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Targeted modifications of neomycin and paromomycin: Towards resistance-free antibiotics?

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ABSTRACT

Despite their clinical importance, saving numerous human lives, over- and mis-uses of antibiotics has created a strong selective pressure on bacteria, which induces the emergence of (multi)resistant strains. Antibioresistance is becoming so pregnant that since 2017, WHO lists bacteria threatening most human health (AWaRe, ESKAPE lists), and those for which new antibiotics are urgently needed. Since the century turn, this context is leading to a burst in the chemical synthesis of new antibiotics, mostly derived from natural antibiotics. Among them, aminoglycosides, and especially the neomycin family, exhibit broad spectrum of activity and remain clinically useful drugs. Therefore, numerous endeavours have been undertaken to modify aminoglycosides with the aim of overcoming bacterial resistances.

After having replaced antibiotic discovery into an historical perspective, briefly surveyed the aminoglycoside mode of action and the associated resistance mechanisms, this review emphasized the chemical syntheses performed on the neomycin family and the corresponding structure activity relationships in order to reveal the really efficient modifications able to convert neomycin and its analogues into future drugs.

This review would help researchers to strategically design novel aminoglycoside derivatives for the development of clinically viable drug candidates.

Keywords (Max 10):

antibiotics, bacterial resistance, neomycin, paromomycin, chemical modifications,

Abbreviations:

AAC, aminoglycoside-acetyltransferases; AcCoA, acetylcoenzyme A; AG, Aminoglycosides; AHB, 4-amino 2-hydroxybutyryl; ANT, aminoglycoside-nucleotidyltransferases; APH, aminoglycoside-phosphotransferases; AWaRe, Access, Watch and Reserve; DCC, dicyclohexyl carbodiimide; ESKAPE, *Enterococci, Staphylococcus aureus, Klebsellia pneumoniae, Acinetobacter, Pseudomonas aeruginosa, Enterobacter* ; FDA, American food and drug administration; Fmoc, fluorenylmethoxycarbonyl; Neo, neomycin; NHS, *N*-hydroxysuccinimide; Par, paromomycin; R&D, Research & Development

1 Antibiotics and resistances

The discovery of antibiotics has been one of the major advances in bioorganic chemistry and medicine in the early 20th century, but the rate of antibiotic discovery and development faded since the 70's, with most pharmaceutical companies stopping their antibacterial R&D programs. However, during this period, bacteria (multi) resistant to antibiotics emerged and started spreading out, threatening human life. The search for antibacterial drugs able to fight these resistant bacteria has thus become vital, motivating a revival of antibiotic chemistry mostly driven by academic groups.

Hemisynthesis starting from natural aminoglycosides has led to the commercialization of new antibiotics such as amikacin, arbekacin and very recently plazomicin, but such advances have not yet led to new drugs on aminoglycosides of the neomycin family. Therefore, the objectives of this review are to describe the known chemical transformations on the neomycin core and to look at their impact on their antibiotic properties.

The aim is to provide readers with an overview of the modifications that really induce improved antibiotic activity, especially against resistant bacteria strains.

1. 1. A brief history

Derived from the concept of *antibiosis* first proposed by J-P. Vuillemin as early as 1889 [1], Pasteur's observations and comments (see below) [2], the terms *antibiotic* and *antibiotic substance* or *agent* were first proposed by S. Waksman in 1941, but only published with a clear definition in 1947 [3].

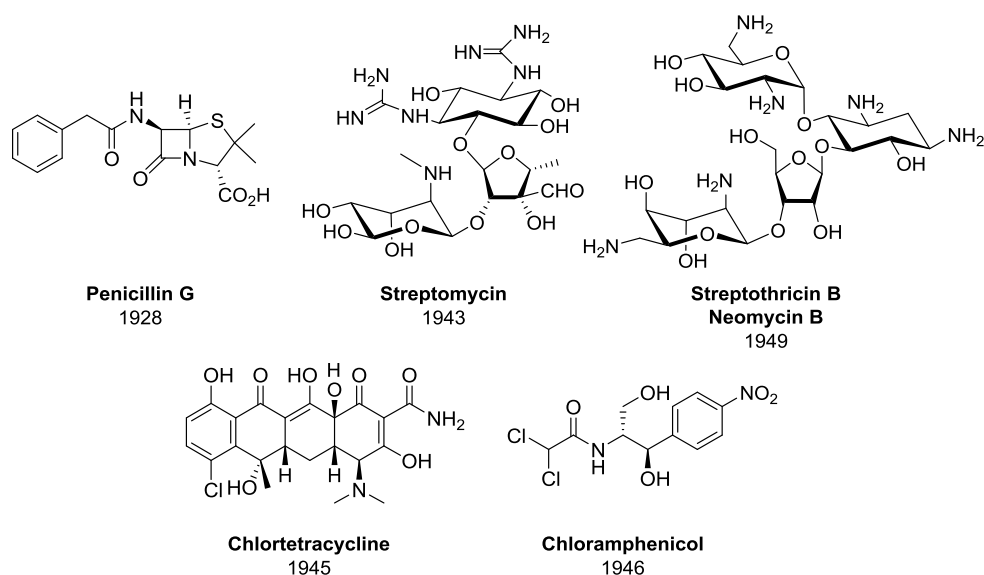
In the XIXth century, within his seminal demonstration that bacteria can be the cause of disease, L. Pasteur was the first to study the effect of moulds and bacteria on other microorganisms and he even clearly realised the therapeutic interest as stated by his writing [2]. Soon after, in 1885, V. Babes was most probably the first to realise that substances from microorganisms can alter the development of bacteria, as exemplified by his sentence: *bacteria of a known species produce chemical substances or modify the culture medium in such a way as to harm bacteria of other species* [4]. Following his work on anthrax, R. Emmerich was the first in 1899 to

prepare an antibacterial extract, called pyocyanase, from *Pseudomonas (Ps.) pyocyanea*, which was bactericidal to suspensions of *Bacillus (B.) anthracis*, *B. typhosum*, *Corynebacterium diphtheriae* and *Yersinia pestis* [5]. This extract was already employed in clinic, mostly as antiseptic. Various groups then applied the same strategy and experimented the use of various bacterial products to treat diseases with some success, such as Vaudremer's extracts of *Aspergillus fumigatus* and Rappin's extracts of *B. subtilis* in tuberculosis [6,7]. Serendipity played a key role in these works, as several accidental contaminations led to the discovery of microorganisms, whose lysate, culture broth or extract proved active against other microorganisms, especially pathogenic ones. The well-known discovery of penicillin G from *Penicillium notatum* by A. Fleming in 1928 is a typical example [8].

In a related but more targeted approach, the 1st compound active against *Streptococcus* infection, named prontosil, was discovered in the early 30's upon screening azoic dyes from the IG Farben company [9]. Soon after, a group of chemists, pharmacologists and bacteriologists at the 'Institut Pasteur' in Paris demonstrated that prontosil was a prodrug and that its active part was the sulfonamide and not the dye moiety as initially thought [10]. This discovery set up the emergence of the broadly active sulfamide antibiotics.

In the meantime, the value of penicillin G as drug was recognized and thus purified and produced in large quantities first for clinical evaluation in 1941 [11], and then for large scale production in order to mostly treat injured soldiers during World War II. The success of penicillin G saving numerous people really set the search for new antibiotics through a more systematic exploration of various natural sources. The latter rapidly led to the discovery of other antibiotics (Scheme 1). Streptomycin, the first anti-tuberculosis agent, was discovered in 1943 from *Streptomyces (Str.) griseus* by Waksman and coll. upon systematic screening of bacterial culture supernatants looking for the presence of *Mycobacterium tuberculosis* inhibitory activity [12]. A few years later, the same new antibiotic was independently discovered by Umezawa and coll., who named it streptothricin B [13], and by Waksman and Lechevalier who termed it neomycin [14]. The first member of the tetracycline family of antibiotics, chlortetracycline, was discovered in 1945 from *Str. aureofaciens* and developed at Lederle Laboratories to treat ocular infections and

dermatitis [15,16], and in 1947, chloramphenicol was isolated from *Str. venezuelae* and set up against typhoid fever [17].



Scheme 1. The first discovered naturally occurring antibiotics.

Within two decades, various new antibiotics were identified following extensive investigations from the pharmaceutical companies and academia, and most of them were more or less rapidly exploited as clinical agents to treat different diseases (Figure 1). Unfortunately, after this ‘golden age’, only a few new antibiotics were validated as therapeutic agents, mostly due to lower interest from pharmaceutical industries. However, a surge occurred during the last decade due to increasing resistances (see section 1.3 and 2.4) and 9 new antibiotics or combinations of them have been approved by the FDA in 2018 and 2019 in contrast to none between 2003 and 2007, but none of them belongs to structurally new antibiotics (Figure 1) [18,19,20]. Started around 2009, this revival of interest may lead to better adjusted structures or to structurally new antibiotics.

Timeline of the Discovery & Introduction of Antibiotics

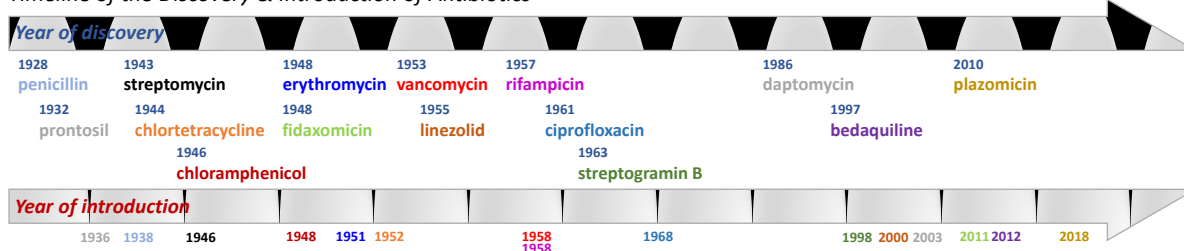


Figure 1. Chronology of the major antibiotic discoveries and of their 1st application (adapted from [18]).

1. 2. The different antibiotic classes and their modes of action

Over the years, various structurally different antibiotics were identified, and they were originally classified according to their structures. Once their mode of action identified, another classification based on the antibiotic target has been set up [21,22]. So far, only four main modes of action have been identified and thus, antibiotics of very different structures can target the same key biological process (Figure 2). Accordingly, antibiotics can act by:

- . Inhibiting the bacterial cell wall biosynthesis,
- . Interfering with protein biosynthesis,
- . Altering the bacterial membrane permeability,
- . Inhibiting the folic acid biosynthesis, which ultimately acts on the nucleoside biosynthesis and thus on DNA and RNA formation and interfering with DNA and RNA function.

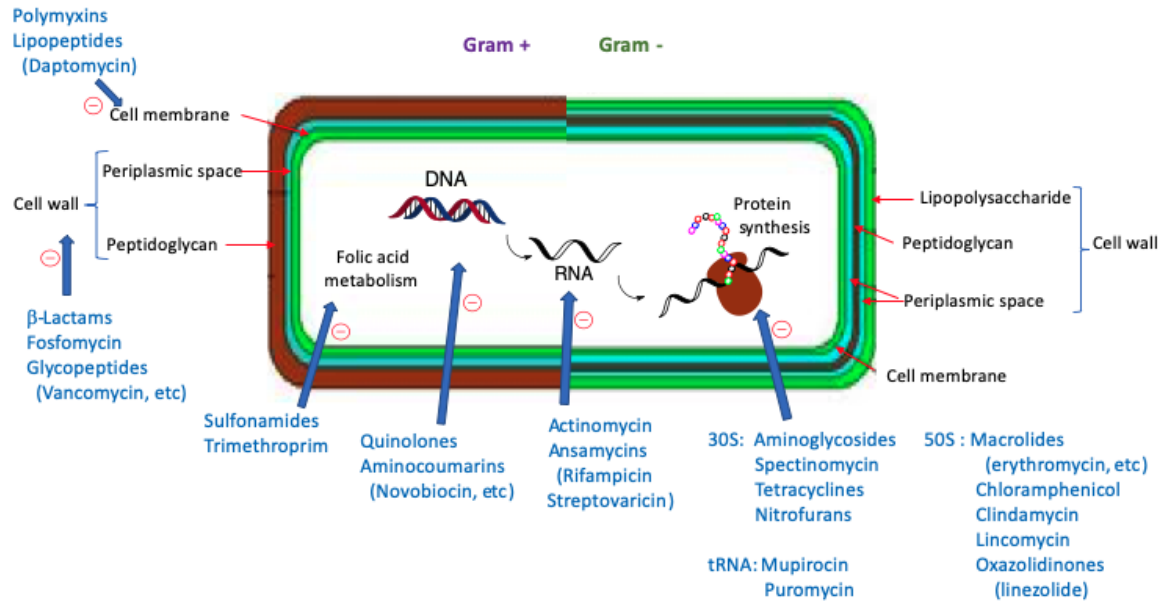


Figure 2. The main classes of antibiotics and their target

1. 3. Apparition of resistances to antibiotics

The success gained from antibiotic treatments during World War II and thereafter unfortunately led to massive and uncontrolled human consumption of antibiotics during the following decades. This behaviour, but also the abusive use of antibiotics in animal farming, led to the emergence of resistant bacterial strains [23]. The latter phenomenon is a normal adaptive behaviour, which usually occurs over millennia during evolution [24,25]. However, the over-use of antibiotics within a short time has created a strong selective pressure on bacteria species, and they adapt themselves to this new environment, becoming increasingly resistant to those antibiotics.

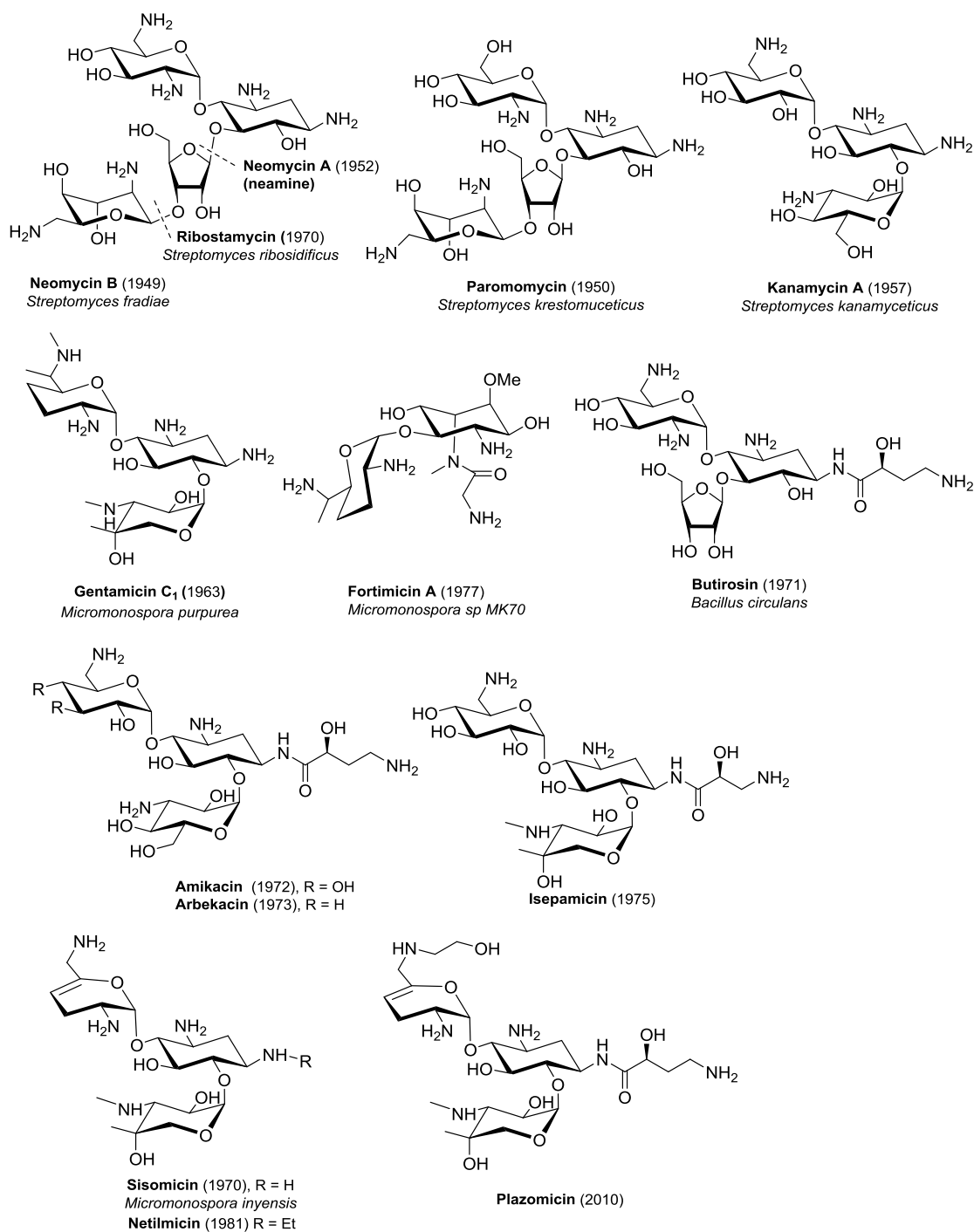
Unfortunately, this phenomenon is rapidly spreading over clinically useful antibiotics, threatening humanity health as recognized by the World Health Organisation (WHO). For example, in USA, more than 2.8 million infections due to resistant bacterial strains are reported each year, inducing more than 35 000 deaths [26], while in Europe more than 670 000 similar infections led to 33 000 deaths in 2015 [27]. Furthermore, bacterial strains which are now resistant to several antibiotics already exist, inducing even more problems, especially in hospital environment with nosocomial diseases.

Such antibioresistance is becoming so pregnant that WHO listed in 2017 twelve bacteria threatening most human health, and published the first list of bacteria for which new antibiotics are urgently needed [28]. Besides, another antibiotic classification has been set by WHO in order to promote better and pertinent uses of antibiotic at various levels. In this classification dubbed AWaRe, antibiotics are organised in three groups, called Access, Watch and Reserve, according to the level of resistance they encountered and thus how to use them [29].

2 Aminoglycosides

2. 1. Aminoglycosides as antibiotics

Aminoglycosides (AG) are among the first discovered antibiotics (see section 1.1 and Scheme 1). The first ones are streptomycin (1943), commercialized in 1946 as the first antituberculosis agent [12] and neomycin (1949) [14]. Kanamycin and gentamicin were isolated and characterized respectively in 1957 and 1963 [30,31], opening up a large family of natural compounds (Scheme 2). The latter rapidly grew during the 60s, and was enlarged with hemisynthetic compounds, some of them being commercialized in the early 70s, such as amikacin [32], isepamicin [33], and arbekacin [33]. Only a few emerged after this period and very recently, plazomycin was approved in 2018 to treat urinary infections [34].

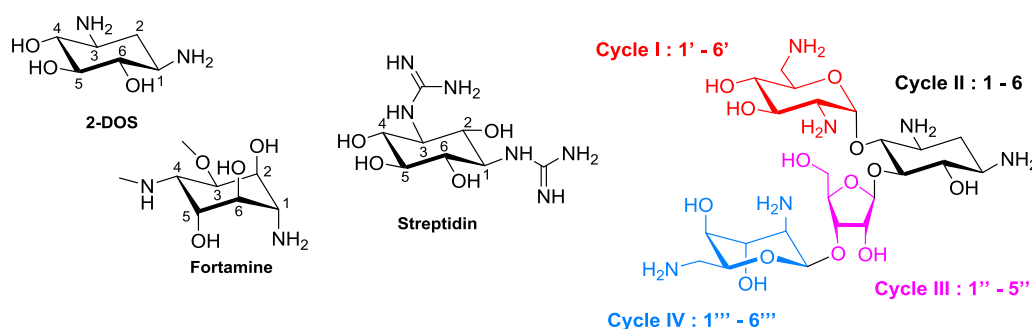


Scheme 2. Structures of the main natural and hemisynthetic aminoglycosides (date of isolation or hemisynthesis)

2. 2. The aminoglycoside family

AG originated from actinobacteria (actinomycetes), but depending on the producing species, the end name changes from 'mycin' if they come from *Streptomyces* species to 'micin' if they come from *Micromonospora* species.

Their structures correspond to pseudo di- to tetrasaccharides, as they always contain an aminocyclitol and at least one aminoglycoside, so their generic name. The aminocyclitol nature defines the sub-family: most AG, especially the clinically useful ones, contain the 2-deoxystreptamine motif (2-DOS), while streptomycin contains the streptidine motif and fortimicins exhibit the fortamine moiety (Scheme 3, left).



Scheme 3. Structures of the central aminocyclitol motif in aminoglycosides (left) and aminoglycoside numbering, based on the central aminocyclitol motif with neomycin as typical aminoglycoside (right).

The 2-DOS based AG differ by the substitution on the aminocyclitol motif. This central carbocycle can indeed be either only substituted at position 4 or 5, or doubly substituted at position 4 and 5 or 4 and 6, such as in kanamycin and tobramycin or in paromomycin and neomycin (Scheme 2).

Furthermore, a specific numbering has been defined to unambiguously described the various aminoglycosides (Scheme 3, right).

2. 3. Aminoglycoside mode of action

Due to their polyaminated structures, AG exist as polycation at physiological pH and they thus strongly interact with polyanionic compounds in cells. They thus interact with the anionic components of bacterial cell wall and membrane, that is

phospholipids and teichoic acid in Gram-positive bacteria, and lipopolysaccharides in Gram-negative bacteria [35,36,37]. Such interactions alter the permeability of the cell wall and membrane and facilitate AG penetration into the bacteria cytosol through an energy-dependant process [38,39]. Once in the cytosol, AG can bind to DNA and RNA sequences, but usually in a non-specific manner, again due to charge interactions, although exceptions have now been recognised [40,41].

However, the main AG target is the 30S sub-unit of bacterial ribosome (Figure 3b), at the place where mRNA is translated to protein sequence, more precisely at the aminoacyl-tRNA acceptor site, the so-called A site (Figure 3a) [42,43,44]. The latter is acting as a switch: in its normal conformation ('off'), the corresponding rRNA exhibits a highly preserved helix conformation (H44), but when a mRNA codon matches with a tRNA codon, their binding induces further interactions with A site adenines 1492 and 1493, which force them to be outside of the H44 helix (Figure 3c). In this 'on' conformation, tRNA can be transferred to the next site, allowing the amino acid it carries to be added to the nascent protein [45,46]. When AG bind to A site, they force adenines 1492 and 1493 to be outside of the H44 helix, therefore even without match between them, a tRNA codon can enter and bind to mRNA and thus any amino acid can be added, ultimately leading to aberrant protein (Figure 3c) [47,48,49]. Such codon misreading induces the accumulation of these aberrant proteins and finally leads to cell death.

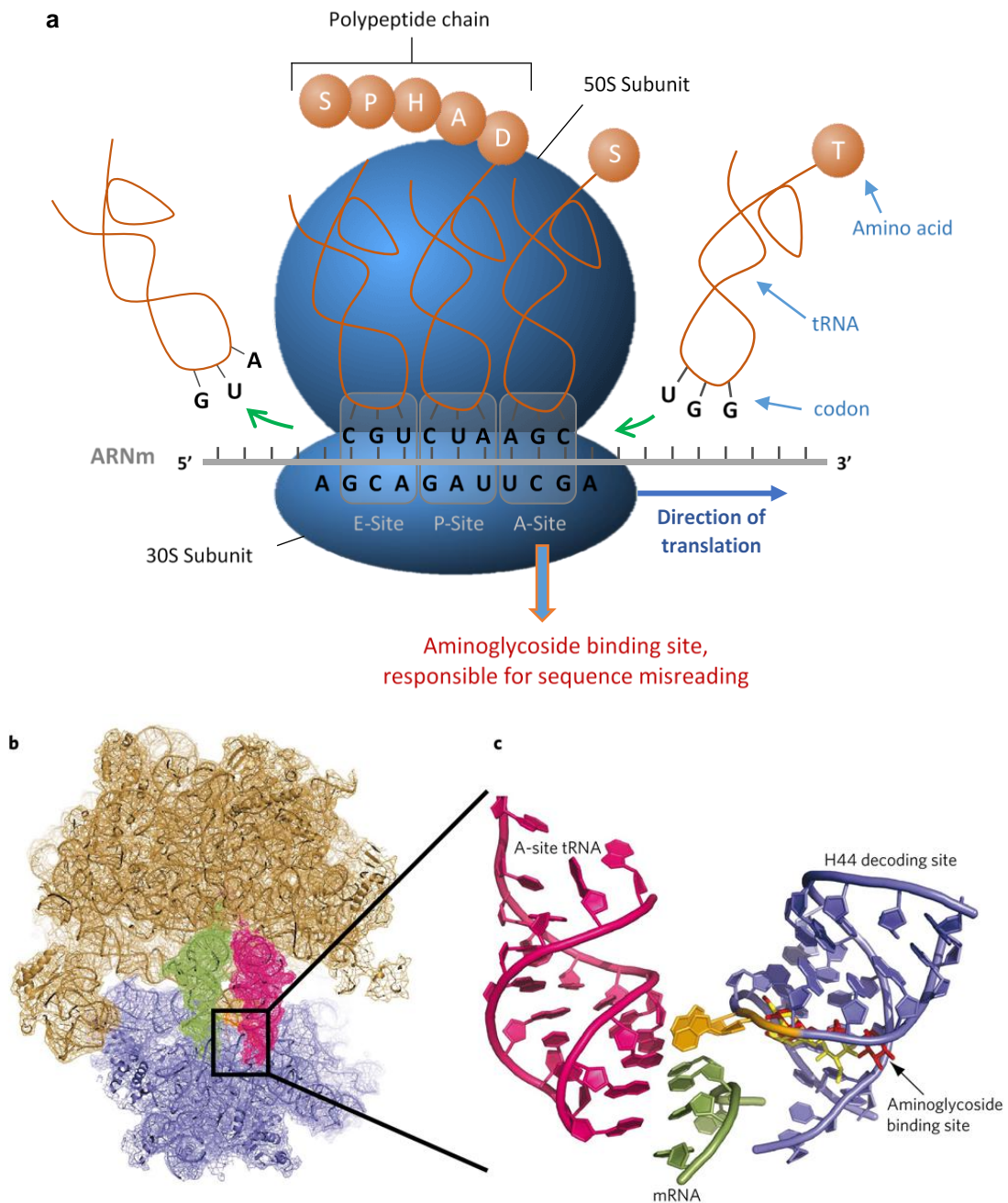
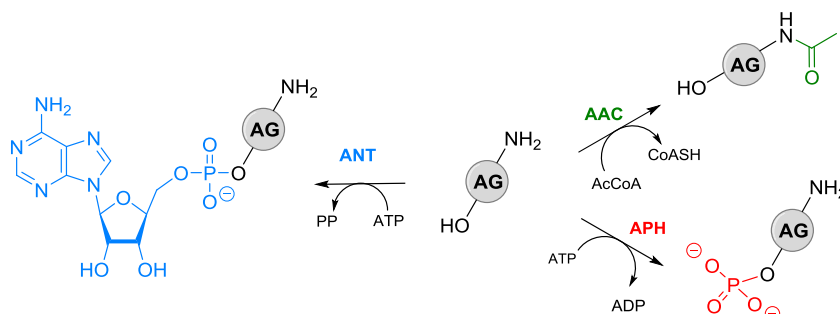


Figure 3. Aminoglycoside mode of action: a) Schematic representation of translation of mRNA (grey) to peptides (orange spheres) and proteins on ribosome (blue); b) Site A location within *E. coli* ribosome; c) Interaction of tRNA (pink) with mRNA (green) codons, which induces the externalisation of adenines 1492-1493 (orange) from the ribosome H44 helix (violet); The same externalisation also occurs but permanently in the presence of aminoglycosides, e.g., neomycin (in red), inducing codon misreading. Taken from Ref. [49] with permission from Springer-Nature.

2. 4. Resistance to Aminoglycosides

Due to their highly functionalised structures, AG are quite vulnerable to enzymes able to modify their various hydroxy and amino groups. Not so surprisingly, bacteria often produced such enzymes, which convert AG to derivatives unable to properly bind to A site, and thus to inactive compounds. Upon decades of antibiotic misuse, at least 50 of these aminoglycoside modifying enzymes (AME) have been identified worldwide [37,50]. These AME can be classified according to the chemical modification they promote, the position where this(these) modification(s) occur(s) and the resistance profile they provide to the bacteria. The three main AME classes are (Scheme 4):

- . aminoglycoside-acetyltransferases (AAC) transfer to an amine an acetyl group from acetylcoenzyme A (AcCoA);
- . aminoglycoside-phosphotransferases (APH) transfer to a hydroxy group a phosphate from adenosine triphosphate (ATP);
- . aminoglycoside-nucleotidyltransferases (ANT) transfer to a hydroxy group an adenosine phosphate moiety from ATP.



Scheme 4. The 3 main classes of aminoglycoside modifying enzymes.

Besides this main deactivating process, some bacteria have also developed a process which impedes AG penetration or expulses aminoglycosides. The latter use proteinic complex acting as efflux pumps [51].

Another deactivating process also exists, probably since the origin of actinomycetes. To defend themselves from the AG they release, these bacteria have developed RNA methyltransferase enzymes (RMT), which methylate their own ribosomal RNA.

This process is slowly emerging, as the first example is known since 2003 [52] and only a few RMT have so far been detected in resistant bacteria [53,54].

Combining several deactivating processes, six genera of bacteria are particularly of concern regarding resistance to clinically useful AG. They have been dubbed the ESKAPE bacteria from their name (Table 1).

Bacteria	Main resistance mechanisms
<i>Enterococci</i>	AAC(6')-Ie/APH(2'')-Ia, APH(3')-IIIa
<i>S. aureus</i>	AAC(6')-Ie/APH(2'')-Ia, ANT(4')-Ia APH(3')-IIIa
<i>K. pneumoniae</i>	AAC(6')-Ib, ACC(3)-I, ANT(2'')
<i>Acinetobacter</i>	Efflux, APH(3')-VI, AAC(6')-I, AAC(3)-I/II, ANT(2'')-I, RMT
<i>P. aeruginosa</i>	Efflux, AAC(6')-II, ANT(2'')-I, APH(3')-II, AAC(3)-I
<i>Enterobacter</i>	AAC(6')-Ie, AAC(3)-I, ANT(2''), RMT

Table 1. The ESKAPE bacteria and their resistance mode of action.

2. 5. Toxicity of aminoglycosides

Despite the emergence of resistances, AG are still clinically useful. However, some of them exhibit renal and ototoxicity, especially upon prolonged treatment.

Studies have revealed that AG are low-protein binding drugs and that they are finally filtered more or less rapidly through kidney without being metabolized in the body. Most of the administered dose (~90% of dose) is finally excreted into urine [55]. Unfortunately, a part of the eliminated AG (~5-10% of dose) can be uptake by nephron cells in the renal cortex and their accumulation in these cells induce their death with the same mode of action as in bacteria. Nevertheless, this nephrotoxicity can be reversible as the concerned cells can be regenerated. Furthermore, using single daily dose has proven less toxic than using multiple doses [56].

Ototoxicity involved internal ear cells, in which mutation of the mitochondrial ribosome RNA in some humans renders this rRNA similar to those of bacteria, and thus susceptible to AG attack inducing cell death [57]. Leading to hearing loss, this toxicity is thus highly patient dependent, with an incidence of 1 in 385 [58].

2. 6. Fighting resistances to aminoglycosides

AG exhibit broad spectrum of activity; they are active against a wide range of aerobic Gram-negative bacilli, many staphylococci, mycobacteria, and some streptococci. They are used to treat serious infections caused by aerobic Gram-negative bacteria, hospital acquired enterobacteriaceae and *Ps. aeruginosa* infections, urinary tract infections, complicated intra-abdominal infections, dermal infections, ear infections and septicemia. It is thus worth keeping as much as possible their efficiency as antibiotics, overcoming resistances while minimising toxicity.

