosphere. A small amount of ethyl acetate was added. To this methanol (2.2 mL) was carefully added via syringe. N-(Cbz)-3’-allyl-paromomycin 8 (200 mg, 0.2 mmol, 1 eq) was added in solution in methanol (3 mL). Acetic acid (43 µL, 0.8 mmol, 5 eq) was added and the reaction was stirred. The flask was evacuated till the solvent starts to boil, then carefully backfilled with nitrogen (x3). A balloon of hydrogen was attached and the reaction flask was evacuated until solvent began to boil and then backfilled with hydrogen (x3). After 2 days no conversion to the desired product was observed and UPLC-MS indicated partial deprotection. The reaction mixture was therefore filtered over celite, fresh Pd/C (48 mg, 0.05 mmol, 0.3 eq) and Pd(OH)2 (31.8 mg, 0.05 mmol, 0.3 eq) were added, and the reaction was placed under hydrogen again. The reaction mixture was stirred at rt. After overnight the reaction was partially converted to the desired product. However, still some (partially) protected paromomycin remained. Therefore, it was decided to add more catalyst in a slurry in ethyl acetate (+ 0.2 eq more of both Pd/C and Pd(OH)2/C) and also more acetic acid was added (+ 5 eq). The reaction stirred overnight and this time full conversion was obtained according to UPLC-MS. The hydrogen balloon was then closed off and detached. The flask was filled with nitrogen. The reaction mixture was filtered over a Whatman filter and the filter was washed with water. The resulting water solution was stirred for one hour with MP-TMT resin and filtered. The water layer was freeze-
Dried to get the final product as a white powder (322 mg, 0.49 mmol, 22%). Purity, as determined by qNMR, was 10% (qNMR experiment with internal standard).

\[ ^1H \text{ NMR} (600 \text{ MHz, D}_2\text{O}) \delta 5.59 (d, J = 4.5 \text{ Hz, 1H}), 5.39 (d, J = 2.9 \text{ Hz, 1H}), 5.29 (d, J = 1.8 \text{ Hz, 1H}), 4.55 - 4.49 (dd, 1H), 4.36 - 4.29 (m, 2H), 4.24 (t, J = 3.1 \text{ Hz, 1H}), 4.21 - 4.17 (m, 1H), 3.98 - 3.91 (m, 2H), 3.89 (d, J = 3.1 \text{ Hz, 1H}), 3.88 - 3.83 (dd, J = 6.9, 2.8 \text{ Hz, 1H}), 3.82 (dt, J = 3.0, 1.3 \text{ Hz, 1H}), 3.80 - 3.73 (m, 2H), 3.72 - 3.61 (m, 2H), 3.60 - 3.53 (td, 2H), 3.43 (dd, J = 13.6, 6.8 \text{ Hz, 2H}), 3.36 (dt, J = 13.5, 4.2 \text{ Hz, 1H}), 3.30 (ddt, J = 13.8, 7.6, 3.7 \text{ Hz, 1H}), 2.42 (dt, J = 12.7, 4.6 \text{ Hz, 1H}), 1.79 - 1.74 (m, 1H), 1.61 - 1.49 (m, 2H), 1.34 - 1.21 (m, 2H), 0.93 (t, J = 7.1 \text{ Hz, 3H}). \]

\[ ^{13}C \text{ NMR} (151 \text{ MHz, D}_2\text{O}) \delta 100, 95.7, 95.6, 81.4, 73.3, 73.29, 73.1, 72.7, 70.3, 69.6, 67.7, 67.3, 60.9, 60.2, 59.8, 50.9, 50, 49.2, 47, 46.9, 41.7, 40.4, 23.2, 18.4, 16.8, 13.4. \]

HRMS (ESI+) calculated for C_{26}H_{52}O_{14}N_{5} ([M+H]^+): 658.3511, found: 658.3506.

