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Comprehensive Review of Chemical Strategies for the Preparation of New Aminoglycosides and their Biological Activities

Nishad Thamban Chandrika^a and Sylvie Garneau-Tsodikova^{a,*}

^aDepartment of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, KY, 40536-0596, USA

Abstract

A systematic analysis of all synthetic and chemoenzymatic methodologies for the preparation of aminoglycosides for a variety of applications (therapeutic and agricultural) reported in the scientific literature up to 2017 is presented. This comprehensive analysis of derivatization/generation of novel aminoglycosides and their conjugates is divided based on the types of modifications used to make the new derivatives. Both the chemical strategies utilized and the biological results observed are covered. Structure-activity-relationships based on different synthetic modifications along with their implications for activity and ability to avoid resistance against different microorganisms are also presented.

1. INTRODUCTION

Aminoglycosides (AGs) are broad-spectrum antibiotics used for the treatment of infections caused by both Gram-positive and Gram-negative bacteria.¹ Based on the substitution pattern of their 2-deoxystreptamine (2-DOS) ring, AGs are classified as 4,5-disubstituted 2-DOS AGs (*e.g.*, neamine (NEA), neomycin B (NEO), paromomycin (PAR), paromamine (PRM), and ribostamycin (RIB)), 4,6-disubstituted 2-DOS AGs (*e.g.*, arbekacin (ABK), amikacin (AMK), dibekacin (DBK), gentamicin (GEN), geneticin (G418), isepamicin (ISP), kanamycin A and B (KANA and KANB), nebramine (NEB), netilmicin (NET), plazomicin (PLZ, formally known as ACHN-490), sisomicin (SIS), and tobramycin (TOB)), and other monosubstituted 2-DOS AGs (*e.g.*, apramycin (APR) and streptomycin (STR)) (Fig. 1). Since the discovery of STR in 1943, the extensive use of AGs has resulted in the emergence of strains that are resistant to normal doses of these antibiotics.

A number of genetic, biochemical, and structural studies have been carried out to understand the mechanisms by which bacteria resist the action of AGs.²⁻⁵ It was found that reduction in the intracellular concentration of antibiotics by efflux pumps or through reduced membrane

*Correspondence to: sylviegsodikova@uky.edu, Phone: 859-218-1686.

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Conflicts of Interest

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permeability as well as decreased affinity of the drug for its target bacterial ribosome, either by modification of the drug or of the ribosome, are major causes of bacterial resistance to AGs. Additionally, it was discovered that structural modifications of AGs by AG-modifying enzymes (AMEs) are by far the most clinically significant mechanisms of resistance to these antibiotics. There are three major classes of AMEs: AG *N*-acetyltransferases (AACs), AG *O*-phosphotransferases (APHs), and AG *O*-nucleotidyltransferases (ANTs). The reactions catalyzed by these monofunctional enzymes are usually regioselective.⁶⁻⁹ However, a monofunctional versatile AAC, the enhanced intracellular survival (Eis) protein, with the ability to multiacetylate different amine positions of AGs,¹⁰ was recently found in *Mycobacterium tuberculosis*,¹¹⁻¹⁵ other *Mycobacteria*,^{16, 17} *Bacillus anthracis*,^{17, 18} *Anabaena variabilis*,^{17, 19} *Kocuria rhizophila*,¹⁷ *Gordonia bronchialis*,¹⁷ and *Tsukamurella paurometabola*.¹⁷ In addition to these monofunctional enzymes, the emergence of various bifunctional AMEs,²⁰ such as AAC(6')-Ie/APH(2'')-Ia from *Staphylococcus aureus*,²¹⁻²⁵ ANT(3'')-Ii/AAC(6')-IId from *Serratia marcescens*,²⁶⁻²⁸ AAC(3)-Ib/AAC(6')-Ib' from *Pseudomonas aeruginosa*,^{29, 30} and AAC(6')-30/AAC(6')-Ib from *P. aeruginosa*,³¹ has been reported. In the past 20 years, a significant amount of research has focused on developing new AG derivatives, which still can maintain their affinity for the ribosome target, while evading structural modification by AMEs.

Along with the ever-growing problem of resistance, nephrotoxicity and ototoxicity have been associated with the prolonged use of these antibacterial agents. Combined with the fact that resistance has developed to all classes of antibiotics currently available, the broad applicability of AGs in the treatment of bacterial infections has prompted a renewed interest in the use of AGs among the scientific community. As a result of this renaissance of AGs, multiple review articles covering the various resistance mechanisms of AGs in details,³²⁻³⁴ the renaissance of these antibiotics and their various usages,^{1, 35, 36} carbohydrate-derived macrocyclic compounds,³⁷ and small molecules targeting RNA³⁸ have been published lately. The past 20 years have seen a steady increase in the number of research articles related to AGs, as exemplified by a systematic breakdown of search results for the term AG in PubMed and SciFinder from 1995 to 2015 (Fig. 2) (*Note*: the data from 2016 were still not all compiled in these databases at the time of publication). These data point to the rise in efforts being made by the scientific community towards solving the problems associated with the use of AGs.

To tackle the problems associated with AG resistance and toxicity, various chemical strategies were developed. These chemical methodologies include: (i) modifying the AG cores, (ii) introducing various alkyl/aryl substituents and (iii) acyl substituents at different positions of AG scaffolds, (iv) developing AG heteroconjugates, (v) developing AG homo- and heterodimers, (vi) synthesizing conformationally-restricted AGs, (vii) generating novel AGs *via* glycodiversification, and (viii) developing new AGs by chemoenzymatic reactions (Fig. 3). In this review, we will summarize the tremendous amount of chemistry related to the 8 above strategies towards development of AGs for application in treatment of bacterial, fungal, and viral infections, as well as genetic disorders associated with premature termination codons (PTCs) (*e.g.*, Duchenne muscular dystrophy (DMD) and cystic fibrosis (CF)) (Table 1). In addition to primarily focusing on the detailed chemistry to help scientists in this field of work to better appreciate the methodologies available to them, we will

provide information about the reasoning behind the modifications made and the biochemical and biological data resulting from these modifications. We believe that by summarizing the interesting chemistry and the overall work done on AGs, this review will not only provide the scientific community with an up-to-date chemical reaction repository for AG synthesis, but will also serve as a great introduction/compendium to anyone who, in the future, would like to work in the field of AGs.

2. CHEMICAL STRATEGIES FOR THE DERIVATIZATION/GENERATION OF NOVEL AGs AND AG-DRUG CONJUGATES ALONG WITH THEIR BIOLOGICAL ACTIVITIES

The presence of multiple amino and hydroxyl groups with similar reactivity profiles renders the derivatization/generation of new AGs a tedious synthetic endeavor for chemists. However, in recent years, in addition to chemistry related to modification of the core of AGs, there has been a great output of inventive strategies for the chemical modification of specific previously unexplored positions on the scaffold of these antibiotics. From an in-depth analysis of the literature on AG syntheses, it is clear that irrespectively of the AG scaffold, the most frequently modified positions remain either primary alcohols or amines, whereas the modification of secondary functional groups continues to be a relatively uncharted territory. Interestingly, in most manuscripts, the biological data collected on these modified AGs is not directly correlated to the specific position changed. After focusing on the chemical methodologies available, we will very briefly highlight some of these observations.

In an attempt to provide an overall summary of all the different strategies for functionalizing AGs, the different chemical modifications performed at various positions of the 18 AGs present in the studies covered in this review (ABK, AMK, APR, DBK, G418, GEN, ISP, KANA, KANB, NEA, NEB, NEO, NET, PAR, PRM, RIB, SIS, TOB) are summarized in Fig. 3 and Table 1. To simplify the gigantic number of possible reactions used to diversify AG scaffolds, we first present, in a concise manner, general strategies for some of the most frequently used reactions (Figs. 4,5). The most common strategy for diversifying the 5''-hydroxyl of NEO as well as the 6''-hydroxyl of AMK, ABK, KANA, KANB, and TOB, consists of first introducing a good leaving group (*e.g.*, triisopropylbenzenesulfonyl (TIBS) of **1a/1b** and **2a/2b-6a/6b**) at these most frequently modified positions (Fig. 4A). The introduction of the TIBS moiety proceeds in 2 steps: (1) the protection of all amino groups with Boc (**a**) or Cbz (**b**), followed by (2) the introduction of TIBS at the primary alcohol. The TIBS leaving group can then be effectively and systematically displaced by various nucleophiles (*e.g.*, amines and thiols) to yield either the desired final products (**18**) or intermediates (**7**, **8**, **12**, **14**, and **15**) for further structural modifications (Fig. 4B). A large portion of the literature present modifications of the 5''-hydroxyl of NEO by nucleophilic displacement of the TIBS of **1a/1b** by various (i) dithiols (reaction **A**), (ii) azides (reaction **B**), and (iii) amino thiols (reaction **F**) (Fig. 4B). Reactions with dithiols produce intermediates **7**, which are an integral part of the formation of both homo- and heterodimers *via* disulfide linkages. Reactions with NaN₃ generate the 5''-azidated NEO derivative **8**. This key intermediate can be converted to the 5''-amino NEO derivative **9** using Pd(OH)₂/C, H₂ or Me₃P (reaction **C**), which can be functionalized by using amide coupling with a variety of

RCO₂H or RCOCl (reaction **D**) to form biologically relevant molecules **10**. The azide group of **8** can alternatively be modified to triazoles by copper-catalyzed click reactions (reaction **E**) with alkynes to form AG heteroconjugates **11**. Reactions with amino thiols generate intermediates **12** (reaction **F**) on the way to their corresponding isothiocyanate derivatives **13** (reaction **G**), which provide a conjugation point for different biologically relevant amines to form thiourea derivatives. Reactions **A-D** have also extensively been used to generate ABK, AMK, KANA, KANB, and TOB derivatized at their 6''-position (compounds **14-17**). Similarly, the displacement of the TIBS group at the 6''-position of various 4,6-disubstituted 2-DOS AGs by thiols (reaction **H**) generate thioethers **18**. The strategy to derivatize the 5''-position of PAR (Fig. 5A) is highly similar to that described for the modifications of the 5''-position of NEO (Fig. 4A) with the only difference residing in the need of an additional protection step of the 4' and 6'-hydroxyl groups by using acetal formation with PhCH(OMe)₂ prior to the introduction of the TIBS group at the 5''-position to form **19a/19b** (**a** represents Boc and **b** represents Cbz). Another prominent strategy frequently used in the literature for single-step modification of AGs is presented in Fig. 5B. This strategy relies on a base-catalyzed reaction of AGs with various *N*-hydroxysuccinimide (NHS) esters to result in analogues with an acyl group at the 6'-, 6'''-, or 6',4'''-amines of AGs. In the case of AMK, KANA, KANB, NEA, NET, SIS, and TOB, acylation at the 6'-position (compound **20**) is commonly observed. With AMK (compound **21**), acylation at the 6'- and 4'''-amines is also commonly found, whereas with PAR (compound **22**), only acylation at the 6'''-amine have been performed.

The general strategies highlighted in Figs. 4 and 5 extensively focused on modifications of both primary amines and hydroxyls. Aside from these most frequently used modifications, there are specific reactions intended for modifying secondary positions of different AGs, which will be discussed in details in the upcoming sections. Before we concentrate on the numerous chemical reactions reported in the literature for functionalizing AGs, emphasis will be given to various methods developed for the modification of AG cores.

2.1. Modification of AG cores

As the amino and hydroxyl groups of AGs play a key role in their interactions with the A-site of the 16S ribosomal RNA (rRNA), which ultimately results in inhibition of protein synthesis, their modifications could have a great effect on the activity of AG derivatives.^{39, 40} The relatively unexplored modifications of AG cores are centered around changes in these critical amino and hydroxyl moieties and include (i) conversion of amino groups into azides (Fig. 6A), (ii) conversion of hydroxyl functionalities into azides, amines, or aldehydes (Figs. 4,6B), (iii) conversion of allylic amines into α,β -unsaturated aldehydes (Fig. 7), (iv) removal of hydroxyl groups to result in either unsaturated or saturated rings (Fig. 8), and (v) epimerization of hydroxyl moieties (Fig. 9).

The selective modifications of AGs normally require multistep synthesis.⁴¹⁻⁴⁴ Therefore, a method for a one-step conversion of an amino group into an azide would deserve special attention. The lower basicity of the amino group at the 3-position of numerous AGs was exploited for the successful regioselective introduction of an azide group by using a diazo-transfer reagent (imidazole-1-sulfonyl azide) in one chemical step without the need for

protection of the other amines or hydroxyls.⁴⁵ This methodology was successfully applied to regioselectively (up to 98%) azidate the AGs AMK, APR, NEA, NEO, PAR, and RIB (Fig. 6A). Since the amino group at the 3-position is a target for the AAC(3) resistance enzymes, this facile one-step modification could be extremely useful for generating new derivatives that could potentially overcome bacterial resistance resulting from the action of these enzymes and remains to be explored.

In most instances, the conversion of primary alcohols into azides and then into amines is a stepping-stone for the introduction of different substituents at various positions on AG scaffolds, which is the topic of the following sections. However, these basic transformations have been used, in two cases, exclusively to modify the core of AGs. In the first occasion, by using the general reaction **B** presented in Fig. 4B, the 6"-azidated KANA and TOB analogues **15** were generated from **4a** and **6a**, respectively (Fig. 4A). These analogues were used to develop a clever microarray strategy for the rapid evaluation of the parent compounds as potential substrates for ANT(2") and APH(3') resistance enzymes.⁴⁶ More specifically, the methodology consists of clicking the azidated compounds with an alkyne covalently attached to agar slides prior to analysis of rRNA binding by using a fluorescently-labeled rRNA A-site mimic. This simple azidation of the core of AGs provides an alternative method to the traditional low-throughput *in vivo* and *in vitro* assays. In the second case, the replacement of hydroxyl moieties by amines on the core of various AGs was performed to explore the importance of electrostatic interactions for the binding between RNA and AGs and to identify a common recognition pattern.⁴⁷ The 5"-amino-NEO, 6"-amino-KANA and 6"-amino-TOB analogues were synthesized from **1b**, **4b**, and **6b** (Fig. 4A) by using reactions **B** and **C** (Fig. 4B). These analogues were tested for their inhibition of the hammerhead ribozyme, and the results demonstrated that a larger number of amino groups on an AG core correlates with an increase in inhibitory activity as well as an increase in RNA binding.

A more unique core modification consists of conversion of hydroxyl groups into aldehydes. In a beautiful structure-activity-relationship (SAR) study focusing on the exploration of the shallow-groove recognition of ribosomal-decoding-site RNA, a series of 6'-modified PRM derivatives (**24**, **25**, **27**, **28**, **29**, **31**, and **32**) were synthesized by making use of aldehyde chemistry (Fig. 6B).⁴⁸ The common intermediate **23** comprising an aldehyde in place of the hydroxyl at the 6'-position of PRM was synthesized in 6 steps from PAR. The 6'-aldehyde of intermediate **23** was utilized for the (i) oxidation to a carboxylic acid to give **24**, (ii) oxidation followed by coupling with ammonia to form **25**, (iii) reaction with MeMgBr to form **27**, (iv) Wittig reaction followed by oxidation to diol derivative **28**, and (v) homologation reaction to form the key 6'-cyano compound **26**, which was further derivatized. Intermediate **26** was subjected to complete oxidation followed by coupling with ammonia to form **29**. The controlled reduction of **26** resulted in the conversion of the cyano group into an aldehyde in **30**, which was further reduced to form **31** as well as **32** as a result of reaction with MeMgBr. These minor modifications at the 6'-position resulted in a dramatic reduction in the ability of these compounds to bind to the decoding site of their RNA target. This SAR study revealed an exquisite sensitivity of the 6'-position of PRM towards substitution, which emphasized the role of this group in RNA target recognition.

The use of an α,β -unsaturated aldehyde intermediate has also been exploited during the total synthesis of verdamicin C2 and C2a (Fig. 7). In this work, a rare, but very interesting allylic oxidation of the primary azide **33** derived from SIS was utilized to form the 6'-aldehyde derivative **34**, which was then subjected to Grignard reaction with MeMgBr. The secondary hydroxyl group of **34** was then subjected to nucleophilic substitution reaction with NaN₃ to form verdamicin C2 (**37**) and C2a (**38**).⁴⁹ α,β -Unsaturated aldehydes could be used in the future for developing novel scaffolds from readily available AGs containing allylic amines.

In addition to their potential use in the preparation of AGs containing double bonds in their core, α,β -unsaturated aldehydes can also be utilized to synthesize 3',4'-dideoxygenated AGs (Fig. 8A). By using α,β -unsaturated aldehydes, analogues of NEO, PAR, and RIB, where ring I was transformed into that found in SIS, were produced with the aim of evading the action of AMEs such as APH(3') and ANT(4').⁵⁰ PAR was first protected with Cbz groups prior to the formation of the 4',6'-*O*-benzylidene derivative, capping of the remaining hydroxyls with methylcarbonates, and removal of the benzylidene to provide the 4',6'-diol **39**. The 4'-hydroxyl of **39** was converted into a mesylate **40** in 3 steps and subjected to Parikh-Doering oxidation/ β -elimination to give intermediate **41**. The heavily functionalized analogue **41** was then subjected to deoxygenation to produce the 3',4'-dideoxygenated intermediate **42**. The 6'- α,β -unsaturated aldehyde was then converted into an alcohol or an amine followed by global deprotection to give 3',4'-dideoxygenated PAR or 3',4'-dideoxygenated NEO, respectively. The 3',4'-dideoxygenated RIB analogue was generated by removal of ring IV of 3',4'-dideoxygenated NEO prior to global deprotection. The NEO analogue displayed MIC values ranging from 0.5 to 2 $\mu\text{g/mL}$ against *Escherichia coli*, *P. aeruginosa*, *Klebsiella pneumoniae*, and *S. aureus*. This methodology was used for synthesizing various SIS-butirosin and SIS-butirosin-NEO hybrids to circumvent the actions of AMEs.⁵¹ To extend upon the formation of dideoxygenated AGs, a novel methodology was developed to synthesize 3',4',3'',4''-tetra-deoxy-NEO and PAR analogues (Fig. 8B). This synthetic strategy relied on a novel application of the chemoselective Garegg-Samuelsson deoxygenation reaction in combination with *N1*-modifications *via* oxazolidinones for efficient access to promising antibiotics analogues.⁵² Both NEO and PAR were protected with Cbz groups and their primary alcohols were capped with trityl and silyl moieties, respectively. The resulting intermediates retained 6 secondary hydroxyls, 4 of which, involved in *trans*-diols, were then subjected to deoxygenation to provide intermediates **43** and **45**. Compounds **43** and **45** were subjected to either global deprotection (**44** and **46**) or *N1*-amide substitution to synthesize novel analogues of NEO and PAR. Scaffold **46** with *N1*- γ -amino- β -hydroxybutyrate (*N1*-AHB) substitution yielded a molecule with 64-fold increase in activity compared to the currently clinically employed AMK and GEN, which also contain a *N1*-AHB substituent.