To reach these goals, several strategies have been developed, mostly around escaping to AME, which is the prevalent mechanism of resistance to AG. The main approaches are thus to design new AG and/or modify AG at sites known to be targeted by AME (Figure 4). These strategies will be detailed in the following sections, taking neomycin and its 6'-hydroxy analogue – paromomycin– as AG archetype.

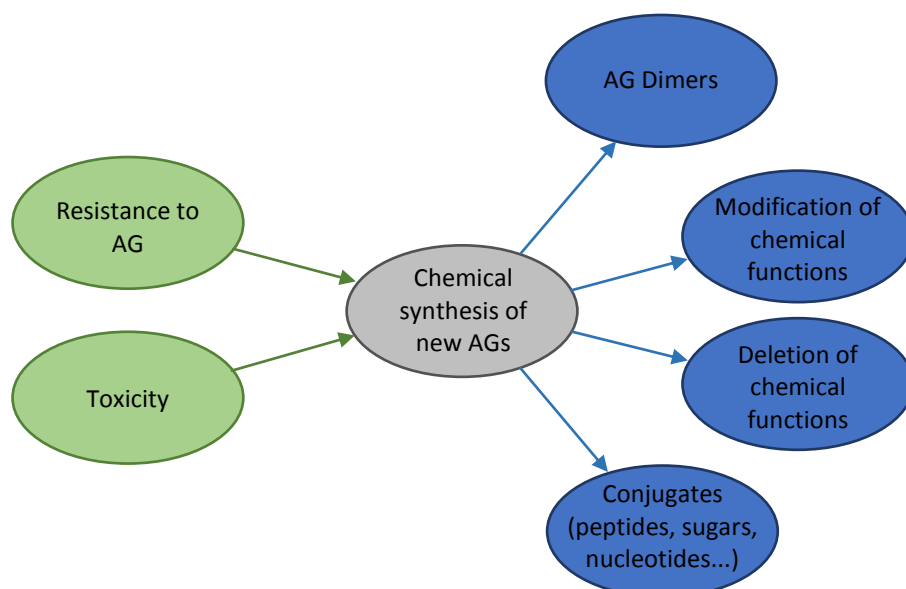
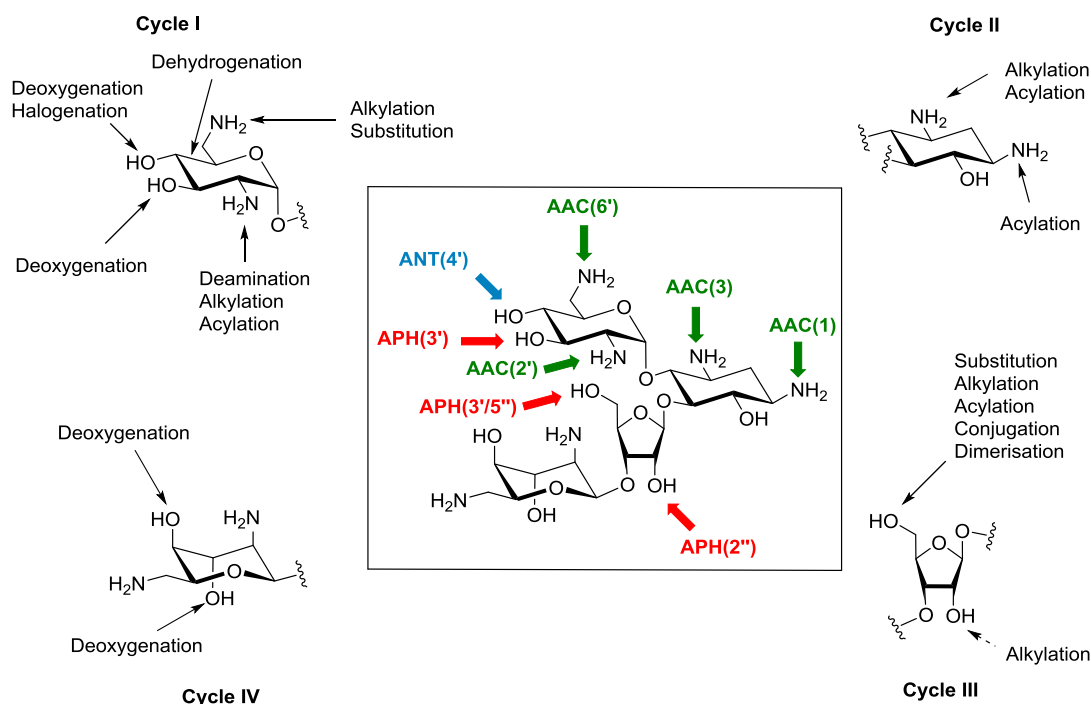


Figure 4. Strategies to fight aminoglycoside resistance and decrease toxicity.

3. Towards new antibiotics: Neomycin and related antibiotic modifications

To overcome resistances, numerous efforts have been made to modify the structure of AG [59]. This work was partly successful with the 2-DOS 4,6-disubstituted family of AG, whose extensive modifications ended up with the commercialisation of amikacin and arbekacin (Scheme 2), but not so far with the 2-DOS 4,5-disubstituted family. Neomycin, paromomycin and ribostamycin belong to the latter family.

Neomycin exhibits one of the broadest spectra of activity within the AG family. It is commercially available, cheap, and thus it is a source of choice for obtaining libraries of new derivatives. The 6'-hydroxy natural analogue paromomycin has also been used to produce new derivatives. The various modifications done on both will be described here, according to the affected carbohydrate or cyclitol unit (Scheme 5). For each one, the resulting activity on bacteria will be reported. When the antibacterial properties are superior to neomycin, we will also describe, if studied, the impact of structural modification on toxicity.



Scheme 5. Strategies developed on neomycin to minimise resistance (sites targeted by AME in colour) and toxicity.

3. 1. Functionalisation of ring I

Whatever the AG family, rings I and II are the key part for binding to A site RNA. The functional groups on these rings establish numerous H-bond with the various rRNA parts, often directly or sometimes through water molecule relay (Figure 5) [60]. Rings I and II are thus the most important for rRNA recognition, binding, and stabilization. Indeed, rings I and II are responsible for most of the direct interactions with A site (9 out of 10 possible). Ring III is at the heart of the AG arrangement and positioning in A site. O-4'' and O-5'' interact directly with N-2' of ring I and directly or not with the rRNA backbone, while O-2'' interacts directly with the rRNA backbone or indirectly with the N-2''' of cycle IV. In contrast, Ring IV has only one electrostatic interaction with the A site.

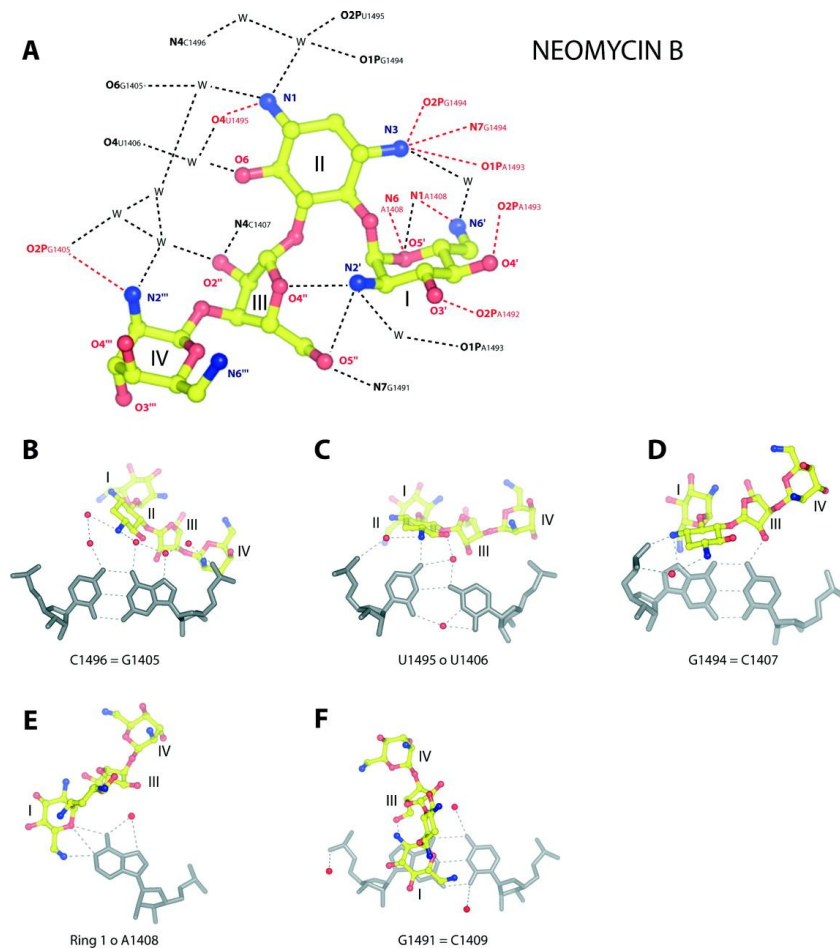


Figure 5. Neomycin B binding mode: Contacts established by neomycin in *E. coli* A site, observed from X ray structure of complexes between neomycin and oligonucleotides containing the decoding A site from *E. coli* rRNA (H-bond as dashed line; W: water). Taken from Ref. [60] with permission from Oxford University Press.

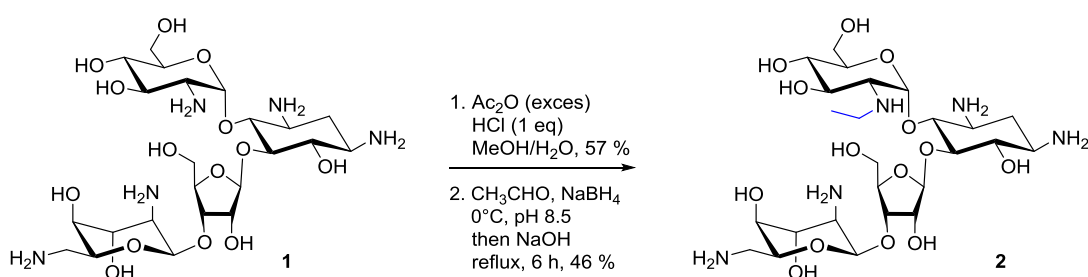
In the neomycin family, ring I is the most targeted by AME (see Scheme 5). Each position on this ring can be acylated, phosphorylated or grafted with a nucleotide by a specific bacterial enzyme. It is thus worth modifying these positions to avoid AME modification and antibiotic activity neutralisation.

3. 1. 1. Modification at position 2'

Some groups attempted modifying position 2'. The latter is indeed relatively easy to keep free, due to intramolecular H-bond, and as an amine, it is more nucleophilic than all the hydroxy groups and thus more reactive.

In 1978, the Arcamone group was able to selectively acetylated all amino groups of paromomycin, except the 2' amino group. Reductive amination of the latter with acetaldehyde provided the 2'-*N*-ethyl *N*-peracetylated paromomycin. A simple deacetylation led to the 2'-*N*-ethyl paromomycin **2** with a reasonable 26% overall yield (Scheme 6) [61].

This 2'-*N*-alkylated product exhibits the same antibiotic activity as the parent antibiotic against standard strains of *Staphylococcus aureus* and *Salmonella abortus-equi*, but a 4-times lower activity against standard strain of *E. coli*. Nevertheless, it proved twice more effective against strains resistant to the AME APH(3')-I and ANT(2'') (Table 2).

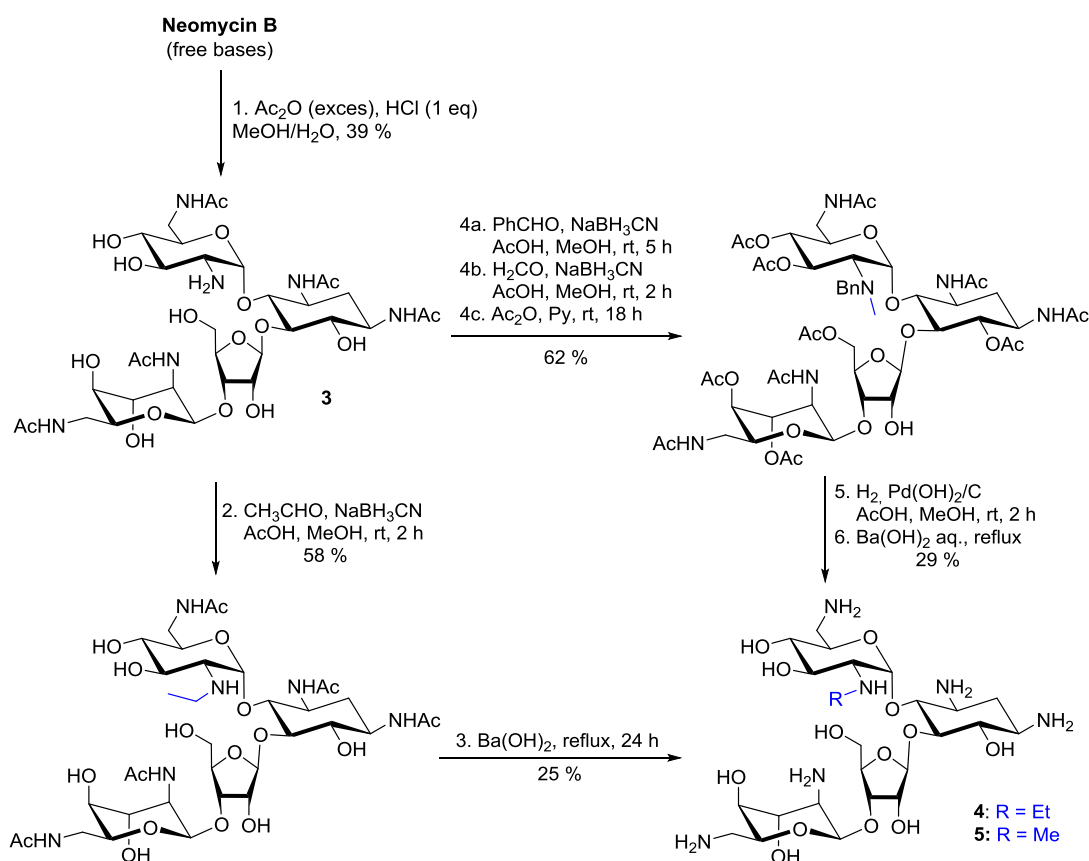


Scheme 6. Selective alkylation of the 2' amino group of paromomycin through reductive amination.

bacterial strain	AME	Par	2
<i>S. aureus</i> 209P		3.1	3.1
<i>S. abortus equi</i>		25	25
<i>E. coli</i> B		6.2	25
<i>E. coli</i> K12-R112	APH(3')-I	250	125
<i>E. coli</i> K12-W677	ANT(2'')	250	125

Table 2. MIC ($\mu\text{g/mL}$) of 2'-*N*-ethyl paromomycin **2** vs its parent paromomycin (Par).

The same strategy was recently applied to neomycin B (Scheme 7) [62]. Selective *N*-acetylation provided compound **3**, although in modest yield (39%), and reductive amination as well as the final deacetylation conditions must be adjusted to this substrate. The 2'-*N*-ethyl neomycin B **4** was thus obtained with a modest 6% overall yield. Unfortunately, the introduction of a methyl group was even more problematic and required a longer sequence involving two successive reductive aminations with benzaldehyde and then with formaldehyde and acetylation of the hydroxy groups. Hydrogenative debenzoylation and deacetylation provided the 2'-*N*-methyl neomycin B **5** with a low overall yield (7%).



Scheme 7. Selective alkylation of the 2' amino group of neomycin B through reductive amination.

Various biological investigations were performed on these 2'-*N*-alkyl neomycin B derivatives and compared to related compounds from paromomycin and ribostamycin (Table 3). The 2'-alkylated neomycin B compounds exhibit activity similar to their parent on a variety of strains, and interestingly, they keep a good activity on *E. coli* resistant strains with AAC(2') AME. The comparison with paromomycin analogues shows that replacing the 6'-amino group by a hydroxy group leads to lower activity, while those derived from ribostamycin, lacking cycle IV, lost their activity. Furthermore, 2'-alkylation on neomycin B and paromomycin result in significant increases in selectivity for bacterial ribosome vs human cytosolic or mitochondrial ribosomes, and even on A1555G mutant mitochondrial ribosomes responsible for ototoxicity (see section 2.5); they should thus be less toxic.

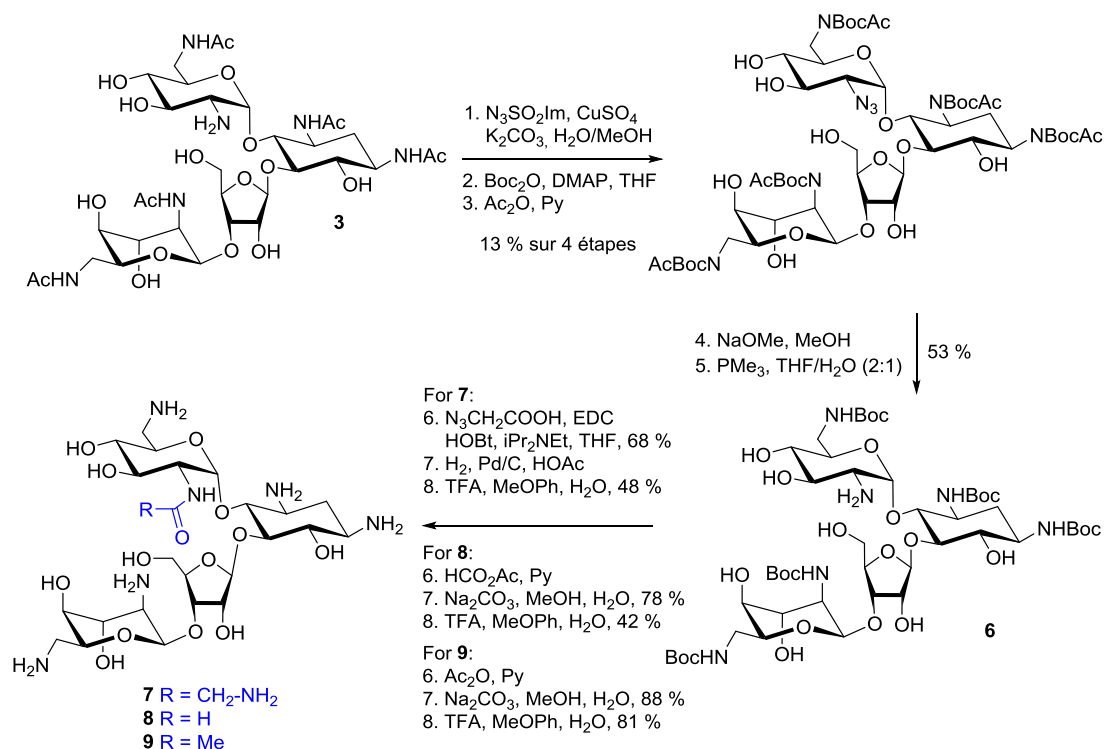
Bacterial strain	AME	Neo	4	5
MRSA		0,5	0,5	0,5
<i>E. coli</i> AG001		1	1–2	2
<i>E. coli</i> AG003		1	1	1
<i>K. pneumoniae</i>		0,25–0,5	0,5	0,5
<i>E. cloacae</i>		1	0,5–1	0,5–1
<i>A. baumannii</i>		1–2	1	1
<i>E. coli</i> AG106	AAC(2')-Ia	16	1	2
<i>E. coli</i> pH434	AAC(2')-Ib	> 64	2	2–4

Table 3. MIC ($\mu\text{g/mL}$) of 2'-*N*-alkylated compounds **4**, **5** vs the parent neomycin (Neo).

The same intermediate **3** was exploited to convert the free 2' amino group to an azide. Further *N*- and *O*-protection followed by deacetylation and Staudinger

reduction provided the pentaBOC compound **6**. The remaining free 2' amino was then acylated with various chains (Scheme 8).

The antibiotic property of the resulting compounds was evaluated (Table 4). Only the 2'-*N*-formyl derivative **8** keeps an activity similar to its parent, and as expected, it is better on AAC(2') resistant strains. It is worth noticing that the larger the acyl moiety, the lower is the antibiotic activity.



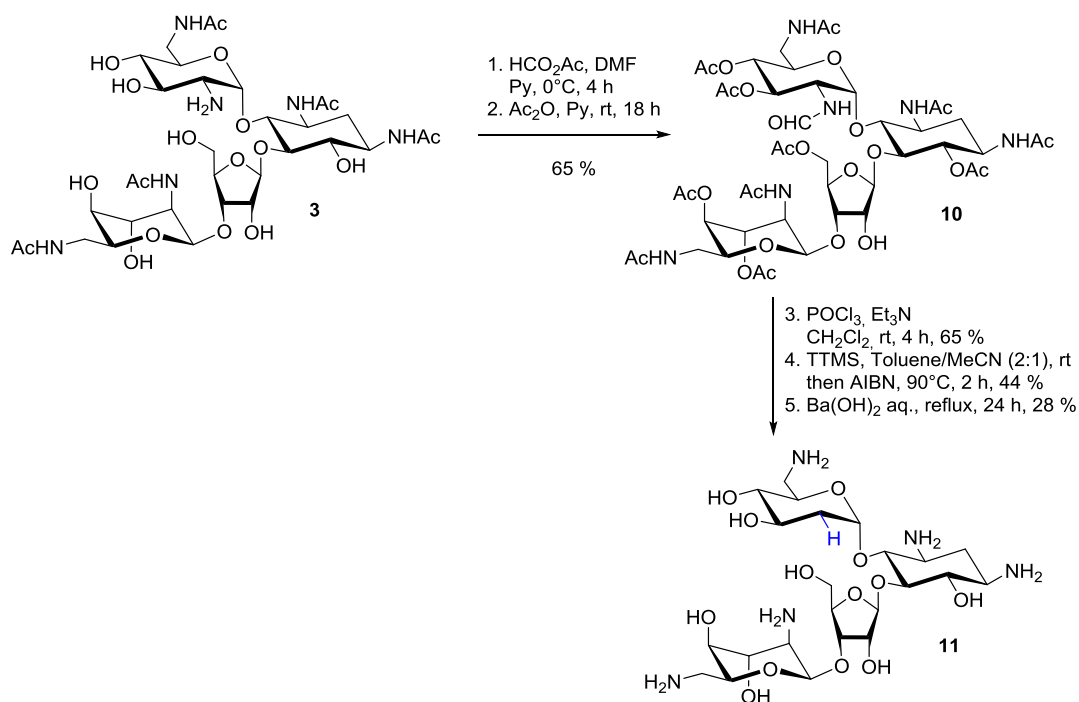
Scheme 8. Selective acylation of the 2' amino group of neomycin B

Bacterial strain	AME	Neo	7	8	9
MRSA		0.5	4	2–4	> 128
<i>E. coli</i> AG001		1	16	4–8	> 128
<i>E. coli</i> AG003		1	32	4–8	> 128
<i>K. pneumoniae</i>		0.25–0.5	n.d.	1–2	n.d.
<i>E. cloacae</i>		1	n.d.	2	n.d.
<i>A. baumannii</i>		1–2	n.d.	2	n.d.
<i>E. coli</i> AG106	AAC(2')-Ia	16	16	8	n.d.
<i>E. coli</i> pH434	AAC(2)-Ib	> 64	16–32	8–16	n.d.

Table 4. MIC ($\mu\text{g/mL}$) of 2'-*N*-acylated compounds **7-9** vs the parent neomycin (Neo).

With the same intermediate **3**, the same group was also able to deaminate the 2' position of neomycin and looked at its effect on the antibiotic activity (Scheme 9 and Table 5). The free 2' amino group of **3** was *N*-formylated and the so-formed formamide **10** was converted to an isocyanate. A Barton deamination, promoted by tris(trimethylsilyl)silane (TTMS) [63] and azobis(isobutyronitrile) (AIBN), followed by deacetylation provided the 2'-deaminoneomycin **11** with a modest overall yield of 5%.

The latter exhibits almost the same activity as its parent, including against multiresistant *S. aureus* (MRSA) and as expected, it keeps a good activity against AAC(2') resistant *E. coli* strains. Removing the amino group is thus an interesting strategy to escape to such AME. As for compounds **4** and **5**, the deamination of the 2' position of neomycin increased the selectivity for prokaryotic over eukaryotic ribosomes, as observed in model system. These compounds should thus exhibit lower toxicity compared to neomycin B.



Scheme 9. Selective deamination of the 2' amino group of neomycin B.

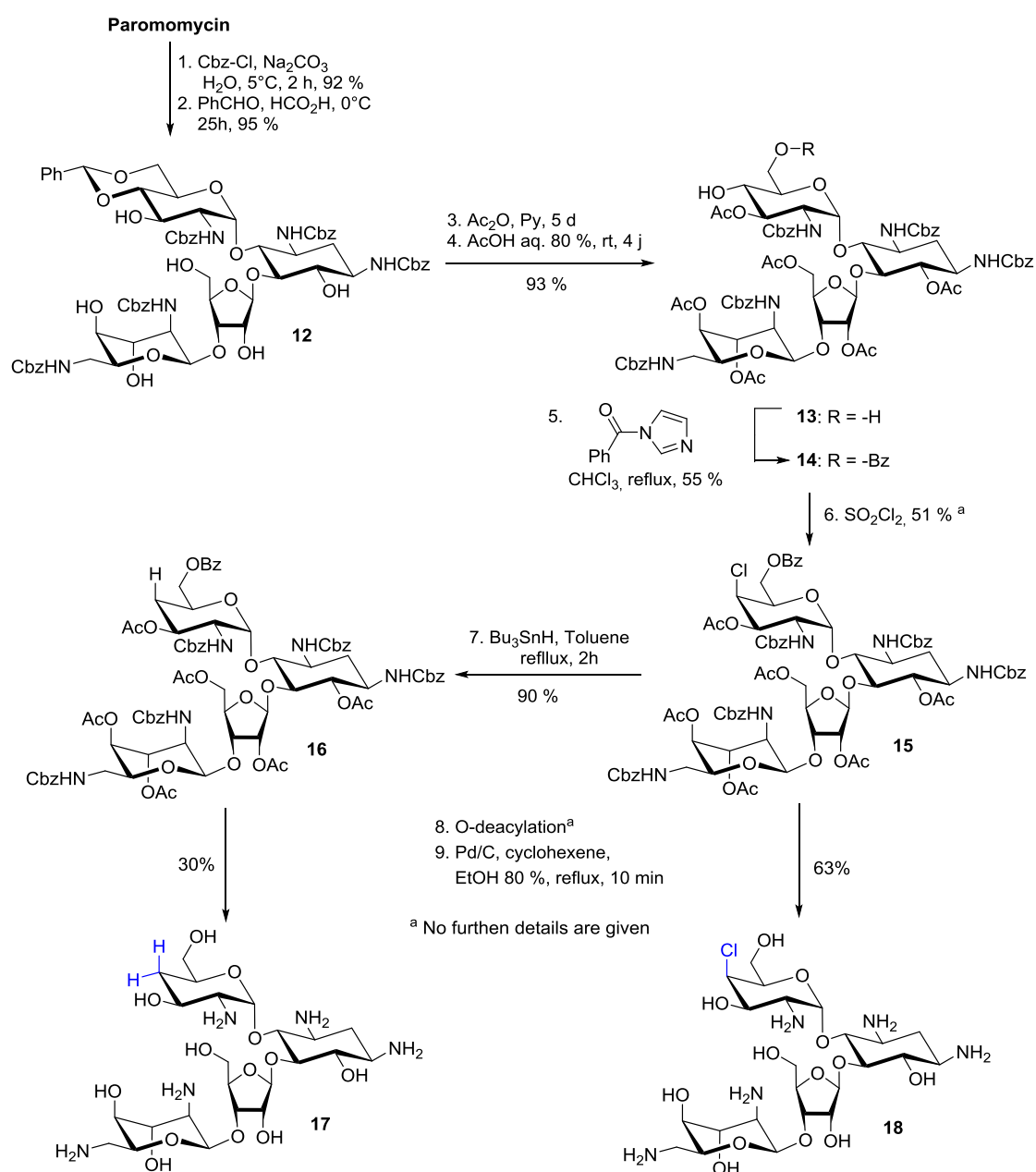
Bacterial strain	AME	Neo	11
MRSA		0,5	1
<i>E. coli</i> AG001		1	2
<i>E. coli</i> AG003		1	1–2
<i>K. pneumoniae</i>		0,25–0,5	0,5
<i>E. cloacae</i>		1	1
<i>A. baumannii</i>		1–2	1
<i>E. coli</i> AG106	AAC(2')-Ia	16	2
<i>E. coli</i> pH434	AAC(2')-Ib	> 64	4

Table 5. MIC ($\mu\text{g}/\text{mL}$) of 2'-deaminated neomycin **11** vs the parent neomycin (Neo).

3. 1. 2. Modification at position 4'

Chloration, fluoration and deoxygenation at position 4', as well as alkylation, have been achieved by different groups.

The Arcamone group carried out the synthesis of 4'-deoxyparomomycin **17** in 1981 through its 4'-chlorinated derivative **15**. (Scheme 10) [64]. Once *N*-protected by benzyloxycarbonyl groups, paromomycin was protected at position 4' and 6' as a benzylidene acetal, providing compound **12**. Alcohol acetylation and cleavage of the benzylidene acetal led to the corresponding 4',6'-diol **13**, which was then selectively benzoylated at the primary position by *N*-benzoyl imidazole. The free hydroxy group left at position 4' was converted into the 4'-epi-chloro-4'-deoxy **15** derivative by sulfuryl chloride. Radical dehalogenation efficiently led to the 4'-deoxy derivative **16**. Deacetylation and hydrogenolysis of both derivatives provided the 4'-deoxy and the 4'-chloro paromomycin analogues **17** and **18** respectively.



Scheme 10. Selective chloration and deoxygenation of the 4' position of paromomycin.