**3’-allyl-Kanamycin x4TFA (SBI209)**

To a suspension of 3’-allyl-(Boc)-kanamycin 9 (100.2 mg, 0.1 mmol, 1 eq) in CH_{2}Cl_{2} (1.3 mL), triisopropylsilane (89 µL, 0.4 mmol, 4 eq) and trifluoroacetic acid (0.7 mL, 8.7 mmol, 80.7 eq) were added via syringe. The reaction mixture turned clear and was left to stir at rt for 1 h. UPLC-MS analysis showed completion of the reaction after 1 h. Toluene (2 mL) was added and the mixture was concentrated in vacuo to get the final product as a grey solid (100 mg, 0.1 mmol, 66%). Purity, as determined by qNMR, was 66% (qNMR experiment with internal standard).

\[ ^1H \text{ NMR} (600 \text{ MHz, D}_2\text{O}) \delta 5.82 (ddt, J = 17.5, 10.2, 7.6 \text{ Hz, 1H}), 5.45 (d, J = 4.4 \text{ Hz, 1H}), 5.27 - 5.19 (m, 2H), 5.11 (d, J = 3.6 \text{ Hz, 1H}), 4.11 (ddd, J = 10.7, 8.0, 3.2 \text{ Hz, 1H}), 3.97 - 3.92 (m, 2H), 3.88 - 3.82 (m, 2H), 3.79 (d, J = 4.9 \text{ Hz, 3H}), 3.60 (dt, J = 9.9, 2.6 \text{ Hz, 1H}), 3.54 - 3.46 (m, 2H), 3.44 (dd, J = 13.4, 3.2 \text{ Hz, 1H}), 3.16 (dd, J = 13.4, 8.0 \text{ Hz, 1H}), 2.59 - 2.46 (m, 2H), 1.91 (q, J = 12.5 \text{ Hz, 1H}). \]

\[ ^{13}C \text{ NMR} (151 \text{ MHz, D}_2\text{O}) \delta 132.3, 119.9, 100.6, 95.8, 83.9, 77.6, 72.7, 72.4, 68.1, 67.4, 66.7, 66, 65.3, 59.8, 54.9, 49.9, 47.7, 40.6, 38.7, 37.3, 27.4. \]

HRMS (ESI+) calculated for C_{26}H_{52}O_{14}N_{5} ([M+H]^+): 525.2772, found: 525.2765
3',4''-diallyl-Kanamycin x4TFA (SBI214)

To a suspension of 3',4''-diallyl-(Boc)-kanamycin 10 (112.5 mg, 0.1 mmol, 1 eq) in CH2Cl2 (1.4 mL), triisopropylsilane (96 µL, 0.5 mmol, 4 eq) and trifluoroacetic acid (0.7 mL, 9.4 mmol, 80.7 eq) were added via syringe. The reaction mixture turned clear and was let to stir at rt for 1 h. UPLC-MS analysis showed completion of the reaction after 1 h. Toluene (2mL) was added and the mixture was concentrated in vacuo to get the final product as a white solid (70 mg, 0.07 mmol, 30%). Purity, as determined by qNMR, was 48% (qNMR experiment with internal standard).

1H NMR (600 MHz, Methanol-d4) δ 5.85 (ddt, J = 17.7, 10.2, 7.5 Hz, 2H), 5.53 (d, J = 4.3 Hz, 1H), 5.34 – 5.11 (m, 4H), 4.13 – 4.04 (m, 2H), 3.99 – 3.86 (m, 3H), 3.73 (ddd, J = 10.2, 8.3, 4.2 Hz, 1H), 3.65 – 3.51 (m, 4H), 3.39 (dd, J = 13.0, 2.9 Hz, 1H), 3.00 (dd, J = 13.1, 9.2 Hz, 1H), 2.50 (m, 4H), 2.06 (q, J = 12.4 Hz, 1H).

13C NMR (151 MHz, Methanol-d4) δ 134.1, 132.2, 121, 120, 101.4, 96.4, 86, 77.8, 76.9, 76.6, 74.1, 73.2, 69.4, 68.6, 68.2, 67.2, 60.7, 54.7, 51, 49.8, 42.5, 39.6, 38.9, 28.7. HRMS (ESI+) calculated for C24H45O11N4 ([M+H]+): 565.3085, found: 565.3079

3'-propargyl-Kanamycin x4TFA (SBI233)

To a suspension of 3'-propargyl-(Boc)-kanamycin 11 (15 mg, 0.02 mmol, 1 eq) in CH2Cl2 (0.2 mL), triisopropylsilane (13 µL, 0.07 mmol, 4 eq) and trifluoroacetic acid (0.1 mL, 1.3 mmol, 0.7 eq) were added via syringe. The reaction mixture turned clear and was left to stir at rt for 1 h. UPLC-MS analysis showed completion of the reaction after 1 h. Toluene (0.5 mL) was added and the mixture was concentrated in vacuo to get the final product as a light orange solid (9 mg, 9.2 µmol, 37%). Purity, as determined by qNMR, was 63% (qNMR experiment with external standard calibration).