Finally, another interesting approach for the modification of the core of AGs involves epimerization of various centers on these antibiotics. Examples of ABK analogues with their 5- or 4"-hydroxyl groups epimerized or epimerized/substituted by various amines and halogens have been reported. For the synthesis of 5-deoxy-5-epi-substituted ABK analogues, all amines of ABK were first protected using Boc anhydride followed by controlled acetylation of all hydroxyl moieties with the exception of that at the 5-position

(Fig. 9A). The 5-hydroxyl group was then converted to the corresponding mesylate **47**, which was then subjected to nucleophilic substitution reaction with various groups (OH, Cl, NH₂, NHMe, N(Me)₂, NH(CH₂)NH₂, and NHCH₂Ph) followed by global deprotection to yield compounds **48-54**. Compounds **48**, **49**, **50**, and **53** showed good antibacterial activity against *P. aeruginosa* and *S. aureus*. In particular, compounds **48** and **53** displayed potent activity against *S. aureus*, including methicillin-resistant *S. aureus* (MRSA) expressing the bifunctional AME AAC(6')-Ie/APH(2'')-Ia.⁵³ For the synthesis of 4"-deoxy-4"-episubstituted ABK analogues, ABK was subjected to Boc protection, 4",6"-ketal formation, and controlled acetylation to form **55** (Fig. 9B). The ketal group was then cleaved, the 6"-hydroxyl tritylated, and the 4"-hydroxyl reacted with Tf₂O to result in **56**. The 4"-OTf was subjected to nucleophilic substitution with different groups (OH, NH₂, Cl, and H) to form compounds **57-60**. Analogue **57** displayed antibacterial activity against *P. aeruginosa*, *S. aureus*, and MRSA equivalent to that of the parent ABK. This methodology was further expanded by epimerizing both the 5- and 4"-positions, which resulted in analogues active against both MRSA and *P. aeruginosa*.⁵⁴ A similar strategy was used to synthesize various 4'-epimers of PAR (Fig. 9C).⁵⁵ Intermediate **61** was synthesized from PAR in 4 steps, which included conversion of amine to azide followed by acetal protection, benzylation, and reductive opening of the benzyldine ring. The 4'-hydroxyl was converted to triflate **62**, which was then subjected to nucleophilic substitution with Bu₄NN₃ followed by global deprotection to afford the 4'-amine epimer **63**. The oxidation of **61** followed by reduction with NaBH₄ resulted in the 4'-hydroxyl epimer **64**. This methodology was further exploited to synthesize various analogues of PAR.⁵⁵

Here, we have summarized some of the important methods developed for modifying the core of various AGs in order to improve their efficacy. In addition to the above mentioned core modifications, there are other methods reported in the literature. The 4'-keto analogue of KANA was synthesized from KANA in 11 steps to overcome the action of APH(3')-Ia.⁴² Various analogues of NEO with modifications on rings II, III, and IV involving reactive epoxide and epoxide-aziridine intermediates were also reported.⁵⁶ Using allylic rearrangement, a convenient method was developed for the synthesis of various 2',3'-dideoxy-KANB and -NEO, which would otherwise be difficult to synthesize.⁵⁷ In another report, methods were developed to selectively cleave ring I of PAR followed by alkylation of its 4-hydroxyl group to form spatial mimics of ring I.⁵⁸ Finally, a series of 5-substituted PRM mimics were synthesized and tested for their antibacterial activity.⁵⁹ Even though the core modification of AGs represents an interesting and important strategy for developing novel AG analogues, as discussed in this section, there are only few methods reported in the literature. As multistep syntheses are in most cases required to modify the core of AGs, their decoration (*e.g.*, alkylation, arylation, acylation, and heteroconjugation) has been gaining in popularity.

2.2. Preparation of alkylated and arylated AGs

One of the most common AG decorations consists of generating amphiphilic AGs by addition of alkyl or aryl groups to their cores. By combining the cationic nature of AGs with the hydrophobic properties of alkyl groups, the resulting amphiphiles can interact with bacterial membranes *via* non-specific interactions, such as hydrophobic and electrostatic. A

reason for the popularity of the alkylation modification stems from the limited number of synthetic steps involved in the process and, therefore, the ease at rapidly gathering SAR data. This type of peripheral decoration is usually carried out to provide AGs capable of (i) selectively targeting bacterial and fungal membranes (*Note*: as in the case of alkylated AGs), (ii) improving activity, and (iii) avoiding inactivation by AMEs. In fact, some of the newer amphiphilic alkylated AG derivatives generated were demonstrated to disrupt the membrane of various prokaryotes, and were found to be poor substrate of AMEs, while displaying superior activity against a wide range of AG-resistant bacterial strains when compared to their parent AG counterparts.

For ease of comprehension, we classify these alkylated/arylated molecules into five classes based on the types of their covalent attachment to AGs: (i) thioether, (ii) ether, (iii) amine, (iv) triazole (generated by click chemistry), (v) amide, and (vi) carbamate linkages.

2.2.1. Preparation of alkylated and arylated AGs via thioether linkages—To begin with, alkyl chains were introduced onto KANB, PAR, and TOB *via* thioether linkages by using standard nucleophilic substitution reactions in which an electrophilic center on the AG was displaced with a thiol nucleophile (Figs. 10,11). In order to selectively target bacterial membranes, novel 6"-alkylated thioether TOB analogues were synthesized along with their *S*-oxidized (sulfoxide and sulfone) derivatives and tested for their activity against 21 Gram-positive and Gram-negative bacterial strains (Fig. 10).^{60, 61} Derivatives with C₁₂, C₁₄, and C₁₆ linear alkyl chains showed potent activity against most TOB-resistant bacterial strains tested and were found to act by targeting bacterial membranes while displaying low hemolytic activity when tested against rat erythrocytes. In addition to the alkylated TOB derivatives, numerous novel 6"-aromatic thioether TOB analogues were synthesized and were found to inhibit bacterial growth and ribosomal translation better than TOB in *E. coli*.⁶² Arylation therefore appears to be a promising avenue to pursue to enhance the ribosome-targeting activity of AGs. A subset of these TOB analogues with linear alkyl chain substituents were investigated for their *in vitro* antifungal activity.⁶³ Analogues with C₁₂ and C₁₄ displayed promising antifungal activity against *Candida albicans* strains, with MIC values ranging from 1.95 to 62.5 µg/mL and 1.95 to 7.8 µg/mL, respectively. These compounds also disrupted the fungal membrane. These alkylated TOB derivatives were also found to act synergistically with various azole antifungal agents.⁶⁴ In another study, 5 amphiphilic KANB analogues were synthesized by attaching medium-length to long linear alkyl chains (C₈, C₁₀, C₁₂, C₁₄, and C₁₆) at the 6"-position of this AG.⁶⁵ The C₈ and C₁₀ analogues showed no activity, C₁₂ and C₁₄ displayed promising antibacterial and antifungal potency, and C₁₆ was found to be the most potent against fungi, but did not display activity against bacteria. KANB derivatives also exhibited chain length-dependent antifungal activity against fungi in the order C₈ < C₁₀ < C₁₂ < C₁₄ < C₁₆. *In vitro* antifungal combination studies of these compounds with the antifungal agent posaconazole showed remarkable synergetic interactions, indicating the promise of these molecules for new antifungal combination therapies. Another study also explored the use of the parent AG PAR as an antifungal agent against *Phytophthora* and *Pythium* species.⁶⁶ A KANA derivative with a C₈ alkyl chain tethered to the 6"-position in a sulfone linkage termed K20 was discovered to display some antifungal activity against *Cryptococcus neoformans*, select *C. albicans*, and

Fusarium graminearum, while devoid of antibacterial activity.⁶⁷ K20 was also found to be synergistic with azoles.⁶⁸

In addition to the mono-alkylated TOB derivatives presented in Fig. 10, homo- and hetero-dialkylated TOB analogues, also with thioether linkages, have been reported (Fig. 11A).⁶⁹ Their syntheses involved site selective nucleophilic displacement of protected alcohols by thiols. Homo 4',6"-dithioethers **70** were synthesized by nucleophilic substitution of both 4', 6"-OTBS groups at elevated temperature, whereas reactions performed at room temperature resulted in the formation of 6"-thioethers **71**. The hetero 4',6"-dithioethers **72** were produced by displacing the 4'-OTBS group of mono-thioethers **71** with various thiols at elevated temperature. When a TOB molecule containing OTBS groups at the 2"- and 6"-position was generated, reactions with thiols at elevated temperature resulted in homo 3",6"-dithioethers **74** as a result of the NHBoc group at the 3"-position forming an aziridine intermediate. These compounds were found to be active against MRSA and *Streptococcus pyogenes*. Similarly, homo 6',5"-dialkylated PAR derivatives **75** were synthesized with linear hydrophobic chains (C₆-C₈) (Fig. 11B).⁷⁰ Among these, the di-C₈ PAR analogue was found to be the most potent. However, the di-C₇ PAR analogue demonstrated the least hemolytic activity. When comparing the di-C₈ PAR with its mono-C₁₆ counterpart, it was found that the di-C₈ was much better, as the mono-C₁₆ showed poor antibacterial activity against all bacterial strains tested and caused 100% hemolysis of rat erythrocytes. These results indicate that using a dialkylation approach may potentially be more favorable than mono-alkylation for the design of AG-based cationic amphiphiles. The dialkylation method remains to be further exploited.

2.2.2. Preparation of alkylated and arylated AGs via ether linkages—In addition to the thioether connections discussed in section 2.2.1, ether linkages have also been extensively reported. In fact, alkylation *via* ether linkages is actually the most common form of chemical modifications found in the literature related to AGs. Hydroxyl groups present on AGs serve as nucleophiles for substitution reactions with various alkyl halides. A number of 2"-O-alkyl PAR derivatives were synthesized and their binding to the rRNA decoding site studied (Fig. 12A).^{71, 72} Their syntheses proceeded *via* a precursor of **19b** (Fig. 5), which was first protected at the 5"-position with a TBDMS group prior to a highly regioselective allylation of the 2"-hydroxyl group to afford **76**. Compound **76** was then benzoylated and, after an oxidative cleavage of the allyl group, the resulting aldehyde was reacted with various amines to afford 28 analogues of **78** after deprotection. These non-toxic analogues were tested against *S. aureus* and *E. coli* and, with a few exceptions, all new analogues showed potent inhibitory activity equal to or better than that of PAR. Co-crystallization of selected analogues with the A-site of *E. coli* RNA revealed a dramatic change in the orientation of rings III and IV in the bound state, which explained the high tolerance for the substitution at the 2"-position. In another report, a series of 4'-O-alkyl (**79**) and 4',6'-O-acetal (**80**) PAR derivatives were synthesized to increase their selectivity for bacterial ribosomes (Fig. 12B).^{73, 74} These compounds were found to be potent antibacterial, while displaying excellent selectivity for their ribosomal target and little to no ototoxicity upon chronic administration in guinea pigs.

Besides the 4'-*O*-substituted PAR derivatives presented in Fig. 2, a variety of 4'-*O*-alkyl KANA and KANB derivatives as well as 4"-*O*-ethyl KANB were also synthesized (Fig. 13).⁷⁵ Modifications at the 4'-position of KANB proved fatal to the antiribosomal and antibacterial activity of the derivatives. Ethylation of KANB at its 4"-position resulted in some loss of antiribosomal and antibacterial activity. These results are the opposite of what was observed with the 4'-*O*-alkylated PAR derivatives, which caused only minimal loss of activity, but resulted in a significant increase in selectivity for the ribosome with concomitant loss of ototoxicity.⁷³ An important lesson from these studies is that one cannot extrapolate observations from one AG scaffold to another. Additionally various 4"- and 6"-alkylated (C₄, C₆, C₇, C₈, C₉, C₁₀, C₁₂, and C₁₄) KANA analogues were synthesized by nucleophilic substitution at these positions. The analogues with a C₈ alkyl chain displayed strong antifungal activity despite being poor in terms of their antibacterial activity.⁷⁶ To achieve the regioselective incorporation of alkyl groups at the 4"- and 6"-positions of KANA, the traditional approach involving the protection of the 4"- and 6"-positions, the protection of the remaining hydroxyl groups, the selective removal of the protecting groups at the 4"- and 6"-position, followed by the alkylation and global deprotection was used.

In another study, various antibacterial amphiphilic TOB derivatives were found to induce immunomodulatory response at concentrations that were non-toxic to host cells. Six novel TOB analogues alkylated at the 5-position (**87**) were synthesized in 4 simple and high yielding steps (Fig. 14A).⁷⁷ To begin with, all amino and hydroxyl groups, with the exception of the 5-hydroxyl, were protected with Boc and TBDMS, respectively, prior to substitution reactions with various alkyl halides and final deprotection. These compounds were demonstrated to boost innate immune response in plastic-adherent human macrophage-like THP-1 cells and to selectively control inflammatory responses induced in presence of endotoxin to prevent septic shock. The combination of antibacterial activity and immunomodulatory response could be useful for anti-infective therapy. Similarly, based on molecular modeling, various ether-linked basic groups were introduced at the 5-position of TOB to mimic rings III and IV of PAR (Fig. 14B).⁷⁸ Acetylation of penta-*N*-Cbz-TOB with acetic anhydride in pyridine afforded a partially acetylated derivative with the 5-hydroxyl group remaining unprotected. This position was then allylated prior to oxidative cleavage and reductive amination with various amines to generate TOB analogues **88** along with their guanidino counterparts. Some of the non-guanidinylated analogues were as active as TOB against *E. coli*, and some of the guanidinylated analogues showed good activity against *P. aeruginosa*. Another AG scaffold that was extensively alkylated *via* ether linkages is NEA (Fig. 14C).⁷⁹ The synthesis of NEA analogues alkylated at the 5-position was carried out in 10 steps from NEO. Intermediate **89** was prepared from NEO by azidation, peracetylation (for ease of purification), deacetylation, benzylation, selective cleavage of the ribofuranosyl bond, and allylation of the free 5-hydroxyl group. To generate the NEA derivatives **90**, oxidative cleavage and reductive amination with various amines were performed. In another study, allyl intermediate **89** was ozonolyzed and oxidized to form an intermediate acid **91**, which was then subjected to parallel synthesis using various aliphatic, aromatic amines, and cyclohexylcarbodiimide bound to macroporous polystyrene resin to yield NEA derivatives **92**.⁸⁰ Analogues **90** and **92** were studied (i) for correlation of antibiotic activity with *in vitro* inhibition of translation, and (ii) as potential antitumor agents that target oncogenic RNA

sequences. A linear correlation between *in vitro* translation inhibition assay and antibiotic activity was observed for these NEA analogues. Furthermore, these analogues displayed, for the first time, high affinity for oncogenic Bcr-Abl and PAX3-FKHR single breakpoints. Based on the affinity of these synthetic AGs for RNA, it is reasonable to believe that AGs can potentially act as regulators of gene expression.

In addition to the modification of NEA at its 5-position by reductive amination starting from NEO (Fig. 14C), modification of the 6-position of this AG was also carried out, although differently, by reductive amination starting directly from NEA (Fig. 15).^{81, 82} After Cbz protection, NEA was subjected to cyclohexylidene protection of its hydroxyl groups at positions 5 and 6 and by further protection of its hydroxyl groups at positions 3' and 4' with chloromethyl methyl ether (MOM) to yield **93** (Fig. 15A). The cyclohexylidene ring was deprotected and treated with NaH to afford a cyclic carbamate where the resulting free hydroxyl and the amino group of the carbamate were protected with MOM and Boc, respectively. The cyclic carbamate was cleaved and the 6-hydroxyl group was allylated and oxidized to form intermediate **95**. Various amines were introduced at the 6-position *via* reductive amination followed by introduction of a number of acyl groups at the 1-position to afford the NEA derivatives **96**. These NEA derivatives showed considerable antibacterial activity against *E. coli*, *S. aureus*, *Enterococcus faecium*, *P. aeruginosa*, and *Enterobacter cloacae*, and analyses of 4 of these analogues with APH(3')-Ia and AAC(6')-Ie/APH(2'')-Ia indicated that these compounds were poor substrates of AMEs. In addition to the mono-alkylated NEA molecules (Fig. 15A), various 6,3'-di- and 6,3',4'-trialkylated NEA analogues where the hydroxyl moieties of NEA are capped with phenyl, naphthyl, pyridyl, and quinoyl rings were prepared in 2 steps from NEA (Fig. 15B).^{83, 84} The 6,3',4'-tri-2-naphthylmethylene NEA derivative was found to be active against sensitive and fluoroquinolone-resistant *S. aureus* strains. Moreover, when compared to NEO, the 6,3',4'-tri-2-naphthylmethylene NEA derivative showed a lower ability to decrease [³H]leucine incorporation into proteins in *P. aeruginosa*, which indicated that the 6,3',4'-tri-2-naphthylmethylene NEA derivative acts by a mechanism that is not inhibition of protein synthesis. The 6,3',4'-tri-2-naphthylmethylene NEA derivative also showed low toxicity in eukaryotic cells, and the role of the 6'-amino functionality was studied by comparing compounds **98** with the exact PRM counterparts. This work revealed that lipophilicity is necessary for strong antibacterial activity.