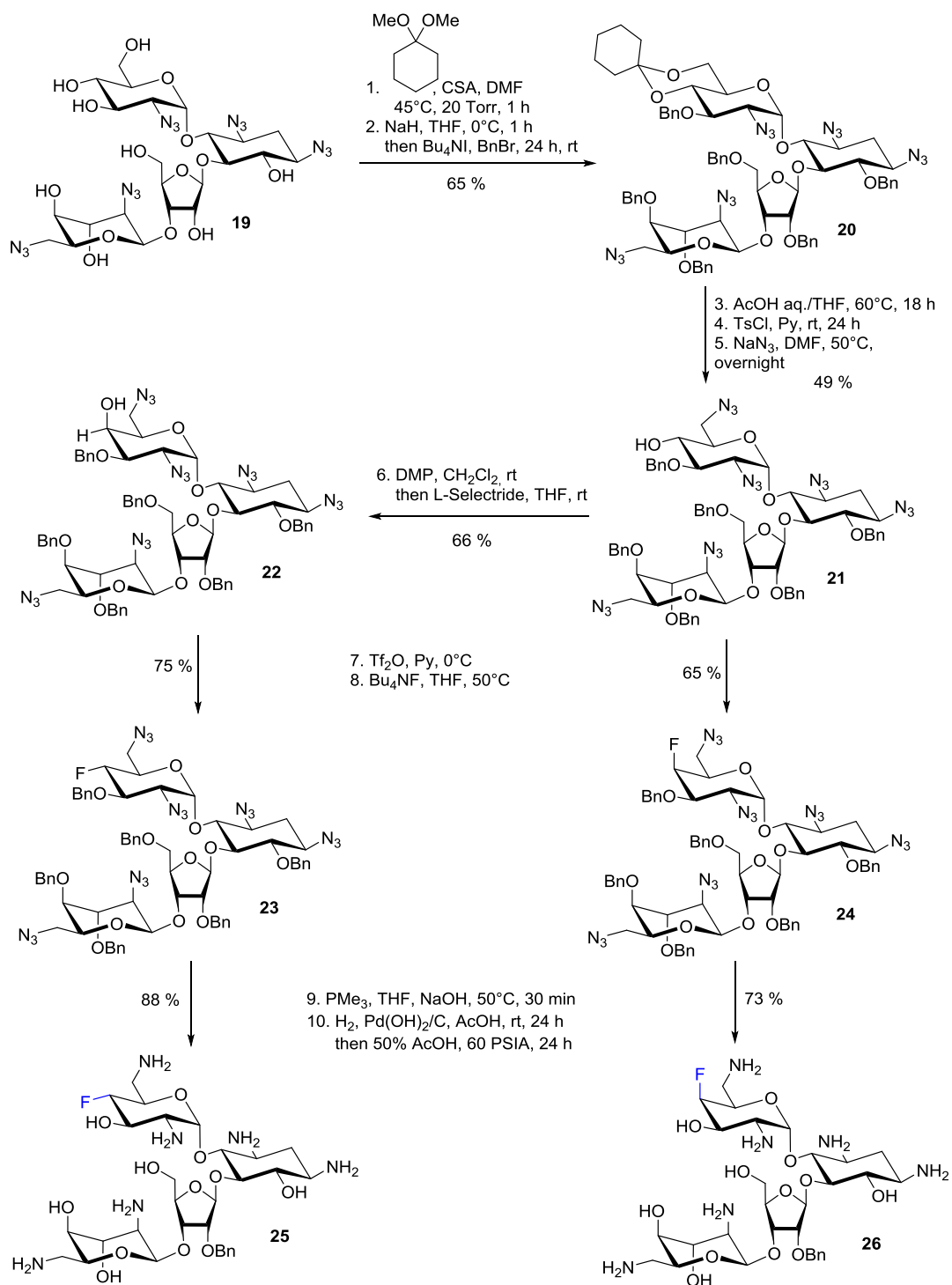
The activities of the latter were evaluated with different bacterial strains (Table 6). The 4'-deoxy derivative **17** exhibits activity as good as paromomycin against standard strains of *S. aureus* and *E. coli*, but much better activity against *S. epidermidis* FK 109 harbouring ANT(4'). The 4'-chloro derivative **18** is less efficient. It should be noted that, although still very moderate, the activity of **17** against *E. coli* K12 with APH(3')-II AME is better than that of paromomycin, while the AME target position is different. Again, the 4'-chloro derivative **18** did not lead to useful activity. These results revealed that it is better to deoxygenate the 4' position than to replace the hydroxyl with a chlorine atom.

Bacterial strain	AME	Par	17	18
<i>S. aureus</i> FDA 209P		1,56	1,56	6,25
<i>S. epidermidis</i> FK 109	ANT(4')	> 50	3,12	12,5
<i>E. coli</i> K 12		6,25	12,5	50
<i>E. coli</i> K 12 (R 112)	APH(3')-I	> 200	> 200	> 200
<i>E. coli</i> K 12 (R 148)	APH(3')-II	> 200	50	200

Table 6. MIC ($\mu\text{g/mL}$) of 4'-chloro or deoxy paromomycin **17** and **18** vs the parent paromomycin (Par).

Despite these results, the Hanessian group synthesized the 4'-fluoro analogue of neomycin and its epimer (Scheme 11) [65]. Starting from the azido masked paromomycin **19**, the same strategy was applied. The 4' and 6' hydroxyl groups were first protected as a cyclohexylidene acetal; the other hydroxyl groups were then benzylated to yield the fully protected derivative **20**. Once the acetal hydrolysed in acidic conditions, the free 6' primary hydroxyl was sulfonylated with *p*-toluene sulphonyl chloride (TsCl), then substituted with an azide to yield the key intermediate **21** carrying a single free hydroxy group at the 4' position.

Oxidation with Dess-Martin periodinane (DMP), followed by reduction with L-Selectride led to the 4'-*epi*-hydroxy derivative **22**. For both epimers **21** and **22**, the free 4'-hydroxy group was converted to a leaving group by sulfonylation with triflic anhydride and then substituted by a fluorine atom with tetrabutylammonium fluoride in good yields. The corresponding 4'-deoxy-4'-fluoro **23** and 4'-deoxy-4'-*epi*-fluoro **24** derivatives were then fully deprotected by azide Staudinger reduction and benzyl hydrogenolysis, providing in high yields the 4'-deoxy-4'-fluoroneomycin B **25** and 4'-deoxy-4'-*epi*-fluoroneomycin B **26**.



Scheme 11. Selective fluorination of the 4' position of neomycin B.

The activity of these fluoro epimers was evaluated against bacterial strains of *E. coli*, *S. aureus* and *P. aeruginosa* (Table 7). Both epimers exhibit an activity very similar to that of neomycin B on *E. coli* and *S. aureus* strains to which they are susceptible.

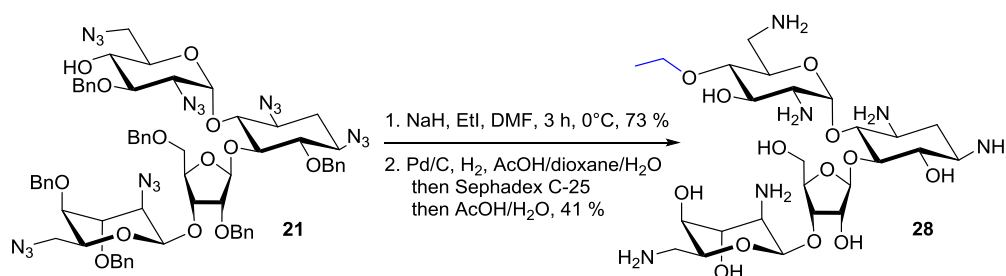
It therefore appears that the replacement of the hydroxy group at the 4' position of neomycin B by a fluorine atom does not alter the affinity of the compounds for the A site of these bacteria. Interestingly, this structural modification makes it possible to escape the action of ANT(4',4'') enzymes. Nevertheless, APH(3') enzyme cannot be blocked in *S. aureus* strain. However, for *P. aeruginosa* strains producing such APH(3')-IIb enzyme, it is worth noticing that only the 4'-fluoro epimer **26** is able to overstep this AME. This effect could be due to the presence of an axial fluorine atom at 4' position, which reduces the nucleophilicity of the 3' hydroxy group through stereoelectronic effect [65], and thus limits AME phosphate transfer. When equatorial, this fluorine atom may prevent the compound from reaching its target in *P. aeruginosa* bacteria.

Bacterial strain	AME	Neo	25	26
<i>E. coli</i> ATCC 25922		0,5	1	1
<i>E. coli</i>	AAC(6')-I	0,5	1	2
<i>S. aureus</i> ATCC 29213		0,5	1	0,5
<i>S. aureus</i>	ANT(4',4'')-I	> 32	2	1
<i>S. aureus</i>	ANT(4',4'')-II	> 32	1	1
<i>S. aureus</i>	APH(3')-III	> 32	> 32	> 32
<i>P. aeruginosa</i> ATCC 27853	APH(3')-IIb	> 32	> 32	2
<i>P. aeruginosa</i>	APH(3')-IIb	8	> 32	1
<i>P. aeruginosa</i>	AAC(6')-II, APH(3')-IIb	4	> 32	4

Table 7. MIC ($\mu\text{g/mL}$) of 4'-epi-fluoro or 4'-fluoro derivatives **25** and **26** vs the parent neomycin (Neo).

Instead of introducing a halogen atom at position C-4', the Crich group attempted to alkylate this position, capitalising on the increased selectivity for bacterial vs human ribosome of 2'-alkylated neomycin and paromomycin derivatives (see section 3.1.1) [66]. From the perazido perbenzyl paromomycin 4',6'-acetal, the azido neomycin

derivative **21** was prepared and its free 4'-hydroxy group was ethylated under basic condition affording compound **27**. After concomitant azide hydrogenation and benzyl hydrogenolysis, compound **28** was rapidly obtained (Scheme 12).



Scheme 12. Selective synthesis of a 4'-ethylated neomycin derivative.

As expected, this modification restored the activity on *E. coli* strain bearing the ANT(4', 4'') AME gene. In addition, the described antibacterial properties showed improved activities against two clinical strains of MRSA that are unaffected by neomycin (See section 3.5.5, Table 21). These encouraging results were counterbalanced by a low ribosomal selectivity, suggesting only a small decrease in toxicity. Nevertheless, they demonstrated the potential of such functionalisation and led the Crich group to embark on a research program to methodically study polyfunctionalised analogues (See section 3.5).

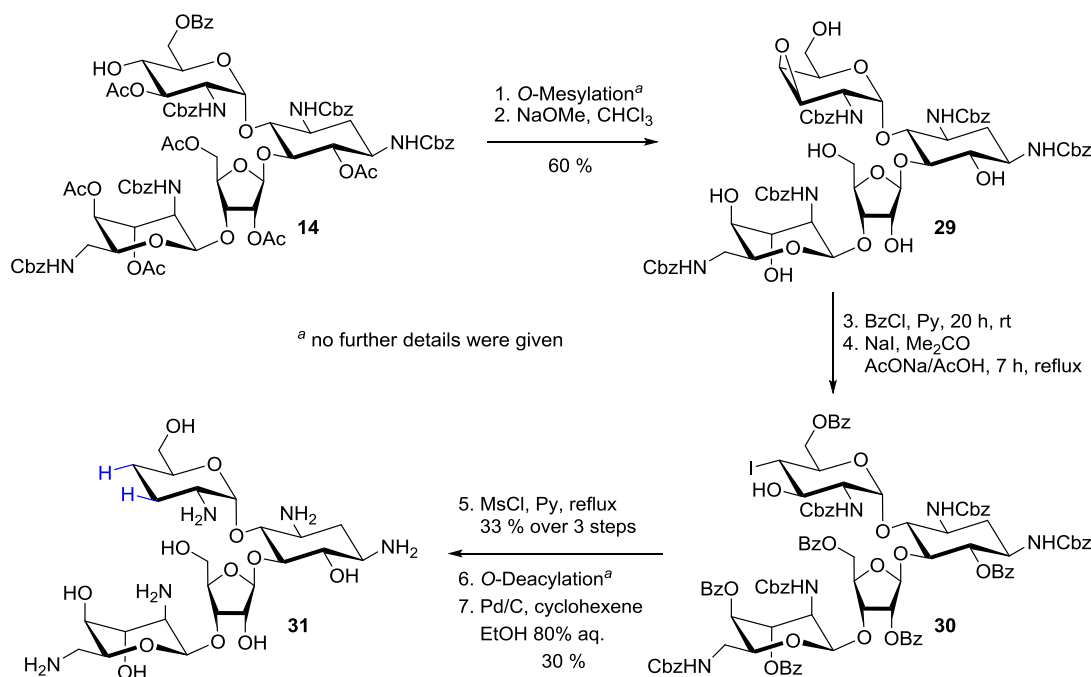
3. 1. 3. Modification at position 3' and 4'

The deoxygenation of the 3' and 4' positions has been extensively explored on 4,6-disubstituted aminoglycosides, but to a more limited extent on aminoglycosides of the neomycin family.

Exploiting the 4',6'-deprotected perCbz peracetylated paromomycin derivative **14** mentioned earlier (see Scheme 10), the Arcamone group was able to prepare the 3',4'- dideoxyparomomycin **31** with 6% yield over 7 steps (Scheme 13) [67].

Starting from the **14**, mesylation of the 4' hydroxy group and treatment with sodium methoxylate led to the 3',4'- β -epoxy deacetylated derivative **29**. Re-esterification of the hydroxy groups with benzoyl chloride and epoxide regioselective opening with

sodium iodide provided the 4'-iodo derivative **30**. Mesylation of the free hydroxy group at 3' position under harsh conditions then allowed the formation of a double bond in the 3',4' position by concomitant elimination of mesyl and iodine, according to Lemieux et al. [68]. Finally, general deprotection and olefin hydrogenation led to 3',4'-dideoxyparomomycin **31**.



Scheme 13. Selective deoxygenation of the 3',4' positions of paromomycin.

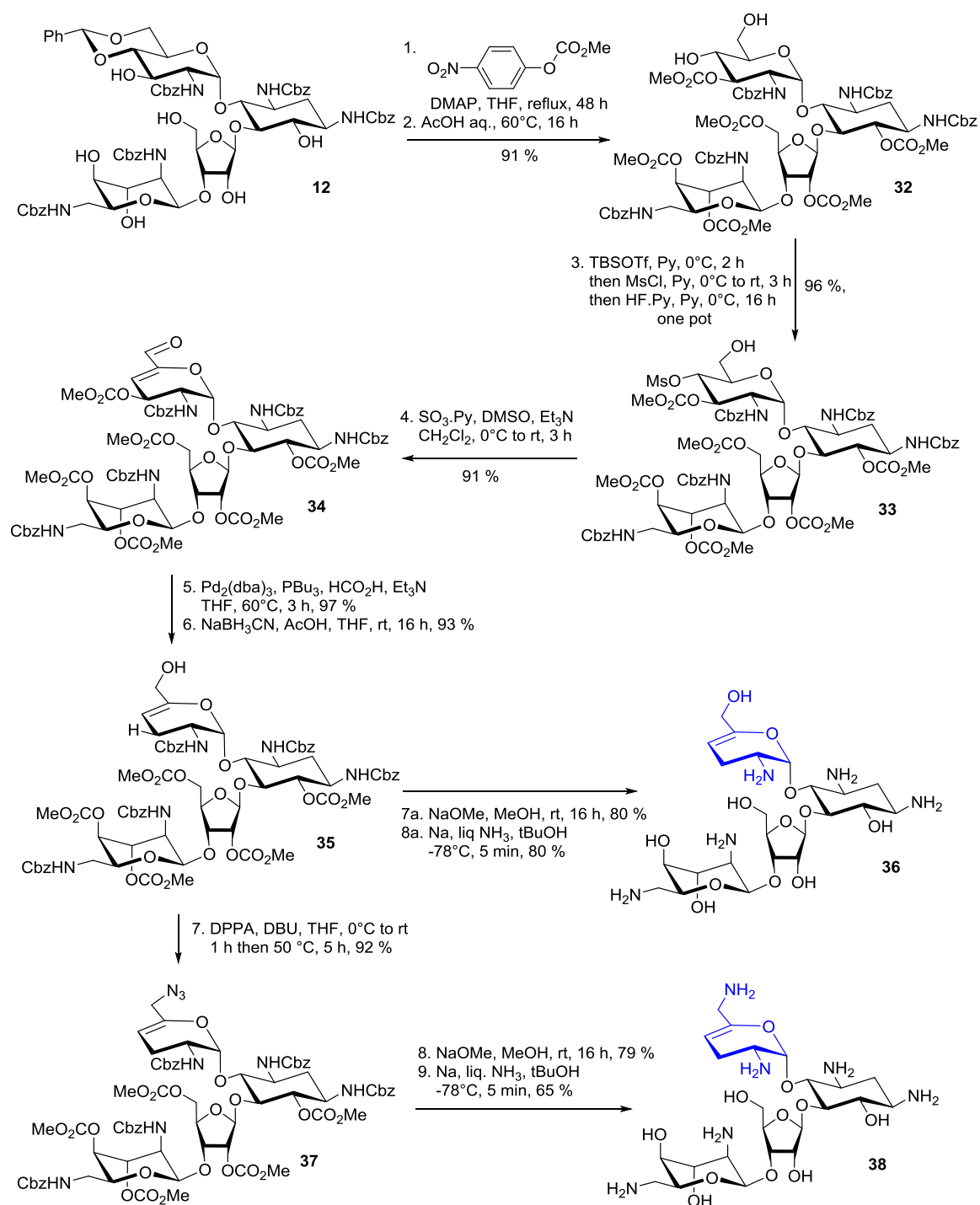
This 3',4'-dideoxyparomomycin **31** is as effective as paromomycin on standard strains of *S. aureus* and *E. coli*, and interestingly retains good activity against the ANT(4') producing strain of *S. epidermidis* and reasonable activity against an APH(3')-II producing strain of *E. coli* (Table 8).

Bacterial strain	AME	Par	31
<i>S. aureus</i> FDA 209P		1,56	1,56
<i>S. epidermidis</i> FK 109	ANT(4')	> 200	3,12
<i>E. coli</i> K 12		6,25	12,5
<i>E. coli</i> K 12 (R 148)	APH(3')-II	> 200	25

Table 8. MIC ($\mu\text{g/mL}$) of 3',4'-dideoxy derivatives **31** vs the parent paromomycin (Par).

More recently, Hanessian's group synthesized similar neomycin and paromomycin derivatives by a pathway that allows the introduction of an unsaturation between the 4' and 5' carbons (Scheme 14) [69]. This structural modification allows the resulting cycle I to mimic that of sisomicin, a natural aminoglycoside antibiotic used in hospitals (see Scheme 2).

Used as starting material, the known *N*-Cbz 4',6'-benzylidene paromomycin **12** (see Scheme 10) was *O*-protected as carbonate and its acetal cleaved to provide the 4',6'-diol **32**. Silylation of the free 6' primary hydroxyl, mesylation of the 4' hydroxyl and desilylation provided intermediate **33**. The single free 6' hydroxyl of **33** was then converted to aldehyde by Parikh-Doering oxidation, which also allowed β -elimination of the adjacent mesylate group, thus leading to the α,β -unsaturated aldehyde **34**. A very effective Tsuji-Trost palladium-catalysed allylic deoxygenation, followed by aldehyde reduction provided the allylic alcohol **35**. Full deprotection, achieved through a Zemplen reaction and Birch reduction, led to the expected paromomycin mimic of sisomicin **36**. Interestingly, the intermediate **35** could also be processed to a neomycin mimic **38** through substitution of the 6' hydroxyl with an azide and deprotection.



Scheme 14. Selective deoxygenation of the 4',5' positions of paromomycin and neomycin.

The activity of the derivative **38** is identical to that of neomycin against standard strains (*S. aureus*, *E. coli* and *K. pneumoniae*) (Table 9). This observation confirms the hypothesis that the hydroxy groups at positions 3' and 4' are not essential for

binding to the bacterial A site and that deoxygenation of these positions does not decrease the affinity of the compound for its target. In addition, MIC results obtained against resistant strains confirm that the absence of these 3' and 4' hydroxy groups does not allow the action of APH(3') and ANT(4') enzymes. In contrast, the paromomycin analogue **36** shows a decreased activity, although a low activity remains with the ANT(4') from *S. aureus* strain. It seems once again that the presence of an amino group in the 6' position is necessary to maintain good antibacterial activity.

Sisomicin-neomycin hybrid **38** exhibits very interesting antibacterial activity, even against resistant strains, but it requires an 11 steps synthesis; it was nevertheless obtained with a good 31% overall yield.

Bacterial strain	AME	Neo	36	38
<i>S. aureus</i> ATCC 29213		1	8	1
<i>E. coli</i> ATCC 25922		2	> 64	2
<i>K. pneumoniae</i> ATCC 10031		0,5	> 64	0,5
<i>P. aeruginosa</i> ATCC 27853	APH(3')	32	> 64	≤ 1
<i>S. aureus</i>	ANT(4')	> 64	16	1

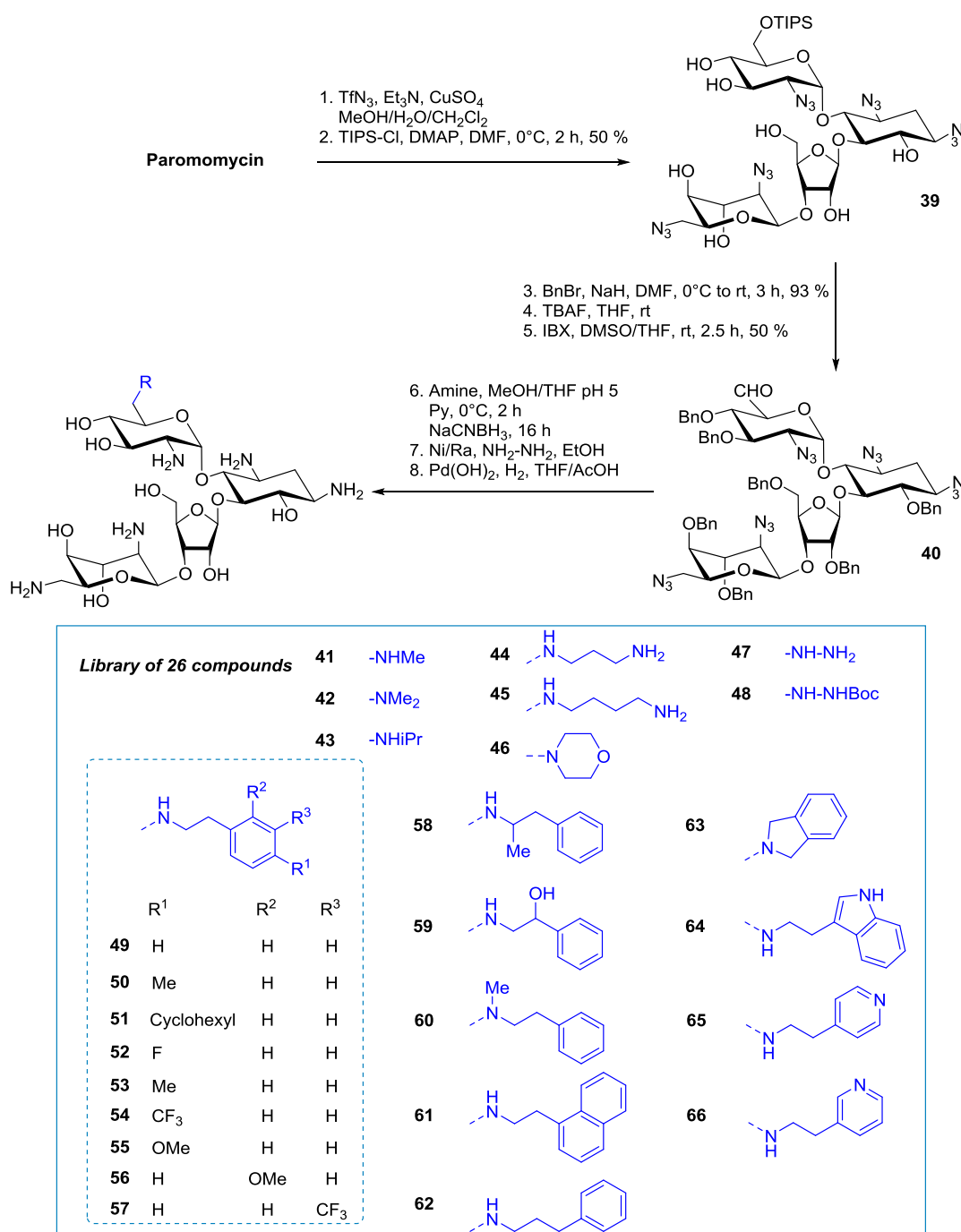
Table 9. MIC ($\mu\text{g/mL}$) of 3',4'-dideoxy 4',5'-deshydro derivatives **36** and **38** vs the parent neomycin (Neo).

3. 1. 4. Modification at position 6'

Despite the availability and reactivity of the 6' position, only a few approaches have so far been achieved to modify this position.

One of the developed strategies relied on the reactivity difference of the two primary alcohols of paromomycin, due to the different environment of these hydroxy groups (Scheme 15) [70]. To take benefit of this particularity, paromomycin was first converted to perazido paromomycin using the explosive trifluoromethanesulphonyl azide as diazo transfer reagent. Chemoselective protection was achieved using

triisopropylsilyl chloride as bulky silylating reagent and dimethylaminopyridine as base in dimethylformamide at 0°C. The desired protected alcohol **39** was obtained in 50% yield. After protection of the remaining hydroxy groups and deprotection of the sole silyl ether, the so-obtained alcohol was oxidized with IBX giving the desired aldehyde **40** in 46 % yield over two steps. From the latter, a library of 26 neomycin analogues **41-66** was obtained through a 3-step sequence: reductive amination, alcohol deprotection and amino deprotection. Apolar, polar, heterocyclic and aromatic groups were introduced using this method.

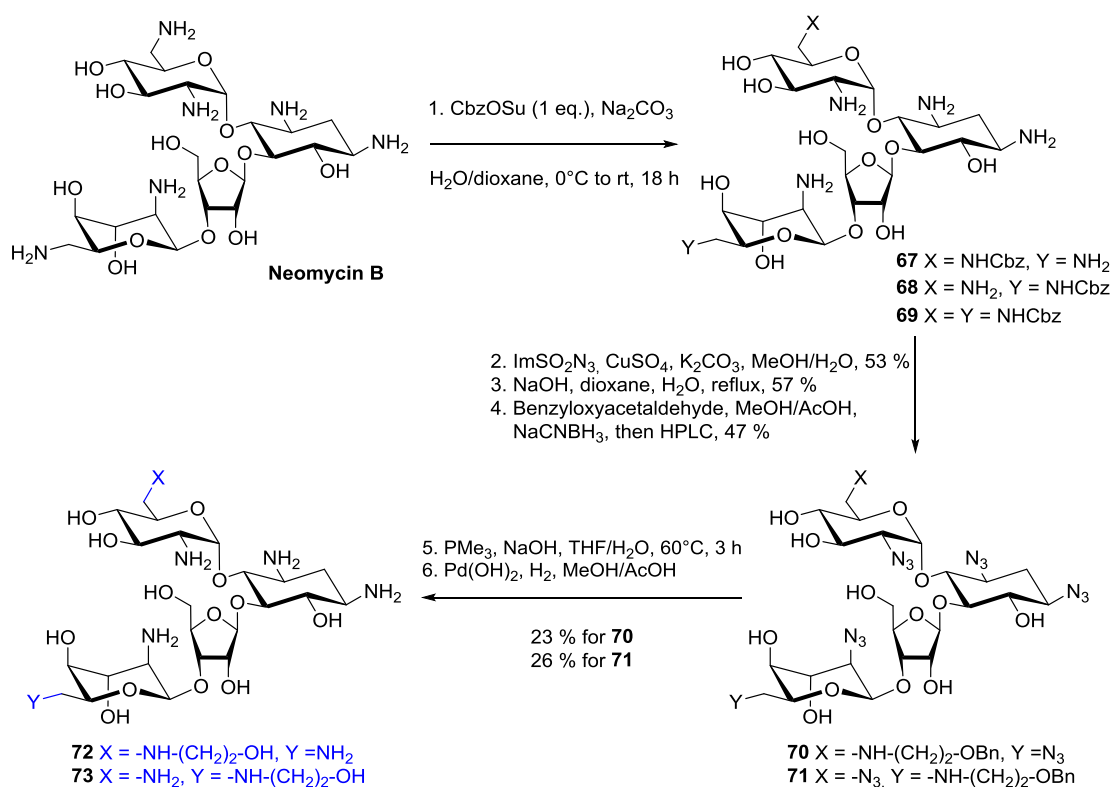


Scheme 15. Synthesis of a library of 6'-alkylated neomycin derivatives

All compounds were tested in order to determine their antibacterial activity against *E. coli* and *S. aureus* strains. With the exception of the morpholino and hydrazine derivatives **46** and **48**, all compounds showed some antibacterial activities but no parent aminoglycoside was used for comparison purpose.

Another approach relied on the mono-protection of the 6'-amine of neomycin. Such mono-protection is barely described in the literature but nevertheless possible. Inspired from Kawaguchi protocol [71], the groups of Crich and Vasella indeed developed conditions able to perform the mono-protection of neomycin B in a single step from neomycin B sulfate salt (Scheme 16) [66]. Treatment of neomycin B with one equivalent of benzyloxycarbonyloxysuccinimide and 6 equivalents of sodium carbonate as base in aqueous dioxane at 0°C during 6h and then at room temperature for 12 hours gave an inseparable mixture of the monoprotected regioisomers **67** and **68** with 31 % yield, and the diprotected neomycin **69** in 30 % yield. The mixture **67** and **68** was then treated with imidazole-1-sulfonyl azide in the presence of a catalytic amount of copper sulfate, which afforded a mixture of pentaazides. Basic benzyloxycarbonyl hydrolysis followed by reductive amination with the benzyl protected 2-hydroxyacetaldehyde gave after HPLC separation compounds **70** and **71** in 23 and 26 % yield respectively. Staudinger reduction of azides and hydrogenolysis of the benzyl group then ensured full deprotection and gave the neomycin derivatives, **72** and **73** respectively, carrying a 2-hydroxyethyl chain at 6' and 6''' position respectively.

The antimicrobial activity of **72** was determined on *E. coli* strains carrying various specific resistances (see section 3.5 and Table 21). However, it proved similar to neomycin B, except that the activity was restored against an AAC(6') resistant strain of *E. coli*, as expected from a 6'-modified neomycin. However, the antibacterial activity of **73** is very similar to that of the parent neomycin, probably due to the fact that N-6''' is not engaged in AG binding to A site (see Figure 5).



Scheme 16. Synthesis of 6'- and 6'''-alkylated neomycin derivatives through monoprotection of neomycin.

In a totally different but interesting approach, some of the enzymes able to modify AG (AME; section 2.4) have been directly used. Among them, AAC(6') enzyme was applied to selectively acylate the 6' position of neomycin (See section 3.6).

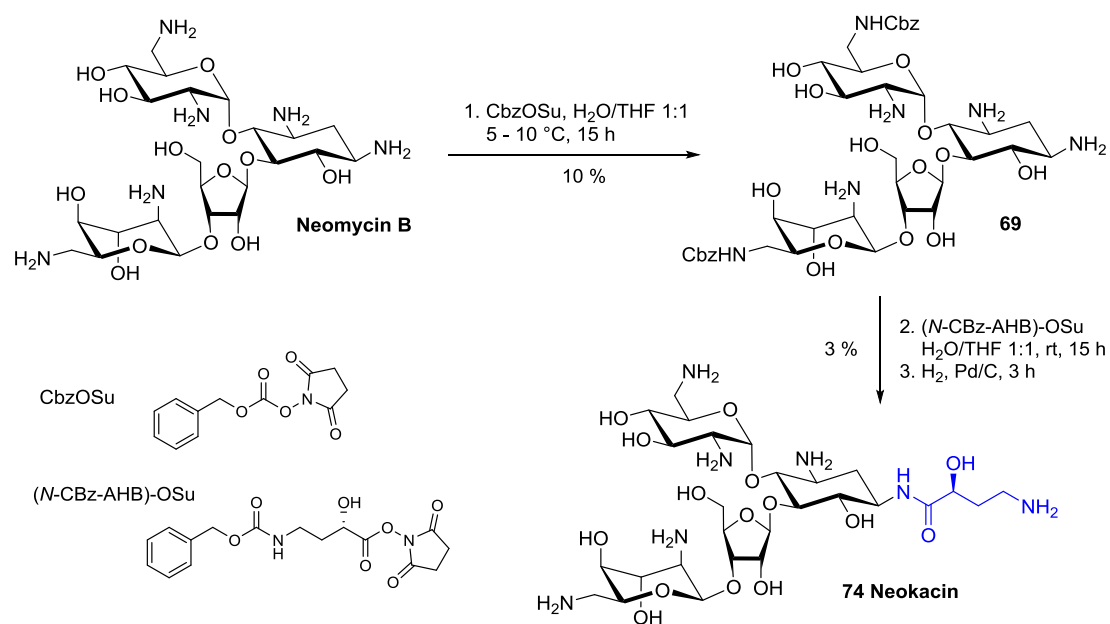
3. 2. Functionalisation of Ring II

In contrast to ring I and despite its importance in A-site binding, ring II is – so far – less targeted by AME, and only *N*-acetyltransferases targeting the amino group at position 1 or 3 are known.