1H NMR (600 MHz, Methanol-d4) δ 5.54 (d, J = 4.4 Hz, 1H), 5.10 (d, J = 3.7 Hz, 1H), 4.15 (td, J = 9.7, 2.8 Hz, 1H), 3.98 (d, J = 4.4 Hz, 1H), 3.90 (m 5H), 3.81 (dd, J = 10.7, 3.7 Hz,
\( ^1 \text{H} \) NMR (600 MHz, Methanol-\( \text{d}_4 \)) \( \delta \): 5.51 (d, \( J = 4.3 \) Hz, 1H), 5.08 (d, \( J = 3.7 \) Hz, 1H), 4.09 (td, \( J = 9.5, 2.9 \) Hz, 1H), 3.90 (d, \( J = 2.3 \) Hz, 1H), 3.89 – 3.87 (m, 1H), 3.85 (dd, \( J = 7.8, 4.7 \) Hz, 2H), 3.80 (dd, \( J = 10.7, 3.6 \) Hz, 1H), 3.71 (t, \( J = 9.4 \) Hz, 1H), 3.67 – 3.63 (m, 2H), 3.50 (t, \( J = 9.8 \) Hz, 3H), 3.42 – 3.36 (m, 2H), 3.36 – 3.32 (m, 1H), 3.01 (dd, \( J = 13.0, 9.2 \) Hz, 1H), 2.48 (dt, \( J = 12.3, 4.2 \) Hz, 1H), 2.02 (q, \( J = 12.3 \) Hz, 1H), 1.80 – 1.66 (m, 5H). \( ^{13} \text{C} \) NMR (151 MHz, Methanol-\( \text{d}_4 \)) \( \delta \): 101.8, 96.6, 85.8, 77.1, 76.6, 75.3, 74.4, 70.1, 69.5, 68.7, 67.6, 67.3, 62, 56.8, 51.3, 42.6, 37, 28.7, 18.4, 15.4, 14.9. HRMS (ESI+) calculated for C\(_{21}\)H\(_{39}\)O\(_{11}\)N\(_4\) ([M+H]^+): 527.2928, found: 527.2923

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**3’-propyl-Kanamycin x4TFA (SBI238)**

To a suspension of (Boc)-3’-propyl-kanamycin 12 (22 mg, 0.02 mmol, 1 eq) in CH\(_2\)Cl\(_2\) (0.3 mL), triisopropylsilane (19 µL, 0.1 mmol, 4 eq) and trifluoroacetic acid (0.2 mL, 1.9 mmol, 80.7 eq) were added via syringe. The reaction mixture turned clear and was left to stir at rt for 1 h. UPLC-MS analysis showed completion of the reaction after 1 h. Toluene (1 mL) was added and the mixture was concentrated in vacuo to get the final product as a yellow solid (17 mg, 0.017 mmol, 36%). Purity, as determined by qNMR, was 47% (qNMR experiment with external standard calibration).