In addition to the di- and trialkylated NEA analogues, numerous multialkylated NEB, PAR, and TOB analogues were synthesized. The amino groups of these alkylated AGs were either (i) left untouched, or converted to (ii) *N*-formyl, (iii) *N*-dimethyl, (iv) guanidine, or (v) imidazole (Fig. 16). TOB-derived cationic amphiphiles with free amines were prepared in 3 steps from TOB (Fig. 16A).⁸⁵ TOB was first transformed into penta-azido TOB **100**, which was then etherified at all 5 hydroxyl groups to yield **101**. Global deprotection of **101** yielded **102**. The pseudo-disaccharides NEB analogues **103** and **104**, also with untouched amines, were synthesized from **101** by acid-catalyzed selective cleavage. In general, for these NEB and TOB derivatives, potent antibacterial activity was accompanied with higher erythrocyte hemolytic activity, with the exception of the TOB analogue **102** with benzyl ethers, which displayed lower hemolytic activity. The trialkylated NEB analogue with C₇ side chains

displayed the broadest spectrum of antibacterial activity and was at least 32 times more effective than TOB and gramicidin D as an antibacterial agent. Since NEB-derived cationic amphiphiles exhibited antibacterial activity against both Gram-positive and Gram-negative pathogens, the 4 amines on these amphiphiles were converted to *N*-formyl, *N*-dimethyl, guanidine, and imidazole (Fig. 16B).⁸⁶ Among these modifications, the conversion of amines to formamides led to complete loss of antibacterial activity, whereas their conversion to guanidines and imidazoles resulted in loss of activity against Gram-negative, but not Gram-positive bacteria. *N*-Dimethylation of the amines of NEB amphiphiles resulted in membrane disruptors that demonstrated a general improvement in antibacterial activity and notably, broadened their antibacterial spectrum from exclusively Gram-positive strains to include several Gram-negative strains. Intrigued by the broad-spectrum activity caused by the *N*-dimethylation of NEB, similar modifications were performed on cationic amphiphiles derived from PAR and TOB.⁸⁶ Alkylated NEB analogues **107**, where amines were converted into imidazoles were explored for their potential antifungal activity in addition to their usual antibacterial activity (Fig. 16C).⁸⁷ These NEB derivatives with imidazole and with *n*-pentyl, *n*-hexyl, and *n*-heptyl aliphatic chains were found to exhibit unique selectivity towards the fungal strain *Candida glabrata*. They were also found to disrupt fungal membrane, but not erythrocytes. From these studies, one learns that imidazole-decorated cationic amphiphiles show promise for the development of less toxic membrane-disrupting antifungal drugs. This avenue remains to be further explored.

2.2.3. Preparation of alkylated AGs via amine linkages—The 1-position of AGs is known to be one of choice for derivatization of these drugs. To generate additional NEA derivatives, compound **108** (resulting from 2 reaction steps from **93** in Fig. 15A) was subjected to chlorination followed by dehalogenation to afford the 5-deoxy derivative **109** (Fig. 17).⁸⁸ The amine at the 1-position of **109** was subjected to reductive amination with various aldehydes to form the NEA derivatives **110**. Although several of these analogues were active as inhibitors of bacterial translation, none of them showed antibacterial potency superior to that of NEA. The use of this methodology where the amino group on AGs is reacted with different aldehyde *via* reductive amination remains poorly explored and leaves much to be done with modifications of the 1-position of 4,5-disubstituted 2-DOS AGs.

2.2.4. Preparation of alkylated and arylated AGs via triazole linkages—Even though not as popular as ether and thioether linkages for synthesizing alkylated/arylated AGs, triazole linkages also exist. 5"-alkylated/arylated NEO and 6"-alkylated TOB analogues linked *via* a triazole are reported in the literature. The NEO analogues **111** were synthesized from **1b** using general reactions **B** and **E** (Fig. 4A,B), whereas the TOB analogues **112** were synthesized from **6a** (Fig. 4A) using NaN₃ followed by click reaction (Fig. 18).^{61, 89} The triazole-linked alkylated NEO analogues were found to be moderately active against *S. aureus* and *E. coli*, whereas the triazole-linked arylated NEO analogues did not display antibacterial activity. A selected triazole-linked alkylated NEO analogue was found to be a poor substrate of APH(3')-IIIa, while still exhibiting activity against NEO-resistant bacterial strains. In the case of TOB derivatives, an analogue with a C₁₄ linear aliphatic chain exhibited the most potent antibacterial activity, which did correlate with its

hemolytic activity. Since this methodology was not extensively explored in the literature, it opens up a window of opportunity for further investigations.

2.2.5. Preparation of alkylated AGs via amide linkages—In addition to triazole linkages, KANA, NEO, and TOB derivatives were prepared with amide linkages (Fig. 19).⁹⁰⁻⁹⁴ The alkyl substituents were introduced at the 5"-position of NEO and at the 6"-position of KANA and TOB by using general reactions **B**, **C**, and **D** (Fig. 4B). In the case of TOB, the analogue with a C₁₆ linear aliphatic chain displayed good activity against Gram-positive bacteria, but reduced activity against Gram-negative ones. A similar trend was observed for NEO analogues with C₁₆ and C₂₀ chains. The MIC values of the NEO C₁₆ analogue against MRSA, methicillin-resistant *Staphylococcus epidermidis* (MRSE), and vancomycin-resistant *enterococci* (VRE) were comparable to that of clinically used antiseptics. Guanidinylation of the polycationic head groups in NEO enhanced the activity of the derivatives against GEN-, KANA-, and NEO-resistant *P. aeruginosa*, while reducing hemolytic activity. Dilipid analogues of NEO exhibited a 2- to 4-fold reduction in activity against most bacterial species when compared to monolipid NEO derivatives. With both NEO and TOB, the replacement of the C₁₆ lipid tail by a fluorinated lipid tail resulted in reduced hemolytic activity.

2.2.6. Preparation of alkylated and arylated AGs via carbamate linkages—Last but not least, carbamate linkages have been explored as an option for synthesizing alkylated/arylated cationic amphiphiles. Various AMK, KANA, NEA, and NEO with all of their hydroxyl groups substituted were synthesized in 2 steps (Boc protection followed by reaction with various isocyanates) (Fig. 20).⁹⁵ These analogues were tested against Gram-positive and Gram-negative bacterial strains. NEO analogues with phenyl and benzyl groups, and AMK with *n*-hexyl chains exhibited improved antibacterial activity against NEO-resistant strains compared to their parent AGs. Unsubstituted phenyl rings in the form of polyphenyl carbamates or polyphenyl ethers consistently exhibited higher antibacterial activity when compared to their *para*-substituted counterparts. As these appear highly promising, it would be interesting (i) to see how other AG scaffolds would fair with such modifications, and (ii) if a synthetic methodology could be developed to regioselectively incorporate different alkyl/aryl groups linked *via* carbamates.

2.3. Preparation of acylated AGs

In section 2.2, the impact of alkylation/arylation of AGs on their antibacterial and antifungal activity, on their mechanism(s) of action, and on their ability to avoid the action of AMEs was made clear. Another approach that has been implemented by scientists to overcome the action of AMEs, while increasing antibacterial activity is the *N*-acylation of AGs. Various reports on the mono-, di- and multi-*N*-acylation of AGs are found in the literature. *N*1-Acylation, especially derivatization with AHB, has proven to be one of the most successful strategies for overcoming resistance to AGs. More recently, 6'-*N*-acylation was revealed as another possible strategy to improve the properties of AGs. The synthesis of *N*-acylated AGs can be categorized based on the method used for their preparation: (i) single-step regioselective mono-*N*-acylation, (ii) multistep regioselective mono-*N*-acylation using activated esters or acids, and (iii) multi-*N*-acylation.

The single-step regioselective mono-*N*-acylation method was used to derivatize the 4,5-disubstituted 2-DOS AGs NEO and PAR. In order to achieve high regioselectivity (89% to 99%), the *N*-acylation of NEO in up to 83% yield was performed in the presence of various RNA aptamers in combination with NHS esters (Fig. 21).⁹⁶ It is important to note that in the absence of RNA aptamers, a mixture of di-, tri-, and tetra-*N*-acylated NEO products was generated. In the case of NEO, the *N*-acylation occurred at the 6'''-position. Interestingly, the use of isocyanate as a substrate, in place of the NHS esters, resulted in urea bond formation at the 2'''-position of both NEO and PAR.

For the preparation of mono- and di-*N*-acylated 4,6-disubstituted 2-DOS AGs as well as PAR derivatives, the NHS ester coupling method was also used (Fig. 22).⁹⁷ However, in contrary to what was found with NEO, in these cases, RNA aptamers were not necessary to achieve high regioselectivity. By using base-catalyzed reactions with corresponding NHS esters, 6'''-*N*-glyciny-*PAR*, 6'''-*N*-AHB-*PAR*, 6'-*N*-glyciny-*TOB*, and 6'-*N*-AHB-*TOB* were synthesized from their parent AGs. When tested against AAC(6')- or AAC(3)-expressing bacteria, as well as other pathogenic bacterial strains, some of the analogues demonstrated improved antibacterial activity when compared to their parent AG counterparts. Improvement in biological activity of the *N*-acylated analogues was found to be highly dependent on the specific AG and acyl group combination used. To follow up, various glyciny-, carboxybenzyl-, and AHB mono- and diderivatization at the 1-, 6'-, and/or 4'''-amines of the AGs AMK, KANA, NET, SIS, and TOB were carried out.⁹⁸ Both 6'-*N*-glyciny-*NET* and *SIS* analogues exhibited the best overall MIC values, and they also were minimally modified by AMEs. Therefore, *NET* and *SIS* appear to be promising scaffold for further *N*-acylation studies. NHS esters were also used to synthesize a variety of *N*-hydroxyurea derivatives of KANA **130** (Fig. 23A).⁹⁹ These *N*-hydroxyurea analogues maintained antibacterial activity against *S. aureus*, *P. aeruginosa*, and *E. coli*. The corresponding *N*-hydroxyurea analogues of *TOB* were also synthesized and were found to be equal in activity to *TOB* with a slight superiority in activity over that of *AMK*. An attempt to correlate the ribosomal binding affinities of these KANA and *TOB* analogues with their antibacterial activity did not result in a linear relationship. A highly relevant novel *N*-acylated AG is *PLZ*, a neoglycoside that was synthesized from *SIS* in 8 steps, one of which involves a key NHS coupling reaction with the *N1* amino group (Fig. 23B).¹⁰⁰ *PLZ* was tested against a panel of Gram-negative and Gram-positive pathogens, many of which harbored AG resistance mechanisms. *PLZ* inhibited the growth of AG-resistant *Enterobacteriaceae* (MIC₉₀ 4 µg/mL), with the exception of *Proteus mirabilis* and indole-positive *Proteae* (MIC₉₀ 8 µg/mL and 16 µg/mL, respectively). *PLZ* was more active alone *in vitro* against *P. aeruginosa* and *Acinetobacter baumannii* isolates containing AMEs than against those with altered permeability/efflux. This compound has advanced into clinical development as a new AG antibacterial agent, demonstrating that AGs still hold a lot of promise and their derivatization should be continued.

Another approach explored for the synthesis of *N*-acylated AGs was the selective coupling of amino groups of AGs with a variety of carboxylic acids in the presence of coupling reagents. 4''- and 6''-*N*-acyl derivatives of *ABK* were synthesized by regioselective coupling of this AG with a variety of aliphatic and aromatic carboxylic acids (Fig. 24).¹⁰¹ Penta-*N*-

Boc-6"-*O*-triisopropylbenzenesulfonyl ABK was obtained from penta-*N*-Boc ABK via selective sulfonylation of the 6"-hydroxyl group, which was then subjected to reactions **B** and **C** (Fig. 4B) to yield the 6"-amine in high yield. The *N*-acylation of **135** with carboxylic acids was carried out in the presence of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) to generate the 6"-*N*-acylated derivatives **136** (Fig. 24A). In order to generate a 4"-*N*-acyl-4"-epi derivative **139** of ABK, a slightly modified reaction sequence was employed (Fig. 24B). The reaction of **137** with methanesulfonyl chloride (MsCl) yielded the 4"-*O*-mesyl derivative, which was then subjected to substitution reaction with NaN₃ followed by reduction to generate the 4"-amine derivative **138**. The coupling of the 4"-amino group with an AHB moiety resulted in the bis-AHB derivative **139**. The 6"-*N*-acylated derivatives, which introduced an AHB residue showed potent antibacterial activity against *S. aureus*, including ABK-resistant MRSA, whereas the 4"-*N*-acyl-4"-epi derivative **139** was less active than ABK against Gram-negative bacteria such as *E. coli* and *P. aeruginosa*.

Finally, multi-*N*-acylation was carried out to generate various *N*-aryl/acylated *O*-sulfonated analogues of APR, KANA, and NEO.¹⁰² AG analogues synthesized to mimic highly sulfonated glycosaminoglycan were evaluated for their ability to selectively inhibit similar bacterial and human topoisomerases. Synthetically, these analogues were generated in 2 steps. AGs were first converted to their *N*-aryl/acylated derivatives prior to sulfonation with a pyridine sulfur trioxide complex (Fig. 25). It was demonstrated that both the structures of the AGs and of the *N*-aryl/acyl moieties were responsible to impart selective inhibition of different topoisomerases with varied mechanisms of action. All studies in this section reveal that *N*-acylation can provide an efficient method for developing new AG analogues for treating clinically relevant bacterial strains.

2.4. Synthesis of AG heteroconjugates

The introduction of a variety of alkyl as well as acyl groups onto AGs and their impact on antimicrobial activity were explored in details in sections 2.2 and 2.3. The idea of conjugating small molecules and FDA-approved drugs instead of functional groups to AGs is now explored in this section covering AG heteroconjugates. The sheer number (>70) of research articles related to this field of research demonstrates the popularity of this topic. In this section, we will explore the fact that covalently linking AGs to other molecules can result in conjugates (i) with improved antibacterial spectrum, (ii) with improved inhibition of bacterial translational properties, (iii) with decreased affinity for AMEs, (iv) that can serve as tools for better understanding binding of AGs to DNA/RNA, and (v) that can be used as probes for understanding their mechanism(s) of action. In order to simplify the amount of information available we divide this section into (i) AG-bisubstrate inhibitors, (ii) AG-drug conjugates, (iii) AG-small molecule conjugates, and (iv) AG-amino acid and AG-peptide conjugates. Each subsection will emphasize the chemistry and biology behind the AG heteroconjugates synthesized.

2.4.1. Synthesis of AG-bisubstrate inhibitors—The most common mechanism of resistance to AGs is enzymatic modification by AMEs. Therefore, strategies must be developed to nullify that threat. A strategy explored to overcome resistance to AMEs

consists of using inhibitors against these resistance-causing enzymes. Bisubstrate inhibitors mimicking the tetrahedral intermediate resulting from the attack of an AG onto the thioester carbonyl of acetyl coenzyme A (AcCoA) in the active site of the resistance enzyme during acetylation could be a good way to overcome the action of AACs. Naturally occurring AGs are complex molecules, and their regioselective modifications remain challenging. An efficient two-step procedure using *N*-(2-bromoacyl)oxy-5-norborene-*endo*-2,3-dicarboximides **142** and CoA was developed for the regio- and chemoselective derivatization of unprotected AGs (*e.g.*, KANA, NEA, and RIB) at the 6'-amino group to develop bisubstrate inhibitors **143** of the common AAC(6') enzymes (Fig. 26A).¹⁰³ This synthetic methodology is advantageous, as it does not require orthogonal protection/deprotection manipulations. The AG (KANA, NEA, and RIB)-bisubstrate inhibitors with *n* = 2 demonstrated a 200-fold increase in AAC(6') binding when compared to AcCoA and a gradual decrease in binding was observed with longer linkers. Eleven additional NEA-truncated CoA bisubstrate inhibitors **147** were also prepared (Fig. 26B).¹⁰⁴ A protected phosphate group was introduced onto the primary alcohol of D-pantethine to generate **145**, which was then conjugated with the 6'-activated AG **146** to yield **147** after global deprotection. Binding studies with AAC(6')-Ii indicated that inhibition of the enzyme by NEA-CoA bisubstrates heavily relies on the integrity of the AG part of the molecule. Truncation of the CoA moiety of these bisubstrates revealed that the pantetheinyl group is not sufficient for recognition by the enzyme and at least one phosphate group is needed. The potency of these bisubstrate inhibitors improved when the pyrophosphate moiety of CoA was replaced by β -carbonyl group mimics, such as malonate and acetoacetate.

As a next generation, various sulfonamide, sulfoxide, and sulfone (Fig. 27A),¹⁰⁵ as well as phosphonate-linked (Fig. 27B)¹⁰⁶ AG-CoA conjugates were synthesized. Five new AG-CoA bisubstrates containing sulfonamide, sulfoxide, and sulfone linkers were synthesized starting with NEA (Fig. 27A).¹⁰⁵ The sulfonamide-containing bisubstrate **151** was prepared by reacting 2-bromoethanesulfonyl chloride with Boc-protected NEA **149** to yield the vinyl sulfonamide **150**, which was then reacted with CoA. The sulfoxide- and sulfone-containing bisubstrates **153** and **154** were generated by partial as well as complete oxidation of the amide-linked NEA analogue **152**. Unexpectedly, although the sulfonamide of **151** was expected to better mimic the tetrahedral intermediate involved during the reaction with AACs than the amide of **152**, bisubstrate **151** showed poorer inhibition of AAC(6')-Ii than **152**. Among the 6'-amide-linked bisubstrate inhibitors, the inhibition efficiency was found to be **152** > **154** > **153**. When attempting to generate phosphonate-linked NEA-bisubstrate analogues, reactions failed. However, when Boc-protected PRM was reacted with methyl vinylphosphonyl chloride and then subjected to Michael reaction with CoA, the phosphonate-linked PRM-CoA conjugate **157** was obtained (Fig. 27B).¹⁰⁶ Bisubstrate **157** was a much weaker inhibitor than the corresponding 6'-amide-linked bisubstrates **152**.

To evaluate the effect of *O*-to-*N* substitution (highlighted with blue boxes) in the pantetheine arm on the efficacy of NEA-CoA bisubstrate inhibitors, derivatives with 4'-aminopantetheine were prepared (Fig. 28A).¹⁰⁷ Compound **158** was subjected to reductive amination followed by reaction with diketene to yield **159**. The disulfide dimer was reduced *in situ* by using DTT, and the free thiol generated was subjected to nucleophilic substitution

reaction with **160** to yield **161**. Analogues **162** (where $n = 1-3$) were prepared in the similar manner where amines with different linkers were introduced during the reductive amination step. The substitution of the oxygen atom by a nitrogen in the pantetheine arm proved to be detrimental to the AAC(6') inhibitory activity of these compounds. Interestingly, it was observed that similar analogues (**163** with acetoacetate or ADP) with the oxygen atom in the pantetheine arm (Fig. 28B), although active against purified AAC(6') enzymes, were not active against bacteria containing these enzymes. NEA-pantetheine analogues **163** with a terminal hydroxyl moiety devoid of inhibitory activity against AAC(6') were cleverly utilized as prodrugs capable of penetrating bacterial cell wall and utilizing the CoA biosynthetic pathway of the cells to generate active *in cellulo* AAC(6') inhibitors.¹⁰⁸ This ingenious prodrug activation strategy could be more widely used in the future for developing various biologically active AG-CoA bisubstrate inhibitors.