3. 2. 1. Modification at position 1

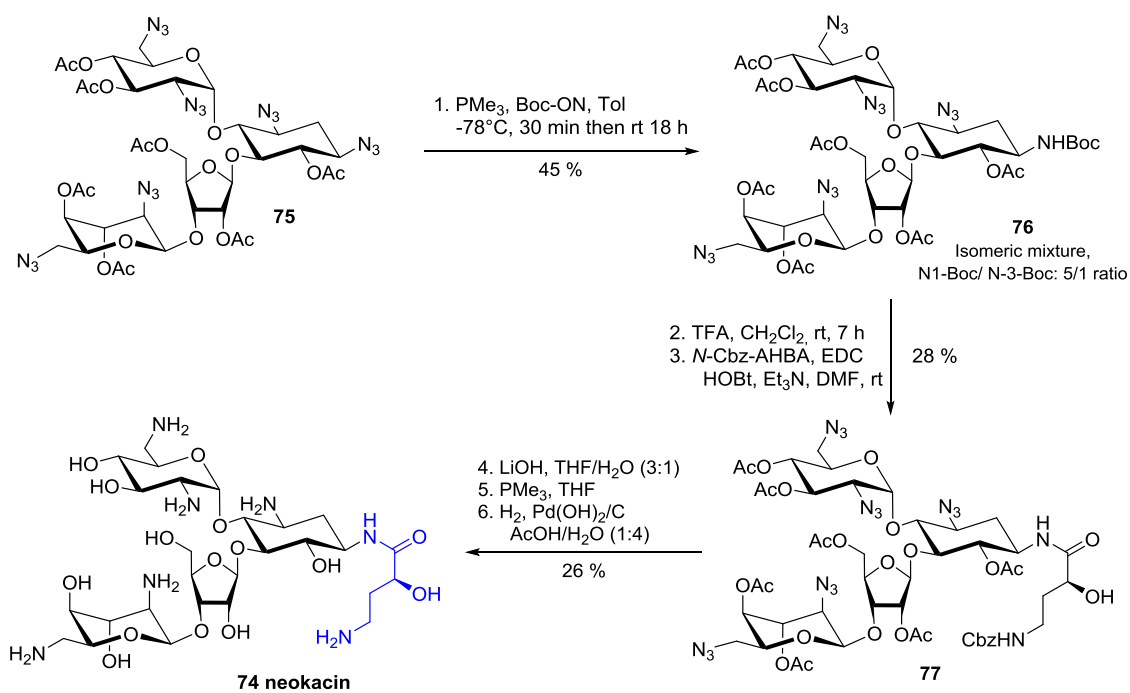
Known since 1972, butirosins are naturally occurring ribostamycin derivative carrying at position 1 a (S)-4-amino 2-hydroxybutyryl (AHB) chain [72]. Produced by *Bacillus circulans*, butirosin A and B have shown activity against a variety of resistant bacteria [73]. As the presence of this chain confers some resistance to resistant strains, different semi-synthetic AG with the AHB chain grafted on the 1 amino group were produced, with some success at least in the 4,6-disubstituted 2-DOS family of AG. Indeed, amikacin, derived from kanamycin in 1972, arbekacin derived from dibecacin in 1973 are still used in hospitals together with the more recently introduced plazomicin from sisomicin in 2010 (see Scheme 2). Nevertheless, several 4,5-disubstituted 2-DOS AG were also modified at position 1, especially with AHB chain for the same reason.

Soon after the discovery of butirosins, an AHB chain was grafted to neomycin B. In 1974, the Bristol-Myers company proposed the first synthesis of this modified neomycin, later called neokacin (Scheme 17) [74]. Neomycin treatment with two equivalents of benzyl succinimidyl carbonate (CbzOSu) led to the 6',6'' *N*-dibenzoyloxycarbonyl neomycin **69**, which was then submitted to one equivalent of the succinimidyl ester of (S)-4-benzoyloxycarbonylamino-2-hydroxybutyric acid. The resulting mixture was then deprotected by catalytic hydrogenation, which provided the *N1*-AHB neomycin **74** (neokacin) but in very low yield (0.3%) despite a very short sequence. The lack of selectivity and the resulting purifications were responsible for this result.



Scheme 17. Synthesis of neokacin, neomycin modified at position 1 with the (S)-4-amino 2-hydroxybutyryl (AHB) chain, typical of butirosin.

33 Years later, another route to neokacin based on a selective Staudinger reduction of the perazido neomycin [75], was proposed by the group of Chang (Scheme 18) [76]. The known perazido neomycin **75**, prepared from neomycin in two steps with a 45% yield was treated by 1.1 equivalent of trimethylphosphine in the presence of 2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile (Boc-ON). This reaction provided in 45% yield a 5:1 mixture of two inseparable regioisomers, in which the major one corresponded to the N1-Boc derivative **76** and the other to the N3-Boc isomer. After carbamate cleavage, the resulting free amines were engaged in peptide coupling with the (S)-4 benzyloxycarbonylamino-2-hydroxybutyric acid (N-Cbz-AHBA). At this stage, the two resulting regioisomeric amides could be separated by chromatographic column and the required derivative **77** were isolated in modest yield. Successive deprotections finally led to neokacin, whose name was given by the Chang's group. The overall yield of this synthesis was rather low (1.5% over 8 steps), again due to poor selectivity and to the ensuing separations.



Scheme 18. Synthesis of neokacin through the selective reduction of perazido neomycin.

Neokacin activity was evaluated against different strains of *E. coli*, *K. pneumoniae*, *S. aureus*, and *P. aeruginosa* (Table 10). This compound is effective against neomycin B-susceptible bacteria and remarkably, against *K. pneumoniae* strains, unlike neomycin. Remarkably, neokacin remains effective against the *E. coli* TG1 strain which carries the pTZ19U-3 plasmid encoding for APH(3')-I, although the targeted position of this enzyme and the modified position are different. However, N1-acylation did not improve activity against MSRA.

Bacterial strain	AME	Neo	74
<i>E. coli</i> ATCC 25922		4–8	8–16
<i>E. coli</i> TG1		4–8	8
<i>E. coli</i> TG1 (pSF815)	AAC(6')/APH(2'')	4	4
<i>E. coli</i> TG1 (pTZ19U-3)	APH(3')-I	> 32	2
<i>K. pneumoniae</i> ATCC 700603		> 32	4
<i>K. pneumoniae</i> ATCC 13883		4–8	2
MRSA ATCC 33591		> 32	> 32
<i>S. aureus</i> ATCC 25923		1–2	2–4
<i>P. aeruginosa</i> ATCC 27853		> 32	16

Table 10. MIC ($\mu\text{g}/\text{mL}$) of neokacin **74** vs the parent neomycin (Neo).

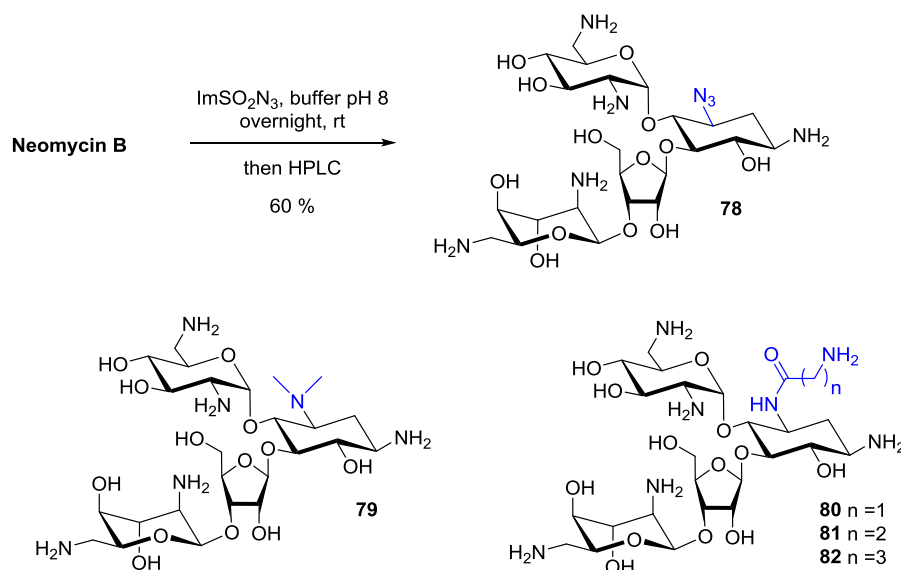
As for the selective acylation of the 6' position of neomycin (see section 3.1.4), enzymes responsible for the introduction of the AHB chain in the butirosin biosynthesis have been applied to selectively introduce this motif to a few AG, including neamine, paromamine and ribostamicine derivatives (see section 3.6).

3. 2. 2. Modification at position 3

In a reverse approach, the regioselective diazotation of aminoglycosides has been investigated, because such route could offer a fast and simple access to valuable building blocks [77].

To carry out this process (Scheme 19), imidazole-1-sulfonyl azide was used as diazotransfer reagent and water as solvent, but at controlled pH to take benefit of the different basicity of the amines. It was found that for neomycin B, pH 8 (phosphate buffer) provided the optimum regioselectivity; nevertheless, HPLC purification was required. Such process gave the valuable intermediate **78** with 60% yield. Without further details, this patent also claimed the synthesis of four *N*-3 modified derivatives

79-82. The antibacterial activities were also determined, and surprising results were mentioned. While these compounds show poor efficiency on non-resistant *E. coli* strain, they exhibit antibacterial activity on *E. coli* strain bearing ACC-3 resistance, as expected, except for compound **82**. The *N*-3 dimethylated neomycin **79** showed slightly better activity than neomycin B.



Scheme 19. Synthesis of *N*-3 modified neomycin through regioselective azidation

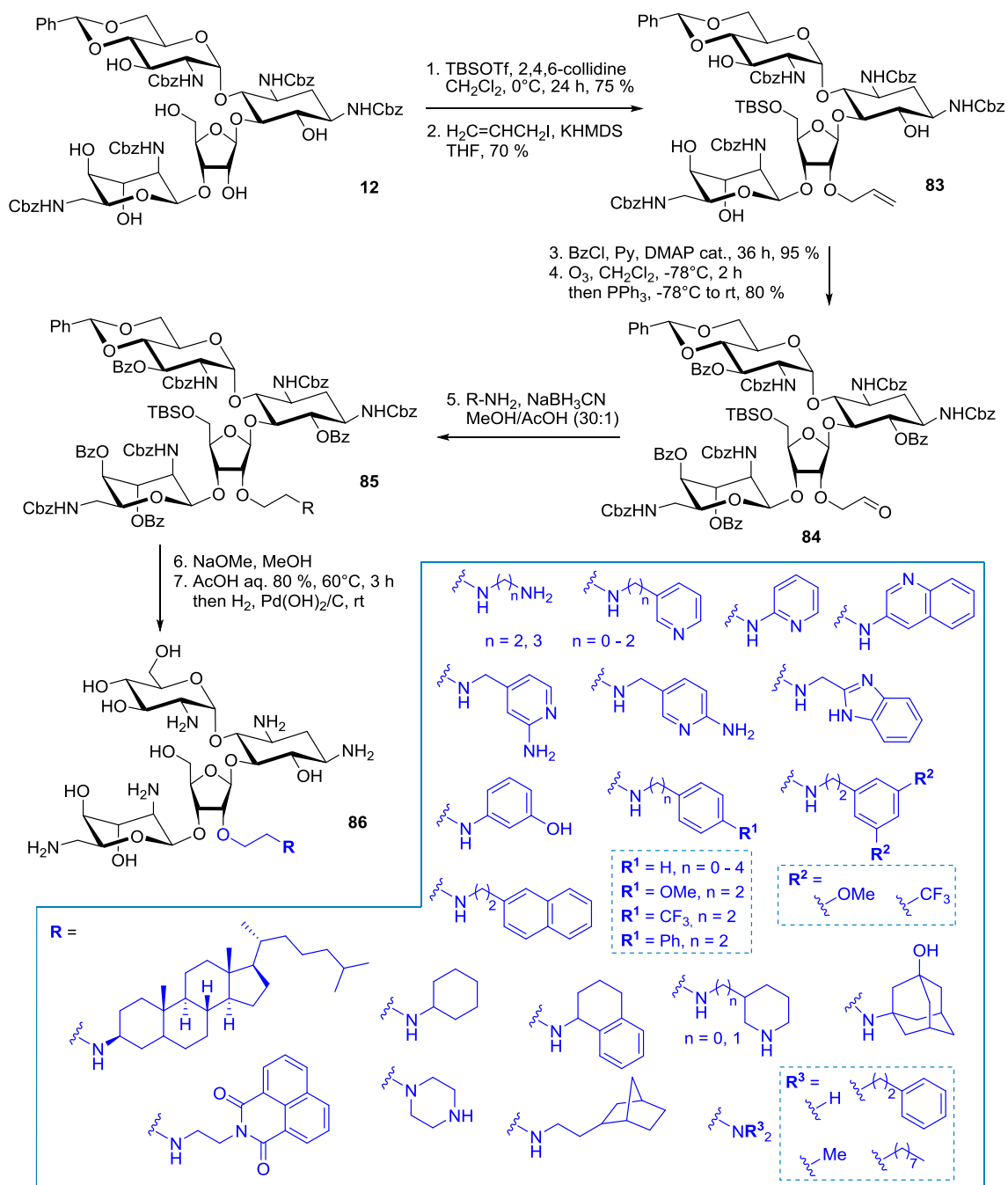
In an alternative and interesting approach, one of the acylating AME enzymes, AAC(3), was applied to selectively acylate the 3 position of neomycin (see section).

3. 3. Functionalisation of Ring III

As for ring II, cycle III is less targeted by AME than cycle I. Its two free hydroxy groups are only the target of phosphotransferases. In contrast, crystal structures revealed that ring III could force neomycin to adopt a special conformation within the ribosomal A site [60]. Thus, a plethora of studies aimed at modifying the 5'' hydroxy group have been described and surprisingly, only a few studies targeted the 2'' hydroxy group.

3. 3. 1. Modification at position 2''

Grafting allyl chain at 2'' position as a handle for further modifications was described by the Hanessian's group. (Scheme 20). For this approach, the *N*-per(benzyloxycarbonyl)-4',6'-benzylidene paromomycin **12** (see Scheme 10) was used as starting material. Its primary 5'' hydroxyl, the most reactive of the remaining hydroxy groups, was protected and then a selective Williamson allylation of the 2'' position was remarkably achieved in the presence of potassium bis(trimethylsilyl)amide (KHMDs) [78]. The derivative **83** was thus obtained with a good 70% yield. After protection of the remaining hydroxy groups, ozonolysis of the allyl group led to aldehyde **84**, ready for reductive amination. Various nitrogen chains were thus introduced, producing after full deprotection a set of more than thirty derivatives in 11 steps [79].



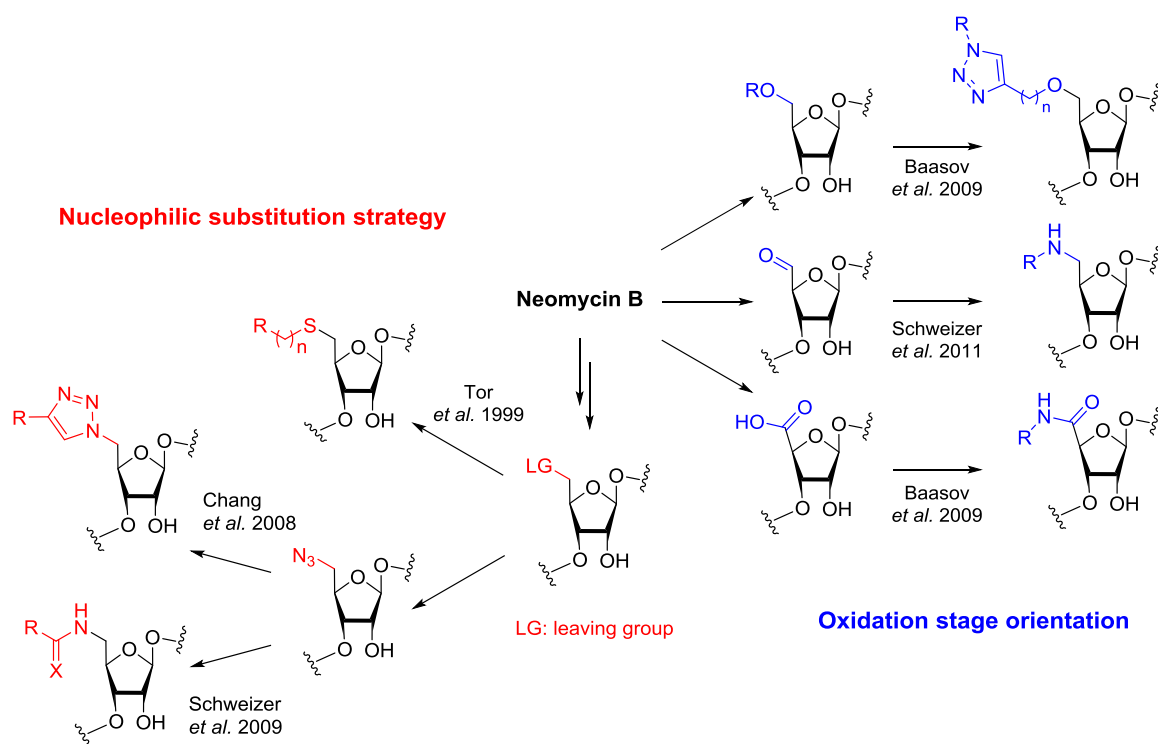
Scheme 20 Synthesis of 2'-alkylaminoethyl derivatives of paromomycin.

Unfortunately, these modifications do not affect antibacterial activity against a standard strain of *S. aureus* (ATCC 13709), while only the smallest, aliphatic, and polar functions appear to be active against *E. coli* (ATCC 25922). Most of these derivatives are still modified by APH(3')-IIIa, but 4 to 9 times slower than the parent paromomycin, although some of them remain unaffected by this enzyme. The most promising compound, carrying a phenylaminoethyl chain, does not appear to have

antibacterial activity against paromomycin-resistant strains, regardless of the resistance mechanism (AAC(3), AAC(2'), APH(3'), ANT(4'), AAC(6') or efflux).

3. 3. 2. Modification at position 5''

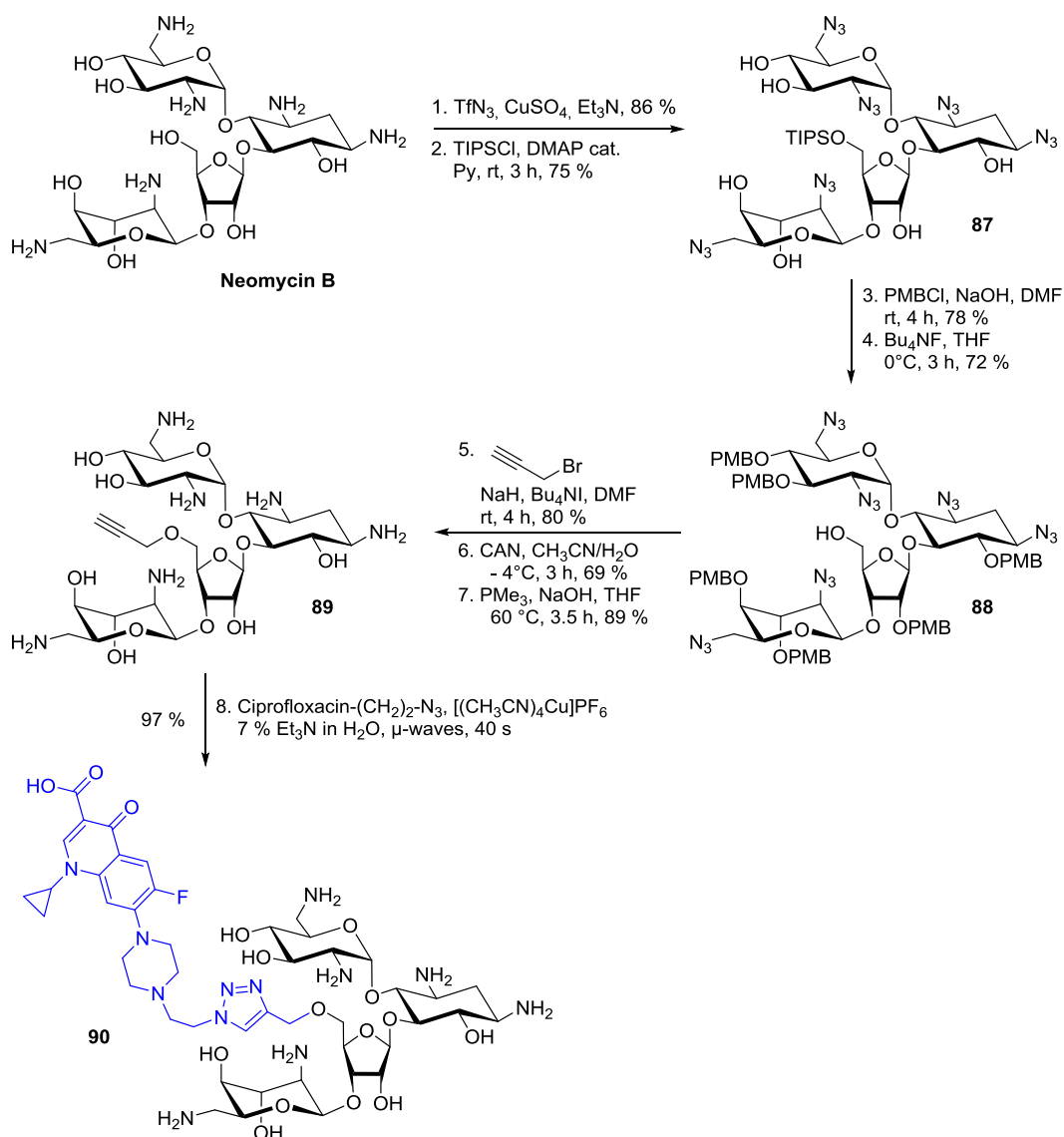
Based on the reactivity difference between the 2'' and 5'' hydroxyl groups, research groups focused on modifications at the primary and more reactive 5'' position. Two main approaches have been developed. The first is based on the synthesis of key intermediates with different oxidation stages of the 5'' carbon (alcohol, aldehyde, carboxylic acid) and the second, on the synthesis of an intermediate that can undergo nucleophilic substitution (Scheme 21).



Scheme 21. The divergent routes to 5''-modified neomycin derivatives.

3. 3. 2. 1 Modification at position 5'' through different oxidation stages of the hydroxy group

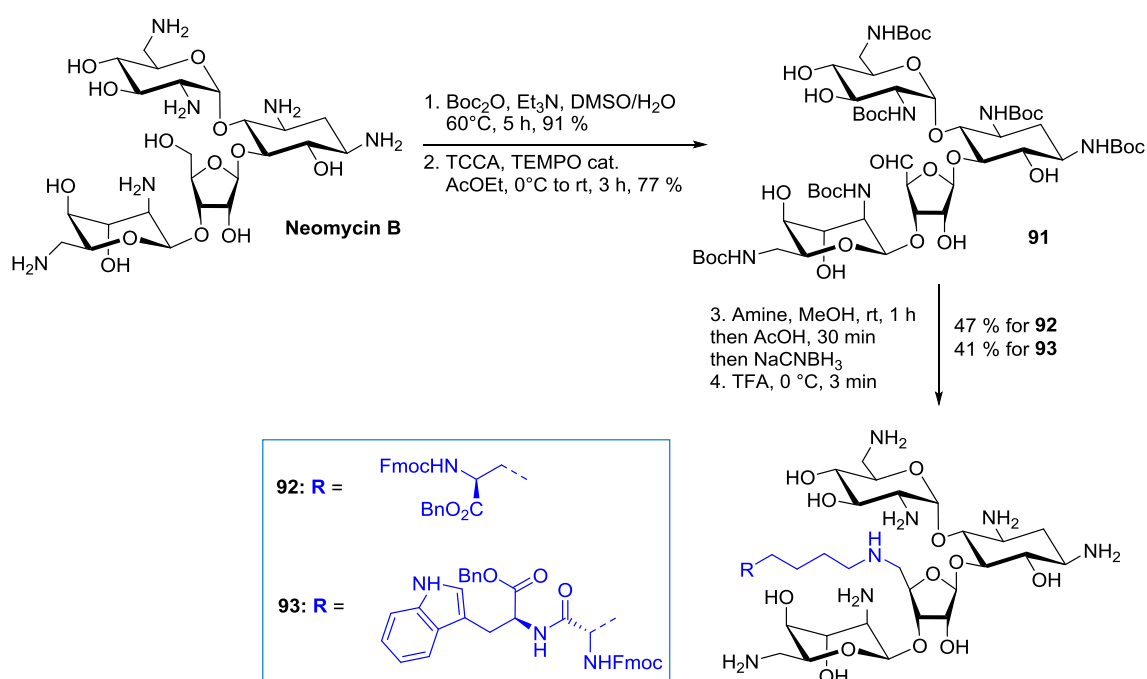
In 2009, Baasov's team developed a strategy that allows alkylating the 5'' alcohol by a propargylic chain (Scheme 22) [80]. Direct chemoselective propargylation of the 5'' hydroxyl was unfortunately not possible, due to the higher nucleophilicity of the amino groups. Neomycin B amino groups were thus first protected as azides, and the more reactive primary 5'' hydroxyl has to be protected, here as a large silyl ether, leading to **87**. The secondary alcohols were then protected with *para*-methoxybenzyl (PMB) groups and the 5'' hydroxyl was deprotected to lead to alcohol **88**. The latter was alkylated by propargyl bromide under basic conditions. Finally, deprotection of the alcohols by ceric ammonium nitrate (CAN) and Staudinger azide reduction yields the 5'' O- propargylated neomycin B derivative **89** in good overall yield (18% over 7 steps). The latter was then 'clicked' with an azido derivative of the fluoroquinolone antibiotic ciprofloxacin to afford compound **90**.



Scheme 22. The synthesis of 5''-ciprofloxacin-neomycin conjugate through click chemistry.

Combining two kind of antibiotics acting at different stages of bacteria life cycle, this fluoroquinolone-neomycin conjugate **90** was expected to be better than each one. Active against standard strains of both Gram-positive and Gram-negative bacteria, conjugate **90** indeed shows antibacterial activity superior to neomycin B on each of the strains tested. However, its activity remains lower than ciprofloxacin alone (Table 12).

Taking benefit of the higher reactivity of the primary 5'' hydroxyl group in neomycin B, the group of Schweizer was able to selectively oxidize this alcohol into aldehyde (Scheme 23) [81]. The *N*-BOC protected neomycin was submitted to trichloroisocyanuric acid (TCCA) and a catalytic amount of 2,2,6,6 tetramethylpiperidine-1-oxyl (TEMPO) in ethyl acetate, leading to aldehyde **91** in good yield (77%). Reductive amination with protected lysine and a protected tryptophanyl lysine peptide, followed by full deprotection provided the neomycin conjugate **92** and **93**.



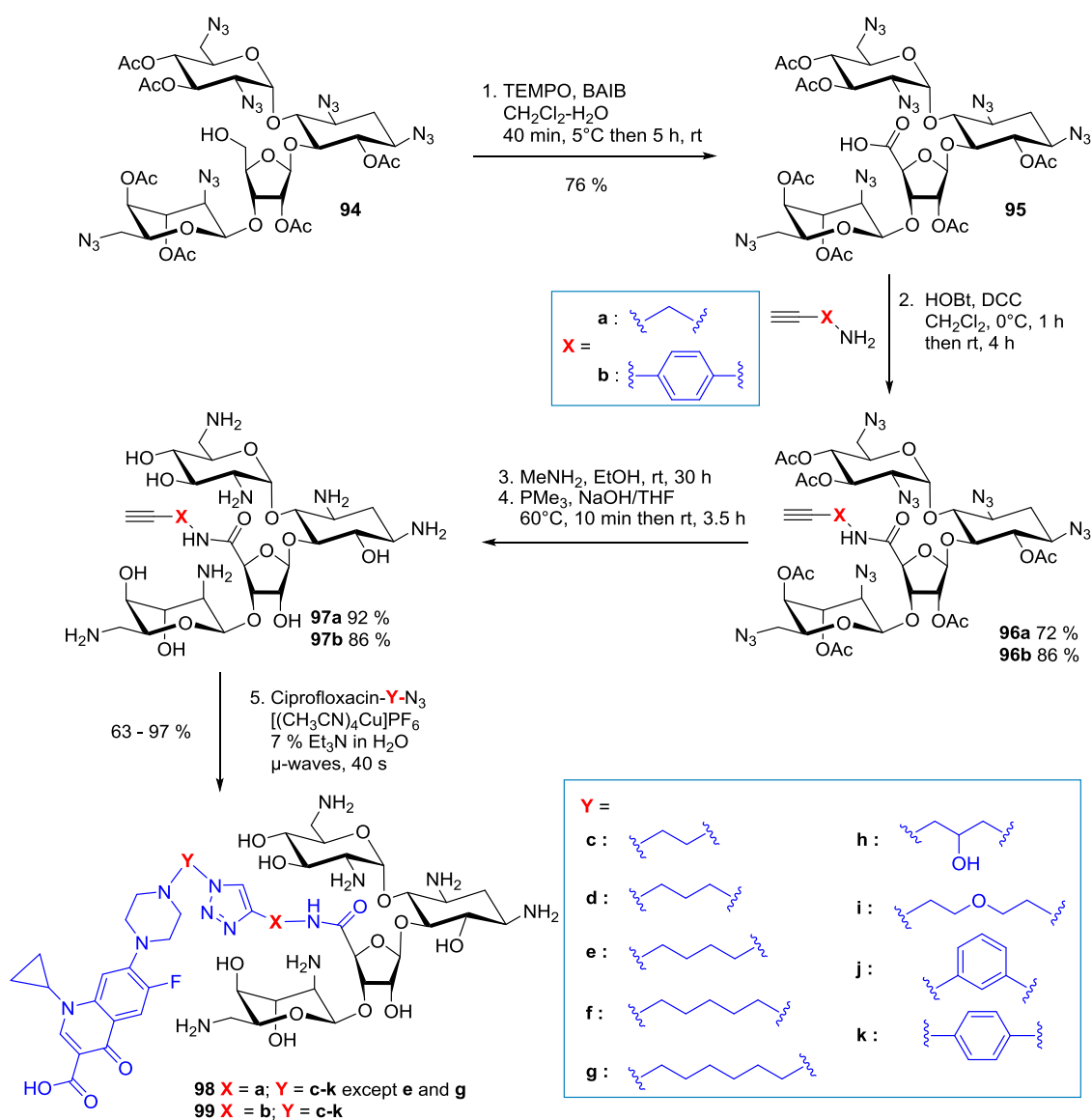
Scheme 23. Synthesis of 5''-peptidyl neomycin conjugates.

Evaluated against Gram-positive and Gram-negative strains, these two peptidyl-neomycin conjugates exhibit a decreased antibacterial activity compared to their parent (Table 11). Nevertheless, the dipeptidyl conjugate **93** offers an activity superior to neomycin B against *MRSA* and *P. aeruginosa* CAN-ICU 62308, but these values are still insufficient to consider it as a possible drug candidate.

Bacterial strain	Neo	92	93
<i>S. aureus</i> ATCC 29213	1	16	8
<i>MRSA</i> ATCC 33592	256	128	32
<i>S. epidermidis</i> ATCC 14990	0,25	4	4
SERM (CAN-ICU) 61589	0,25	4	4
<i>S. pneumoniae</i> ATCC 49619	8	128	64
<i>E. coli</i> ATCC 25922	2	32	16
<i>E. coli</i> CAN-ICU 61714	4	32	16
<i>P. aeruginosa</i> ATCC 27853	512	> 256	128
<i>P. aeruginosa</i> CAN-ICU 62308	512	> 256	64

Table 11. MIC ($\mu\text{g/mL}$) of peptidyl-neomycin conjugate vs the parent antibiotics.

Such oxidative route to 5'' modification was already reported by the Baasov group but up to the acid stage (Scheme 24) [80]. To do so, the neomycin amino groups were masked as azide and the hydroxy groups were acetylated, except for the one at the 5'' position [82]. This free hydroxy group was then oxidized to the carboxylic acid **95** in the presence of TEMPO and [bis(acetoxy)iodo]benzene (BAIB). Peptide coupling with propargylamine or 4-ethynylaniline under classical conditions then provided amides **96a** and **96b** in high yields. Their deprotection led to acetylenic neomycin derivatives **97a-b**, which were conjugated again to ciprofloxacin azides through click reaction. By adjusting the azide linker, a library of 15 conjugates were thus obtained.