\( ^1 \text{H} \) NMR (600 MHz, Methanol-\( \text{d}_4 \)) \( \delta \): 5.51 (d, \( J = 4.3 \) Hz, 1H), 5.08 (d, \( J = 3.7 \) Hz, 1H), 4.09 (td, \( J = 9.5, 2.9 \) Hz, 1H), 3.90 (d, \( J = 2.3 \) Hz, 1H), 3.89 – 3.87 (m, 1H), 3.85 (dd, \( J = 7.8, 4.7 \) Hz, 2H), 3.80 (dd, \( J = 10.7, 3.6 \) Hz, 1H), 3.71 (t, \( J = 9.4 \) Hz, 1H), 3.67 – 3.63 (m, 2H), 3.50 (t, \( J = 9.8 \) Hz, 3H), 3.42 – 3.36 (m, 2H), 3.36 – 3.32 (m, 1H), 3.01 (dd, \( J = 13.0, 9.2 \) Hz, 1H), 2.48 (dt, \( J = 12.3, 4.2 \) Hz, 1H), 2.02 (q, \( J = 12.3 \) Hz, 1H), 1.80 – 1.66 (m, 5H). \( ^{13} \text{C} \) NMR (151 MHz, Methanol-\( \text{d}_4 \)) \( \delta \): 101.8, 96.6, 85.8, 77.1, 76.6, 75.3, 74.4, 70.1, 69.5, 68.7, 67.6, 67.3, 62, 56.8, 51.3, 42.6, 37, 28.7, 18.4, 15.4, 14.9. HRMS (ESI+) calculated for C\(_{21}\)H\(_{39}\)O\(_{11}\)N\(_4\) ([M+H]^+): 527.2928, found: 527.2923
To a solution of neomycin trisulfate (20 g, 0.02 mol, 1 eq) in H₂O (117 mL) a solution of K₂CO₃ (10.64 g, 0.07 mol, 3.5 eq) in 72 mL of H₂O was added. Then THF was added (129 mL) followed by the dropwise addition of N-(benzyloxy-carbonyl-oxy)-succinimide (49.3 g, 0.2 mmol, 9 eq) in THF (243 mL) over 30 min. A white suspension is formed which is let stir at room temperature overnight. After completion (TLC in CH₂Cl₂: MeOH 8:2, detected with anisaldehyde stain) the reaction was quenched with glycine (14.86 g, 0.2 mmol, 9 eq) in a saturated solution of NaHCO₃. The resulting mixture was stirred for 1 hour at room temperature. THF was then removed in vacuo. Next, the mixture was basified with 1M NaOH to pH 9. The resulting mixture was then extracted with EtOAc (3 x mL). The combined organic layers were dried over MgSO₄, filtered, and concentrated in vacuo. The white solid was purified by flash column chromatography in CH₂Cl₂: MeOH 9:1 as the eluent with a prior wash of DMC with 25% aqueous ammonia to improve the separation between neomycin B and neomycin C. The desired product, neomycin B, was obtained as a light yellow foam (25 g, 0.02 mol, 86%).

**1H NMR** (400 MHz, Methanol-d₄) δ 7.36 – 7.24 (m, 30H), 5.21 – 5.14 (m, 2H), 5.14 – 4.98 (m, 13H), 3.99 (s, 1H), 3.92 (q, J = 7.0, 5.0 Hz, 4H), 3.83 (s, 2H), 3.81 – 3.68 (m, 3H), 3.61 (td, J = 9.8, 3.4 Hz, 4H), 3.51 (dd, J = 5.6, 3.6 Hz, 2H), 3.46 (d, J = 7.2 Hz, 2H), 3.37 (s, 2H), 3.22 (t, J = 9.4 Hz, 2H), 1.96 (d, J = 12.5 Hz, 1H), 1.45 – 1.31 (m, 1H).

**13C NMR** (101 MHz, Methanol-d₄) δ 159.2, 159.1, 158.7, 138.2, 137.7, 129.61, 129.54, 129.51, 129.47, 129.43, 129.40, 129.02, 129, 128.97, 128.94, 128.9, 128.82, 128.8, 128.6, 104.3, 100.5, 95.7, 87.2, 78.1, 75.8, 75.5, 74.9, 74.5, 73.7, 72.8, 72.2, 71.5, 67.9, 67.7, 67.6, 61.5, 60.4, 57.9, 53.7, 51.4, 49.9 49.6, 49.4, 49.2, 43.2, 26.3, 20.9, 16.7, 14.5. **HRMS (ESI+)** calculated for C₇₁H₈₂O₂₅N₆Na ([M+Na]+): 1441.5222, found: 1441.5237.
Method 1: \(N\)-(Cbz)\text{-}6-neomycin B (1 eq) was dissolved in DMSO. Benzoquinone (3 eq) was added. Upon dissolution, [(neocuproine)PdOAc]\(_2\)(OTf)\(_2\) (2.5 mol\%) was added and the dark orange solution was stirred overnight at room temperature. Upon 50% conversion to the desired product (TLC CH\(_2\)Cl\(_2\): MeOH 9:1, stained with anisaldehyde) the reaction was quenched by the addition of H\(_2\)O (total H\(_2\)O: DMSO v:v 5:1), the resulting suspension was filtered and washed with water. The residue was freeze-dried. The resulting brown solid was purified by manual column chromatography (CH\(_2\)Cl\(_2\): MeOH 9:1).