2.4.2. Synthesis of AG-drug conjugates—In this subsection, clinically relevant antibiotics with modes of action differing from that of AGs were covalently linked to AGs at positions known to not be important for binding of the AG to its 16S rRNA target. These conjugates were designed with the purpose of expanding (i) their spectrum of activity, (ii) their capacity to inhibit different parts of the ribosome or different bacterial targets, and (iii) their ability to decrease the efficacy of AMEs. Examples of drugs that have been linked to AGs include the 23S rRNA-targeting chloramphenicol (CAM) and linezolid (LNZ) (Fig. 29), the 50S rRNA-targeting clindamycin (CLN) (Fig. 30A), as well as the DNA gyrase-targeting ciprofloxacin (CIP) (Fig. 30B).¹⁰⁹⁻¹¹²

In an attempt to enhance the specificity towards RNA targets, CAM and LNZ were covalently linked to NEO by using spacers of different lengths (3, 6, and 9 carbons). These conjugates were synthesized in a total of 6 steps starting from NEO. The OTIBS group at the 5''-position of **1a** (Fig. 4A) was first displaced by alkyl-dithiols of different lengths to afford **164**, which was then covalently linked to CAM or LNZ *via* nucleophilic substitution reactions to afford conjugates **165-167** and **168**, respectively (Fig. 29).^{113, 114} Conjugates **165-168** displayed enhanced site-selective binding to several RNA targets, such as Rev response element (RRE), *trans*-activation response element (TAR), thymidylate synthase (TS), iron responsive element (IRE), as well as 16S and 18S A-sites. Compounds **166** and **168** (where $n = 6$) exhibited nanomolar binding to RRE and TAR (10-fold better than that of NEO). Similarly, compound **166** displayed nanomolar binding to the 16S rRNA, which is 20-fold higher than that of NEO. Conjugate **168** exhibited enhanced binding to the 18S rRNA when compared to that of NEO. However, the results of antibacterial and *in vitro* translation inhibition assays for these conjugates did not correlate well with their improved K_d values. Among the designed molecules, conjugate **168** was later used as a tool to select LNZ-specific hairpin RNA from *E. coli* genomic RNA.¹¹⁵ RNA footprinting analysis showed that **168** bound to both RNA stem (AG part of the conjugate) and loop (LNZ portion of the conjugate) regions. These findings suggest that conjugation of small molecules to strong RNA binders can serve as a new strategy to elucidate small molecule specific RNA loop sequences that are otherwise difficult to identify.

CAM was also linked to the AG TOB, and so was CLN. The TOB-CAM and TOB-CLN conjugates, along with CAM-CAM, CAM-CLN, and CLN-CLN homodimers (9 compounds in total), were synthesized with the intent of generating improved ribosome-targeting antibiotics.¹¹⁶ These homo- and heteroconjugates were synthesized by using a disulfide bond formation reaction (Fig. 30A). A bis(2-mercaptoethyl) ether unit was attached to each of the chosen ribosome-targeting antibiotics. Once attached to TOB, treatment with 2,2'-dipyridyl disulfide yielded **172**. The reaction between **172** and **173** followed by global deprotection resulted in conjugate **171**. Of the 9 compounds generated, TOB and CLN homodimers, and TOB-CLN conjugate **171** displayed broader antibacterial activity than either of the parent drugs alone. The *in vitro* prokaryotic translation inhibition potency exhibited by conjugate **171** was between that of TOB and CLN. Compound **171** was 2.5-5 times less prone to deactivation by AAC(6')-Ie/APH(2'')-Ia and AAC(6')-Ib than TOB itself.

In addition to ribosome-targeting antibiotics, the fluoroquinolone CIP, which targets the GyrA subunit of DNA gyrase, was attached to the AG NEO *via* a series of triazole-containing linkers (Fig. 30B).¹¹⁷ The antibacterial activity of these conjugates was determined against both Gram-positive and Gram-negative bacterial strains (*e.g.*, *E. coli*, *Bacillus subtilis*, and MRSA). Various predesigned linkers with terminal azido group were introduced at one end of CIP **175**, and were subjected to click reaction with an alkyne functionality present at the 5''-position of NEO **174**. The majority of these conjugates were significantly more potent than NEO itself, and they were found to be more potent inhibitors than CIP in supercoiling assays with DNA gyrase, relaxation assays with TopoIV, and in *in vitro* transcription/translation assays with *E. coli* S30 extract system. The nature of the spacers separating NEO from CIP greatly influenced the antibacterial activity, and a significant delay in resistance formation was observed in both *E. coli* and *B. subtilis* for the NEO-CIP conjugates over each drug individually or in a 1:1 mixture.

Other examples of AG-drug conjugates include the coupling of AGs, such as NEA and ISP, at their 6'-position, with the β -lactam antibiotic cephalosporin.¹¹⁸ Preliminary biological evaluation of these conjugates indicated that they are good substrates for β -lactamases, indicating that linking β -lactams to AGs might not be an optimal way to generate AG-drug conjugates. In another study, CAM was linked to NEO at the 6''-position, which was then conjugated to phage coat proteins at the 6'-position *via* 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) chemistry.¹¹⁹ The phages complexed with polyclonal antibodies that served as the targeting moieties resulted in complete growth inhibition towards *S. aureus*, *S. pyogenes*, and *E. coli*, and an improvement in potency by a factor of 20,000 was observed when compared to the free CAM.

2.4.3. Synthesis of AG-small molecule conjugates—In addition to being conjugated to FDA-approved drugs, AGs have also been linked to other biologically relevant small molecules (*e.g.*, intercalators, nucleobases, DNA/oligonucleotides, fluorescent molecules, and polyethylene glycol (PEG)) by using variety of chemical reactions. These AG-small molecule conjugates were designed to (i) better aim at therapeutically important biological targets (DNA and RNA forms), (ii) enhance antibacterial activity, (iii) improve biofilm inhibition, (iv) enhance cellular uptake, (v) demonstrate differential cytotoxicity, and (vi)

target oncogenic microRNAs (miRNAs). This subsection is further divided based on the type of reaction used to attach small molecules to AGs: (a) thiocarbamate formation, (b) triazole formation, (c) amide formation, and (d) nucleophilic substitution reaction.

2.4.3a. Synthesis of AG-small molecule conjugates via thiocarbamate linkages: As a part of developing new compounds towards recognition of nucleic acids, AGs were conjugated to a variety of small molecule intercalators and oligonucleotides *via* thiocarbamate linkages (Figs. 31-33).¹²⁰ A significant amount of work has been invested in the study of DNA triplexes, because of their potential as biological tools and therapeutic applications. AGs are known for their groove recognition of nucleic acid triple helices, and among them, NEO is the most effective AG for stabilizing DNA triple helices. Examples of NEO conjugated to benzo(*f*)quino(3,4-*b*)quinoxaline (BQQ) (**179**),¹²¹ bisbenzimidazole (**180**),¹²²⁻¹²⁴ bisbenzimidazole and pyrene (**181**),¹²⁵ pyrene alone (**182** and **183**),¹²⁶ anthraquinone (**184**),^{127, 128} and naphthalene diimide (**185**),¹²⁶ *via* thiocarbamate linkages have been reported (Fig. 31). The following assumption was made during the design of these conjugates: the amino groups on rings I, II, and IV of NEO are necessary in stabilizing and recognizing the triplex groove. This assumption was made based on the fact that AGs without any of these amines did not stabilize triplexes as efficiently.¹²⁹ Therefore, the 5''-hydroxyl on ring III was selected as the linkage point for the intercalating units. The OTIBS group of **1a** (Fig. 4A) was displaced by cysteamine, and the product was further reacted with 1,1-thiocarbonyl di-2(1*H*)-pyridone in order to convert the amino group into an electrophilic (isothiocyanate) intermediate **177**. Intermediate **177** was broadly used for the conjugation of NEO to intercalator amines to yield **178** after global deprotection. Among the available pentacyclic intercalators, BQQ-based ones are some of the best for triplex binding selectivity and potency.^{130, 131} For this reason, NEO was conjugated to BQQ (**179**) and the NEO-BQQ conjugate was found to be more potent in stabilizing DNA triple helices than NEO or BQQ alone, or a 1:1 combination.¹²¹ Computational modeling of NEO-BQQ **179** docked with DNA triplex showed that BQQ stacked between the triplex bases, while NEO stayed bound in the larger triplex W-H groove. NEO-bisbenzimidazole conjugates linked *via* a thiocarbamate were also synthesized by conjugating **177** with DNA minor groove binding ligands such as **180b** and **180c**.^{122, 123} These NEO-bisbenzimidazole conjugates showed remarkable stabilization of DNA duplexes compared to the individual parent compounds. Conjugate **180b** exhibited enhanced thermal stability for B-DNA when compared to conjugate **180c**. These data suggest that the linker between the AG and the bisbenzimidazole groups plays an important role in delivering both binding moieties to the grooves. Additional NEO-bisbenzimidazole conjugates (**186**) were designed to evaluate the effect of the length and composition of linkers on the binding of G-quadruplex derived from the *C-myc* promoter sequence (Fig. 32).¹²⁴ Even though conjugates **186** and **180a,d,e** exhibited stronger binding to *C-myc* quadruplex than their individual components, a case for duplex to quadruplex selectivity was never established. This DNA recognition work was then expanded to attach intercalators **181-185** to NEO derivative **178** *via* thiocarbamate linkages (Fig. 31). Overall, the work on these conjugates provided a measure of quantification of triplex binding in the order: BQQ > anthraquinone > naphthalene diimide > pyrene.¹²⁶

Other examples of AG conjugates with thiocarbamate linkages include KANA/NEO-oligonucleotide conjugates, which were designed with the goal of targeting specific RNA sequences. KANA/NEO-DNA 2mer conjugates were efficiently synthesized by first displacing the OTIBS group of **4a/1a** (Fig. 4A) by cysteamine prior to reaction with 1,1-thiocarbonyl di-2(1*H*)-pyridone to afford the electrophile (isothiocyanate) intermediate **187** (Fig. 33A).¹³² Intermediate **187** was conjugated to **188** to yield **189** after global deprotection. Similarly, NEO was covalently linked to a 7mer oligonucleotide to generate conjugate **190**, which was utilized for sequence-specific targeting of RNA (Fig. 33B).¹³³

2.4.3b. Synthesis of AG-small molecule conjugates via triazole linkages: Another method widely used to conjugate small molecules to AGs is click chemistry, which inserts a triazole at the linkage point. NEO-nucleobase conjugates with triazole linkages (**192**) were prepared by converting NEO to **191** using reaction **B** (Fig. 4B) prior to attachment by copper-mediated click reaction of natural (*e.g.*, adenine, uracil, cytosine, and guanine) and artificial nucleobases modified by introduction of an alkyne in their structures (Fig. 34A).¹³⁴ These conjugates were found to inhibit the production of the oncogenic miRNA-372 and -373 by binding to their respective pre-miRNAs. In addition, some of these compounds displayed anti-proliferative activity towards gastric cancer cells. Guanidinylated NEO-NHS ester conjugates with triazole linkages were also prepared for use in the study of heparan sulfate-dependent transport of active enzymes to lysosomes (Fig. 34B). NEO analogue **193** was coupled with alkyne NHS ester **196** to yield **194**. Compound **194** was guanidinylated prior to click reaction with azide NHS ester **197** to form the NHS activated guanidinylated NEO analogue **195**, which was then conjugated to two lysosomal enzymes, β -D-glucuronidase and α -L-iduronidase. Conjugation did not interfere with enzyme activity and enabled the binding of the enzymes to heparan sulfate.¹³⁵

Additional guanidinylated NEO conjugates with triazole linkages include those that are biotinylated at their 5''-position as well as acylated at their 3-position (Fig. 35).¹³⁶ These conjugates were prepared to study the internalization mechanism(s) of amphiphilic guanidinoglycosides by cell-surface FRET analysis. Their syntheses began with Boc protection of all amino groups of NEO prior to azidation of the 5''-position to afford **198**. After deprotection, all amino groups, with the exception of that at the 3-position, were converted to Boc protected guanidino groups (**199**). The biotin derivative **201** was then clicked to the azide group at the 5''-position of **199** prior to amide coupling of the amino group at the 3-position with acyl halides comprised of alkyl chains of various lengths to give **200**. The alkyl chains introduced were found to directly interact with cell membrane, which in turn improved the cellular uptake of NEO-biotin conjugates **200** when compared to the NEO-biotin conjugates lacking alkyl groups.

Another very interesting example of usage of click chemistry with AGs is that where a somewhat bigger molecule (PAMAM-G4 dendrimer) was attached to NEA, NEO, and PAR.¹³⁷ As an example of their syntheses, PAR sulfate was first neutralized with amberlite IRA-400-OH⁻ resin and then treated with NHS ester **205** prior to Boc protection to functionalize the 6'''-position of the AG (Fig. 36). Click reaction of **202** with propargyl derivative **206** led to the formation of **203**, which possessed an isothiocyanate group, which

was used to anchor PAR to PAMAM-G4 dendrimer. The resulting multifunctional gene vector possessed significantly reduced cytotoxicity as well as enhanced transfection efficiency when compared to the PAMAM-G4 dendrimer itself.

Two RNA binding molecules, NEO and bisbenzimidazole, were conjugated *via* click reaction to yield conjugates **207-209** (Fig. 37).¹³⁸ NEO-bisbenzimidazole conjugates displayed enhanced potency for downregulating miR-27a levels when compared to the individual parent molecules. Out of all analogues, NEO-bisbenzimidazole with one triazole moiety involved in the linker exhibited nanomolar binding for miRNA, which emphasized the effect of the linker length and composition on RNA binding. The NEO-monobenzimidazole analogue **208** showed reduced activity, which suggested the importance of the bisbenzimidazole in miRNA binding. Examples of NEO and PRM conjugated to oligonucleotides *via* triazole linkages are also found in the literature.^{139, 140} These conjugates were synthesized by solution-phase and solid-supported methods to study their affinity for DNA duplexes.

2.4.3c. Synthesis of AG-small molecule conjugates *via* amide linkages: The most prominent reaction used for conjugating small molecules to AGs is by far amide coupling. Amide linkages have been used to generate AG-small molecules for many different purposes, including improving (i) ribosomal binding (Fig. 38A), (ii) cytoplasmic delivery of bioactive cargos (Fig. 38B), (iii) biofilm disruption activity (Fig. 38C), (iv) nucleic acid binding (Figs. 39-42), (v) transfection efficiency (Fig. 43A), and (vi) anticancer activity (Fig. 43B).

To improve the ribosomal binding affinity of TOB, a TOB-EDTA complex was synthesized *via* amide coupling. TOB analogue **6a** (Fig. 4A) was treated with cysteamine to afford intermediate **210**, which was then coupled with EDTA using EDCI and HOBt (Fig. 38A).¹⁴¹ When a complex of analogue **211** with Fe^{II} was formed, it was found that ribozyme inhibition was greatly improved.

In the case of drug delivery applications, facilitating the uptake of bioactive molecules into living cells is of substantial interest. For biological macromolecules, effective delivery depends on minimal exposure to conditions that may disrupt their activity. Thus, using AG derivatives for the transport of therapeutically important molecules (*i.e.*, bioactive cargos) inside bacterial cells is really appealing. To exploit the use of AGs for cellular uptake, a NEO analogue **212** was conjugated to biotin by using HATU and HOBt to afford conjugate **213** (Fig. 38B).¹⁴² A guanidinylated counterpart of conjugate **213** was successfully used for cytoplasmic delivery of bioactive cargos, such as streptavidinylated saporin and phycoerythrin.

Bacteria such as *E. coli*, *S. aureus*, *Streptococcus pneumoniae*, *P. aeruginosa*, and *Haemophilus influenzae* are known to cause biofilms. Biofilms are complex functional communities of one or more species of microorganisms that are encased in extracellular polymeric substances and attached to both a solid surface and to each other. The resulting biofilms are complex matrices, which makes it difficult for antibacterial agents to reach the pathogens embedded in these networks.^{143, 144} To improve biofilm disruption activity, TOB

was conjugated to PEG in a single-step reaction (Fig. 38C). The TOB-PEG conjugate **214** was then used for treating *P. aeruginosa* biofilms.¹⁴⁵ The MIC of the TOB-PEG conjugate **214** (13.9 µg/mL) against *P. aeruginosa* in liquid cultures was higher than that of TOB (1.4 µg/mL). However, conjugate **214** was found to be ~3.2-fold more effective in eliminating *P. aeruginosa* biofilms than TOB itself.