Scheme 24. Synthesis of a library of 5''-ciprofloxacin-neomycin conjugates.

As for conjugate **90** (see Scheme 22), these conjugates combining two kind of antibiotics were expected to have better activities than each one. In these cases, they were designed with various linkers in order to optimise their respective positioning in A-site for better antibacterial activity. Unfortunately, and as for conjugate **90**, these conjugates show good antibiotic activity against standard or resistant bacterial strains, better than that of neomycin B, but lower than that of ciprofloxacin alone (Table 12). Nevertheless, they retain very good activity against resistant APH(3')-IIIa *E. coli* strains, and good to intermediate activity against APH(3')-I or Ia strains and against a bifunctional AAC(6'')/APH(2'') strain. These

hybrid aminoglycosides also have an interesting activity against neomycin B-resistant *MRSA*.

In contrast to what was expected, the length of the linear aliphatic linker (Y; **98a/(c-d)**, **98a/f** and **99b/(c-g)**) has no influence on the antibacterial activity. If this Y linker is functionalised, a better activity was observed, an alcohol (**98a/h** and **99b/h**) being better than an ether (**98a/i** and **99b/i**). Hybrids containing an aromatic spacer at position Y (**98a/(j-k)** and **99b/(j-k)**) have a better activity, especially the *para*-phenyl substituted derivative **99b/k** which showed the highest activity against all the bacterial strains tested. Surprisingly, the only conjugate with an activity spectrum similar to that of **99b/k** is conjugate **90** which contains the shortest spacers at the X (-CH₂-) and Y (-CH₂-CH₂-) positions.

Bacterial strain	AME	Cipro	Neo	90	98a/h	98a/j	98a/k	99b/k
<i>E. coli</i> R477-100		0,02	24	3	6	6	6	1,5
<i>E. coli</i> ATCC 25922		0,02	48	3	3	6	6	3
<i>E. coli</i> AG100B	APH(3')-I	0,05	384	12	12	12	12	3
<i>E. coli</i> AG100A	APH(3')-I	< 0,005	96	0,38	0,75	0,75	1,5	0,75
<i>E. coli</i> XL1 blue		0,1	6	3	6	6	6	3
<i>E. coli</i> XL1 blue <i>pSF815</i>	AAC(6')- APH(2'')	0,38	> 384	12	48	24	6	24
<i>E. coli</i> XL1 blue <i>pET9d</i>	APH(3')-Ia	0,1	96	12	12	6	6	3
<i>E. coli</i> BL21		< 0,005	6	0,2	0,2	0,4	0,4	0,4
<i>E. coli</i> BL21 <i>pETSACG1</i>	APH(3')-IIIa	< 0,005	48	0,2	0,2	0,4	0,4	0,4
<i>B. subtilis</i> ATCC 6633		0,02	1,5	0,75	3	3	3	1,5
<i>MRSA</i> ATCC 43300		0,2	384	6	12	6	3	3

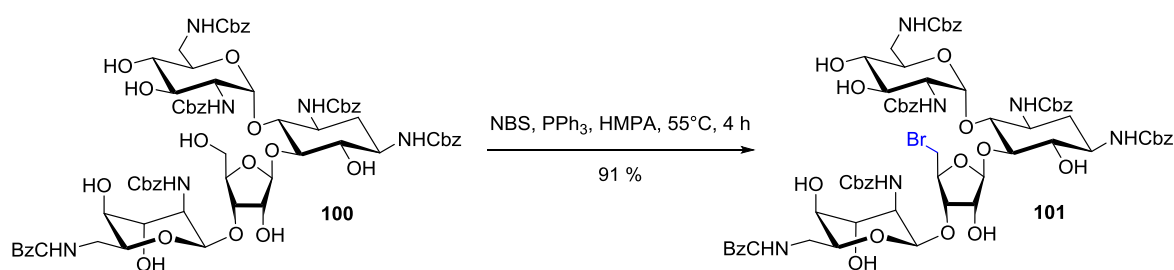
Table 12. MIC ($\mu\text{g/mL}$) of fluoroquinolone-neomycin conjugates vs the parent antibiotics.

3. 3. 2. 2 Modification at position 5'' through nucleophilic substitution

As mentioned above, alternative routes to functionalise the neomycin 5'' position have also been developed taking advantage of the single primary hydroxy group at this position. Based on nucleophilic substitution, these approaches relied on

selectively inserting a leaving group at this position. Surprisingly, only three leaving groups have so far been used: a bromide, the large 2,4,6-triisopropylbenzenesulfonate (-OTIBS) group and a tosylate (-OTs).

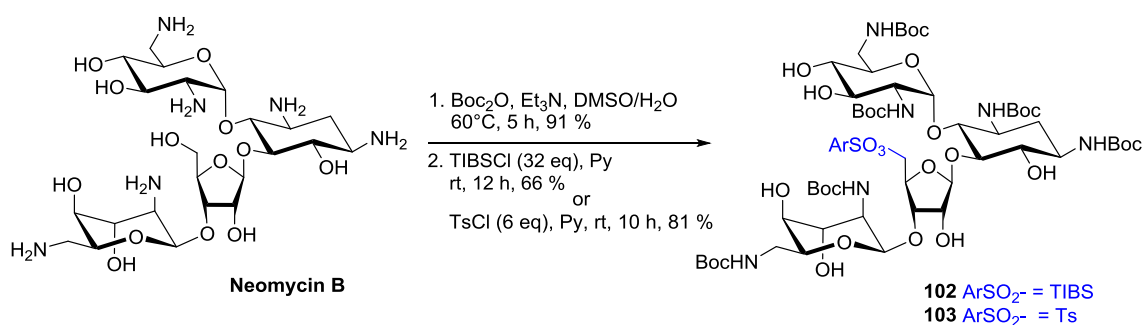
In 1977, Hanessian's group was the first to report on this strategy using the reaction they set up for more classical carbohydrates [83,84]. Starting from the per-*N*-Cbz neomycin **100**, selective bromination was achieved through the combined action of *N*-bromosuccinimide (NBS) and triphenylphosphine in hexamethylphosphoramide (HMPA) with a high 91% yield (Scheme 25). Although very efficient, this reaction has never been reproduced or improved in the literature, probably due to the required HMPA, a mutagenic solvent [85].



Scheme 25. Synthesis of a 5''-bromoneomycin derivative.

Introduced by Khorana and Lohrmann for selective coupling of deoxyribonucleotides [86], the bulky 2,4,6-triisopropylbenzenesulfonyl (TIBS) group has been applied by the Tor group in 1999 to the selective activation of the 5'' hydroxy group of neomycin B and other AG towards dimeric derivatives [87]. Once the neomycin amines protected with *tert*-butoxycarbonyl groups, the single primary 5'' hydroxyl was selectively sulfonated by a large excess (32 equivalents) of TIBSCl in pyridine, giving intermediate **102** with a 66% yield (Scheme 26). This method is today the most widely used for the 5'' functionalisation of neomycin B. Several research groups have since taken up this route for the elaboration of new derivatives by substitution with thiols or azides.

More recently, the Schweizer group also applied the same sequence but with the simple *para*-toluenesulfonyl chloride [88]. A good selectivity was achieved as the sulfonate **103** was obtained with 81% yield (Scheme 26), but surprisingly, only one other group applied this method for the formation of probes for bacteria imaging [89].

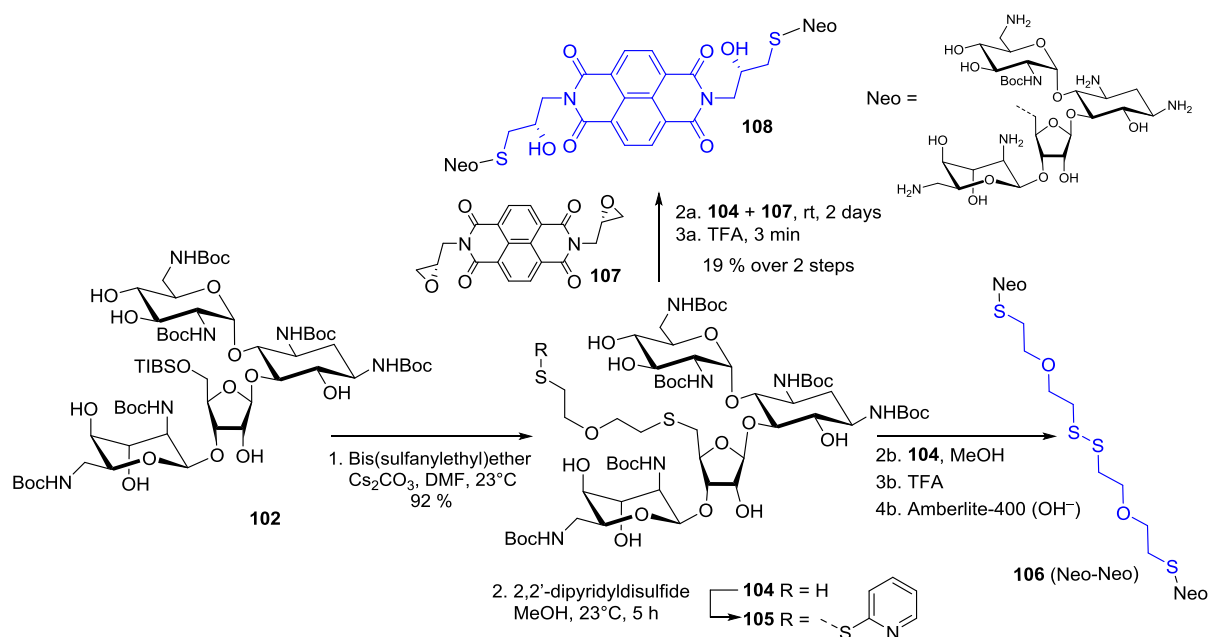


Scheme 26. Synthesis of 5''-O-sulfonyl neomycin derivatives.

From the 5''-OTIBS perBOC neomycin derivative **102** mentioned above, several groups were able to introduce various sulfur chains at this position for various applications.

The first example showing the replacement of the oxygen atom with a sulfur atom was described by Tor and coll. in 1997 [90]. This strategy allowed the preparation of neomycin (Scheme 27) and tobramycin dimers, but also of mixed heterodimers (e.g. neomycin-tobramycin) [87]. The synthesis simply relied on the treatment of **102** with bis(2-sulfanylethyl)ether in DMF in the presence of caesium carbonate, providing the sulfanylthioether derivative **104**, which could then be elaborated to various AG dimers. The neomycin homodimer **106** was thus obtained by simply mixing **104** and **105** in methanol, followed by BOC deprotection. Dimer **106** showed better affinity than neomycin and a few other dimers (Neo-Tob and Tob-Tob; Tob for tobramycin) towards various small RNA sequences (wild type 27 nt A-site 16S rRNA and 46 nt 'dimerized' A-site 16S rRNA) [91].

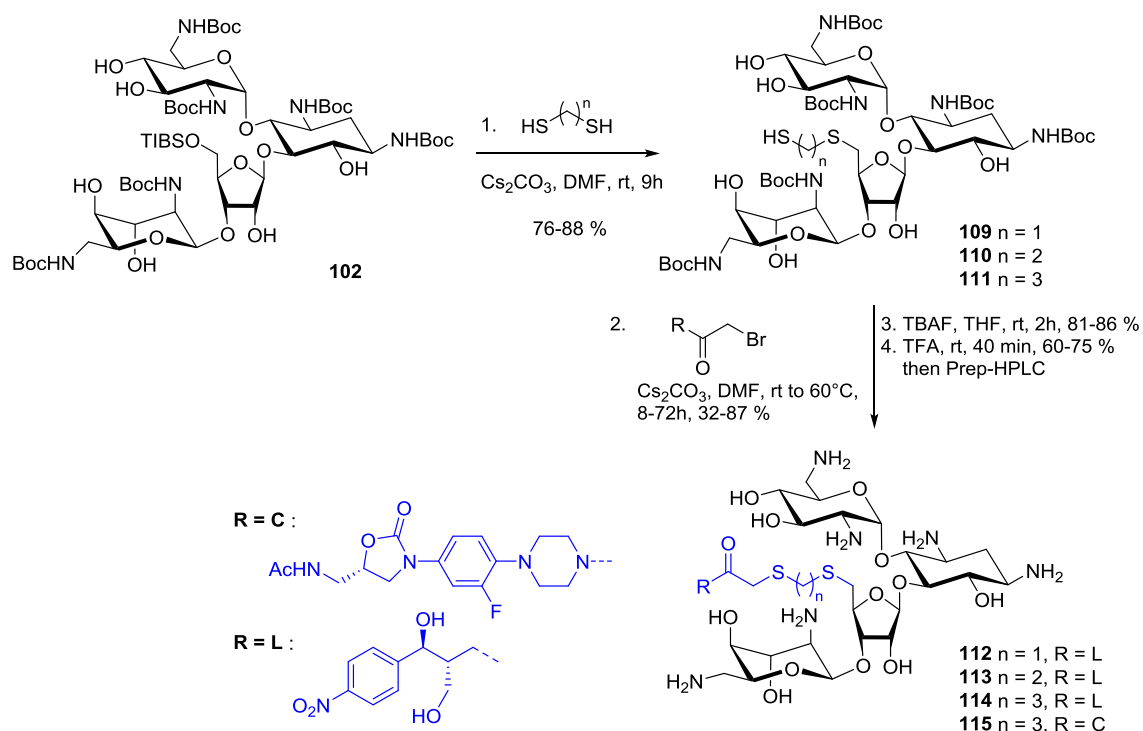
Homodimers could also be produced by exploiting either the nucleophilic or redox properties of the thiol group in **104**. With the goal of introducing an intercalating agent, *N*-glycidyl naphthalene diimide **107** was opened up by the thiol **104**, providing after deprotection the dimer **108**, although in modest yield. Such addition of an intercalator moiety induced better binding affinity for the same A site models of dimers **108** than neomycin alone or its homo dimer **106** [92].



Scheme 27. Synthesis of neomycin homodimers linked by sulfanyl ether.

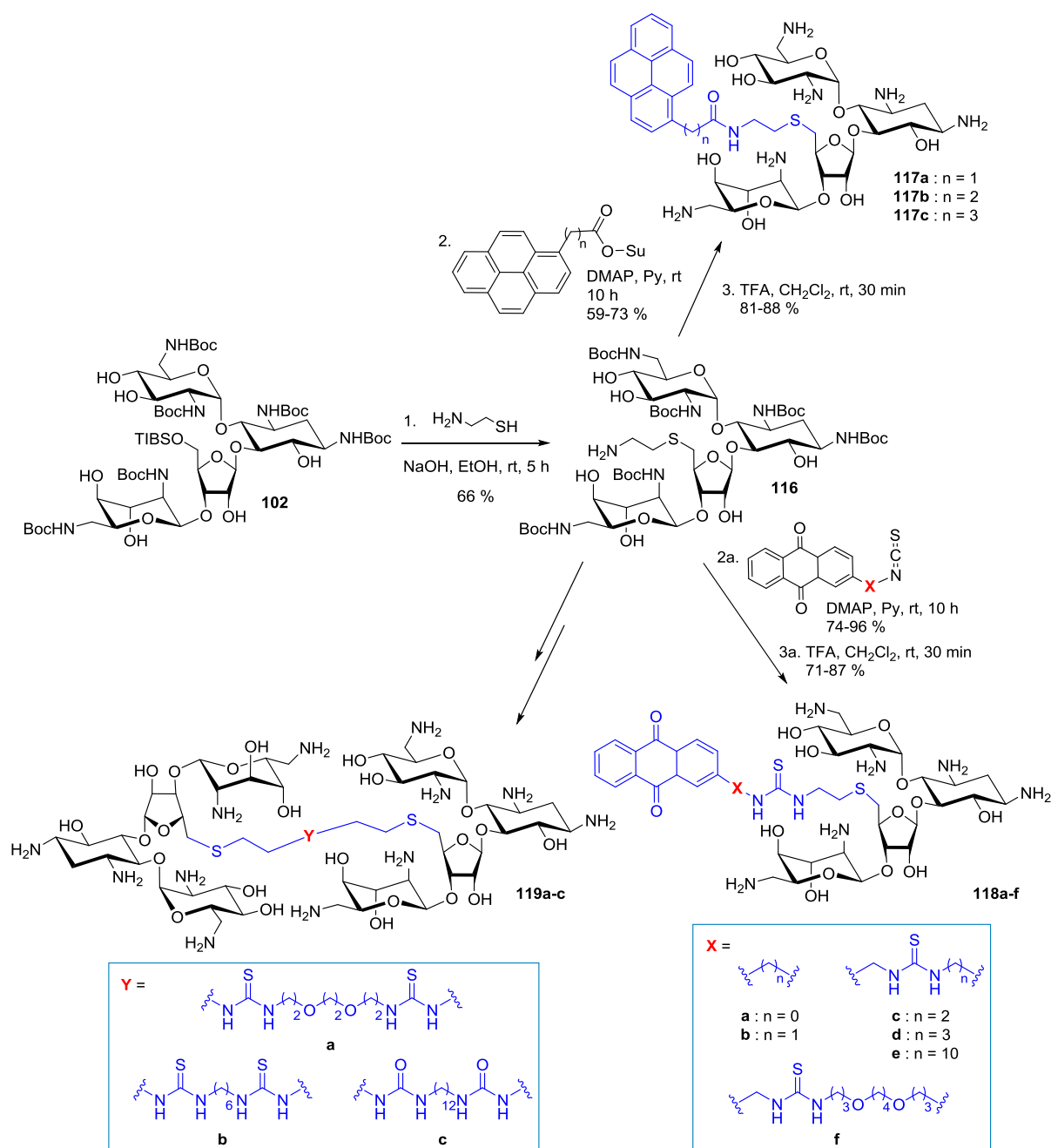
Using a similar methodology, α,ω -alkyl dithiols of variable chain lengths were introduced by substitution of the 5''-arylsulfonate moiety in **102**. The residual sulfanyl group was then coupled to bromoacetylated compounds derived from other antibiotic families (Scheme 28) [93]. After a very short deprotection sequence, three linezolid conjugates **112-114** and one chloramphenicol conjugate **115** were obtained.

As for related conjugates combining different antibiotics (see **90**, **98-99**, or neomycin-tobramycin), the antibacterial activity of these compounds was expected to be better than each one. However, none of them showed better activity than neomycin on various bacteria strains [94].



Scheme 28. Synthesis of linezolid or chloramphenicol-neomycin conjugates linked through alkyldithiols.

With the aim of targeting various RNA sequences as well as improving antibiotic activity, several groups replaced the 5''-OTIBS group by the 2-aminoethylsulfanyl chain (Scheme 29). Such displacement was achieved by submitting **102** to a large excess of 2-aminoethanethiol under basic conditions in ethanol. The so-produced amino thioether derivative **116** allowed to graft intercalating agents on the so-introduced free amine, either directly [95] or through amide [96] or thiourea [97] formation, and thus to produce conjugates **117a-c** and **118a-f**. Using the same methods, neomycin dimers linked through thiourea linkage **119a-c** were also prepared and studied [98,99].



Scheme 29. Synthesis of acridine, pyrene or anthraquinone-neomycin conjugates from 5''-aminoethylsulfanyl neomycin derivative.

Evaluated against different bacterial strains, including *S. aureus* and *E. coli*, the pyrene-neomycin conjugates generally proved less active than neomycin B on neomycin B-susceptible strains, except on neomycin B-resistant *MRSA* strains (Table 13). The results obtained with *MRSA* exhibiting two AMEs, an ANT(4') and the bifunctional AAC(6')/APH(2''), indicate that conjugation of pyrene to neomycin B may overcome some AG resistance mechanisms. It appears that **117** conjugates are

modified less rapidly by AMEs than neomycin B, but this does not give them sufficient antibacterial activity to become useful antibiotics.

Surprisingly, the standard ATCC 25923 *E. coli* strain, susceptible to neomycin B, is resistant to **117** conjugates. The latter nevertheless retain activity on the resistant TolC *E. coli* strain, in which efflux was mutated.

The antibiotic activity of the six anthraquinone-neomycin conjugates **118a-f** was evaluated against 14 bacterial strains by measuring the inhibition of bacterial growth and the minimal inhibitory concentration. These compounds proved less effective than neomycin, except against *S. epidermis*, and none of them were effective against neomycin B-resistant strains.

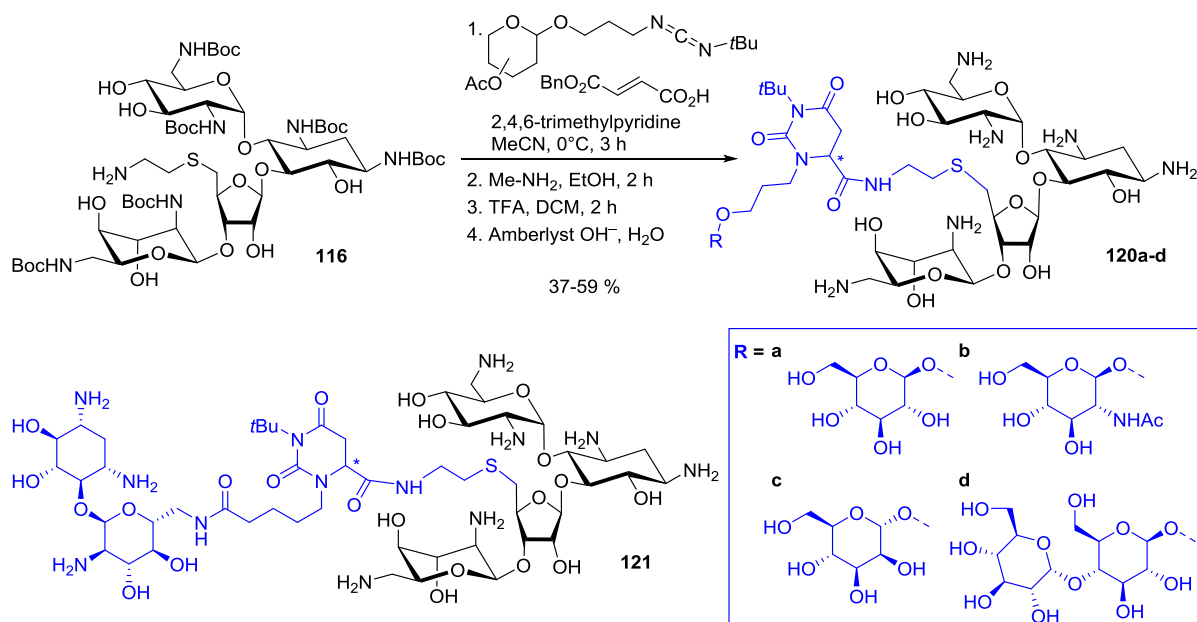
The same was true for the thiourea linked dimers.

Bacterial strain	Resistance	Neo	117a	117c
<i>S. aureus</i> ATCC 25923		1,56	12,5	12,5
<i>S. aureus</i> NorA	Efflux	1,56	6,25–12,5	6,25–12,5
<i>S. epidermidis</i> ATCC 12384		1,56	6,25	6,25
MRSA ATCC 12384		400	12,5	12,5
MRSA A960649	ANT(4') & AAC(6')/APH(2'')	100	12,5	12,5
MRSA SU-5		> 200	25	25
MRSA M0602		> 400	25	25
<i>B. anthracis</i> BA852		1,56	6,25	6,25
<i>M. smegmatis</i>		0,78	6,25	6,25
<i>E. coli</i> ATCC 25923		3,13	>25	>25
<i>E. coli</i> TolC	Efflux	1,56	6,25	6,25
<i>E. coli</i> H4H		400	>25	>25

Table 13. MIC ($\mu\text{mol/mL}$) of pyrene-neomycin conjugates vs the parent neomycin.

In another but interesting approach, various carbohydrates were grafted on intermediate **116** through its 5''-aminoethylsulfanyl chain [100]. Such process was

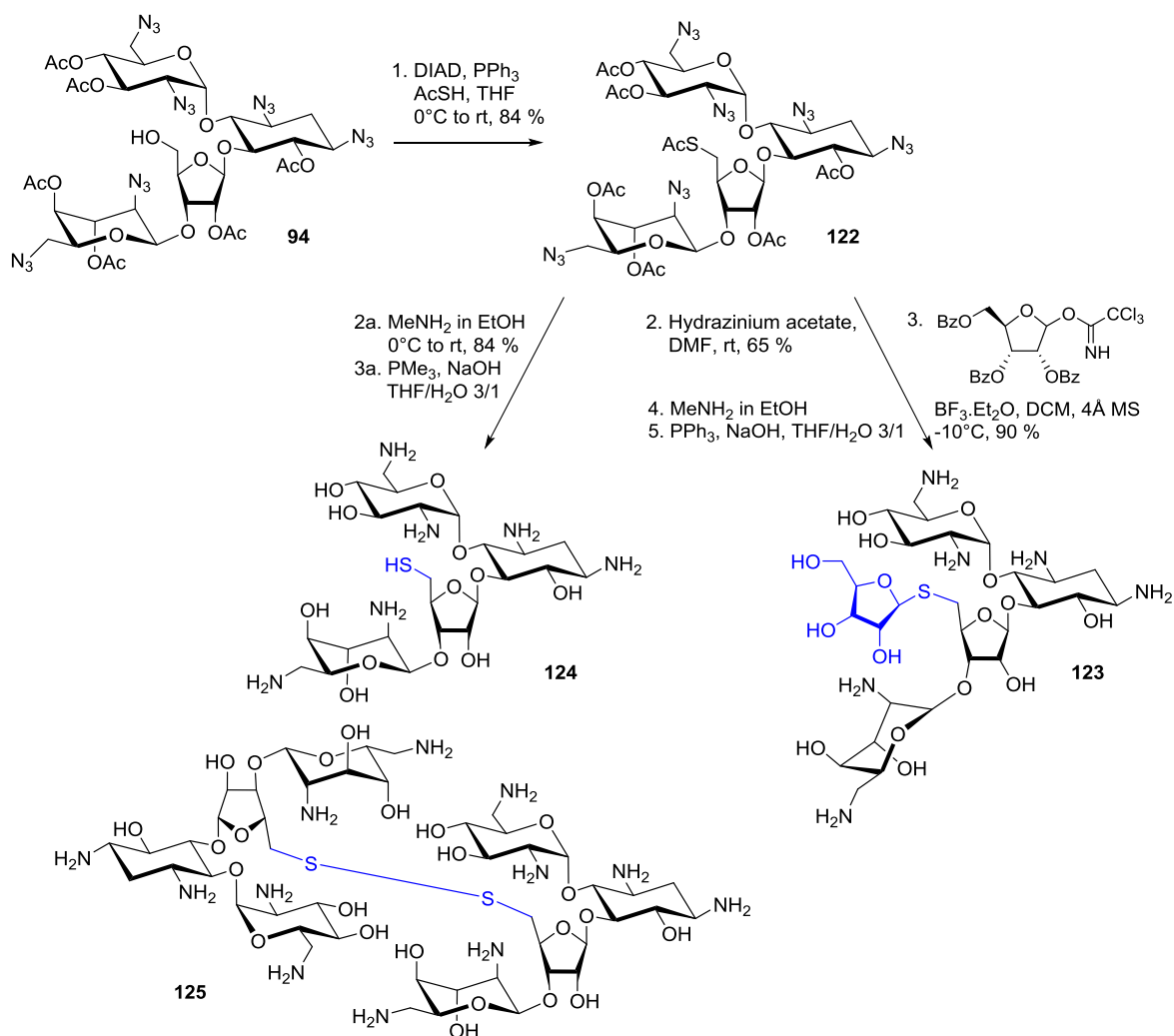
based on an elegant multicomponent domino reaction (Scheme 30). Reaction of fumaric monoester with an *in situ* prepared glycosyl carbodiimide led to the formation of a substituted 2-(*tert*-butylimino)-1,3-oxazin-6-one. The latter was opened by the free amine of **116** and the resulting ureidoester amide recycled to a dihydropyrimidine-2,4-dione carboxamide with reasonably good yields (62 to 78%), but as a mixture of two diastereoisomers. Total deprotection of these conjugates was achieved using a small sequence: aminolysis of the carbohydrate esters and acid-mediated amine deprotection. The free amino conjugates **120a-d** were then obtained with a basic resin. This strategy was also employed for the preparation of a neamine-neomycin dimer **121** with 37% overall yield. However, and despite the efficiency of this sequence, the antibacterial properties of these conjugates were disappointingly low compared to the neomycin parent.



Scheme 30. Synthesis of carbohydrate- or neamine-neomycin conjugates from the 5''-aminoethylsulfanyl derivative **116**.

Other carbohydrates have been introduced at the 5'' position, but in a more direct way through glycosylation or thioglycosylation (Scheme 31) [101]. For the latter, a protected 5''-deoxy-5''-sulfanyl neomycin derivative **122** was prepared from intermediate **94** through a Mitsunobu reaction with thioacetic acid. The selective deacetylation with hydrazinium acetate, followed by S-glycosylation with D-ribosyl

trichloroacetamide under trifluoroboron-etherate catalysis, afforded a β -thioglycoside which, after complete deprotection, gave the thiosugar conjugate **123**. 5''-Deoxy-5''-sulfanylneomycin **124** was also obtained in 2 steps from **122**, after removal of all acetyl group and Staudinger azide reduction. However, this sequence led to a 1:3 mixture of the desired product **124** and the corresponding disulfide dimer **125** in 88% yield. The separation of this mixture was carried out by exclusion chromatography.



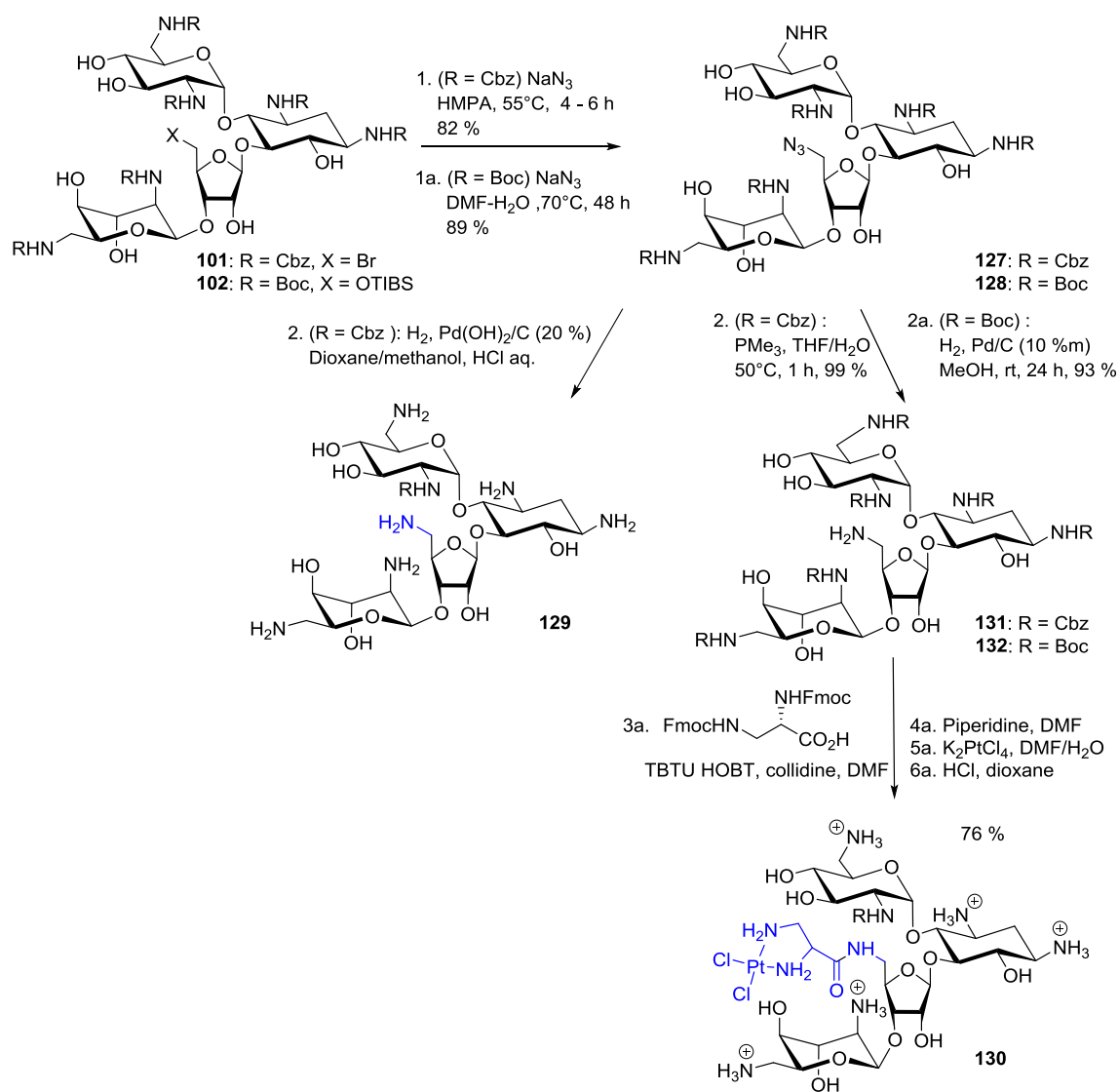
Scheme 31. Synthesis of a thioribosyl-neomycin conjugate.