Method 2: \(N\)-(Cbz)\text{-}6-neomycin B (1.90 g, 1.34 mmol, 1 eq) was dissolved in MeOH (4.5 mL). Benzoquinone (152 mg, 1.41 mmol, 1.05 eq) was added. Upon dissolution, [(neocuproine)PdOAc]\(_2\)(OTf)\(_2\) (35 mg, 0.03 mmol, 2.5 mol\%) was added and the dark orange solution was stirred overnight at room temperature. Upon 50% conversion to the desired product (TLC CH\(_2\)Cl\(_2\): MeOH 9:1, stained with anisaldehyde) the mixture was concentrated in vacuo. The resulting orange foam was purified by manual column chromatography (CH\(_2\)Cl\(_2\): MeOH 9:1). The desired product (1 g, 52.7 %) was obtained as a light orange foam.

\(^1\)H NMR (400 MHz, Methanol-\(d_4\)) \(\delta\) 7.40 – 7.23 (m, 30H), 5.20 – 4.96 (m, 15H), 4.58 (s, 1H), 4.10 (d, \(J = 9.8\) Hz, 1H), 4.02 (s, 2H), 3.92 (t, \(J = 8.3\) Hz, 4H), 3.84 (s, 1H), 3.72 (d, \(J = 11.9\) Hz, 1H), 3.63 (dd, \(J = 13.0, 4.7\) Hz, 2H), 3.58 – 3.45 (m, 5H), 3.38 (m, 4H), 1.95 (d, \(J = 12.6\) Hz, 1H), 1.40 (d, \(J = 12.6\) Hz, 1H). \(^13\)C NMR (101 MHz, Methanol-\(d_4\)) \(\delta\) 212.1, 159.2, 159, 138.2, 138.1, 129.6, 129.54, 129.5, 129.48, 129.45, 129.4, 129, 128.99, 128.95, 128.9, 128.8, 128.6, 100.44, 100.4, 92.3, 87.2, 78, 75.8, 73.4, 71.5, 67.9, 67.8, 67.6, 64.6, 63.2, 57.8, 54, 52.4, 49.9, 45.6, 42.5, 40.8, 32.8, 28.8, 21.5. HRMS (ESI-) calculated for C\(_{71}\)H\(_{59}\)O\(_{25}\)N\(_6\) ([M-H]\(^+\)): 1415.5100, found: 1415.5126
3'-epoxy-N-(Cbz)\textsubscript{6}-Neomycin B (20) *

Note: Diazomethane is potentially explosive. It is required to work behind a blast shield and use glassware without scratches and ground joints.

Diazomethane stock solution was prepared as follows: in an Erlenmeyer 3 g KOH in 4.5 mL H\textsubscript{2}O and 30 mL Et\textsubscript{2}O were added. The solution was stirred and cooled to 0°C. 1-methyl-3-nitroguanidine (3.3 g) was added portion-wise. The ether layer turned yellow upon the addition of the guanidine. 3'-keto-N-(Cbz)\textsubscript{6}-neomycin B (240 mg, 0.17 mmol) was dissolved in abs. EtOH (6mL). 3-4 mL from the stock solution of diazomethane were added carefully. After 2h a flow of N\textsubscript{2} was applied to the solution mixture which was left to stir overnight. A thick green gel was obtained. This was purified by flash column chromatography in CH\textsubscript{2}Cl\textsubscript{2}: MeOH 9:1 as the eluent. The desired product was obtained as a dark green solid (18 mg, 0.01 mol, 7.4%).