Recognition of nucleic acids is important for our understanding of their structures as well as their interactions with proteins. To improve binding of AGs to nucleic acids, a NEO-fluorescein conjugate **215** was prepared by coupling **212** to an activated fluorescein-NHS ester **216** (Fig. 39A).¹⁴⁶ Conjugate **215** was used for binding studies with RNA duplex, A and B form DNA duplexes, DNA-RNA hybrids, triple helical nucleic acids, and four-stranded nucleic acids. To further explore the details of molecular recognition by AGs, a novel NEO-methidium conjugate **217** was synthesized by linking intercalator **218** to **212** by using DCC-mediated amide bond formation (Fig. 39B).¹⁴⁷ Conjugate **217** was found capable of selective recognition of a DNA-RNA hybrid duplex. Amide coupling was also exploited for conjugating metal complexes to AGs. Novel amino- and guanidinoglycosides conjugated to Pt^{II} were developed to study their exclusive RNA selectivity (Fig. 39C).¹⁴⁸ NEO analogue **219** was coupled to **221** by using TBTU and HOBt. The Fmoc groups were cleaved, and the resulting intermediate was treated with potassium tetrachloroplatinate to afford conjugate **220** after global deprotection. The corresponding guanidinylated NEO-Pt^{II} conjugate was also prepared, and both of these novel NEO-Pt^{II} conjugates were found to generate site-specific, RNA-selective, covalent cross-links to the RRE. In another study, PAR-thiazole orange and PAR-pyrene conjugates were synthesized to evaluate their binding affinity for the 16S rRNA.¹⁴⁹ The thiazole orange conjugate **224** was synthesized in 2 steps and was coupled to PAR at its 6'''-position (Fig. 40A). These compounds were observed to bind the truncated A-site with higher affinity than PAR, and the shorter spacer groups resulted in greater affinity for the A-site. β-carboline, a ring system observed in many naturally occurring alkaloids, was conjugated to NEA to enhance its affinity to and specificity for RNA. The hydroxyl group at the 5-position of **226** was replaced with a variety of diamine linkers prior to coupling with β-carboline-3-carboxylic acid to generate conjugates **228** (Fig. 40B). Some of these conjugates were found to have better affinity for the *E. coli* 16S rRNA and human 18S rRNA, as well as better antibacterial activity against *P. aeruginosa* than NEA.¹⁵⁰ Nucleotides (*e.g.*, T and U) have also been conjugated to NEA (Fig. 41).^{151, 152} The hydroxyl at the 5-position of NEA analogue **229** was converted to its corresponding triflate prior to substitution reaction with various diamine linkers. The resulting analogues were then coupled with the mono- and dinucleotide acids **231** and **232**. The nucleobase played a significant role in binding of conjugates **233** to the 16S rRNA and shorter linkers between NEA and the nucleotides favored their 16S rRNA binding. However, the NEA-dinucleotide conjugates **234** exhibited weak binding affinity for the 16S, 18S, and TAR RNA. Additional NEA analogues bearing A, C, G, and T with a lysine or an arginine as a linker were prepared as inhibitors of HIV TAR-Tat (*trans*-activator of transcription).^{153, 154} NEO- and RIB-oligonucleotide conjugates were also synthesized *via* solid-support for targeting HIV-1 TAR RNA.¹⁵⁵ Various aromatic rings were also conjugated to NEA and the binding of the resulting conjugates to the RRE RNA was examined (Fig. 42A). Among these, NEA-pyrene conjugates displayed strong binding affinity (submicromolar) for the

RRE RNA.¹⁵⁶ Additional 5-, 4'-, and 5,4'-NEA-small molecule conjugates were synthesized in 7 steps *via* amide coupling reactions (Fig. 42B).¹⁵⁷ Most of the conjugates with modifications at the 4'-position displayed better affinity and selectivity for TAR RNA when compared to derivatives modified at the 5-position. In this series, the most promising conjugate was found to be the 4'-NEA-histidine derivative. Intramolecular amide coupling was similarly used to tether NEO to three different aromatic platforms (acridine (**240**), quinacridine (**241**), and phenanthroline (**242**)) (Fig. 42C). The NEO-capped aromatic platforms **239** adopted highly flexible structures without conformational restriction and were found to exhibit high affinity for DNA quadruplexes.¹⁵⁸

To develop an efficient alternative to recombinant viruses for gene transfer, efforts were made towards synthetic gene delivery systems. Among these nonviral systems, cationic lipids have emerged as an attractive option for gene delivery. NEO and PAR were derivatized with lipids to generate novel cationic lipids, which were then evaluated for their transfection efficiency.¹⁵⁹ The amino intermediate **212** was coupled to either *N*-succinyl-dioleamine or cholesteryl chloroformate to generate **243** and **244**, respectively (Fig. 43A). The alkyl and cholesterol conjugates, when formulated with dioleoylphosphatidylethanolamine (DOPE), mediated high transgene expression both *in vitro* in different cell lines and *in vivo* in mouse airways. These results demonstrate that AGs (*Note*: KANA-lipid conjugates were also prepared¹⁶⁰) can be used as gene delivery vectors.

To overcome the toxicity and drug resistance of cisplatin and Pt^{II}-related analogues, efforts were aimed at developing organometallic complexes for improved anticancer activity. As described above, AG-Pt^{II} complexes have been used to increase the RNA binding affinity of AGs. To improve anticancer activity, AG-Ru^{II} complexes have been produced. More specifically, a Ru^{II} arene complex was conjugated to NEO and its guanidynylated counterpart for such application (Fig. 43B).¹⁶¹ The guanidynylated NEO-Ru^{II} conjugate was found to be more toxic to cancer cell lines than the NEO-Ru^{II} conjugate, despite its higher accumulation in normal cells when compared to cancer cells.

2.4.3d. Synthesis of AG-small molecule conjugates by nucleophilic substitution

reactions: Last but not the least, examples of AG-small molecule conjugates synthesized *via* nucleophilic substitution reactions are reported in the literature. These AG conjugates generated *via* nucleophilic substitution were used for different applications including, improving (i) cellular uptake (Fig. 44A), (ii) inhibition of AMEs (Fig. 44B), and (iii) nucleic acid binding (Figs. 45,46). Heavily-charged large biomolecules usually exhibit poor bioavailability due to decreased cellular uptake. To begin with, BODIPY was conjugated to both NEO and TOB as well as to their corresponding guanidinoglycosides to analyze the efficacy in cellular uptake by eukaryotic cell cultures of the resulting conjugates (Fig. 44A).¹⁶² The introduction of a dithiol linker to intermediate **1a** (Fig. 4A) yielded analogue **247**, which was then subjected to substitution reaction with BODIPY halide to yield **248**. Both NEO- and TOB-BODIPY conjugates displayed poor cellular uptake. However, upon guanidinylation, the cellular uptake of the NEO- and TOB-BODIPY conjugates were enhanced by 20- and 10-fold, respectively. In a separate study, to overcome the most common mechanism of resistance to AG antibiotics, AMEs, NEA-adenine bisubstrate inhibitors **249** were synthesized by tethering the 3'-hydroxyl of NEA to the 5'-hydroxyl of

adenosine by using different linkers (Fig. 44B).¹⁶³ These NEA-adenine bisubstrate inhibitors were found to efficiently inhibit APH(3').

To improve the binding of AGs to nucleic acids, a series of AG-acridine conjugates with various linkers were synthesized and evaluated for their RRE specificity.¹⁶⁴⁻¹⁶⁶ Beginning with **1a** (Fig. 4A), linkers were introduced onto NEO by substitution reactions with either NaN₃, dithiols, or aminothiols (Fig. 45). The resulting AG intermediates were then conjugated to acridine *via* substitution nucleophilic aromatic reaction to yield **250**, **251**, and **252** after global deprotection. When compared to NEO, NEO-acridine conjugates were proven to be potent inhibitors of RRE. Conjugate **250**, with the shortest linker, displayed optimal RRE specificity when compared to NEO. Similar KANA- and TOB-acridine conjugates were found to have slightly lower RRE affinity, but better RRE specificity.

These AG-acridine conjugates were followed up by PAR- and NEO-nucleobase conjugates designed to confer selectivity to the A-site by interacting with the unpaired nucleotides in its binding pocket (Fig. 46).¹⁶⁷ The tosyl group at the 5"-position of PAR analogue **255** was replaced with various nucleobases (*e.g.*, adenine, cytosine, and uracil) to yield the AG-nucleobase conjugates **256** (Fig. 46A). NEO-nucleobase conjugates were prepared similarly. Interestingly, in contrary to the expected increased affinity for the A -site, these conjugates were found to bind the A-site more weakly than their parent AG counterparts. However, these conjugates bound TAR RNA more tightly than the parent AGs. Another AG derivative was synthesized by replacing ring I of NEO with a thymine moiety in order to form Watson-crick hydrogen bonds with adenine 280 of viral RNA (Fig. 46B).¹⁶⁸ Derivative **257** was synthesized from NEO in 6 steps. This compound was subjected to oxidative cleavage to form **258**, which was treated with DMSO and acetic anhydride to produce a methyl thiomethyl ether that was converted to chlorinated analogue **259**. Finally, the chloro group of **259** was replaced with thymine to afford compound **260**. Conjugate **260** was found to bind the HIV-1 DIS viral RNA with high specificity when compared to NEO.

2.4.4. Synthesis of AG-amino acid and AG-peptide conjugates—Another approach explored to counter the emergence of drug-resistant bacteria involved conjugating AGs to amino acids or peptides.¹⁶⁹ These conjugates were designed with the goals of improving their (i) RNA binding, and (ii) antibacterial activity.

To explore the importance of AG-peptide conjugates on RNA binding, a library comprised of 215 NEO-peptide conjugates was synthesized *via* a rapid solid-phase method (Fig. 47A).¹⁷⁰ The 5"-amino group of the protected NEO **261** was reacted with glutaric acid to generate intermediate **262**, which was then coupled with peptides **266** generated by solid-phase synthesis. Some of these NEO-peptides bound strongly and selectively to the *E. coli* A-site model with up to 28 times greater affinity than to the human model A-site rRNA. Another set of NEO-peptide nucleic acid (PNA) conjugates was designed to improve RRE RNA binding (Fig. 47B).¹⁷¹ Twenty PNAs **268** were linked to NEO derivative **267** *via* nucleophilic substitution reaction. The resulting conjugates **269** were found to exhibit 5-fold increased affinity for the RRE RNA when compared to the parent AG NEO. A split-and-pool combinatorial method was used for the synthesis of another library comprised of 256 NEO-dipeptide conjugates.¹⁷² Out of these, 5 conjugates bound to RRE RNA with

moderately improved affinity and highly improved specificity when compared to NEO. Finally, TOB was also coupled, at its 6''-position, to 12mer peptides. The resulting TOB-peptides displayed enhanced membrane permeability and strong antibiotic activity.¹⁷³

Two novel derivatives of NEA were synthesized by connecting arginine or lysine to its 5-hydroxyl group by using ethylenediamine as a linker (Fig. 48A).¹⁷⁴ The 5-hydroxyl of **270** was displaced with ethylenediamine to form **271**, which was then coupled with Fmoc-Arg(Pbf)-OH or Boc-Lys(Fmoc)-OH in the presence of DCC and HOBt to afford the NEA-amino acid conjugates **272**. The NEA-lysine conjugate exhibited better binding affinity towards TAR RNA when compared to its NEA-arginine counterpart. The amino acid arginine was also linked to GEN C₁, KANA, NEA, NEO, and PAR in studies exploring the binding of AGs to RNA (Fig. 48B).¹⁷⁵⁻¹⁸⁰ These studies showed that, in addition to the AG core, the number of arginines attached to a specific AG, are important in developing potent RNA-binding agents.

Various examples of amphiphilic AG-peptide conjugates were reported in the literature based on the working hypothesis that conversion of AGs into cationic amphiphiles may enhance antibacterial activity against AG-resistant bacterial strains (Fig. 49). Amphiphilic NEO-lysine conjugates were prepared by reductive amination (Fig. 49A).¹⁸¹ The 5''-hydroxyl of NEO was selectively oxidized with trichloroacetic acid (TCAA) to generate aldehyde **277**, which was then coupled with Fmoc-Lys-OBn to yield **278**. It is important to note that the Fmoc and Bn group of **278** have been selectively deprotected and coupled with other amino acids. In most cases, with the exception of MRSA, the NEO-lysine conjugates displayed reduced potency against Gram-positive bacterial strains. Additional NEO- as well as KANA-peptide conjugates were prepared by click chemistry in solution and in solid phase (Fig. 49B).¹⁸² Antibacterial screening of these conjugates demonstrated the importance of the nature of the peptide component. Several conjugates exhibited superior activity against KANA- and NEO-resistant strains when compared to their parent AGs, but surprisingly displayed reduced activity against KANA- and NEO-susceptible strains.

2.5. Synthesis of AG homo- and heterodimers

In addition to the several semi-synthetic AGs designed by core modification (*e.g.*, ABK, AMK, and DBK), by addition of alkyl and/or acyl substituents, as well as by conjugation to small molecules, AG homo- and heterodimers have been explored with the goal of increasing the local concentration of the AG drug in cells. Homo- and heterodimerization of AGs have resulted in broad-spectrum antibacterials capable of evading common modes of AG resistance and binding to RNA with high affinity. The nature of the linker tethering the two AGs, the position at which the linker is attached, and the ease of synthesis have been taken into consideration while designing these AG dimers. The syntheses of AG homo- and heterodimers were categorized based on the type of reactions utilized: (i) disulfide linkage formation, (ii) amide coupling, (iii) carbamate/thiocarbamate linkage formation, (iv) epoxide ring opening, (v) triazole ring formation, (vi) condensation reaction, and (vii) nucleophilic substitution reaction. In addition to synthetic strategies, this section of the review will focus on the effect of the antibacterial and RNA binding properties of the AG dimers prepared.

A variety of AG homo- and heterodimers were synthesized by using a disulfide linkage (Fig. 50). For the preparation of NEO-TOB heterodimer **284**, the OTIBS group at the 5''-position of **282** was displaced with a dithiol linker to generate **283**, which was then reacted with the activated TOB thioether **285** to yield the desired heterodimer. This strategy was also utilized to synthesize various KANA, NEO, and TOB homodimers **286** as well as KANA-TOB and NEO-TOB heterodimers **287** linked *via* the 5''- and 6''-positions.^{166, 183-188} Because of their higher number of positive charges and potential binding to multiple sites, these dimers displayed enhanced affinity for RNA when compared to their monomeric counterparts. The characteristic inhibition curves obtained for dimeric AGs suggest the existence of multiple AG binding sites within the ribozymes's 3D-fold. These dimers exhibited 19-fold increase in binding to A-site 16S rRNA when compared to their monomeric counterparts. Similarly, a NEO-NEO dimer was found to bind the HIV-1 RRE-RNA 17-fold better than NEO itself. Additionally NEO-NEO and NEO-TOB dimers displayed unexpected B-form duplex stabilization.

In contrary to the disulfide linkages that were used to prepare both AG homo- and heterodimers, amide coupling was utilized exclusively to prepare NEA homodimers (Fig. 51). However, the variety of linkers used with the amide coupling strategy was greater. Two NEA molecules were linked *via* amide coupling through *N1* with the aim of targeting the dimerization initiation site (DIS) of HIV-1 RNA (Fig. 51A).¹⁸⁹ Intermediate **289** was synthesized from NEO in 5 steps, which involved the interesting sequence of formation of cyclic carbamate by NaH as well as the selective deprotection of the 5-membered carbamate ring using barium hydroxide. The activated diacid **290** was used to couple two NEA analogues **289** *via* amide coupling to generate homodimer **292**. This homodimer was found to be more efficient at preventing heterodimer formation of RNA DIS fragments than NEA. Other than the *N1*-linked NEA dimers presented in Fig. 51A, NEA dimers linked *via* amide coupling between either the 5- or 4'-position were reported (Fig. 51B).¹⁹⁰ The trityl and *p*-methoxybenzyl protecting groups were used in the synthesis of intermediate **293**, which in the presence of succinic anhydride underwent self-coupling to generate homodimer **294** (an example of the 5-position linkage). The two NEA homodimers were able to inhibit the TAR-Tat at submicromolar concentrations. As the NEA homodimers generated in this way were found to be promising, it would be interesting to further explore this chemical strategy to generate other AG homodimers for such purposes.

Carbamate and thiocarbamate linkages were also widely used in the syntheses of AG homodimers, with NEO being especially exploited.¹⁹¹⁻¹⁹⁴ The formation of NEO homodimers **297** in one step began with either the parent protected NEO **295** or the 5''-cysteamine-modified NEO **296**, which were reacted with a variety of linkers with isocyanate or isothiocyanate groups at both ends (Fig. 52A). The DNA binding and enzymatic activity of these NEO homodimers **297** were explored. It was found that the linker length played an important role in DNA binding, with longer linkers leading to greater binding and lesser modifications by AMEs. A NEA homodimer **300** linked at the 1-positions with carbamate linkages was also prepared (Fig. 52B).¹⁹⁵ However, this compound was synthesized in two steps. Intermediate **298** was treated with ethylenediamine in pyridine to produce the urea derivative **299**, which was subsequently used in the second ring-opening reaction with **298** to

generate the NEA homodimer **300**. This elegant 2-step synthetic approach could be more widely exploited to generate new AG homodimers as well as heterodimers.

Another interesting strategy for the preparation of AG homo- and heterodimers takes advantage of the ease of opening an epoxide ring (Fig. 53).^{196, 197} The epoxides to be opened have been placed either on the linkers or the AGs themselves. For example, NEO homodimers **303** (Fig. 53A) and NEO-TOB heterodimers were conjugated to a naphthalene diimide intercalator by opening the epoxide rings on linker **302**. Homodimer **303** exhibited a 35-fold enhancement in RNA binding affinity when compared to NEO itself. To prepare NEA homodimers **306**, an alternate approach was used. In this case, the epoxide ring was introduced onto NEA by reaction with (*R*)-glycidyl tosylate prior to opening by various linker nucleophiles (Fig. 53B).^{198, 199} A number of these NEA homodimers **306** displayed *in vitro* activity against several bacterial strains, including drug-resistant *P. aeruginosa*.

In the past 20 years or so, click chemistry has become a popular mean to generate compounds for diverse applications. It is therefore not surprising that click chemistry has been used to generate AG dimers for various purposes. It is known that the cellular uptake of monomeric guanidinoglycosides is limited in cells expressing undersulfated heparan sulfate.²⁰⁰ With the idea of providing additional cell surface binding capability to improve cellular uptake, guanidinylated NEO homodimers conjugated to biotin were prepared (Fig. 54).²⁰⁰ The 5''-amine of **307** was coupled with hex-5-ynoic acid under standard peptide coupling conditions prior to the introduction of Boc-protected guanidino groups. The alkyne-containing NEO **308** was conjugated to linker **310** by using copper-mediated click chemistry to afford NEO homodimer **309**, which was found to more easily enter a number of Chinese hamster ovary (CHO-K1) cells.