The antibiotic activity of the thioribosyl-conjugate **123**, the 5''-deoxy-5''-sulfanylneomycin **124**, and the disulfide dimer **125** was evaluated for *B. anthracis* (Sterne strain) by measuring the minimal inhibitory concentration. Compared to the neomycin parent, all compounds exhibit lower activities, with respectively MIC of 8, 5 and 2 $\mu\text{g/mL}$ MIC as compared to the 0,25 $\mu\text{g/mL}$ MIC of neomycin.

In a way similar to the 5''-sulfanyl neomycin B derivatives mentioned above, several groups accessed to 5''-nitrogenated compounds. Indeed, by substituting the sulfonyl leaving group with an azide, it is possible either to obtain substituted triazoles by 'click' reaction with terminal alkynes, or to reduce the azide to an amine, which can be further functionalised.

The first example was reported by Hanessian group in 1977 (Scheme 32), taking benefit of the 5''-bromo neomycin derivative **101** they already prepared (see Scheme 25) [85]. The latter was substituted by NaN_3 in HMPA, leading to derivative **127** with 82% yield. Reduction of the azido group and deprotection of the amines by catalytic hydrogenation provided 5''-deoxy-5''-aminoneomycin B **129**. Interestingly, this compound offers a slightly higher antibiotic activity than neomycin B and allows activity against a strain of *E. coli* (B 1002) that is resistant to kanamycin and neomycin.

Similarly, the Tor group exploited their TIBS derivative **102** to introduce the azido group under safer conditions, which is then also reduced by catalytic hydrogenation in methanol. The resulting amine **132** was functionalised by peptide coupling with the Fmoc-protected (S)-2,3 diaminopropanoic acid. This chain, after deprotection of the Fmoc groups, can chelate a platinum atom and allowed targeting HIV RNA [102].

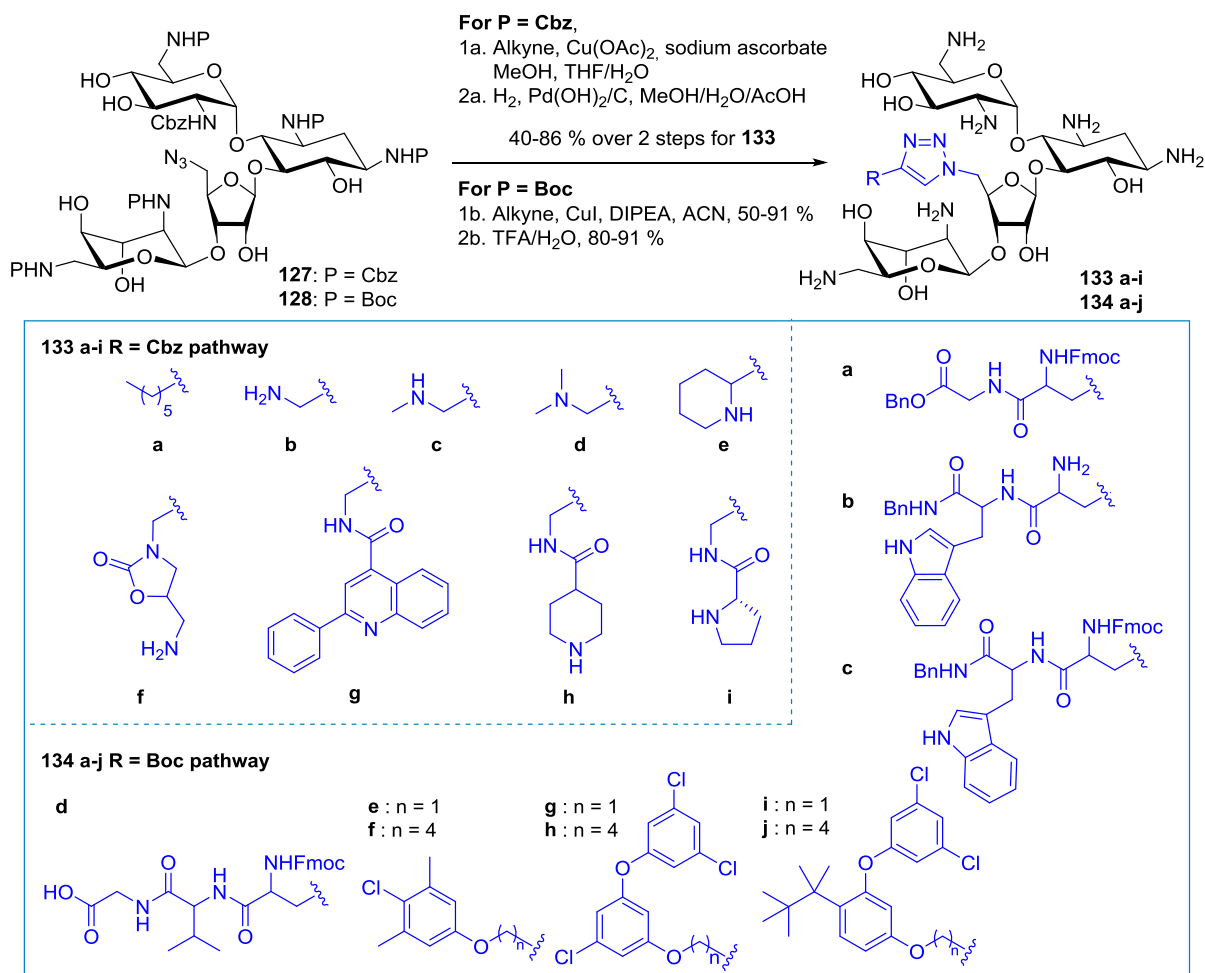


Scheme 32. The synthesis of 5''-amino-5''-deoxyneomycin and derivatives.

The so-introduced azide was also exploited in click reaction with various alkynes. The Chang group was the first in 2008 to use this methodology to produce a library of 9 5''-triazoloneomycin derivatives **133 a-i** (Scheme 33) [103]. The antibiotic activity of these compounds was evaluated against strains of *S. aureus* and *E. coli* (Table 14). Compounds **133** show an activity very close to that of neomycin B against the *S. aureus* strain. However, this activity is more moderate against *E. coli* and not effective against APH(3')-I enzyme.

Similarly, the Schweizer group reported the synthesis of neomycin B derivatives conjugated to peptides [104] and to phenolic derivatives [105] (Scheme 33). Evaluated against bacterial strains of *E. coli*, *S. aureus*, *S. pneumoniae* and *P.*

aeruginosa, these derivatives **134 a-j** showed very poor antibacterial activity. However, some phenolic compounds (**134 h-j**) maintained moderate activity against the MRSA studied, while neomycin B was ineffective.



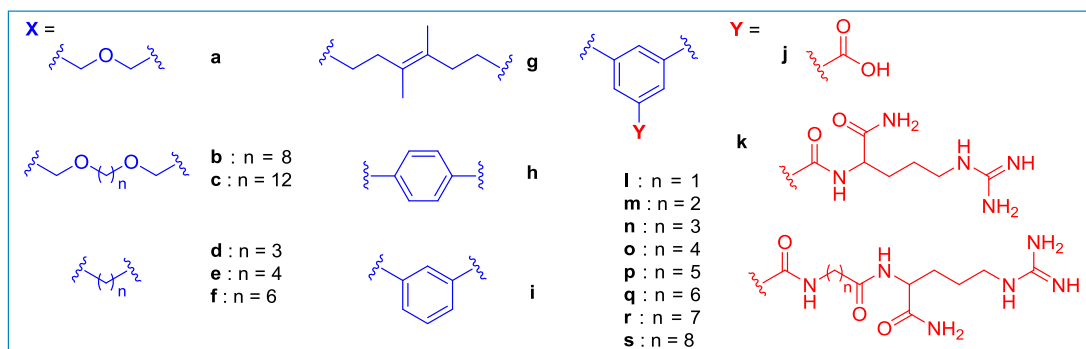
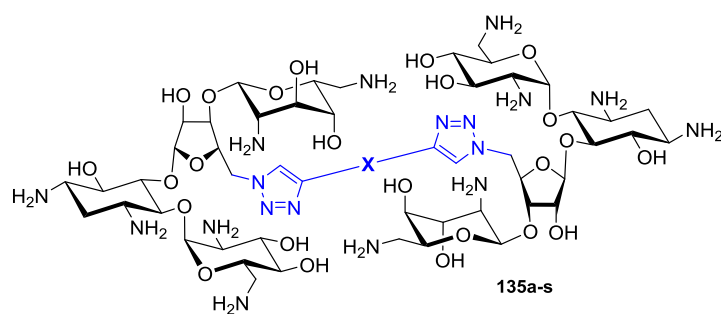
Scheme 33. The synthesis of 5''-triazoloneomycin derivatives.

Bacterial strain	AME	Neo	133a	133b	133c	133d	133e	133f	133g	133h	133i
<i>S. aureus</i> ATCC 25923		1	4	1–2	1–2	2	2	4–8	8	4	2
<i>E. coli</i> ATCC 25922		4	32	8–16	8	8	8–16	32	32	16–32	16
<i>E. coli</i> TG1		4–8	8–16	16	16	8–16	16	16	8–16	16	16
<i>E. coli</i> TG1 pSF815	AAC(6')/APH(2'')	4–8	16	16	16	16	16	32	32	32	16
<i>E. coli</i> TG1 pTZ19U–3	APH(3')–I	>2000	500	1000	>2000	>2000	>2000	>2000	500–1000	>2000	>2000

Table 14. MIC ($\mu\text{g}/\text{mL}$) of 5''-triazolo neomycin derivatives **133** vs the parent neomycin.

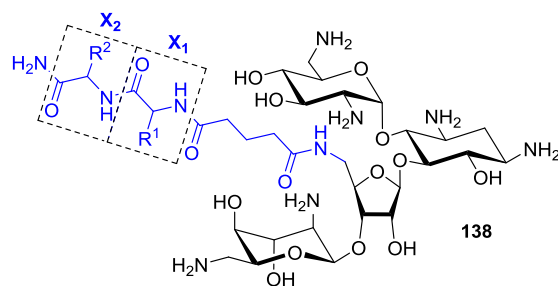
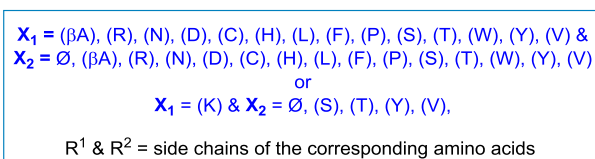
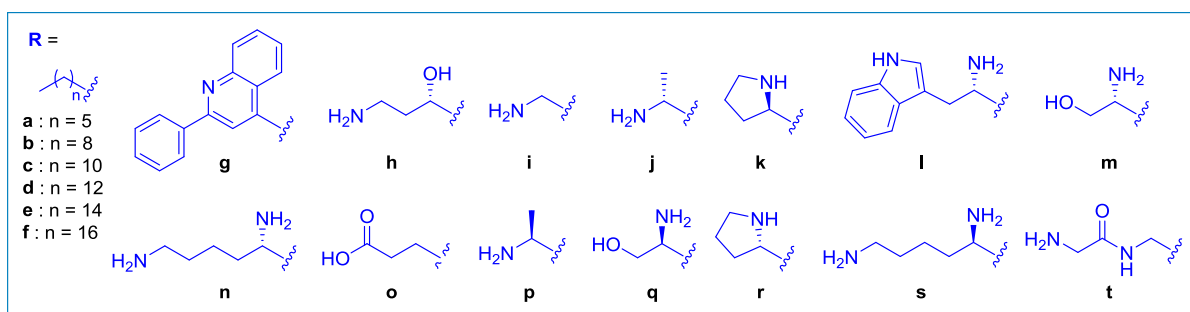
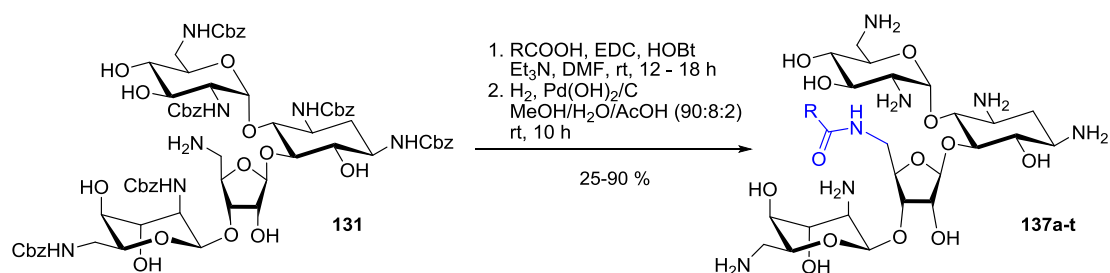
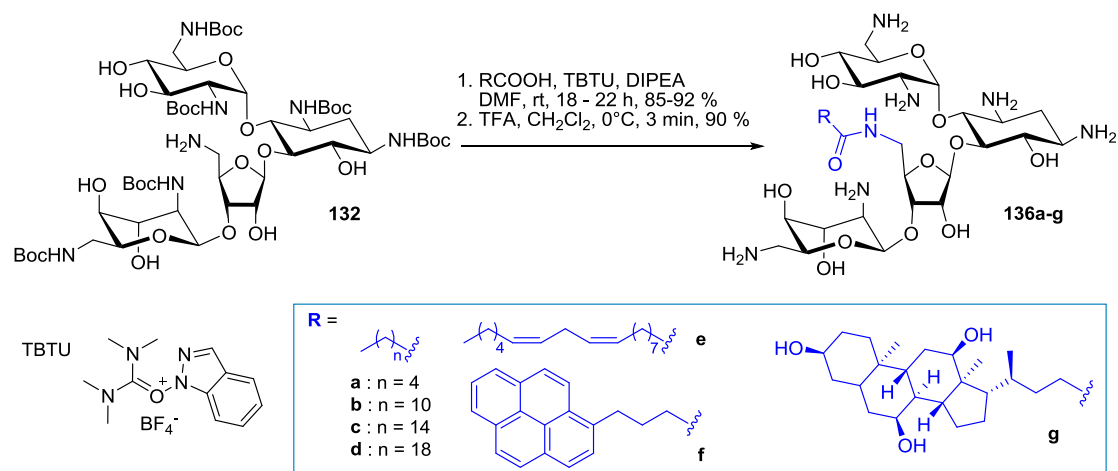
Neomycin dimers were also obtained through this strategy using various dialkynyl alkanes, ethers or aryls (Scheme 34). Initially synthesized to study their interaction with HIV RNA, compounds **135a-i** were also evaluated regarding their antibacterial activity. Compared to neomycin, these dimers retain their activity against *S. aureus* and *S. epidermidis*, but not against other neomycin-sensitive strains [106,107].

In an attempt to increase the antibiotic activity of such dimers, the phenyl group of **135i** was functionalised with L-arginine derivatives [108]. By varying the chain length between the dimer and the arginylated linker, a library of 10 new neomycin dimers **135j-s** were obtained and tested in bacteriology against three Gram-positive bacterial strains (*B. cereus*, *S. epidermidis* and *S. aureus*). However, none of them proved active against *S. epidermidis* and *S. aureus* and the activity is much reduced against *B. cereus*.



Scheme 34. Synthesis of neomycin dimers linked through 5''-triazole chain

As mentioned above, the azido group, once introduced at the 5'' position, could be easily converted to an amino group (see **132** in Scheme 32). The latter can then be engaged in a variety of reactions. Among them, peptide coupling is probably the most represented. A large number of 5''-amido neomycin B derivatives has thus been produced and evaluated for their antibacterial activity (Scheme 35).



Scheme 35. Synthesis of neomycin derivatives with 5'-amide chain.

In 2008, the Schweizer group synthesized a library of lipid-neomycin B conjugates **136a-g** in an attempt to enhance the uptake of AG into bacterial cells. These conjugates were readily obtained using conventional amide formation (Scheme 35, top) and evaluated their antibacterial activity (Table 15) [109].

The measured MICs showed that the nature and the size of the lipidic part of these compounds has a significant influence on the activity. Long saturated aliphatic chains induce a better activity against Gram-positive bacteria, while the pyrene or cholesterol motif leads to a significant activity reduction. Remarkably, compounds **136c** and **136d** show antibacterial activity against neomycin B-resistant MRSA. It appears that conjugation of neomycin B with 16- or 20-carbon lipids results in a strong enhancement of antibiotic activity, while shorter lipids at 6 or 12 carbons only decrease it. In sharp contrast, with *E. coli* strains, the longer the saturated chains are, the more the activity is reduced. This decrease in activity is probably linked to the difference in the bacterial outer membrane. The wall of Gram-negative bacteria provides a barrier against polycationic lipids, while the wall of Gram-positive bacteria can transport cationic lipids to the inner membrane.

However, cationic lipids are well known for disrupting membranes and such mode of action or a combination of them may occur (see below).

Bacterial strain	Neo	a	b	c	d	e	g	f
<i>S. aureus</i> ATCC 29213	2	16	32	4	8	16	16	16
MRSA ATCC 33592	256	> 512	> 512	8	8	32	128	256
<i>S. epidermidis</i> ATCC 1490	1	2	4	2	4	4	8	2
MRSE ATCC 14990	0.5	32	8	2	4	4	16	64
<i>S. pneumoniae</i> ATCC 49619	64	64	128	64	64	128	> 256	> 512
<i>E. coli</i> ATCC 25922	8	16	64	32	128	128	32	32
<i>E. coli</i> ATCC 6174	4	16	64	64	128	64	64	64
<i>P. aeruginosa</i> ATCC 27853	512	> 512	256	128	64	> 512	> 256	256

Table 15. MIC ($\mu\text{g/mL}$) of 5''-lipid neomycin derivatives **136 a-g** vs the parent neomycin.

A similar series of fatty acid was conjugated to neomycin B through the same method by the Chang group and compared to amino acid analogues (Scheme 35, middle) [103,110]. All of them retain neomycin B activity against strains susceptible to it. Interestingly, fatty acid amides with a long lipid chain **137c-f** are more active

than neomycin B on resistant strains, especially against the *MRSA* strain and against *P. aeruginosa* APH(3')-IIb, and the longer the chain, the better the activity. The amino acid chain does not improve activity. It can be noted that the amino acid stereochemistry does not influence the activity of the compound (**137j** vs **p** and **137k** vs **r**).

However, QSAR analyses have suggested that different modes of actions could exist for such lipid-AG conjugates. For relatively short acyl chain, moderate variation of the antibacterial activity occurred, but increasing the chain length (C7-C10) led to an 8- to 16-fold decrease in activity against AG susceptible strains. These conjugates are thus probably acting through interaction with A-site. With longer chain length (C14-C180), the activities against aminoglycoside-resistant strains increased, indicating a different mode of action.

In the latter case, the well-known membrane disrupting role of lipids, especially cationic lipids as are these lipidic AG, may operate.

Although not analysed as such, the same phenomenon could occur with other lipid-AG conjugates (see above [109]).

Despite the latter results, a large library of dipeptidylglutaramide conjugates was prepared by coupling neomycin to 14 mono- and 196 diamino acids via solid phase synthesis (Scheme 35, bottom) [111]. These derivatives were screened for their binding affinity to A site using a rapid test with a fluorescent neomycin B probe (Table 16) [112]. Only 14 compounds showed better affinity than neomycin B. Overall, polar amino acids reduce affinity for A site less than apolar residues. The negatively charged aspartate derivative is the most damaging residue, strongly reducing affinity for A site. Comparing the affinity for *E. coli* A site and human A site, it turned out that conjugates tryptophane-leucine **138WL** and tyrosine-tryptophane **138YW** bind respectively 14 and 28 times better to bacterial A site, and thus should have decreased toxicity. However, most compounds have the same spectrum of activity for various Gram-positive and Gram-negative bacterial strains as neomycin B. They are inactive against neomycin-resistant strains and relatively less active against susceptible strains.

Bacterial strain	AME	Neo	b	c	d	e	f	j	p	k	r
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<i>S. aureus</i> ATCC 25923		1	16	8–16	2–4	2	4	0,5	1	0,5–1	0,5–1
<i>E. coli</i> ATCC 25922		4	32	16–32	8–16	4	4–8	8–16	16–32	16–32	8–16
<i>E. coli</i> TG1		4	n.d.	n.d.	n.d.	2–4	2–4	8–16	16	16	8
<i>E. coli</i> TG1 pSF815	AAC(6')/APH(2'')	4–8	n.d.	n.d.	n.d.	16	32	16	16–32	16–32	16
<i>E. coli</i> TG1 pTZ19U–3	APH(3')-I	> 2000	n.d.	n.d.	n.d.	8	8	> 2000	> 2000	> 2000	> 2000
SARM ATCC 33591		125	125–250	16–32	4–8	2–4	2–4	n.d.	n.d.	n.d.	n.d.
<i>P. aeruginosa</i> ATCC 27853	APH(3')-IIb	64	16–32	16	8	4	8–16	n.d.	n.d.	n.d.	n.d.

Table 16. MIC ($\mu\text{g/mL}$) of selected 5''-lipid and amino acid neomycin derivatives **137** vs the parent neomycin.

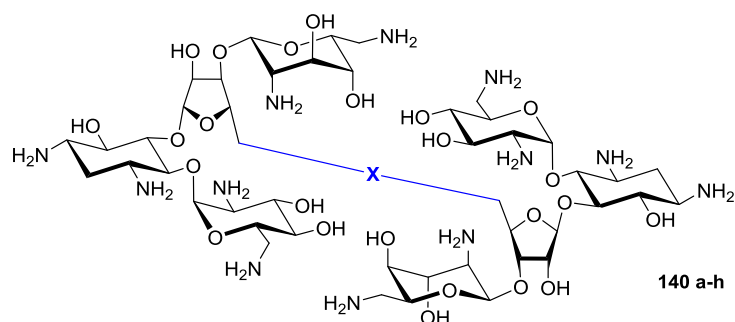
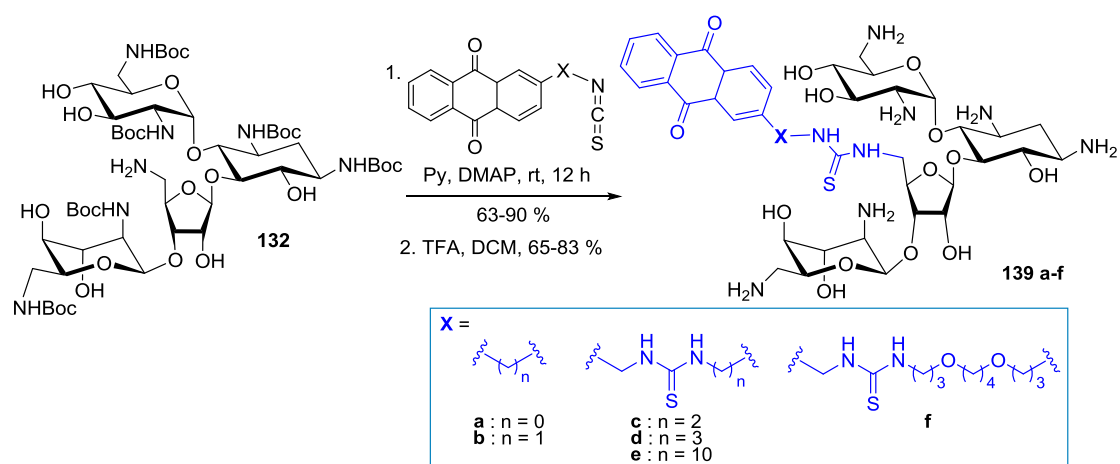
Among the work done to derivatize neomycin at the 5'' position with a thioether linkage and an anthraquinone (see Scheme 27), the Arya group also developed conditions for functionalising a 5''-amino neomycin directly either with an anthraquinone isothiocyanate or with diisothiocyanates. The corresponding anthraquinylythiourea neomycin conjugates **139a-f** [97] or the dimers **140a-h** [98,99] were thus produced (Scheme 36) and their antibacterial properties evaluated with various bacterial strains including *E. coli*, *A. baumannii* and *P. aeruginosa* standards [113].

The anthraquinone-neomycin conjugates do not exhibit significant activity against neomycin-resistant strains such as *MRSA*, *P. aeruginosa* or *K. pneumoniae*. Surprisingly, while neomycin B exhibits 91% inhibition against the *A. baumannii* strain, none of the compounds showed some efficacy against it. If the anthraquinone moiety is directly bound to thiourea (**139a**), the activity sharply drops against *E. coli* strain. In this case, a short linker probably induces lack of flexibility and steric hindrance, preventing the molecule from reaching its target.

Nevertheless, the relative activity rates of AAC(2')-Ic, AAC(3)IV, AAC(6')-Ie and APH(3')-Ia AMEs were compared for the different **139** conjugates to those of neomycin B. The conjugates show good resistance to the AACs studied with relative velocities less than 25% of that of neomycin (50% for **139e**). The only exception is compound **139d** which appears to be modified as rapidly as neomycin B by AAC(2')-

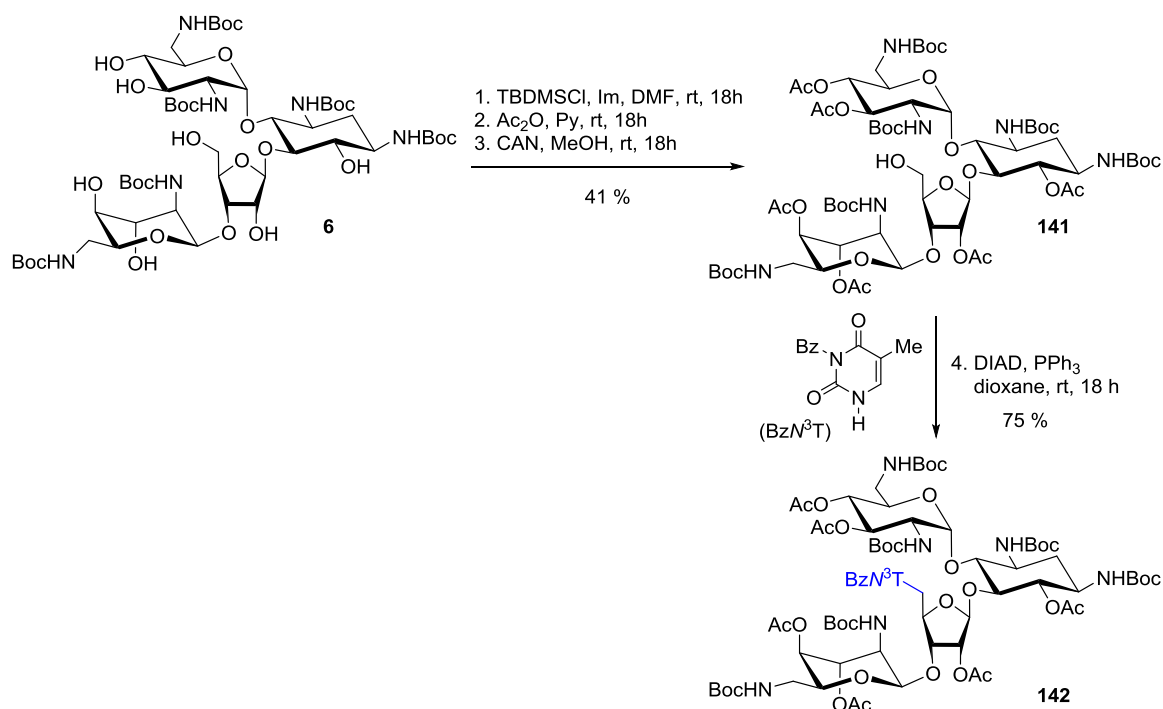
Ic. However, only **139c** resists the action of APH(3')-Ia, **139a** and **139b** are similar to neomycin B, and compounds **139d-f** react 3.5, 2.5 and 1.7 times faster than neomycin B, respectively.

As for other dimers, the dimers **140 a-g** do not exhibit significant activity against five susceptible and three resistant strains to neomycin B, including *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922), *S. marcescens*, *E. cloacae*, and *S. epidermidis*. None of them are better than neomycin.



Scheme 36 Synthesis of neomycin anthraquinone-thiourea conjugates and (thio)urea-linked dimers.

Following the same idea, the group of Fourrey developed a route to introduce thymine or uracil at the 5''-position of neomycin B, with the hope of gaining additional contacts with the target rRNA [114]. Starting from *N*-Boc-protected neomycin B **6**, a direct route was envisaged based on a Mitsunobu reaction, expecting to take benefit of the more reactive 5'' primary alcohol. However, attempts led to a product, that proved more complex than expected (see section 3.4). A more classical approach was then experimented based on a protection-deprotection sequence (Scheme 37). Selective silylation of the 5'' hydroxy group under classical conditions followed by per-acetylation of the remaining hydroxy group provided a fully protected neomycin derivative. The selective deprotection of the 5''-position proved problematic with fluoride-based methods, but this deprotection could be efficiently achieved by applying cerium ammonium nitrate (CAN) in methanol (71% yield). The resulting 5''-free derivative **141** was then engaged in a Mitsunobu reaction with *N*³-benzoylthymine and the conjugated product **142** was obtained with a good 75% yield. Complete deprotection was achieved in two steps upon ammonia treatment to remove both benzoate and acetates, then by trifluoroacetic acid treatment. Although designed to fight antibiotic resistance, this compound and a derivative (see section 3.4 below) have not so far been investigated regarding their biological properties.