\textsuperscript{1}H NMR (600 MHz, Methanol-d\textsubscript{4}) \(\delta\) 7.42 – 7.14 (m, 30H), 5.24 – 4.91 (m, 15H), 4.34 – 4.05 (m, 3H), 4.05 – 3.87 (m, 4H), 3.83 (d, \(J = 5.9\) Hz, 1H), 3.79 – 3.69 (m, 2H), 3.70 – 3.51 (m, 5H), 3.49 (m, 2H), 3.46 – 3.32 (m, 5H), 2.84 – 2.71 (m, 1H), 2.48 (d, \(J = 5.2\) Hz, 1H), 2.00 (d, \(J = 13.3\) Hz, 1H), 1.37 (d, \(J = 40.8\) Hz, 1H). \textbf{HRMS (ESI\textsuperscript{+})} calculated for C\textsubscript{72}H\textsubscript{82}O\textsubscript{25}N\textsubscript{6}Na ([M+Na\textsuperscript{+}]): 1453.522, found: 1453.5213.
2D-COSY
(epoxide signals at 2.44 and 2.78 ppm)

HSQC
(the two epoxide signals correlate with the same carbon signal at 42.8 ppm)

*reaction setup for diazomethane preparation performed together with N. Marinus
To a solution of 3'-allyl-(Boc)-kanamycin (105 mg, 0.11 mmol, 1 eq) in DCE (1 mL, 0.11M), ethylacrylate (37 µL, 0.34 mmol, 3 eq) was added. The reaction mixture was kept in the dark and was heated at 60°C for 1h. After this time the mixture was let cool down to room temperature and Grubbs-Hoveyda 2nd generation (2.14 mg, 0.03 mmol, 0.3 eq) in solution in DCE was added via syringe. The reaction mixture was heated again at 60°C and monitored by UPLC-MS and stopped upon full conversion was achieved after 2 days. The mixture was concentrated in vacuo. Flash column chromatography in CH2Cl2:MeOH (from 0% to 15%) followed. The desired product was obtained as a brown-orange solid (17 mg, 0.017 mmol, 15%).

1H NMR (600 MHz, MeOD) δ 6.98 (dt, J = 15.6, 7.9 Hz, 1H), 5.93 (dt, J = 15.5, 1.4 Hz, 1H), 5.14 (s, 1H), 5.06 (s, 1H), 4.20 – 4.11 (m, 2H), 4.02 (ddd, J = 9.5, 5.1, 2.4 Hz, 1H), 3.94 – 3.88 (m, 1H), 3.80 (ddd, J = 11.7, 2.4 Hz, 1H), 3.73 – 3.62 (m, 3H), 3.56 – 3.45 (m, 4H), 3.43 – 3.36 (m, 3H), 3.33 (s, 1H), 3.23 – 3.16 (m, 1H), 2.64 (d, J = 18.0 Hz, 2H), 2.11 (d, J = 13.0 Hz, 1H), 1.49 – 1.40 (m, 1H), 1.26 (d, J = 12.1 Hz, 4H). 13C NMR (151 MHz, MeOD) δ 168.05, 158.7, 157.5, 145.8, 142.6, 142.1, 136.6, 132.4, 131.9, 131, 126.3, 125.3, 119.7, 114.9, 110, 100.2, 98.8, 80.5, 76.9, 74.5, 72.2, 69.8, 61.4, 56.1, 52.6, 41.9, 37.6, 29, 28.9, 28.8, 21.1, 17.7, 14.6. HRMS (ESI+) calculated for C44H76O21N4Na ([M+Na]+): 1019.4894, found: 1019.4908

To a suspension of (Boc)-3’-ethyl-but-2-enoate-kanamycin (12.3 mg, 0.012 mmol, 1 eq) in CH2Cl2 (0.15 mL), triisopropylsilane (10 µL, 0.05 mmol, 4 eq) and trifluoroacetic acid (0.08 mL, 1.0 mmol, 80.7 eq) were added via syringe. The reaction mixture turned clear and was left to stir at rt for 1 h. UPLC-MS analysis showed completion of the reaction after 1 h. Toluene (1 mL) was added and
the mixture was concentrated \textit{in vacuo} to get the final product as a brown film (10 mg, 0.01 mmol, 85%).