With the goal of generating compounds with improved RNA binding and antibacterial properties, click chemistry was also used to synthesize various NEO homodimers linked at their 5''-position (Fig. 55). It was found that shorter linkers resulted in better affinity for HIV TAR RNA (Fig. 55A).^{201, 202} For the synthesis of arginine-linked NEO homodimers, the azide group at the 5''-position of **313** was coupled to a dialkyne linker with a free carboxyl acid, which proved to be extremely useful for further modifications with a library of arginine analogues to generate **314** (Fig. 55B).²⁰³ Although these arginine-linked NEO homodimers displayed weaker antibacterial activity than NEO, they were found to be poor substrates for several AMEs. These homodimers bound preferentially to bacterial A-sites, with a carbon linker length of 4-6 being optimal. Various amphiphilic AG (KANA and NEO)-peptide homodimers were also synthesized by click chemistry as antibacterial agents (Fig. 55C). The azide group at the 5''-position of **315** was coupled to dialkyne peptide **316** to generate NEO-peptide homodimer **317**.¹⁸² Both of these analogues exhibited superior activity against KANA- and NEO-resistant strains when compared to the parent AGs.

Another useful approach to produce AG homodimers involves imine intermediates.²⁰⁴ An example of such reaction is that of the preparation of a SIS homodimer in 7 steps (Fig. 56A). Oxidation of the allylic azide of **318** with SeO₂ generated a 6'-aldehyde, which upon treatment with trimethylorthoformate led to the dimethyl acetal intermediate **319**. Cleavage of the Cbz group with concomitant reduction of the azides under Birch conditions afforded a

masked 6'-ketal. Treatment of this compound with sulfuric acid liberated aldehyde **320**, which upon neutralization with barium hydroxide underwent self-condensation to produce the SIS homodimer **321** comprising 2 imine moieties. This methodology provides an interesting way to dimerize AGs with allylic azides. It has so far not been widely exploited and therefore remains open to further explorations. In another example, imine formation was used to link two PAR analogues **322** at their 6-" position with quinacridine **323** to generate PAR homodimer **324** (Fig. 56B). The quinacridine linker was also used to prepare KANA as well as TOB homodimers.²⁰⁵ The TOB homodimer exhibited stronger binding to the P6.1 element of human telomerase RNA when compared to TOB, and a similar trend was observed with KANA and PAR, albeit with lower affinity.

Last but not least, a nucleophilic substitution reaction was used to make a PRM homodimer linked *via* the 3'-hydroxyl groups of this AG (Fig. 57).^{206, 207} All the amino groups of PRM were converted to azides prior to treatment with cyclohexanone dimethyl ketal to afford intermediate **325** with a free 3'-hydroxyl group. Treatment of **325** with dibromomethane under basic conditions followed by global deprotection afforded the PRM homodimer **326**. This homodimer did not display antibacterial activity, and increasing the number of amino groups by creating the dimer did not lead to an increase in read-through activity when compared to PRM itself.

2.6. Synthesis of conformationally-restricted AGs

Enzymatic inactivation is the most prevalent source of clinically relevant AG resistance.^{34, 208} AMEs act by chemically modifying AGs, which adversely affects their clinical efficacy.⁶ A chemical approach based on structures of AGs bound to the ribosome was developed to obtain conformationally-restricted AGs capable of evading the action of AMEs while retaining their binding affinity for their ribosome target. In the early stages of method development, thermodynamic and conformational implications of preorganized glycosidic sugar rotamers were explored in details with respect to their binding to lectins.^{209, 210} The efficacy of AG antibiotics can be modified by controlling the number of available conformations, thereby altering their target selectivity. The synthesis of conformationally-restricted analogues of KANA, NEA, NEO, and PAR were reported by using either (1) amide coupling (in the case of NEA; Fig. 58), (2) nucleophilic substitution reactions (in the case of NEO and PAR, Fig. 59; as well as KANA, Fig. 60A), or (3) ring-closing metathesis (in the case of KANA and PAR; Fig. 60B,C).²¹¹⁻²¹⁸ These studies were aimed at (i) developing analogues with substantially improved antibacterial activity and/or effective protection against AMEs, as well as (ii) understanding DNA or RNA-AG specificity. Even though each ring has its own preferred conformation, AGs as a whole exhibit considerable conformational flexibility around their glycosidic bonds. By fixing the spatial orientation of two of the AG rings (rings I and II in NEA and KANA; rings I and III in NEO and PAR; or rings II and IV in PAR) *via* a covalent bond, the entropy required for binding to macromolecules was observed to be significantly different, as it had been paid at the onset by this new covalent bond formation. The initial design of conformationally-constrained AGs was based on the information available from crystal structures of RNA-AG complexes.²¹⁹⁻²²¹ This section of the review will focus on the effect of structural preorganization of AGs on their activity as well as their ribosome and AME binding profiles.

2.6.1. Preparation of a conformationally-restricted AG by amide coupling—

Only one example of synthesis of a conformationally-restricted AG using amide coupling as a strategy to connect two rings has been reported in the literature.²¹⁴ The AG modified in that example was NEA, and the two rings connected were rings I and II (Fig. 58). More specifically, the NEA restricted analogue **328** was prepared by linking the 3- and 6'-amino groups of this AG by using 1,4-butanedione. The copper-catalyzed reaction of NEA (prepared from NEO in 2 steps) with Boc anhydride resulted in protection of the 3- and 6'-amino groups, which then allowed for the modification of the remaining 1- and 2'-amino groups with Cbz to give intermediate **327**. The reaction of **327** with succinic anhydride followed by global deprotection resulted in the conformationally-restricted NEA **328**. Compound **328** was not subjected to biochemical or biological activity testing. It is surprising that this methodology developed about 10 years ago, has not been more widely utilized, as the regioselective AG amino group protection chemistry developed provides a rapid platform for linking two amine moieties present in different rings of AGs.

2.6.2. Preparation of conformationally-restricted AGs by nucleophilic substitution reactions—

In contrary to the amide coupling, which has had limited usage for the synthesis of conformationally-restricted AGs, nucleophilic substitution reactions have been more widely investigated. The proximity of the 2'-amino and 5''-hydroxyl groups in NEO and PAR was exploited for the synthesis of the conformationally-restricted analogues **329** and **330**, respectively (Fig. 59).²¹⁶ More specifically, their syntheses involved intramolecular and regioselective cyclization where the OTBS group at the 5''-position was displaced by the 2'-amino functionality under high dilution conditions. These two analogues were examined for their affinity for different RNA targets, such as the A-site and HIV-1 TAR RNA. Both restricted-NEO **329** and restricted-PAR **330** were found to bind the A-site and HIV-1 TAR RNA with lower affinity than their counterpart parent AGs, and their binding activity was shown to be pH dependent. Interestingly, in contrary to what was originally believed, these conformationally-restricted compounds did not display selectivity towards specific RNA targets, but were instead found to bind with similar affinity to the A-site and HIV-1 TAR RNA. To further explore the use of conformationally-locked AGs to overcome bacterial resistance, NEO analogue **334** comprising a 3-carbon bridge (including the 5''-carbon of the ribose ring of the AG) was synthesized (Fig. 59).²¹⁸ For this analogue **334**, the bridge was introduced at the 5''-position by using Wittig reaction with the 5''-aldehyde generated by oxidation of analogue **331**. The intramolecular nucleophilic cyclization of **333** resulted in compound **334**. Both conformationally-restricted NEO derivatives **329** and **334** maintained reasonable affinity for A-site RNA while exhibiting antibacterial activity against *E. coli*, *S. epidermidis*, *Bacillus cereus*, and *Alcaligenes faecalis*.²¹⁷ However, NEO itself was found to be more potent and to display better antitranslational activity than derivative **329**, indicating that forming a ring involving the nitrogen at the 2'-position is not favorable. Insights into the importance of the free 2'-amino group were gained by buffer and salt-dependent binding studies coupled with high-resolution NMR, which revealed that the 2'-amino group interacts with the phosphate functionality of A1492 for enhancement of stability of the drug-rRNA complex.²¹² Conformational restriction provided complete protection against resistance enzymes in *S. aureus* (ANT(4')) and in *M. tuberculosis* (AAC(2')), as well as partial protection against

modification by the *Enterococcus faecalis* APH(3'). X-ray crystallography was used to determine the 3D structures of the RNA decoding site in complex with both NEO and the conformationally-restricted NEO derivative **329**.²¹³ Based on these crystal structures, it was observed that ring III of derivative **329**, in contrary to that of NEO, did not participate in direct contact with the target site, which established the sensitivity of AG target recognition by the RNA towards even slight modifications. However, other key interactions of AG ligands were found to be undisturbed by the modifications in derivative **329**.

2.6.3. Preparation of conformationally-restricted AGs by ring-closing

metathesis—Two PAR analogues (*Note*: only one of them, **342**, is presented in Fig. 60C) were prepared by tethering the 6-hydroxyl of ring II and the 6''-amine of ring IV with a 5-carbon bridge by using ring closing metathesis.²¹¹ Both synthetic analogues were tested against APH(3')-IIIa and were found to be poorer substrates of this resistance enzyme than PAR. Unlike PAR, which was phosphorylated at both its 3'- and 5''-hydroxyl by APH(3')-IIIa, both PAR analogues were phosphorylated only at their 5''-hydroxyl, as established by NMR spectroscopy. However, these conformationally-restricted PAR derivatives were found to be inactive at concentrations lower than 40 µg/mL against *S. aureus* and *E. coli*. In a different study, a series of conformationally constrained KANA derivatives (**337** and **339**) were synthesized by connecting the 5- and 2'-hydroxyl groups of this AG by using either nucleophilic substitution (Fig. 60A) or ring-closing metathesis (Fig. 60B).²¹⁵ All KANA conformationally-restricted analogues showed decreased activity against *S. aureus* and *E. coli* when compared to KANA itself. However, it was noted that conformational restriction led to higher activity than simple alkylation of hydroxyl groups, as observed when comparing derivative **339** and its precursor intermediate **338**.

In summary, conformationally-restricted AGs were designed to mimic drug-bound RNA complexes. In general, it was observed that (i) although these conformationally-restricted derivatives were not as affected as their parent AGs by AMEs, (ii) their antibacterial activity was lesser than that of the parent drugs. In the cases where RNA binding was investigated, it was overall found that the conformationally-restricted compounds did not bind as well to their target as their parent counterparts. It was also observed that the objective of selectivity between different RNA targets was never materialized. This type of structural modification is relatively unexplored and more systematic SAR studies could be performed before deeming this strategy as ineffective. In the future, while designing conformationally-restricted AGs, more emphasis should be given to selectivity of different RNA targets to AGs, which could provide a path to the design of novel target selective RNA-based therapeutics.

2.7. Preparation of novel AGs by glycodiversification

So far we have explored modifications on different positions on AGs and their impact on biological activity. All of these core and peripheral modifications were carried out on rings I-IV on various 4,5- and 4,6-disubstituted, as well as other monosubstituted 2-DOS AGs. This section will focus on the glycodiversification technique where either (i) a new carbohydrate ring is introduced into 4,5-disubstituted 2-DOS AGs (Figs. 61,62), or (ii) an existing

carbohydrate ring is replaced on 4,6-disubstituted 2-DOS AGs (Figs. 63,64) to generate newer AG analogues.

New carbohydrate rings have been added to the 4,5-disubstituted 2-DOS AGs NEO, PAR, PRM, and G418. The clinically important NEO was modified at its 5''-position by addition of one extra sugar ring in the β -configuration (Fig. 61A).²²² NEO acceptor **343** was readily synthesized from NEO in 4 steps. NIS-promoted coupling of **343** with various thioglycosides furnished the pseudo-pentasaccharides **344** after global deprotection. The NEO analogue with the ribose substituent at the 5''-position exhibited better antibacterial activity against KANA-resistant *E. coli* when compared to NEO. In another study, a variety of mono- and disaccharides were introduced at the 5''-position of NEO, and some of these pseudo-penta- and hexasaccharides displayed better activity than NEO and they were poor substrates of APH(3')-IIIa.²²³ PAR is another AG to which additional carbohydrate rings have been added (Fig. 61B). Interestingly, although almost identical in structure to NEO, the new carbohydrate rings were added to the 4'-position of PAR instead of the 5''-position as in NEO.^{224, 225} Coupling of selectively protected PAR derivative **345** with a series of thioglycosides resulted in α - and β -glycosides **346**. The 4'-*O*- β -D-xylopyranosyl PAR derivative exhibited antiribosomal activity comparable to that of PAR, but was significantly more selective for bacterial ribosome over cytosolic ribosome and A1555G mutant mitochondrial ribosome associated with hypersensitivity to drug-induced ototoxicity. The equatorial glycosides bind the mitochondrial ribosome less tightly than their axial anomers and are therefore the anomers of choice for developing less toxic AGs. The 4'-*O*-glycosylated PAR derivatives were not susceptible to modifications by ANTs (4' and 4''), and their antibacterial activity largely followed the antiribosomal trend with β -anomers exhibiting better antibacterial activity against clinical strains of MRSA and *E. coli* comparable, in most cases, to that of APR and PAR. As addition of carbohydrate rings to the 5''-position of NEO and 4'-position of PAR provided derivatives with great promise, it would be interesting to see how NEO and PAR derivatized at their 4'- and 5''-positions, respectively, would fair. These remain to be explored.

While new carbohydrate rings have been added to a single position on NEO (5''-position) and PAR (4'-position), they were added to 3 different positions (5, 6, and 3') on PRM (Fig. 62A). The resulting PRM pseudo-trisaccharides were prepared for the study of human genetic disorders caused by PTCs. For the construction of pseudo-trisaccharides **350-352**, the appropriately protected PRM acceptors **347-349**, which selectively exposed the 5-, 3'-, and 6-hydroxyl groups, respectively, were subjected to glycosylation reactions with 2 different thioglycoside donors. All of these PRM pseudo-trisaccharides were not active when tested against AG-sensitive *E. coli* and *B. subtilis* strains.^{206, 226} However, they displayed good read-through activity, with **350** displaying better read-through than **352**, which in turn displayed better read-through than **351**. These data demonstrated the importance of the point of attachment of the new ribose ring for such activity. Compound **350** with R = NH₂ was found to have significantly reduced cytotoxicity when compared to PAR and GEN. In an attempt to further improve read-through efficiency, an AHB chain was introduced at the M1-position of **350**. This analogue exhibited higher read-through efficiency and lower toxicity than GEN and was found to potently target all 7 different nonsense

mutations underlying Usher syndrome, CF, DMD, and Hurler syndrome. Similarly, novel G418-derived pseudo-trisaccharides **354** were synthesized by glycosylation of the 5-hydroxyl of **353** with a variety of ribose rings (Fig. 62B).^{227, 228} When compared to **350**, these analogues with an (*R*)-methyl group at the 6'-position exhibited superior read-through efficiency in 6 different nonsense mutation DNA constructs associated with Usher syndrome, CF, DMD, and Hurler syndrome. Carbohydrate rings were also added at the 5-position of NEA to generate the pseudo-trisaccharides pyranmycins **356-358** (Fig. 62C). These analogues were tested against *E. coli*, *S. aureus*, and *Bacillus megaterium*, and among them, **358** was found to be as active as RIB, while **356** and **357** were found to be inactive.²²⁹ A significant amount of work has been carried out to understand the SAR of pyranmycin analogues as it related to antimicrobial activity by using glycodiversification.²³⁰⁻²³³ In addition to being used to generate AGs with activity against bacteria and PTC disorders, glycodiversification was used to produce NEA analogues with fungicidal activity.²³⁴ An analogue with a C₈ alkyl chain was completely devoid of antibacterial activity, but was found to display antifungal activity. This analogue was found to increase the plasma membrane permeability by a mechanism that is influenced by the lipid composition of the fungal plasma membrane.²³⁵

Besides the addition of carbohydrate rings to 4,5-disubstituted 2-DOS AGs, glycodiversification of 4,6-disubstituted 2-DOS AGs by replacement of one of their carbohydrate rings also exist. Many KANB and TOB analogues have been generated by this technique for use as treatment of drug-resistant bacterial and fungal infections (Figs. 63,64).²³⁶⁻²³⁹ The perazido KANB **359** was selectively protected at its 6''-position prior to site selective protection of its 3',4'-diol to give **360** (Fig. 63). Removal of the silyl protecting group and subsequent benzylation of free hydroxyl groups afforded **361**, which upon treatment with NaIO₄ afforded the acceptor **362**. This compound was glycosylated with donor **363** to afford the new KANB scaffold **364** with an amino group in place of the hydroxyl at the 4'-position of the parent KANB. This new KANB scaffold was further modified at its 4'-position. The resulting KANB analogues displayed considerable antibacterial activity against *K. pneumoniae*, MRSA, *P. aeruginosa*, *E. faecalis*, *E. coli*, and *S. epidermidis*, which indicated that modifications at the 4'-position do not have a negative impact on bioactivity. Other KANB analogues synthesized *via* glycodiversification with further modification at the *N1*-position are also reported in the literature.²⁴⁰

Besides the example presented in Fig. 63 where KANB derivatives were generated by replacing ring I of the parent AG with various carbohydrate rings, KANB derivatives produced by adding new rings III to NEA have been reported (Fig. 64A,B).^{236, 237, 239} The NEA acceptor **365** was prepared from NEA in 3 steps, and this acceptor successfully underwent regiospecific glycosylation at its 6-position with a variety of glycosyl donors to yield mixtures of KANB derivatives **366** with the new carbohydrate rings III in the α - or β -configurations (Fig. 64A).^{236, 239} Although the α -glycosidic bond between rings II and III is normally found in AGs, the stereoselective formation of an α -glycosidic bond is challenging since no neighboring group assistance can be used. By testing these KANB derivatives **366** against *E. coli* and *S. aureus* it was found that R₁ = NH₂ is favored, R₂ = NH₂ is better than R₂ = OH, R₃ = NH₂ decreases activity when compared to R₃ = OH or H, and R₄ = H is

preferred to OH and NH₂. Similarly, KANB derivatives **367** with long alkyl chains (C₈H₁₇) at the 2''-, 4''-, 6''-, 2''- and 4''-, as well as 4''- and 6''-positions were synthesized from **365** (Fig. 64B).²³⁷ The attachment of an octyl group at the 4''-position of KANB converted this antibacterial AG into a novel antifungal agent. From this SAR study, it was revealed that the 4''-position of KANB is the optimal site for the attachment of linear alkyl chains to produce antifungal activity. The strategy of adding a carbohydrate ring to a pseudo-disaccharide to generate new pseudo-trisaccharides has also been employed to produce novel TOB derivatives from NEB (Fig. 64C).^{241, 242} The protected NEB **368**, which was itself derived from TOB, was subjected to glycosylation reaction with mono- and disaccharide donors in the presence of benzenesulfonylpiperidine (BSP) and Tf₂O to yield **369**. Many of these TOB analogues displayed an affinity in the nanomolar range against specific RNA sequences.