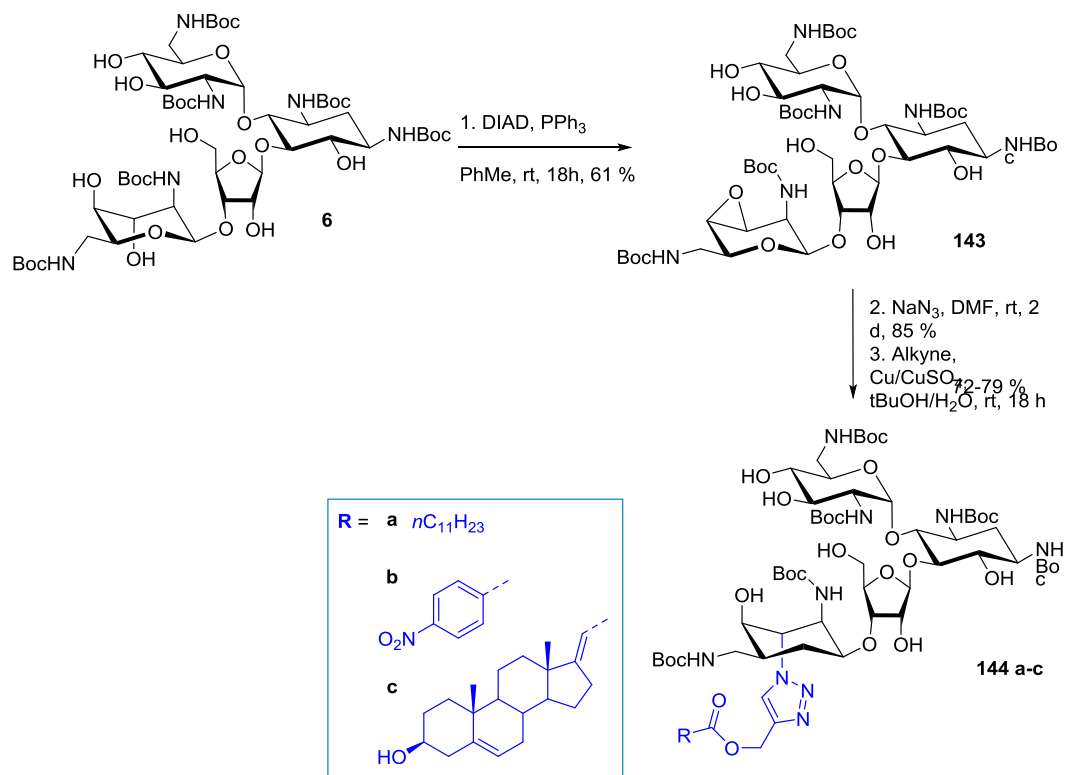
Scheme 37. Synthesis of a 5''-thymine-neomycin conjugate

3. 4. Functionalisation of ring IV

Despite 60 years of synthetic works, examples of ring IV functionalisation remain very scarce. Selective functionalisation of ring IV is a very difficult task due to the similarity of ring I and IV, that makes chemoselective transformations nearly impossible. Furthermore, ring IV is very important for strong binding to ribosomal RNA and thus its modification may alter this binding.

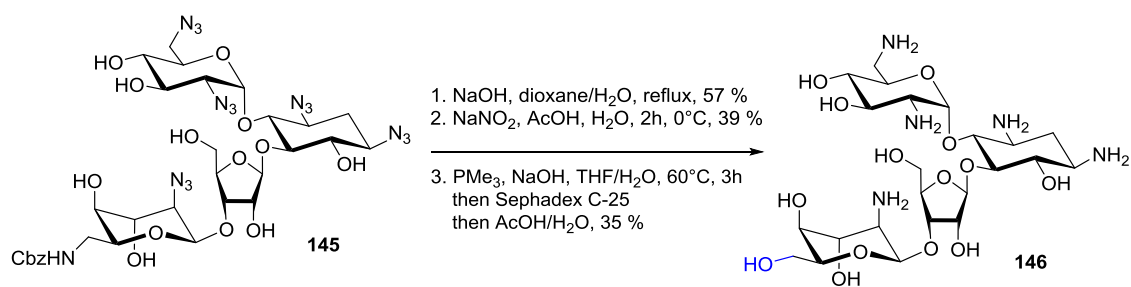
While studying the introduction of a nucleobase at position 5'' (see Scheme 37), Fourrey's group discovered an unexpected modification of ring IV under Mitsunobu conditions [114]. Their initial structural elucidation proved to be incorrect, but further studies revealed the formation of a 3''',4'''-epoxide due to the ⁴C₁ chair conformation and the trans-diaxial orientation of the two alcohols on ring IV (Scheme 38) [115]. This feature is not found in ring I, explaining the selectivity of this process. The key to succeed in such modification relied on 2 additions of diisopropyl azodicarboxylate DIAD (2 equivalents) and triphenylphosphine (2 equivalents) in toluene with a 12 h interval between each addition. Epoxide **143** was then obtained in 61 % yield. Interestingly, this epoxide can be regioselectively opened by sodium azide in DMF; a

subsequent copper-catalyzed formal 1,3-dipolar cycloaddition led to click adducts **144 a-c** in good yield [116].



Scheme 38. Synthesis of 3'''-triazolo neomycin derivatives.

Aiming at mimicking sisomicin and plazomicin, the groups of Vasella and Crich engaged in a large project on modifying paromomycin and neomycin with alkyl, hydroxy or aminoalkyl group (see section 3.1.2 and 3.1.4). Within this work, they reported the formation of a neomycin derivative, that can be viewed as a regioisomer of paromomycin (Scheme 39) [66]. In the monoprotection of neomycin (see Scheme 16), a mixture of compounds was obtained, from which the pure regioisomer *N* 6'''-Cbz neomycin **145** could be isolated after azidation of the remaining amino groups and careful separation. Upon carbamate hydrolysis, treatment with sodium nitrite in aqueous acetic acid, and azide reduction through the Staudinger reaction, 6'''-deamino-6'''-hydroxyneomycin **146** was obtained in 23% overall yield.



Scheme 39. Synthesis of 6''-deamino-6''-hydroxyneomycin.

A few other modifications of ring IV have been mentioned, but always coupled with another modification on rings I-III. These ring IV modifications will thus be discussed within the next section.

3. 5. Multi-Functionalisation

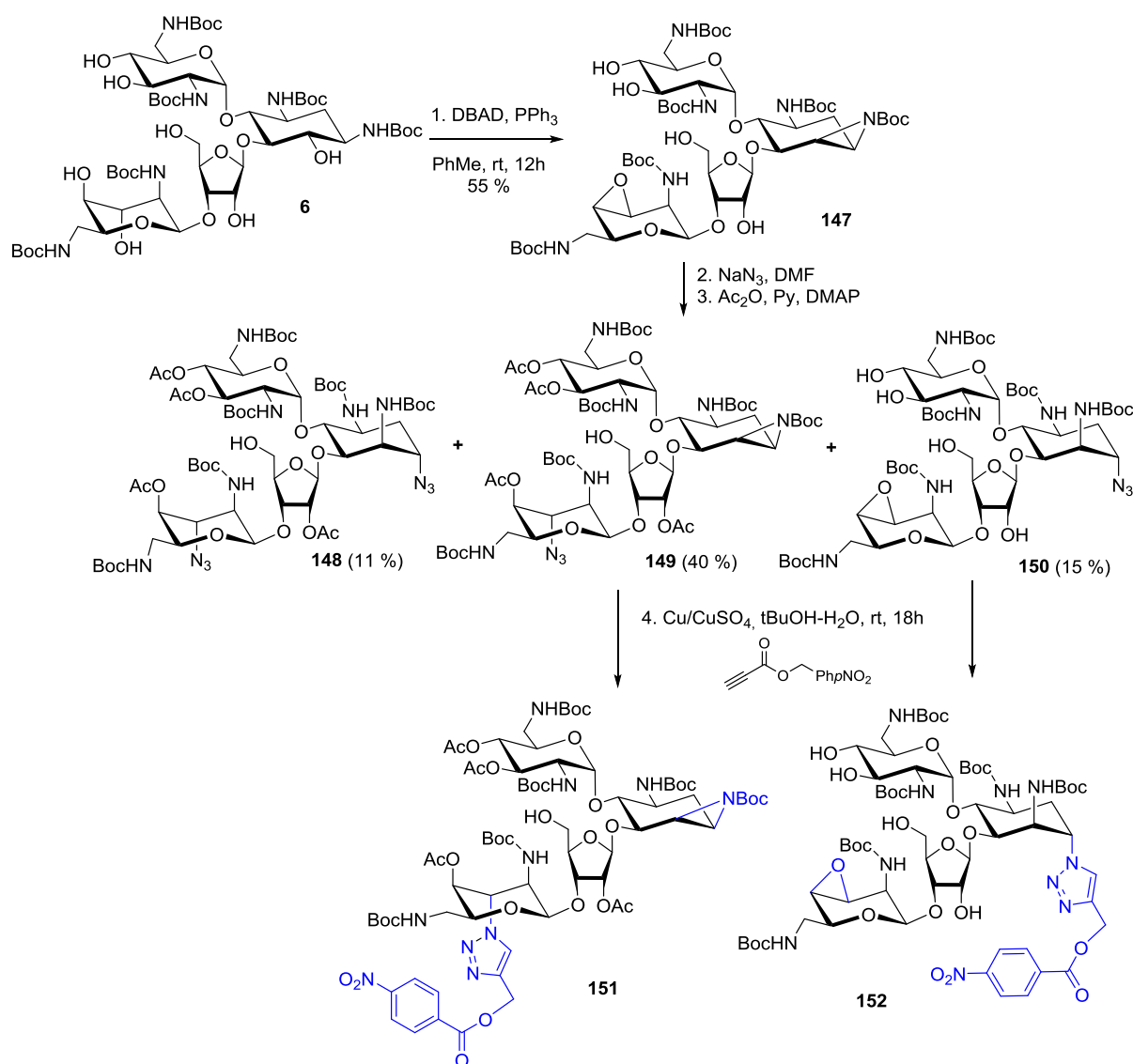
As seen above, selective modifications and functionalisation of neomycin and analogues have efficiently been achieved. However, none of the resulting new aminoglycosides could be turned to compounds useful enough to be commercialised. Combining various functionalisation at different positions on several rings was thus explored in order to improve antibiotic property, counteract resistant strains and minimise toxicity. Among them, the N1-modification with an AHB chain known to enhance activity and decrease the action of some AME (see section 3.2.1) is the most common and is often combined with another or several modifications elsewhere.

3. 5. 1. Modification of ring II and IV

Capitalising on the Fourrey's work and on their own work mentioned above (see section 3.4), the group of Houston and Jenkins attempted introducing hydrophobic esters onto neomycin with the original aim at improving neomycin effectiveness at crossing the waxy, mycolate-rich barrier that envelops *Mycobacterium tuberculosis*. In this context, they found that Mitsunobu reaction could induce the formation of an epoxide within ring IV (see Scheme 38), but also of an aziridine within ring II (Scheme 40) [116]. By adjusting reaction conditions and reagent, they could obtain

up to 55 % of a stable bis-anhydro derivative **147** directly starting from the perBOC neomycin **6**. The latter could be opened by one equivalent of sodium azide in DMF, but mixture of opening products was formed. 'Click' reaction nevertheless provided both the 3'''-triazolo 1,6-aziridinyl derivative **151** and the 1-triazolo 3''',4'''-epoxy derivative **152**.

No biological activity has so far been reported for this family of derivatives.



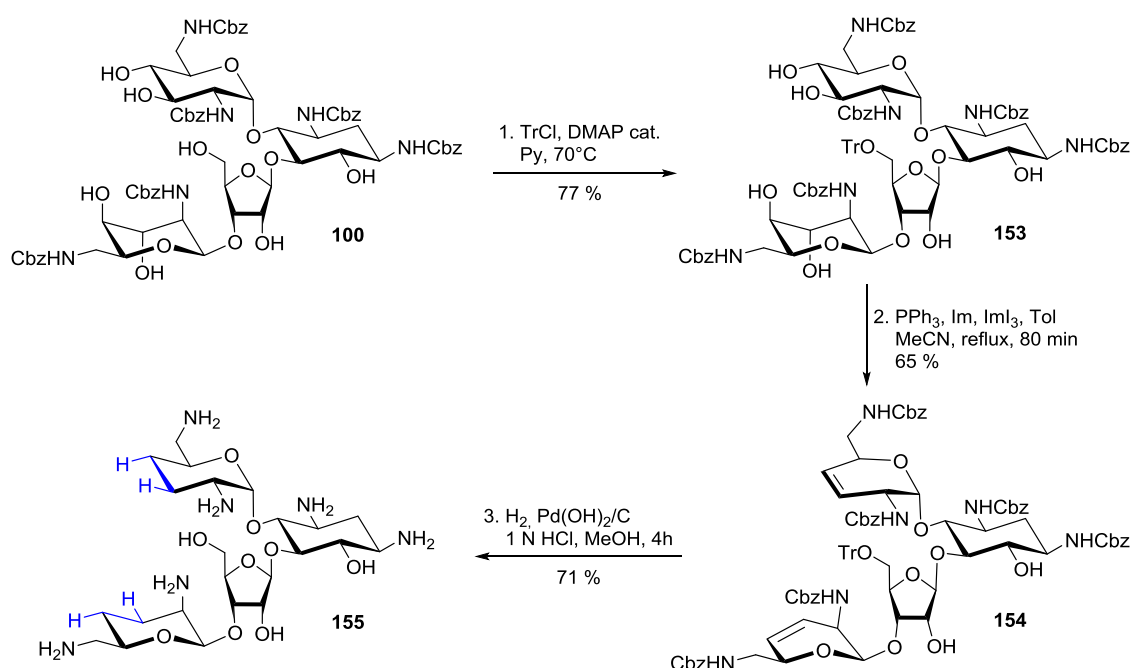
Scheme 40. Synthesis of 3'''-triazolo 1,6-aziridinyl and the 1-triazolo 3''', 4'''-epoxy neomycin derivatives.

3. 5. 2. Polydeoxygenation

Following work on ring I deoxygenation (see section 3.1.3), the Hanessian group was able in 2011 to perform the selective dideoxygenation of rings I and IV diols of neomycin and paromomycin (Scheme 41) [117], using the methodology developed by Garegg and Samuelsson [118], a modification of the original Tipson-Cohen reaction [119].

The single primary hydroxy group of the per *N*-Cbz neomycin was selectively protected with the bulky triphenylmethyl chloride (TrCl) in the presence of DMAP. The resulting product **153** possess six free secondary hydroxy groups, including two *trans* 1,2-diols. Due to their stereochemical arrangement, the latter could be eliminated upon treatment with triphenylphosphine, imidazole and triiodoimidazole, leading to the diene **154** in good yield. Hydrogenation and deprotection then provided the 3',4',3''',4'''-tetradeoxyneomycin B **155** with a yield of 33% over four steps.

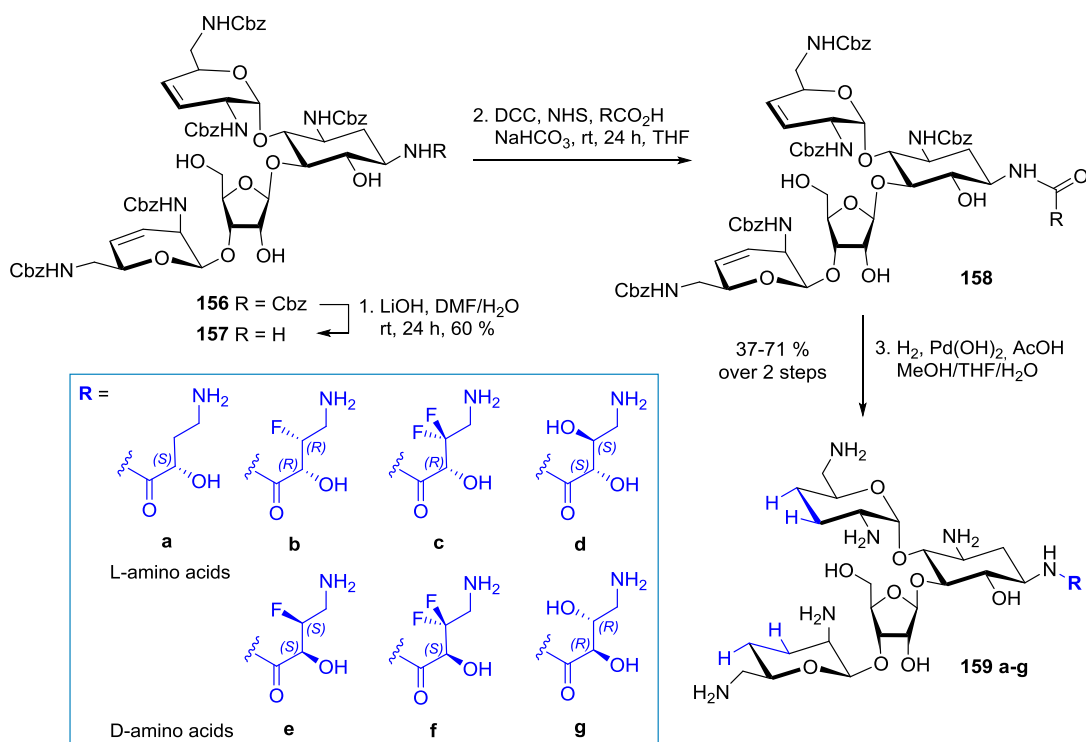
This compound is as effective as neomycin in term of antibiotic activity, and even better on some resistant strains (Table 17). As expected, deoxygenation inhibits the action of ANT(4')-I enzyme, but surprisingly not much the action of APH(3') enzyme despite the absence of hydroxy group at the 3' position. APH(3'/5'') remains capable of modifying the 5'' position.



Scheme 41. Synthesis of 3',4',3''',4'''-tetradeoxyneomycin.

3. 5. 3. Deoxygenation and AHB chain

In an attempt to further improve antibacterial activity, an AHB chain was introduced at position 1 (see section 3.2.1). To do so, the *N*1-Cbz group of building block **156** was selectively deprotected upon LiOH treatment, taking benefit of the adjacent hydroxy group [120]. The resulting amine **157** was then coupled with (S)-4-benzyloxycarbonylamino-2-hydroxybutyric acid in the presence of DCC, triethylamine and *N*-hydroxysuccinimide (NHS). Deprotection provided the 3',4',3''',4'''-tetradeoxyneokacin **159a** with 15% yield over six steps (Scheme 42). As interesting biological results were obtained with this compound (see below and Table 17), further modifications within the AHB chain were introduced [121]. The main goal was to tune the net AG charge, known to correlate in some way to antibacterial action and kidney toxicity [56]. Several D and L-AHB chains containing one more hydroxy and one or two fluoro substituents were thus prepared through Sharpless asymmetric epoxidation of *meso* penta-1,4-dien-3-ol, azide opening of the resulting epoxide and fluorination or not. These chains were then grafted to the amine **157**, as before (see Scheme 41), and the resulting *N*-1 acylated 3',4',3''',4'''-tetradeoxyneomycin derivatives **159b-g** were evaluated for their antibiotic properties against standard and resistant strains from the ESKAPE bacteria and compared to reference AG (Table 17).



Scheme 42. Synthesis of 3',4',3''',4'''-tetraoxygenokacin analogues.

These tetraoxygenated compounds proved as effective as a set of clinical AG antibiotics, including neomycin (Table 17). However, the simple member of this series **155** cannot fight against certain resistant strains, while the analogue **159a** carrying the natural L-AHB chain could. Interestingly, the antibiotic activity against ESKAPE strains expressing various AME is clearly improved over clinical AG antibiotics. The analogues **159b-d** having modified L-AHB chains behave similarly, with the monofluoro **159b** being the best, but those with the D stereochemistry are slightly less efficient, especially the difluoro derivative **159f** and to a less extent the hydroxy derivative **159g**. These differences could be ascribed to different binding modes depending on stereochemistry and to lower pKa of the terminal amine of the AHB chain due to the presence of two β fluorides, as revealed by X-ray structure of a A-site-**159b** co-crystal and modelisation (Figure 6).

Bacteria	Resistance	Neo	Par	Gen	Ami	155	159a	159 (L)			159 (D)		
								b	c	d	e	f	g
<i>E. coli</i>		2	4	0,5	2	0,5	0,5	0,5	0,5	1	1	4	2
<i>S. aureus</i>		0,5	2	0,5	2	0,5	0,5	0,5	0,5	0,5	0,5	1	0,5
<i>K. pneumoniae</i>		0,5	2	0,25	0,5	0,5	0,5	0,5	0,5	0,5	0,5	2	1
<i>A. baumannii</i>		1	4	2	2	0,25	1	0,5	0,5	0,5	0,5	2	1
<i>P. aeruginosa</i>	APH(3')	32	>64	0,5	2	0,25	0,5	0,5	1	0,5	1	4	1
<i>E. cloacae</i>	APH(3')-I, ANT(2'')-I, AAC(6')	>64	>64	32	64	>64	4	2	2	2	4	32	16
<i>E. coli</i>	APH(3')-Ib	64	>64	0,25	0,5	32	0,25	0,25	0,25	0,5	1	4	2
<i>S. aureus</i>	APH(3'/5'')-III	>64	>64	0,5	8	32	1	1	1	1	1	4	2
<i>A. baumannii</i>	AAC(3)-I, APH(3')-VI, ANT(2'')-I	>64	>64	>64	>64	32	2	1	2	2	2	8	2
<i>S. aureus</i>	ANT(4')-I	>64	>64	0,5	64	0,5	1	1	2	1	1	4	2
<i>P. aeruginosa</i>	ANT(4')-II	8	>64	2	32	1	1	1	2	1	2	16	2
<i>E. coli</i>	ANT(2'')-I	2	4	64	4	2	2	2	2	2	2	8	8
<i>P. aeruginosa</i>	AAC(6')-II	8	>64	32	4	2	1	1	2	1	2	16	2
<i>S. aureus</i>	AAC(6')/APH(2'')	>64	>64	>64	64	>64	2	2	4	2	8	16	8
<i>E. coli</i>	AAC(3)-IV	2	8	16	2	2	2	1	1	1	4	8	2

Table 17. MIC ($\mu\text{g/mL}$) of 3',4',3'',4''-tetradeoxyneokacin analogues vs clinically relevant AG (Neo: neomycin, Par: paromomycin, Gen: gentamicin, Ami: amikacin).

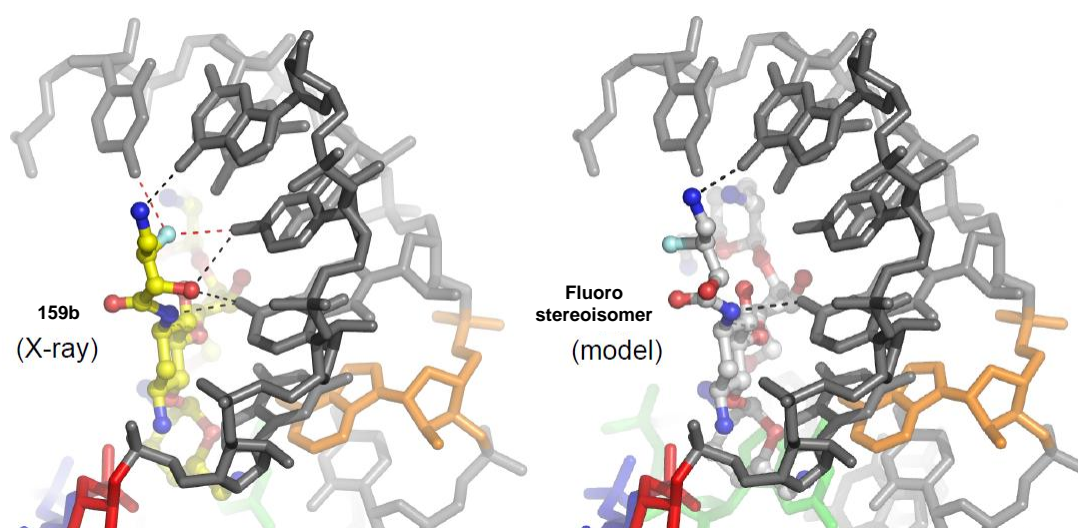


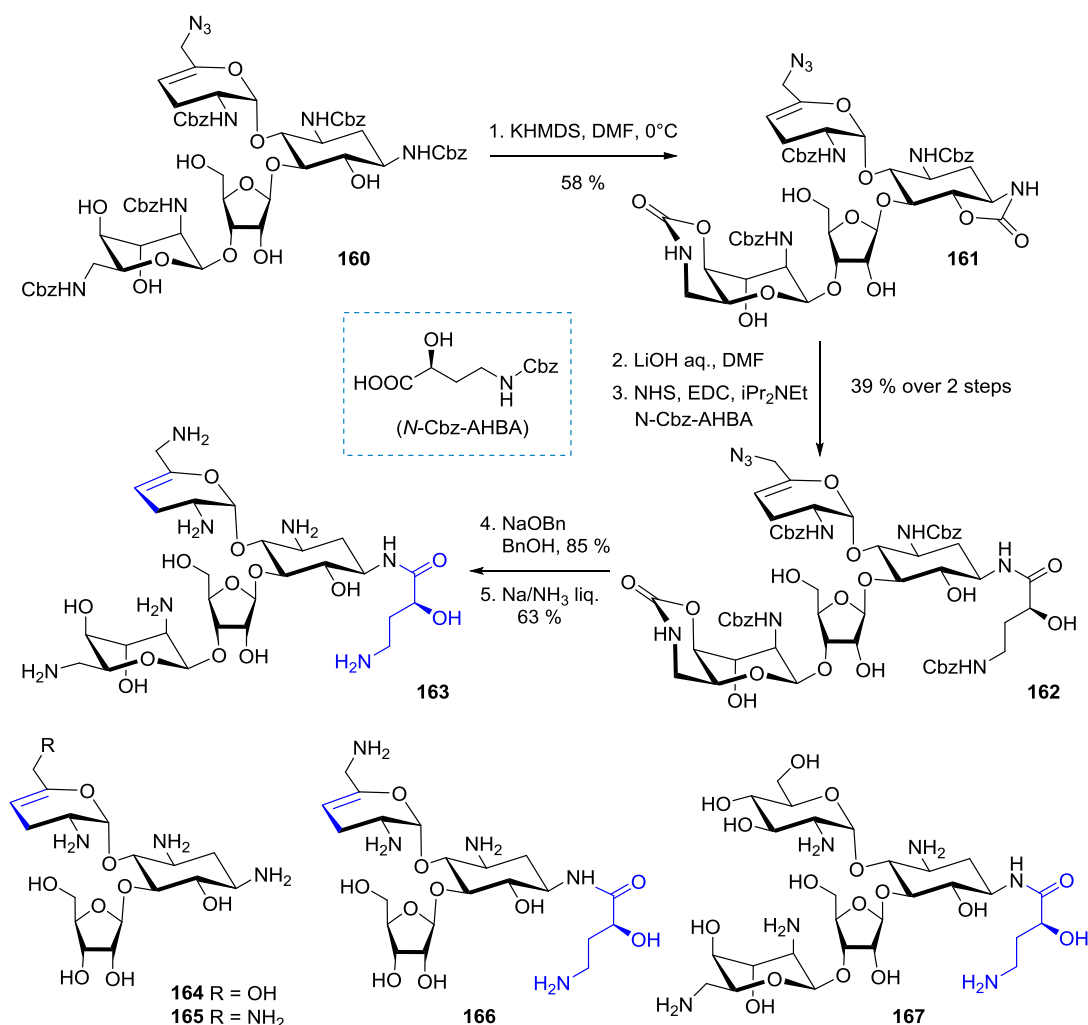
Figure 6. X-ray structure of a A-site-**159b** co-crystal (left) and modelisation of its fluoro stereoisomer (right). Their comparison shows how the (*R*)-fluoride interacts with the rRNA, while the (*S*)-isomer cannot. (A site RNA backbone in grey; AHB chain in yellow, with N in blue, O in red and F in cyan). Taken and adapted from ref. [121] with ACS permission.

Furthermore, the renal cytotoxicity of these 3',4',3'',4''-tetradexoyneokacin analogues was evaluated by measuring the concentration-dependent activation of apoptosis induced by AG accumulation in kidney cells. The results indicate that the monofluoro and hydroxy AHB analogs **159b**, **159d**, **159e**, and **159f** induce apoptosis at a concentration close to that of the AHB analog **159a**. In contrast, the difluoro **159c** and **159f** analogs required approximately twice the concentration, close to that measured for gentamicin, to induce apoptosis. This effect could be correlated to the pKa difference of the terminal amino group of the AHB chain. Difluoro analogs are better tolerated by renal cells than other analogs and therefore have decreased toxicity.

These very interesting results reveal the strong beneficial effect of combining several structural modifications and confirm the key roles of the AHB chain at position 1 and of the pKa of AG amines. Fluorinated 3',4',3'',4''-tetradexoyneokacin derivatives thus exhibit improved antibiotic activity and lower toxicity, and appear as good drug candidates.

In a related approach, the Hanessian group also attempted to combine the interesting features of sisomicin and butirosin within ribostamycin, paromomycin and neomycin analogues (Scheme 43, top) [122]. For that, the 6'-azido-3',4'-deoxy-4',5'-dehydro **160** derivative, obtained in 13 steps (see Scheme 14), was treated with KHMDS in order to get the dicarbamate **161**. The more strained carbamate could be selectively hydrolyzed and the resulting amine was acylated with *N*-Cbz-AHBA to yield the amide **162**. Hydrolysis of the remaining carbamate and deprotection of the amines led to the 1-AHB-4',5'-dehydro-3',4'-deoxyneomycin B derivative **163**. Cleaving ring IV from **160** also led to a ribostamycin analogue, which could then be deprotected or engaged in the same sequence to provide sisomicin–ribostamycin or

butirosin hybrids **165** and **166**. For comparison (see Table 18), the analogous paramomycin derivative **167** was also prepared.



Scheme 43. Synthesis of 4',5'-dehydro-3',4'-deoxyneomycin or -neokacin analogues, and of sisomicin–ribostamycin or -butirosin hybrids.

With a few other derivatives available, these compounds constituted a series (Scheme 43, bottom) in which it was possible to examine multiple structural modifications applied to a single series of aminoglycosides, such as the impact of dideoxygenation of cycle I, the charge of the group in the 6' position, the functionalisation of amines 1 and 6', and the role of cycle IV. The antibacterial activities of these new analogues were compared to antibiotics used in hospitals against a panel of 16 strains of bacteria from the ESKAPE group, representative of pathogens sensitive and resistant to aminoglycosides (Table 18).

Compound		Rib	But	164	165	166	Neo	Par	167	36	38	163
Structure												
Skeleton		Rib	Rib	Rib	Rib	Rib	Neo	Neo	Neo	Neo	Neo	Neo
Dideoxy-deshydro		-	-	+	+	+	-	-	-	+	+	+
N1-Acylation		H	AHB	H	H	AHB	H	H	AHB	H	H	AHB
Function 6'		NH ₂	NH ₂	OH	NH ₂	NH ₂	NH ₂	OH	OH	OH	NH ₂	NH ₂
Bacterial strain	AME											
<i>E. coli</i> ATCC 25922		8	2	>64	64	8	2	4	2	>64	2	0,5
<i>S. aureus</i> ATCC 29213		16	8	>64	16	8	0,5	2	2	8	1	0,5
<i>K. pneumoniae</i> ATCC 10031		2	1	>64	16	4	0,5	2	1	>64	0,5	0,5
<i>A. baumannii</i>		8	1	>64	>64	4	1	4	2	>64	1	0,5
<i>P. aeruginosa</i> ATCC 27853	APH(3')-IIa	>64	>32	>64	16	4	32	>64	8	>64	0,5	0,25
<i>E. cloacae</i>	APH(3')-I, ANT(2'')-I, AAC(6')	>64	32	>64	>64	>64	>64	>64	8	>64	>64	8
<i>E. coli</i>	APH(3')-Ib	>64	1	>64	64	4	64	>64	2	>64	>64	0,25
<i>S. aureus</i>	APH(3'/5'')-IIIa	>64	>32	>64	>64	16	>64	>64	32	>64	16	1
<i>A. baumannii</i>	AAC(3)-I, APH(3')-VI, ANT(2'')-I	>64	>32	>64	>64	8	>64	>64	64	>64	>64	1
<i>S. aureus</i>	ANT(4')-I	>64	>32	>64	16	8	>64	>64	>64	16	1	1
<i>P. aeruginosa</i>	ANT(4')-II	>64	>32	>64	64	8	8	>64	4	>64	1	0,5
<i>E. coli</i>	ANT(2'')-I	8	4	>64	>64	8	2	4	8	>64	2	4
<i>P. aeruginosa</i>	AAC(6')-II	>64	>32	>64	>64	16	8	>64	8	>64	>64	0,5
<i>S. aureus</i>	AAC(6')/APH(2'')	>64	>32	>64	>64	16	>64	>64	32	>64	64	2
<i>E. coli</i>	AAC(3)-IV	>64	2	>64	>64	8	2	8	4	>64	8	1

Table 18. MIC ($\mu\text{g/mL}$) of 3',4'-deoxyneokacin analogues vs parent AG (Neo: neomycin, Par: paromomycin, Rib: ribostamycin).