$^1$H NMR (600 MHz, MeOD) $\delta$ 7.03 (dt, $J = 15.7$, 7.9 Hz, 1H), 5.99 (dt, $J = 15.5$, 1.3 Hz, 1H), 5.51 (d, $J = 4.3$ Hz, 1H), 5.09 (d, $J = 3.7$ Hz, 1H), 4.18 (q, $J = 7.1$ Hz, 2H), 4.16 – 4.10 (m, 1H), 3.89 (ddd, $J = 14.8$, 8.3, 4.5 Hz, 5H), 3.81 (dd, $J = 10.7$, 3.6 Hz, 1H), 3.74 (t, $J = 9.1$ Hz, 1H), 3.69 – 3.62 (m, 2H), 3.60 – 3.47 (m, 5H), 3.42 – 3.35 (m, 3H), 3.01 (dd, $J = 12.9$, 9.4 Hz, 1H), 2.76 – 2.62 (m, 2H), 2.49 (d, $J = 11.6$ Hz, 2H), 2.04 (q, $J = 12.6$ Hz, 1H), 1.60 (dd, $J = 15.0$, 8.3 Hz, 1H), 1.27 (d, $J = 7.1$ Hz, 5H). $^{13}$C NMR (151 MHz, MeOD) $\delta$ 145, 125.6, 96.3, 85.8, 76.7, 75.3, 70.3, 70.1, 69.5, 67.7, 67.2, 62.1, 61.5, 56.8, 52.5, 52.47, 51.3, 38.1, 30.8, 28.7. HRMS (ESI+) calculated for C$_{31}$H$_{60}$O$_{13}$N$_{4}$ ([M+H]+): 597.2978, found: 597.2973

\textbf{N-\textit{(Boc)}-3'-\textit{(2-ethyl-oxirane)-Kanamycin}}

To a solution of 3'-allyl-kanamycin (500 mg, 1 mmol, 1 eq) in CHCl$_3$ (5.4 mL), \textit{m}CPBA (112 mg, 1.2 eq). The reaction mixture was stirred at rt overnight and monitored by UPLC-MS and TLC (CH$_2$Cl$_2$: MeOH 10%) and stained with ninhydrin. After this time no completion was observed, hence 0.6 eq of \textit{m}CPBA was added (56 mg extra). After two hours, full conversion was observed. Flash column chromatography in CH$_2$Cl$_2$: MeOH (15%) gave the desired product in 40 mg, 7.9%. NMR analysis was unclear. Therefore it was decided to proceed with deprotection in TFA using the general procedure as the one described for compound 13 (above). NMR analysis was still troublesome (2D-COSY below) however the desired product(s) seem to be formed as shown from the correlation in 2D-cosy in the region of 2.6 - 1.8 ppm.
To a suspension of N-(Boc)4-3'-keto-Kanamycin (215 mg, 0.24 mmol, 1 eq) in MeCN (15 mL), (ethoxycarbonyl)-methylenetriphenylphosphorane (169.7 mg, 0.488 mmol, 2 eq) was added as the Wittig reagent. After two days not much conversion was observed. Therefore more Wittig reagent was added for a total amount of 678.6 mg (1.95 mmol, 8eq). After overnight, the reaction was stopped and column chromatography followed in CH₂Cl₂: MeOH 15%. Mixed fractions were collected. Due to the complex analysis, it was opted to proceed also in this case with deprotection in TFA using the general procedure as the one described for compound 13. The remaining Wittig reagent could not be removed. However, the product(s) seem to be formed as visible from HSQC below, where the key correlation of carbon and proton of the double bond is observed around 6.10-6.05 ppm and 114-113 ppm.
Protection of AGA with different carbonate-based protecting groups selected in chapter 2

The general procedure for the protection has to be referred to the already reported Boc and Cbz protection of kanamycin (4), paromomycin (2), and neomycin B (18). Here some of the UPLC-MS are reported with the aminoglycoside structure involved in the protection together with the structure of the protecting group that was used. The attempts are reported for 3 of the selected protecting groups (as described in chapter 2) and for 3 aminoglycosides (neomycin, kanamycin, and amikacin). The full mass spectra are firstly reported and then the most relevant peaks are selected and the highlighted mass is then explained in the picture itself by reporting the corresponding modification that has been occurred, unless no mass value was corresponding to partial/full protection. The mass is indicated with M and a subscript number, indicating the amount of protecting group that belongs to that mass value, unless otherwise explained with letters described as “M of x times protected AGA”.

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In this case, it seems like a fully protected amikacin was obtained. However, the peak on the UPLC-MS spectra corresponding to it is one of the minor components, and a zoom-in shows the presence of other mass containing either a partially protected amikacin or unknown species.
M of double protected Kan

[M-2H] 1582.27

M of double protected Kan

[M-2H] 1582.40
References