2.8. Preparation of novel AGs by chemoenzymatic derivatization

Finally, to overcome the resistance caused by AMEs, new strategies involving semi-synthetic modifications of AGs by using enzymes were developed. For example, KANA, NEA, and NEO-based polyamine derivatives were synthesized by using a chemoenzymatic method relying on a regioselective lipase-catalyzed acylation followed by chemical aminolysis.²⁴³ The regiospecific synthetic acylation of densely functionalized AGs normally requires multiple steps. Therefore, the single-step chemoenzymatic acylation methodology is advantageous over traditional chemistry. Butirosin, an AG produced by *Bacillus circulans*, bears the unique AHB side chain, which is introduced by the acyltransferase BtrH and its partner BtrG, and is known to provide protection against several common AG resistance mechanisms. The AG promiscuity of BtrH for NEA, NEO, and PAR was utilized to successfully introduce an AHB side chain regiospecifically onto these AGs (Fig. 65).^{244, 245} Lastly, AMEs themselves have been utilized to generate new AGs. For example, APR was azidoacetylated at its 3-position by using AAC(3).²⁴⁶ The resulting azidoacetylated APR analogue was used in a microarray application using click chemistry. The sequential use of AAC(6')-Ie and AAC(3)-IV, which display broad AG and cosubstrate promiscuity, resulted in the production of novel mono- and hetero-di-*N*-acylated AGs.²⁴⁷ New *O*-nucleotidylated AGs were also prepared by taking advantage of the substrate and cosubstrate promiscuity of ANT(4').²⁴⁸ In sum, the chemoenzymatic derivatization of AGs is beneficial as it allows to rapidly regiospecifically modify AGs.

2.9. Summary of the correlations observed between various modifications of AGs and their biological activities

The different types of structural modifications carried out at numerous positions of AG scaffolds along with their biological and biomedical applications were summarized in Table 1. The biological activities against various Gram-positive and Gram-negative bacteria as well as fungal pathogens based on systematic AG SAR studies were discussed in the above sections. With a few exceptions, AG modifications were dictated by ease of feasibility of synthetic transformations. Therefore, the positions modified were most often primary functional groups. As a result, direct comparisons for a specific modification made at many different positions of the same AG could not be made with the information available in the literature. On the other hand, correlations between (i) different structural modifications at a

specific position of a particular AG, and (ii) identical structural modifications at the same position on various AGs could easily be made based on the work presented herein. These correlations along with their biological implications are summarized below.

Inspiring synthetic methodologies were used for derivatizing the core of ABK, AMK, APR, KANA, KANB, NEO, PAR, PRM, RIB, and TOB at the 3-, 5-, 3'-, 4'-, 6'-, 4'', 5'', 6'', 3'''- and 4'''-positions. The analogues prepared were found to be effective as antibacterial, inhibitors of ribosomes, and RNA binders, and to be poor substrates of AAC(3), APH(3'), ANT(4'), ANT(2''), and AAC(6')-Ie/APH(2'')-Ia resistance enzymes. From this analysis, it can be concluded that the electrostatic interactions caused by the number of amino groups on the AGs influence their activity. Thioether, ether, amine, triazole, amide, and thiocarbamate linkages were used for synthesizing alkylated AG analogues. AMK, KANA, KANB, NEA, NEB, NEO, PAR, and TOB were modified with alkyl and aromatic groups at their 1-, 5-, 6-, 4'-, 6'-, 2'', 4'', 5'', and 6''-positions. Novel thioether-linked KANB and TOB exhibited antibacterial and antifungal activity by disrupting the membrane of the organisms. Antibacterial activity, RNA binding, inhibition of bacterial ribosomes, antitumor activity, and immunomodulatory property, along with inhibition of translation and inhibition of AMEs were observed for ether-linked AGs. Alkylated AGs with amine, triazole, and thiocarbamate linkages were observed to be effective as antibacterial and to be poorly modified by AMEs.

Various analogues of ABK, AMK, APR, KANA, NEO, NET, PAR, SIS, and TOB *N*-acylated at their 1-, 6-, 6'-, 4'', 6'', 4'''-, and 6'''-positions were reported. These analogues were synthesized *via* mono- and multiacylation and found to be efficient inhibitors of bacterial and human topoisomerases and poor substrates of AAC(3) and AAC(6') resistance enzymes. As seen in this review, AG heteroconjugates are some of the most widely covered types of modified AGs studied by the scientific community. AG bisubstrates as well as AG-drug, AG-small molecule, and AG-peptide conjugates were reported for GEN, KANA, NEA, NEO, PAR, RIB, and TOB with modifications at their 5-, 3'-, 4'-, 6'-, 5'', 6'', and 6'''-positions. Examples of molecules conjugated to AGs include CAM, LNZ, CIP, CLN, BQQ, bisbenzimidazole, pyrene, anthraquinone, naphthalene diimide, oligonucleotide, nucleobase, biotin, PAMAM dendrimer, EDTA, PEG, fluorescein, methidium, Pt, thiazole orange, carboline, lipid, Ru, BODIPY, acridine, amino acids, peptides, and peptide nucleic acids. KANA, NEA, and RIB bisubstrate inhibitors linked through their 6'-positions were found to be poor substrates of AAC(6'). NEO- (5'' and 6''') and TOB- (6'') drug conjugates were found to be great antibacterials, poor substrates of AAC(6')-Ib and AAC(6')-Ie/APH(2'')-Ia, and good binders of RRE, TAR, TS, IRE, 16S A-site, and 18S A-site. In the case of small molecule conjugates, KANA (6''), NEA (5, 3', 4', 6'), NEO (5'' and 6'''), PAR (5'', 6'''), and TOB (6', 6'') analogues were successfully used as RNA and DNA binders, ribozyme inhibitors, bioactive cargo transporters, antitumor agents, transfection agents, biofilm inhibitors, cellular uptake enhancers, and as poor substrates of AMEs. KANA (6''), NEA (5), NEO (5''), and TOB (6'') conjugated to amino acids and peptides were synthesized and were found to exhibit excellent RNA binding, ribozyme inhibition, cellular uptake, and antibacterial activity.

KANA-, NEA-, NEO-, PAR-, SIS-, and TOB-based homo- and heterodimers linked *via* disulfide, amide, carbamate, thiocarbamate, ether, triazole, and imine linkages were summarized in section 2.5. The positions manipulated for synthesizing these dimers were the 1-, 5-, 3'-, 4'-, 6'-, 5"-, and 6"-positions. The linkers between AGs were categorically designed for the intended biological applications. The dimers were found to be efficient for RNA binding, inhibition of dimerization initiation (DIS) of HIV-1 RNA, inhibition of human telomerase, enhanced cellular uptake, and avoiding the action of AMEs. However, none of the dimers exhibited antibacterial activity superior to that of the parent AGs. A correlation between efficiency and the positions modified could not be made in the case of these homo- and heterodimers, as only limited structural modifications were explored in the literature. For conformationally-restricted AGs, examples of KANA, NEA, NEO, and PAR were reported with applications as RNA binders and antibacterial agents capable of avoiding the action of AMEs. Various NEO and PAR analogues linked *via* their 2'- and 5"-positions were found to display selectivity for RNA targets, but were observed to be less efficient as antibacterials. Conformationally-restricted KANA and PAR analogues linked through their 5,2'- or 6,6'''-positions were found to be poor substrates of AMEs. In summary these conformationally-restricted AG analogues, although mimicking well the drug bound to RNA targets, were found to be poor antibacterial agents.

Finally, by using glycodiversification methods, new carbohydrate rings were added to NEO, PAR, PRM, and G418 at their 5-, 6-, 3'-, 4'-, and 5"-positions. In general, the derivatives generated displayed good antibacterial activity and selectivity for bacterial ribosomes, and were found to evade the action of APH(3')-IIIa, ANT(4'), and ANT(4"). It is important to note that although the PRM and G418 derivatives were not active against bacteria, they displayed good read-through activity when compared to the parent AGs and exhibited superior read-through efficiency in 6 different nonsense mutation DNA constructs associated with Usher syndrome, CF, DMD, and Hurler syndrome. Various KANB, NEB, and TOB derivatives were generated by replacing ring I of the parent AG with various carbohydrate rings. The resulting analogues were found to be great antibacterials and antifungals.

2.10. Importance of biophysical studies for development of AGs

Although this manuscript is meant to be a comprehensive compendium of the chemical strategies used for the preparation of new AGs, before we conclude this review, it is important to acknowledge that without the extremely important biophysical studies elucidating the ways by which AGs interact with their targets for various biological applications, the rational synthetic optimization of AGs to improve their biological activity presented above would not be possible. Therefore, in this short section, we would like to briefly highlight some of the biophysical work that has been done by using nuclear magnetic resonance (NMR), surface plasmon resonance (SPR), fluorescence-based methods, mass spectrometry, and X-ray crystallography towards understanding the binding of AGs to their targets. Mostly towards the goal of developing AGs as better antibacterial agents and molecules to overcome PTC disorders, the crystal structures of AGs have been solved in complex with the ribosome or part of it.^{5, 72, 74, 207, 219, 220, 249-262} Also with the aim of improving the antibacterial activity of AGs by overcoming the action of AMEs, the crystal structures of AGs in complex with a variety of these resistance enzymes have been

determined.^{15, 263-277} Additional dissections of interactions of AGs with resistance enzymes have also been performed by calorimetric and NMR studies.²⁷⁸⁻²⁸⁰ NMR has also been utilized to decipher the binding affinity of AGs to the HIV-1 RRE towards developing AGs as potential antiviral agents.²⁸¹⁻²⁸³ Fluorescence spectroscopy,²⁸²⁻²⁸⁴ crystallography,²⁸⁵ and SPR²⁸⁶ have also been used towards the latter goal. Mass spectrometry has also been used to determine AG binding specificity for rRNA.²⁸⁷ Additionally, NMR was utilized (i) to explore RNA dynamics and structural plasticity in the presence of AGs,²⁸⁸ (ii) to investigate the selective condensation of DNA by AGs,²⁸⁹ and (iii) to study AG drug susceptibility in *E. coli*.²⁹⁰ More thorough/detailed coverage of interactions of AGs with various targets can be found in other reviews.^{1, 33, 35}

3. CONCLUSIONS AND OUTLOOK

Overall, in this review, an in-depth analysis of 8 types of transformations on 18 different AG scaffolds has been performed. In the past two decades, AGs have experienced a renaissance. The development of PLZ and the creation of the company Achaogen exemplify this renewed interest in this old class of antibiotics. Although a lot of work has been done, as seen in this review, the field of AG research is still widely open and a lot remains to be done to improve on these very important drugs. For example, potential exciting avenues to generate new AGs from scratch and get carbohydrate chemists and biochemists of all generations involved in AG research could rely on some interesting glycosylation techniques,²⁹¹⁻²⁹⁹ masterful and elegant chemical transformations reported for the synthesis of deoxysugar building blocks,³⁰⁰⁻³⁰⁹ as well as engineered enzymes³¹⁰ for their chemoenzymatic preparation. Another potential route that could be explored to revive the use of current AGs against which resistance as already developed, or to improve the chances of new AGs to remain active longer, AME inhibitors could be utilized as adjuvants to AG therapies. AME (*e.g.*, AAC and APH) inhibitors are currently gaining in popularity for such purposes.³¹¹⁻³²³ For the longest time, AGs were only used as antibiotics. Recently, their application has extended to antifungal (human and agricultural), antiviral, anticancer, and PTC disorders. It would not be surprising to see, in a near future, AGs gaining in popularity for a variety of still to be discovered therapeutic, agricultural, veterinary, and industrial applications. We have made great strides since our understanding of AGs targeting the 16S rRNA site of ribosomes, and if scientists from more disciplines (not only chemistry and microbiology, but also biology, biochemistry, engineering, health sciences, and computational chemistry) join forces, we will highly likely discover new mechanisms by which AGs can function. The combined efforts of specialists from different disciplines could push AGs to new territories remaining to be explored. We hope that this review has provided a reference tool for those already working in the AG field (chemists, biochemists, microbiologists, and clinical specialists). We also wish that it has picked the curiosity of others and will provide them, and a new generation of scientists, with an entry point into this fascinating field of research and give them ideas for future research projects.

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ABBREVIATIONS

AAC	aminoglycoside <i>N</i> -acetyltransferase
ABK	arbekacin
ADP	adenosine diphosphate
AG	aminoglycoside
AIBN	azobisisobutyronitrile
AME	aminoglycoside-modifying enzyme
AMK	amikacin
ANT	aminoglycoside <i>O</i> -nucleotidyltransferase
APH	aminoglycoside <i>O</i> -phosphotransferase
APR	apramycin
BODIPY	4,4-difluoro-4-bora-3a,4a-diaza- <i>s</i> -indacene
BSP	benzenesulfinylpiperidine
CoA	coenzyme A
CF	cystic fibrosis
CSA	camphor sulfonic acid
DBK	dibekacin
DCC	dicyclohexyl carbodiimide
DCE	dichloroethane
DHP	dihydropyran
DIPEA	diisopropylethylamine
DIS	dimerization initiation site
DMAP	dimethyl aminopyridine
DMBNH₂	dimethoxybenzylamine
DMD	Duchenne muscular dystrophy
DMT-MM	4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride
2-DOS	2-deoxystreptamine
DTT	dithiothreitol
EDCI	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide

Eis	enhanced intracellular survival
G418	geneticin
GEN	gentamicin
HATU	(1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate)
HCTU	<i>O</i> -(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HF•Py	hydrogen fluoride pyridine complex
HMPA	hexamethylphosphoramide
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	hydroxybenzotriazole
Imd	imidazole
IRE	iron responsive element
ISP	isepamicin
KANA	kanamycin A
KANB	kanamycin B
KHMDS	potassium bis(trimethylsilyl)amide
LG	leaving group (<i>Note</i> R-LG = thioglycoside)
MIC	minimum inhibitory concentration
MOMCl	methoxymethyl chloride
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
MRSE	methicillin-resistant <i>Staphylococcus epidermidis</i>
MsCl	methanesulfonyl chloride
NEA	neamine
NEB	nebramine
NEO	neomycin B
NET	netilmicin
NHS	<i>N</i> -hydroxysuccinimide
NIS	<i>N</i> -iodosuccinimide
NMO	4-methylmorpholine- <i>N</i> -oxide

NMR	nuclear magnetic resonance
PAR	paromomycin
PBS	phosphate-buffered saline
Pd₂(dba)₃	tris(dibenzylideneacetone)dipalladium(0)
PLZ	plazomicin
PNA	peptide nucleic acid
PRM	paromamine
PTC	premature termination codon
PyBOP	(benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate
RIB	ribostamycin
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RRE	Rev response element
SAR	structure-activity-relationship
SIS	sisomicin
SPR	surface plasmon resonance
STR	streptomycin
TAR	<i>trans</i> -activation response element
TAR-Tat	<i>trans</i> -activation response element- <i>trans</i> -activator of transcription
TBAB	tetrabutylammonium bromide
TBAF	tetrabutyl ammonium fluoride
TBAI	tetrabutyl ammonium iodide
TBDMSCl	<i>tert</i> -butyldimethylsilyl chloride
TBDMSOTf	<i>tert</i> -butyldimethylsilyl trifluoromethanesulfonate
TBDPSCI	<i>tert</i> -butyldiphenylsilyl chloride
TBTU	<i>O</i> -(benzotriazol-1-yl)- <i>N,N,N,N</i> -tetramethyluronium hexafluoroborate
TCCA	trichloroisocyanuric acid
TESCI	triethylsilyl chloride
TFA	trifluoroacetic acid

TfN₃	trifluoromethanesulfonyl azide
Tf₂O	trifluoromethanesulfonic anhydride
TIBSCl	triisopropylbenzenesulfonyl chloride
TIEA	triisopropylethylamine
TIPSOTf	triisopropylsilyl trifluoromethanesulfonate
TOB	tobramycin
TrCl	tritylchloride
Troc	trichloroethoxycarbonyl
TS	thymidylate synthase
TsCl	<i>para</i> -toluenesulfonyl chloride
TsOH	<i>para</i> -toluene sulfonic acid
TTBP	tri- <i>tert</i> -butylpyrimidine
VRE	vancomycin-resistant <i>enterococci</i>

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4,5-Disubstituted 2-DOS AGs:

	R ₁
Neamine (NEA)	NH ₂
Neomycin B (NEO)	NH ₂
Paromomycin (PAR)	OH
Paromamine (PRM)	OH
Ribostamycin (RIB)	NH ₂

4,6-Disubstituted 2-DOS AGs:

	R ₁	R ₂	R ₃	R ₄
Arbekacin (ABK)	NH ₂	H	H	AHB
Amikacin (AMK)	OH	OH	OH	AHB
Dibekacin (DBK)	NH ₂	H	H	H
Kanamycin A (KANA)	OH	OH	OH	H
Kanamycin B (KANB)	NH ₂	OH	OH	H
Nebramine (NEB)	NH ₂	H	OH	H
Tobramycin (TOB)	NH ₂	H	OH	H

	R ₁	R ₂
Gentamicin C ₁ (GEN C ₁)	Me	Me
Gentamicin C ₂ (GEN C ₂)	H	Me
Gentamicin C _{1a} (GEN C _{1a})	H	H

AG scaffolds from other types:

Fig. 1.

Structures of AGs discussed in this review. *Note:* the positions that have been modified on these AGs (as reported in Table 1) are indicated by black numbers.

Fig. 2.

Graph showing the increase in the number of publications related to AGs in PubMed (turquoise ovals) and in SciFinder (red ovals) over a 20-year period (1995-2015).

Fig. 3.
Overview of the AG modifications, with an AG as a starting point, discussed in each section (2.1-2.8) of this review.

A. General strategy for introducing an electrophilic center at the 5''-position of NEO and 6''-position of ABK, AMK, KANA, KANB, and TOB

B. General strategies for modifying the 5''-position of NEO and the 6''-position of ABK, AMK, KANA, KANB, and TOB

Fig. 4.

General strategies for **A.** the introduction of an electrophilic center at the 5''-position of NEO and at the 6''-position of ABK, AMK, KANA, KANB, and TOB, as well as **B.** their further modification.