The obtained results show again the necessity of the amine function in the 6' position to maintain an antibacterial activity (compare Par to Neo, **164** to **165** and **36** to **38**). The loss of activity of 6'-OH compounds seems to come from their difficulty to

penetrate bacterial cells, probably linked to the AG charge. Again, the functionalisation of the amine at position 1 offers a better activity against standard strains but also counteracts AME action (compare **Rib** to **But**, **165** to **166**, **36** to **167**, **38** to **163**). As expected, 3',4'-dideoxygenation allows reducing the action of bacterial strains that depend on ANT(4') enzymes (compare **But** to **166**, Neo to **38**). The absence of cycle IV caused considerable damage to activity (compare **Rib** to **Neo**, **165** to **38** and **166** to **163**).

Combining the benefit of 3',4' dideoxygenation, 4',5'-dehydrogenation and *N*1-acylation on a neomycin-type tetrasaccharidic skeleton, compound **163** is the most promising. It retains a very good antibiotic activity even against strains with APH(3'), APH(3'/5''), ANT(4'), ANT(2''), AAC(3), AAC(6') or AAC(6')/APH(2'') enzymes. However, this compound requires 18 steps to be synthesized with a 6% overall yield. Toxicity evaluation is thus necessary to measure the true potential of compound **163** to become an antibiotic drug.

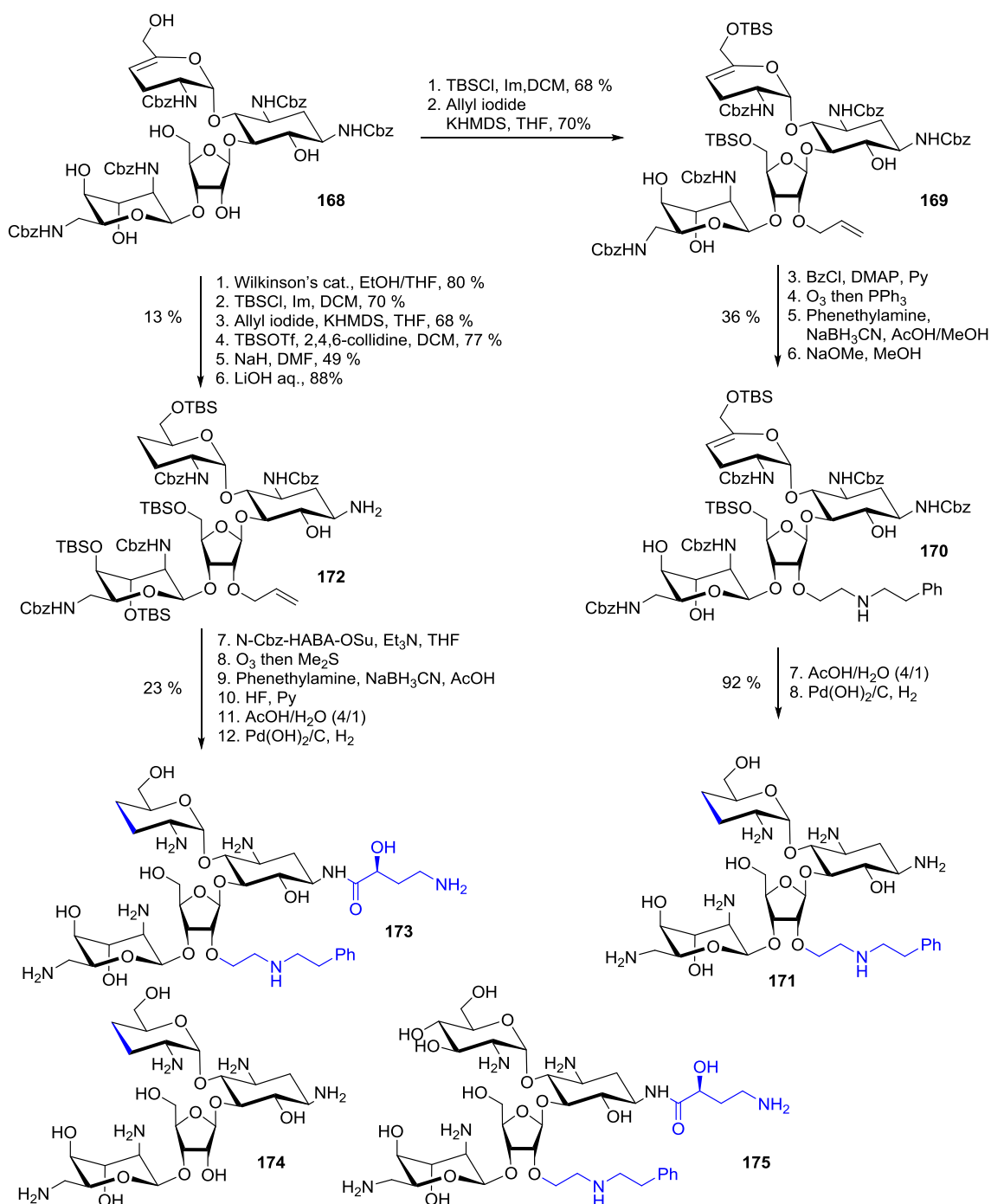
3. 5. 4. Deoxygenation and alkylation (with or without AHB chain)

In a related approach, the Hanessian group attempted to combine the beneficial effect of dideoxygenation at 3',4' positions and of the AHB chain at 1 position with the functionalisation of neomycin B in position 2'' (see section 3.3.1). New paromomycin derivatives including such modifications were thus prepared from the known intermediate **168** (see Scheme 14) and their antibiotic properties examined (Scheme 44) [123].

The primary alcohols of **168** were first selectively silylated, a regioselective *O*-allylation then gave **169**, and the remaining hydroxy groups were protected as benzoate esters. Ozonolysis of the allylic double bond gave an aldehyde, which upon reductive amination with phenethylamine, then global deprotection, provided the expected 2''-*O*-(phenethylamino)ethyl-3',4'-dideoxy paromomycin **171**.

To get the tetra-modified derivative **173**, **168** was treated with Wilkinson's catalyst to selectively reduce the double bond in the presence of the *N*-Cbz groups. The same sequence as above then provided an intermediate with three hydroxy groups

unprotected. Those of ring IV were then selectively protected as silyl ether. Cyclic carbamate formation and its chemoselective cleavage led to **172**. Introduction of the HABA side chain could thus be operated with the corresponding *N*-hydroxysuccinimide ester. Ozonolysis, reductive amination, desilylation and finally hydrogenolysis afforded the analogue **173**. For comparison purpose (see Tables 19-20), the analogous paramomycin derivative **175** was also similarly prepared.



Scheme 44. Synthesis of 2''-O-alkylated 3',4'-dideoxyparomomycin analogues.

The antibiotic properties of these modified compounds were measured against two standard strains of *E. coli* and *S. aureus* and compared to paromomycin, neomycin B, 1-*N*-AHB-paromomycin **167**, neokacin **74** and 3',4'-dideoxyparomomycin **174** (Table 19).

Compound	<i>E. coli</i>	<i>S. aureus</i>	Structural modifications		
			N1-AHB	3'-4'-dideoxy	O-2'' modified
Neomycin B	3-6	1-2	-	-	-
Neokacin (74)	2.5-5.0	2.5-5.0	+	-	-
Paromomycin	3-6	1-2	-	-	-
167	5-10	1.3-2.5	+	-	-
171	40	1-3	-	+	+
173	2.5-5.0	0.6-1.3	+	+	+
174	20-40	5-10	-	+	-
175	0.6-1.3	0.6-1.3	+	-	+

Table 19. MIC ($\mu\text{g/mL}$) of paromomycin derivatives vs parent AG neomycin, paromomycin, neokacin and **167** (ParAHB) for wild type strains of *E. coli* (ATCC25922) and *S. aureus* (ATCC13709).

The di-functionalisation at positions 1 and 2'' (AHB and alkyl chain respectively) induces an interesting decrease in MIC for *E. coli* and to a less extend for *S. aureus*, while the 3',4'-dideoxy and 2''-alkyl modification combination decreases the activity, notably against *E. coli*. On the other hand, the multimodified compound **173** also exhibits interesting activity, especially against *S. aureus*.

Both compounds **173** and **175** were thus further investigated against strains of *E. coli*, *S. aureus* and *S. epidermidis* that are resistant to commonly used antibiotics such as vancomycin, oxymycin, levofloxacin, ciprofloxacin and ceftazidime (Table 20). They both exhibit useful activities but **173** proved very active against all the resistant strains evaluated. Therefore, the combination of the 1-*N*-AHB and 2''-O-(phenylethylamino)ethyl chains with the 3',4'-dideoxygenation of compound **173** produces a potent antibiotic.

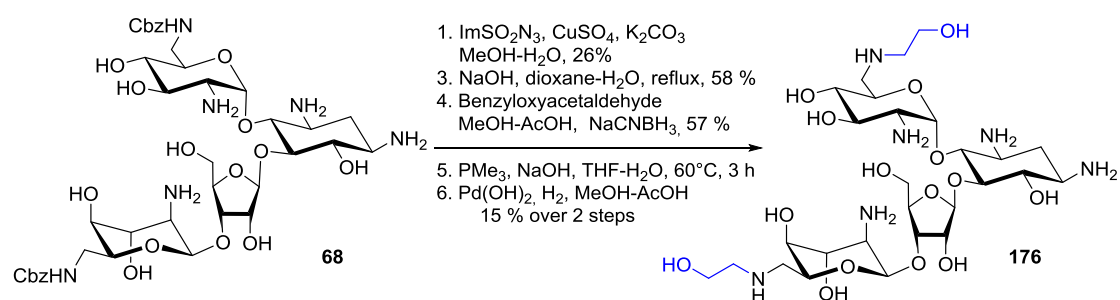
Bacterial strain	sensible to	resistant to	167	G	A	173	175
<i>E. coli</i> ATCC 25922	–	–	8	1	2	8	2
<i>E. coli</i> ATCC 1269687	–	–	8	0.5	1	1	1
<i>E. coli</i> ATCC 1269640	V, C, G	–	16	4	8	8	4
<i>E. coli</i> 1 ATCC 269620	–	V, C, G	8	64	2	4	1
<i>E. coli</i> ATCC 1269621	–	V, C, G	8	64	2	4	2
<i>E. coli</i> ATCC 1269652	–	V, C, G	32	> 128	64	16	8
<i>E. coli</i> ATCC 1269653	–	V, C, G	8	> 128	8	8	2
<i>S. aureus</i> ATCC292213	–	–	4	0.5	2	> 0.12	0.25
<i>S. aureus</i> ATCC1269615	–	V, C, G	>128	32	16	0.25	64
<i>S. aureus</i> ATCC1269616	I	I, C, G	>128	64	16	0.5	128
<i>S. aureus</i> ATCC1269617	–	V, C, G	>128	32	8	0.25	32
<i>S. aureus</i> ATCC1269618	I	I, C, G	>128	64	16	0.5	128
<i>S. aureus</i> ATCC1269619	V	O, C, G	>128	128	16	1	> 128
<i>S. aureus</i> ATCC1269669	O	–	4	0.25	2	0.25	< 0.12
<i>S. aureus</i> ATCC1269670	O	–	4	0.25	1	0.25	< 0.12
<i>S. capitis</i> ATCC1269682	–	–	0.5	0.12	0.5	< 0.12	< 0.12
<i>S. epidermidis</i> ATCC1269663	–	–	1	< 0.12	0.8	< 0.12	< 0.12
<i>S. epidermidis</i> ATCC1269675	–	O, G, L	64	32	8	< 0.12	16
<i>S. epidermidis</i> ATCC1269676	–	O, G, L	0.5	32	4	< 0.12	< 0.12
<i>S. epidermidis</i> ATCC1269677	–	O, G	16	16	4	< 0.12	< 0.12
<i>S. epidermidis</i> ATCC1269680	–	O, G, L	1	64	8	< 0.12	< 0.12
<i>S. warneri</i> ATCC1269686	–	–	0.25	< 0.12	< 0.25	< 0.12	< 0.12

Table 20. MIC ($\mu\text{g/mL}$) of **173** and **175** vs **167** (ParABH), Gentamicin C1 (G), Amikacin (A). Abbreviations: V (vancomycin), I (vancomycin intermediate), C (ciprofloxacin), O (oxymycin), L (levofloxacin), E (ceftazidime).

3. 5. 5. Polyalkylation

In their work on alkylation of paromomycin and neomycin at various positions (see sections 3.1.2 and 3.1.4), the groups of Vasella and Crich attempted to cumulate the so-observed beneficial effects against some AME by combining several alkylations at selected positions.

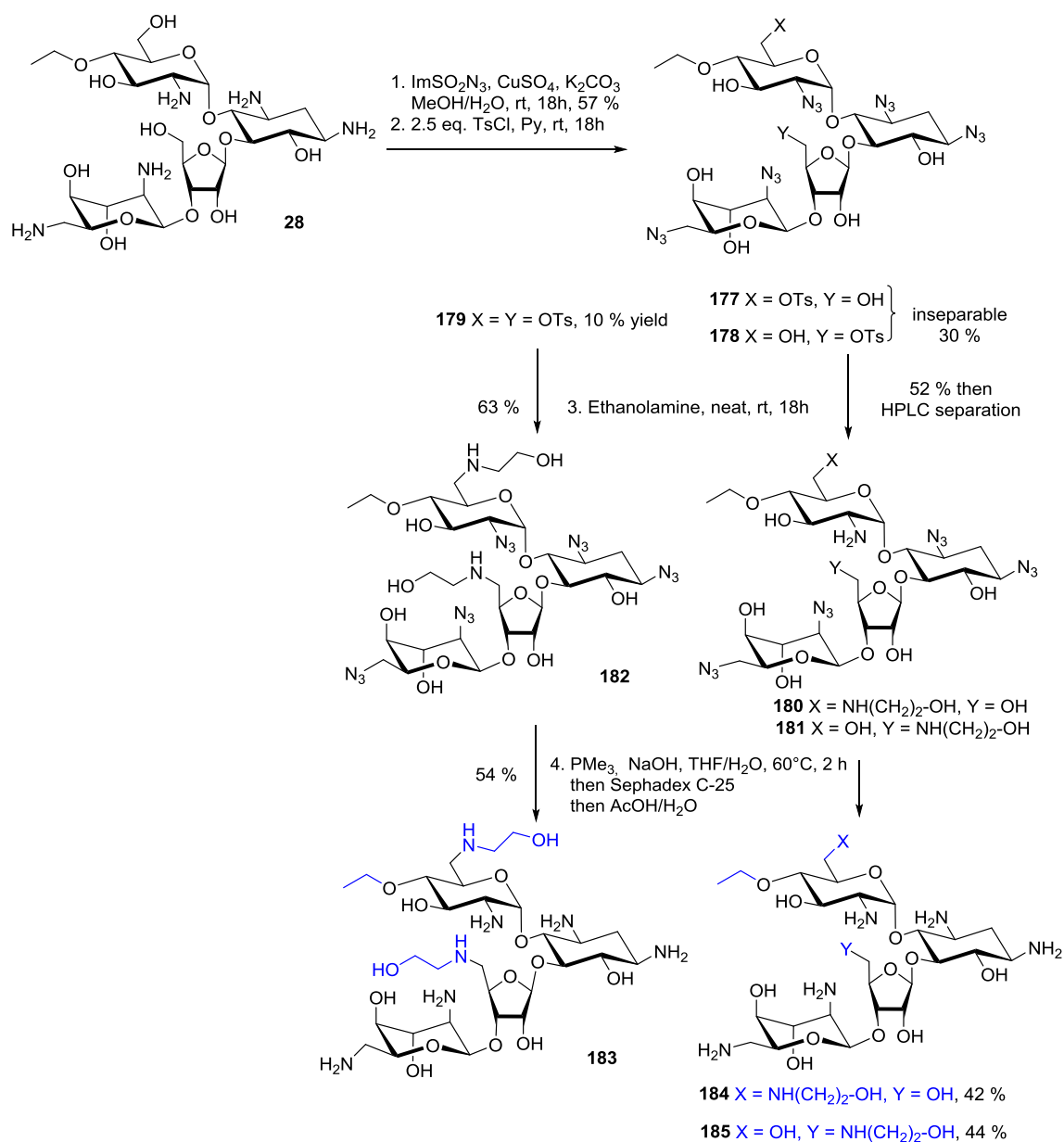
Among them, the neomycin derivative **176** carrying two hydroxyethyl chains at 6' and 6''' position was produced upon direct Cbz protection of the neomycin primary amines, azidation of the remaining amino groups, Cbz deprotection, reductive amination with benzyloxyacetaldehyde and full deprotection (Scheme 45) [66]. This bis-hydroxyethylated derivative proved to be less efficient than neomycin itself (Table 21).



Scheme 45. Synthesis of 6',6'''-bis(2-hydroxyethyl)neomycin.

In a similar manner, the *N*-(2-hydroxyethyl) modification at the 6' position was combined with the 4'-*O*-ethyl alkylation (Scheme 46). Starting from the 4'-ethyl paromomycin **28** (see Scheme 12), azidation of the amino groups and tosylation of the primary hydroxy groups provided a mixture of mono and ditosylated compounds **177-179**. Although the monotosylated compounds could not be separated, the ditosylate could. Each were then treated with ethanolamine, giving the corresponding substitution products **180-182**.

At this stage, preparative HPLC allowed separating the two monosubstituted compounds. They were converted by Staudinger reaction to the neomycin derivative **183** and paromomycin derivatives **184** and **185** in 42 and 44% yield, respectively.



Scheme 46. Synthesis of 4'-ethyl-6'-(2-hydroxyethyl)neomycin, 4'-ethyl-6', 5''-bis(2-hydroxyethyl)neomycin and 4'-ethyl-5''-(2-hydroxyethyl)paromomycin.

Monoalkylated neomycin derivatives **28**, **72**, and **73** (4'-O-ethyl, 6'-N-(2-hydroxyethyl) and 6''-N-(2-hydroxyethyl)neomycins; see Scheme 12 and 16, respectively) showed antibacterial properties very close to that of the parent neomycin, even for ESKAPE pathogens (Table 21). Interestingly, the introduction of the ethyl group on position 4'-O restore activities on MRSA strains (see activities of **28**). Additional studies on ribosomal selectivity showed that this modification is unlikely to reduce toxicity.

The accumulation of these modifications or some combination of this alkylation with others, like respectively in compounds **176** or **183-185**, did not improved antibacterial activities. Among them, compound **184** probably proved to be the best polysubstituted derivatives, but still not as active as neomycin (Table 21).

Bacterial strain	Par	Neo	28	72	73	176	183	184	185
<i>E. coli</i> AG001	4	2	4	4	2-4	16-32	32	16-32	64-128
<i>E. coli</i> AG0055	2-4	1	2-4	2-4	4	16	16-32	8-16	64-128
<i>E. coli</i> AG003	2-4	4	2-4	4	4	16	16-32	8-16	32-64
<i>MRSA</i> AG038	4	1-2	4	4	2	8	16	8-16	32-64
<i>MRSA</i> AG039	> 256	128	2	> 128	> 128	> 128	16-32	8	32
<i>MRSA</i> AG042	> 256	128	2	> 128	> 128	> 128	16	4-8	32
<i>MRSA</i> AG044	4-8	1	1-2	2	2	4	16	4-8	64
<i>K. pneumoniae</i> AG261	1	0.5	0.5	0.5-1	0.5	nd	nd	2-4	nd
<i>K. pneumoniae</i> AG262	1	0.5	1	1	0.5	nd	nd	4	nd
<i>K. pneumoniae</i> AG263	1	1	1	0.5	1	nd	nd	6	nd
<i>E. cloacae</i> AG290	2	1	1	1	1	nd	nd	4	nd
<i>E. cloacae</i> AG291	1-2	1	1	1	0.5	nd	nd	8	nd
<i>E. cloacae</i> AG292	2	1	1	1	0.5	nd	nd	4	nd
<i>A baumannii</i> AG225	4	4	4	2	2	nd	nd	8-16	nd
<i>A baumannii</i> AG226	2-4	4	4	2	2	nd	nd	4-8	nd
<i>A baumannii</i> AG286	1-2	0.5-1	1-2	1	0.5	nd	nd	8	nd

Table 21. MIC ($\mu\text{g/mL}$) of compounds **28**, **72-73**, **176** and **183-185**. (nd: not determined).

3. 6. Chemoenzymatic approaches

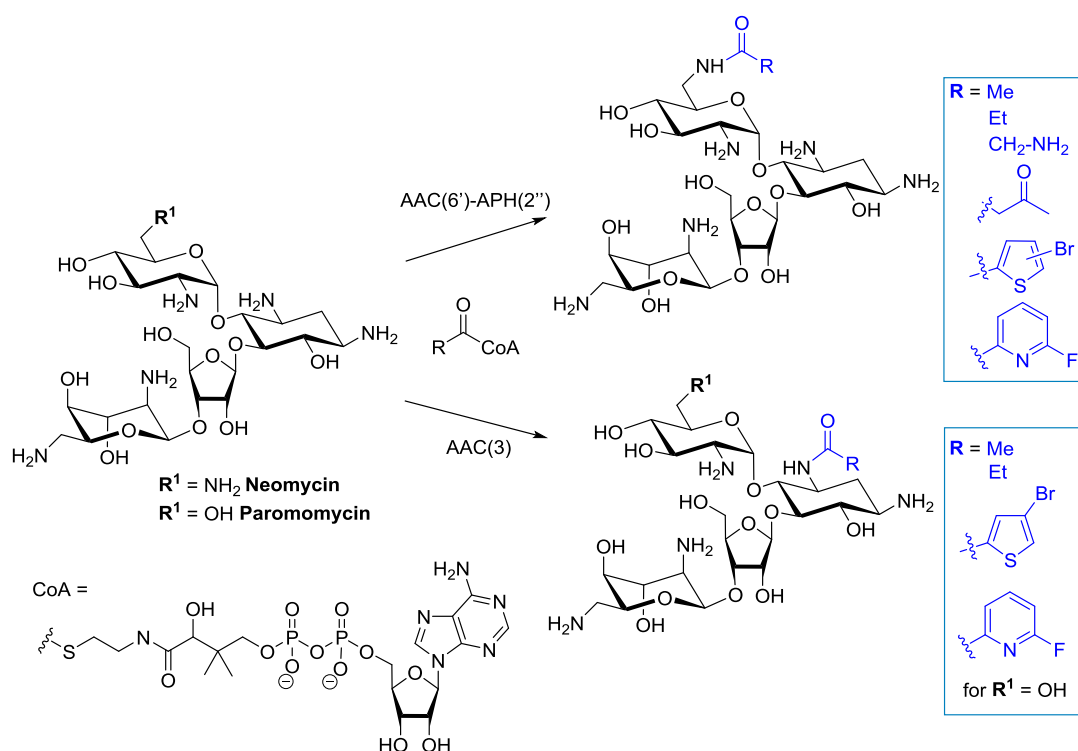
As seen in the preceding sections, conventional chemical modifications of AG, although quite effective, often require multistep sequences. The selective modification at specific site of multifunctionalised AG molecules is the main reason, because protection-deprotection steps are required to achieve such goal. This leads to complex and lengthy syntheses.

In sharp contrast to conventional reagents, enzymes are able to recognize one site of its substrate among others and to modify it with unique selectivity. Although such chemoenzymatic approach to organic synthesis is well known [124,125], only a very few examples have been reported in AG chemistry.

3. 6. 1. Applying AG modifying enzymes

Produced by resistant bacteria, the enzymes able to circumvent AG antibiotic action act by selectively modifying a single AG site (see section 2.4) and among them, four AG acetyl transferases (AAC) have been identified. Each selectively acetylate positions 1, 3, 6' or 2' of neomycin, so their respective name AAC(1), AAC(3), AAC(6') and AAC(2') (see Scheme 5).

In an interesting way, two of these AAC (AAC(3) and AAC(6')-APH(2'')) have been applied in the selective acylation of some AG, including paromomycin and neomycin [126]. These enzymes require an acyl coenzyme A as co-substrate, and only a few are commercially available, although quite expensive (~300 €/5 mg). Nevertheless, 16 of them have been screened together with 7 AG. Not so surprisingly, the native co-substrate of these enzymes (acetylCoA) was readily accepted, but only a few other acylCoA could also react (Scheme 47). Furthermore, double *N*3, *N*6'-acetylations could be performed in a one-pot process, by successive addition of the corresponding AAC and acetylCoA. However, this could only be achieved by adding AAC(6')-APH(2'') first and then AAC(3).

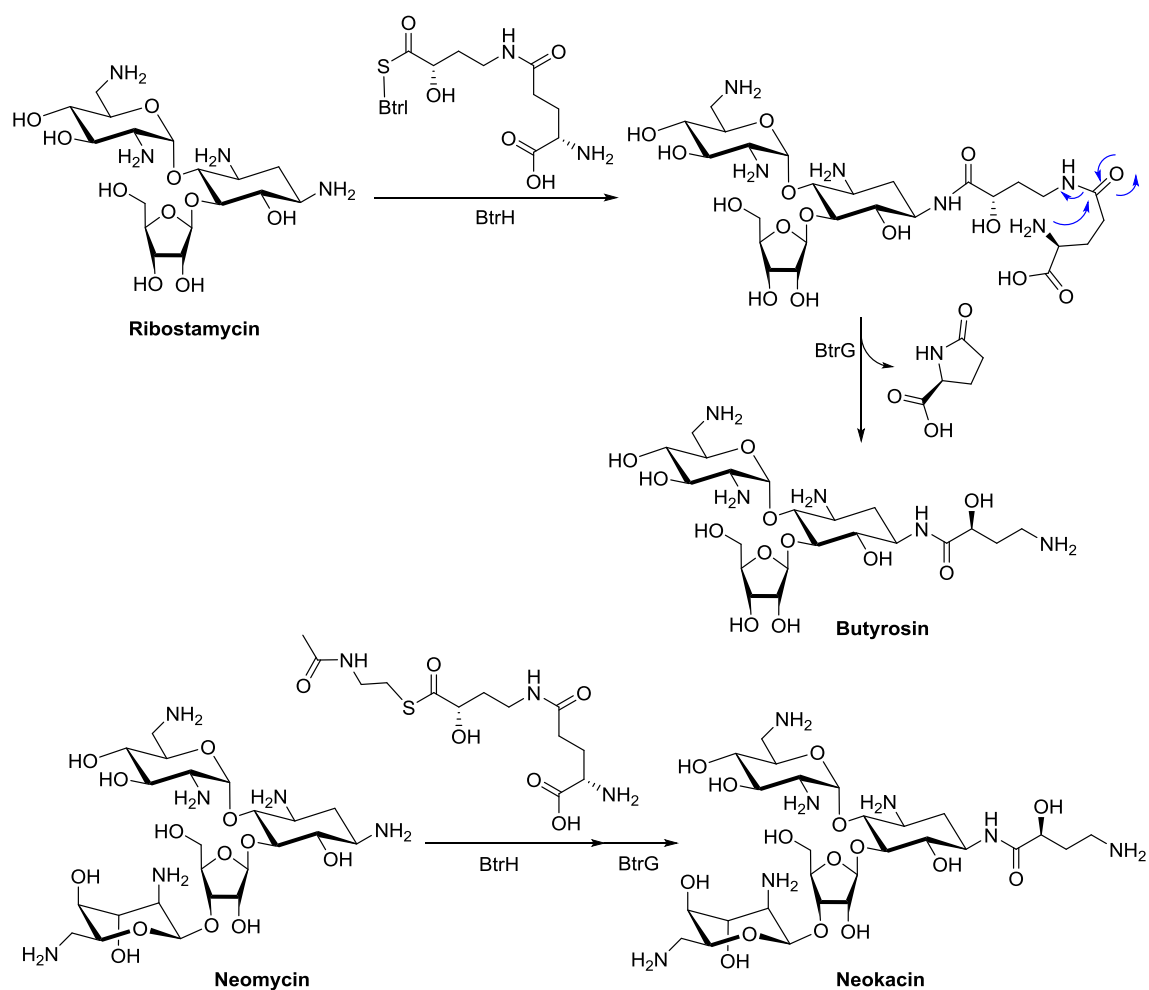


Scheme 47. Enzymatic synthesis of 6'-*N*-acylneomycin and 3-*N*-acylneomycin or 3-*N*-acylparomomycin.

3. 6. 1. Applying enzymes from AG biosynthesis

AG biosynthesis is an obvious source of enzymes able to build up the complex AG molecules. Among them, butirosin biosynthesis has deserved attention because the typical AHB chain of this AG is introduced from an acyl protein carrier in two steps at the biosynthesis end after having built up ribostamycin (Scheme 48 top).

The introduction of AHB chain to AG using these two enzymatic steps has thus been envisaged and successfully experimented [127]. In the set-up process, the natural acyl protein carrying the AHB chain could be replaced by a synthetic *N*-acetylcysteamine thioester reagent. A few neamine and ribostamycin derivatives could be converted to their *N*1-AHB derivatives. Neomycin itself could also be converted using this enzymatic route to its *N*1-AHB derivative, *i.e.* neokacin (Scheme 48 bottom) [128].



Scheme 48. The 2 last steps of the butyrosin biosynthesis (top); Direct enzymatic synthesis of neokacin from neomycin.

Due to the biotechnological tools currently available, engineering of biosynthetic pathway could even be envisaged [129].

Whatever the routes, such enzymatic strategies require to be able to prepare overexpression gene or plasmid constructs, to overexpress the so-selected enzymes and the latter need to be fished out and then purified. So far, these methods allow producing only mg quantities of modified AG.

4 Conclusion

Soon after the isolation of neomycin in 1949 and of its related analogues (paromomycin, ribostamycin) and the determination of their antibiotic properties, considerable efforts have been made to improve these properties and to reduce its toxicity. These efforts gradually faded during the 70's. However, with the emergence of bacterial strains (multi)resistant to antibiotics, chemists have taken over, especially in the last decade, trying to develop new antibiotics able to counteract such resistances.

To achieve this goal, a variety of reaction sequences have been developed to modify one or several of the constitutive rings of these antibiotic family, and that at almost each of the carried functional groups. These reactions have been extensively surveyed in the present review. All this creativity led to a large variety of molecules with one or more modifications, including dimers, hybrids with peptides, nucleosides, other antibiotics, intercalating agents, etc...

Although immense progress in understanding the cellular processes underlying the mode of action of this family of antibiotics has been made, pertinent neomycin modification is still a challenge. Indeed, despite the intensive works done, the present review reveals that only a few compounds have demonstrated their potential and hold out hope for becoming future drugs.

The results described here revealed:

- the requirement of an amino group at position 6' for keeping good antibiotic activity;
- the beneficial effect of acylation at position N1, of alkylation at position 6' and 4', of deoxygenation in ring I;
- combining those modifications at the appropriate position(s) is even more beneficial for improving antibiotic activity;
- tuning the pKa of the amino groups in AG could also be beneficial, probably by altering the overall AG charge and thus some of its interaction within A site, and/or its ability to cross bacteria wall.
- fluorination seems to reduce toxicity.

However, the complexity and length of some of the reaction sequences employed is also hampering the development of new compounds and of future antibiotic drug(s), and only the improvement of existing protocols and the development of new ones will

allow providing the necessary tools to achieve this goal. Avoiding such problems, (chemo)enzymatic approaches have emerged, but so far did not gain enough importance, clearly due to limitation regarding access to the appropriate enzymes.

Declaration of Competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have influence the work reported in this paper.

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