A. General strategy for modifying the 5''-position of PAR **B. General strategy for single-step modification of the 6'-position of AMK, KANA, KANB, NEA, NET, SIS, TOB, 6',4'''-positions of AMK, and 6'''-position of PAR** **Fig. 5.**

General strategies for **A.** the modification of the 5''-position of PAR, and **B.** the single-step modification of the 6'-position of AMK, KANA, KANB, NEA, NET, SIS, TOB, 6',4'''-positions of AMK, and 6'''-position of PAR, and 4'''-position of AMK.

A. Single-step modification of the 3-position of AGs (NH₂ to N₃) **B. Oxidation of the 6'-position of PRM**

Fig. 6. Synthetic schemes for **A.** the single-step modification of the 3-position of AGs (AMK, APR, NEA, NEO, PAR, RIB), and **B.** the oxidation of the 6'-position of PRM and its many subsequent multistep modifications.

Oxidation of an allylic amine into an α,β -unsaturated aldehyde

Fig. 7.
Oxidation of an allylic amine into an α,β -unsaturated aldehyde.

A. Representative example for the synthesis of 3',4'-dideoxygenated PAR, NEO, and RIB **B. Synthesis of 3',4',3''',4'''-tetra-deoxy-NEO and PAR analogues** **Fig. 8.**

Synthesis of **A.** 3',4'-dideoxygenated PAR, NEO, and RIB, and **B.** 3',4',3''',4'''-tetra-deoxy-NEO and PAR analogues.

Fig. 9.
 Synthesis of **A**, 5-deoxy-5-episubstituted ABK, **B**, 4''-deoxy-4''-episubstituted ABK, and **C**, 4'-epimers of PAR.

Synthesis of 6''-thioether KANB and TOB derivatives

Fig. 10. Schemes for the preparation of 6''-thioether KANB and TOB derivatives and their oxidation to sulfoxides and sulfones.

A. Synthesis of homo and hetero 4',6''- and 3'',6''-dithioether TOB derivatives **B. Synthesis of homo 6',5''-dithioether PAR derivatives**

Fig. 11.
Synthesis of **A.** homo and hetero 4',6''- and 3'',6''-dithioether TOB derivatives, and **B.** homo 6',5''-dithioether PAR derivatives.

A. Synthesis of 2''-O-alkyl PAR derivatives **B. 4'-O-alkyl and 4',6'-O-acetal PAR derivatives** **Fig. 12.**

A. Synthesis of representative 2''-O-alkyl PAR derivatives. **B.** Examples of 4'- and 4',6'-O-acetal PAR derivatives.

Synthesis of 4'-O-alkyl KANA and KANB, as well as 4''-O-ethyl KANB derivatives

Fig. 13.
Synthesis of 4'-O-alkyl KANA and KANB derivatives, as well as 4''-O-ethyl KANB.

A. Synthesis of 5-*O*-alkyl TOB derivatives **B. Synthesis of 5-*O*-alkyl TOB derivatives by reductive amination** **C. Synthesis of 5-*O*-alkyl NEA derivatives by reductive amination** **Fig. 14.**

Synthesis of **A.** 5-*O*-alkyl TOB derivatives, **B.** 5-*O*-alkyl TOB derivatives by reductive amination (Note: 5-*O*-alkyl PRM derivatives were also similarly produced), and **C.** NEA analogues by reductive amination.

Fig. 15.

Synthesis of **A**. NEA derivatives alkylated at their 6-position (*Note*: an AHB group is shown at the 1-position, but other *N*-hydroxysuccinimide esters have also been used to derivatized this position), and **B**. NEA aryl ether derivatives (*Note*: PRM aryl ether derivatives were also similarly produced).

A. Synthesis of NEB and TOB ethers **B. Synthesis of NEB ethers** **C. Synthesis of NEB ethers** **Fig. 16.**

Representative schemes for the preparation of **A.** multialkylated NEB and TOB ether derivatives, **B.** multialkylated NEB ether derivatives with modified amine groups (*Note:* PAR and TOB ether derivatives were also similarly produced), and **C.** multialkylated NEB ether derivatives with imidazole decoration.

Fig. 17.
Synthesis of 1-*N*-derivatized NEA.

Fig. 18.
Examples of NEO and TOB derivatives linked *via* a triazole.

KANA, NEO, and TOB derivatives linked *via* amides

AMK, KANA, NEA, and NEO derivatives linked *via* carbamates and ethers **Fig. 20.**

Examples of AMK, KANA, NEA, and NEO derivatives linked *via* carbamates and ethers.

Single-step synthesis of 6'''-acylated NEO analogues

Fig. 21.

Representative scheme for the single-step synthesis of 6'''-acylated NEO analogues (*Note*: PAR derivatives were also similarly produced).

Synthesis of AMK, KANA, NET, SIS, and TOB *N*-acylated analogues

Fig. 22. Scheme for the preparation of AMK, KANA, NET, SIS, and TOB *N*-acylated analogues.

A. Synthesis of KANA *N*-hydroxyurea analogues **B. Synthesis of PLZ** **Fig. 23.**

Representative schemes for the preparation of **A.** KANA *N*-hydroxyurea analogues (*Note:* TOB derivatives were also similarly produced), and **B.** PLZ.

A. Synthesis of 6''-N-acyl ABK derivatives **Representative R =** **B. Synthesis of a 4''-N-acyl-4''-epi ABK derivative** **Fig. 24.**

Schemes for the synthesis of **A**, 6''-N-acyl ABK derivatives, and **B**, a 4''-N-acyl-4''-epi ABK derivative.

Synthesis of *N*-aryl/acylated *O*-sulfonated NEO analogues **Fig. 25.**

Representative scheme for the preparation of *N*-aryl/acylated *O*-sulfonated NEO analogues (*Note*: APR and KANA derivatives were also similarly produced).

A. Representative synthesis of NEA-CoA conjugates **B. Representative synthesis of truncated NEA-bisubstrate inhibitors** **Fig. 26.**

Representative schemes for the preparation of **A.** NEA-CoA conjugates (*Note:* KANA- and RIB-CoA conjugates were also similarly produced), and **B.** truncated NEA-bisubstrate inhibitors.

A. Synthesis of sulfonamide-, sulfoxide-, or sulfone-linked NEA-CoA conjugates **B. Synthesis of a phosphonate-linked PRM-CoA conjugate** **Fig. 27.**

Synthetic schemes for the preparation of **A.** sulfonamide-, sulfoxide-, or sulfone-linked NEA-CoA conjugates, and **B.** a phosphonate-linked PRM-CoA conjugate.

A. Synthesis of NEA-CoA conjugates **B. NEA-pantetheine conjugates** **Fig. 28.**

A. Scheme for the preparation of NEA-CoA conjugates. **B.** Examples of NEA-pantetheine conjugates.

Synthesis of NEO-CAM and NEO-LNZ conjugates

Fig. 29.
Synthetic methodology for the preparation of NEO-CAM and NEO-LNZ conjugates.

A. TOB-CAM and TOB-CLN conjugates **Representative example for the synthesis of a TOB-CLN conjugate** **B. Synthesis of NEO-CIP conjugates** **Fig. 30.**

Representative schemes for the preparation of **A. TOB-CAM and TOB-CLN conjugates**, and **B. NEO-CIP conjugates**.

Synthesis of NEO-small molecule conjugates

Fig. 31.

Representative scheme for the preparation of NEO-small molecule conjugates.

NEO-bisbenzimidazole conjugates

Fig. 32.
Examples of NEO-bisbenzimidazole conjugates.

A. Synthesis of a KANA-oligonucleotide (2mer) conjugate **B. NEO-oligonucleotide (7mer) conjugate** **Fig. 33.**

A. Representative scheme for the preparation of a KANA-oligonucleotide (2mer) conjugate (*Note: a NEO-oligonucleotide (2mer) conjugate was also similarly produced*). **B.** Structure of a NEO-oligonucleotide (7mer) conjugate.

A. Synthesis of NEO-nucleobase conjugates **Representative R =** **B. Synthesis of NEO-NHS ester conjugates** **Fig. 34.**

Schemes for the preparation of **A.** NEO-nucleobase conjugates, and **B.** NEO-NHS ester conjugates.

Synthesis of NEO-biotin conjugates

Fig. 35.
Synthesis of NEO-biotin conjugates.

Synthesis of a PAR-PAMAM-G4 dendrimer conjugate

Fig. 36.

Representative scheme for the preparation of a PAR-PAMAM-G4 dendrimer conjugate
(Note: NEA- and NEO-PAMAM-G4 dendrimer conjugates were also similarly produced).

Structures of NEO-mono- and bisbenzimidazole conjugates

Fig. 37.
Examples of NEO-mono- and bisbenzimidazole conjugates.

A. Synthesis of a TOB-EDTA conjugate **B. Synthesis of a NEO-biotin conjugate** **C. Synthesis of a TOB-PEG conjugate**

Fig. 38. Synthetic schemes for the preparation of **A.** a TOB-EDTA conjugate, **B.** a NEO-biotin conjugate, and **C.** a TOB-PEG conjugate.

A. Synthesis of a NEO-fluorescein conjugate **B. Synthesis of a NEO-methidium conjugate** **C. Synthesis of a NEO-Pt^{II} conjugate** **Fig. 39.**

Synthetic schemes for the preparation of **A**, a NEO-fluorescein conjugate, **B**, a NEO-methidium conjugate, and **C**, a NEO-Pt conjugate.

A. Synthesis of PAR-thiazole orange conjugates **B. Synthesis of NEA-β-carboline conjugates** **Fig. 40.**

Representative schemes for the preparation of **A.** PAR-thiazole orange conjugates (*Note:* NEA-pyrene and PAR-pyrene conjugates were also similarly produced), and **B.** NEA-β-carboline conjugates.

Synthesis of NEA-nucleotide conjugates *via* amide coupling

Fig. 41.

Scheme for the preparation of NEA-nucleotide conjugates *via* amide coupling.

A. NEA-aromatic ring conjugates with amide linkages **B. NEA-small molecule conjugates with amide linkages** **C. NEO-capped aromatic platforms** **Fig. 42.**

Examples of **A.** NEA-aromatic ring conjugates with amide linkages, **B.** NEA-small molecule conjugates with amide linkages, and **C.** NEO-capped aromatic platforms.

A. Synthesis of NEO-lipid conjugates via amide coupling **B. Synthesis of a NEO-Ru^{II} conjugate** **Fig. 43.**

Representative schemes for the preparation of **A.** NEO-lipid conjugates *via* amide coupling (Note: KANA- and PAR-lipid conjugates were also similarly produced), and **B.** a NEO-Ru^{II} conjugate.

A. Synthesis of guanidino-NEO-BODIPY conjugates **B. NEA-adenine bisubstrate inhibitors** **Fig. 44.**

A. Representative scheme for the preparation of guanidino-NEO-BODIPY conjugates (*Note:* guanidine-TOB-BODIPY conjugates were also similarly produced). **B.** Examples of NEA-adenine bisubstrate inhibitors.

Synthesis of NEO-acridine conjugates

Fig. 45.

Representative schemes for the preparation of NEO-acridine conjugates (*Note*: KANA- and TOB-acridine conjugates were also similarly produced).

A. Synthesis of PAR-nucleobase conjugates

B. Synthesis of a NEO derivative-thymine conjugate

Fig. 46.

Representative schemes for the preparation of **A**, PAR-nucleobase conjugates (*Note*: NEO-nucleobase conjugates were also similarly produced), and **B**, a NEO derivative-thymine conjugate.

A. Synthesis of a NEO-peptide library **B. Synthesis of NEO-peptide nucleic acid conjugates** **Fig. 47.**

Schemes for the preparation of **A.** a NEO-peptide library, and **B.** NEO-peptide nucleic acid conjugates.

A. Synthesis of a NEA-arginine conjugate **B. Examples of AG-arginine conjugates** **Fig. 48.**

A. Representative scheme for the preparation of a NEA-arginine conjugate (*Note*: a NEA-lysine conjugate was also similarly produced). **B.** Examples of AG (KANA, NEA, NEO, and PAR)-arginine conjugates (*Note*: GEN C₁-arginine conjugates were also reported).

A. Synthesis of NEO-lysine conjugates **B. Synthesis of NEO-peptide triazole conjugates** **Fig. 49.**

Representative schemes for the preparation of **A.** NEO-lysine conjugates (*Note*: other amino acids have also been conjugated to NEA by using this synthetic methodology), and **B.** NEO-peptide triazole conjugates.

Synthesis of NEO-TOB heterodimers by using a disulfide linkage

Fig. 50. Representative scheme for the preparation of NEO-TOB heterodimers by using a disulfide linkage (*Note*: KANA-KANA, NEO-NEO, TOB-TOB homodimers and KANA-TOB and NEO-TOB heterodimers were also similarly produced).

A. Synthesis of a NEA homodimer via amide coupling **B. Synthesis of a NEA homodimer via amide coupling** **Fig. 51.**

Representative schemes for the preparation of **A,B**, a NEA homodimer *via* amide coupling
(*Note*: other linkers have also been used to similarly produce additional NEA homodimers).

A. Synthesis of NEO homodimers by using carbamate and thiocarbamate linkages **B. Synthesis of a NEA homodimer by using a carbamate linkage** **Fig. 52.**

Schemes for the preparation of **A.** NEO homodimers by using carbamate and thiocarbamate linkages, and **B.** a NEA homodimer by using a carbamate linkage.

A. Synthesis of a NEO homodimer by epoxide ring opening **B. Synthesis of NEA homodimers by epoxide ring opening** **Fig. 53.**

Representative schemes for the preparation of **A.** a NEO homodimer by epoxide ring opening (*Note:* a NEO-TOB heterodimer was also similarly produced), and **B.** NEA homodimers by epoxide ring opening (*Note:* NEB homodimers were also similarly produced).

Synthesis of a NEO homodimer conjugated to biotin via click chemistry

Fig. 54.

Representative scheme for the preparation of a NEO homodimer conjugated to biotin *via* click chemistry (*Note*: a TOB homodimer conjugated to biotin was also similarly produced).

Fig. 55.

Representative schemes for the preparation of **A.** NEO homodimers *via* click chemistry, **B.** arginine-linked NEO homodimers *via* click chemistry, and **C.** a NEO-peptide homodimer *via* click chemistry (*Note:* a KANA-peptide homodimer was also similarly produced).

A. Synthesis of a conformationally-restricted SIS homodimer via imine formation **B. Synthesis of a PAR homodimer linked by a quinacridine intercalator via imine formation** **Fig. 56.**

Representative schemes for the preparation of **A.** a conformationally-restricted SIS homodimer *via* imine formation, and **B.** a PAR homodimer linked by a quinacridine intercalator *via* imine formation (*Note:* analogous KANA and TOB homodimers were also similarly produced).

Synthesis of a PRM homodimer by using nucleophilic substitution reaction

Fig. 57.

Synthetic scheme for the preparation of a PRM homodimer by using nucleophilic substitution reaction.

Synthesis of a conformationally-restricted NEA by amide coupling

Fig. 58.

Synthetic scheme for the preparation of a conformationally-restricted NEA by amide coupling.

Synthesis of conformationally-restricted NEO/PAR derivatives by nucleophilic substitution reaction

Fig. S9.

Schemes for the preparation of conformationally-restricted NEO/PAR derivatives by nucleophilic substitution reaction.

A. Synthesis of conformationally-restricted KANA derivatives by nucleophilic substitution reaction **B. Synthesis of a conformationally-restricted KANA derivative by ring-closing metathesis** **C. Synthesis of a conformationally-restricted PAR derivative by ring-closing metathesis** **Fig. 60.**

Representative schemes for the preparation of **A.** conformationally-restricted KANA derivatives by nucleophilic substitution reaction, **B.** a conformationally-restricted KANA derivative by ring-closing metathesis, and **C.** a conformationally-restricted PAR derivative by ring-closing metathesis (*Note:* a conformationally-restricted PAR derivative with a 5-carbon bridge was similarly produced).

A. Synthesis of NEO pseudo-pentasaccharides via glycodiversification **B. Synthesis of 4'-O-glycoside PAR derivatives via glycodiversification** **Fig. 61.**

Schemes for the preparation of **A.** NEO pseudo-pentasaccharides, and **B.** 4'-O-glycoside PAR derivatives *via* glycodiversification.

Fig. 62.

Schemes for the preparation of **A.** PRM pseudo-trisaccharides, **B.** G418 pseudo-trisaccharides, and **C.** pyranmycin (pseudo-trisaccharide) derivatives *via* glycodiversification.

Fig. 63.
Representative scheme for the preparation of 4'-modified KANB *via* glycodiversification.

A. Synthesis of KANB derivatives via glycodiversification **B. Synthesis of amphiphilic KANB derivatives for antifungal activity** **C. Synthesis of TOB derivatives via glycodiversification** **Fig. 64.**

Scheme for the preparation of **A.** KANB derivatives, **B.** amphiphilic KANB derivatives for antifungal activity, and **C.** TOB derivatives *via* glycodiversification.

Chemoenzymatic modification of NEA, NEO, and PAR by BtrH/BtrG

Fig. 65.

Chemoenzymatic modification of NEA, NEO, and PAR by BtrH/BtrG.

Table 1

Summary of all positions modified on AGs classified by the sections of this review along with the application(s) (App(s)) of the final molecules generated.

Review section	Modification(s)	AG(s)	Position(s) modified	App(s) ^a
2.1	AG cores	ABK	5, 4''	A
		AMK	3	I
		APR	3	I
		KANA	3', 6''	D, J
		NEA	3	I
		NEO	3, 4', 6', 5'', 3''', (3', 4', 3''', 4''')	A, D, I
		PAR	3, 4', 6', (3', 4'), (3', 4', 3''', 4''')	A, D, I
		PRM	5, 6'	A, D
		RIB	3, 6'	I
		SIS	6'	I
		TOB	2'', 4'', 6''	A, D, E, J
2.2	alkylation/arylation			
2.2.1	via thioether links	KANB	6''	A, C
		PAR	(6', 6'')	A
		TOB	4', 3'', 6''	A, B, C, D, I
2.2.2	via ether links	KANB	4''	A, D
		NEA	5, 6, (3, 6'), (6, 3', 4')	A, D, E
		NEB	(5, 4'), (5, 6, 4'), (4', 6')	A, C
		PAR	4', (6', 5'')	A, D
		PRM	(3, 6'), (6, 3', 4')	A
		TOB	5, 6'', (5, 4', 2'', 4'', 6'')	A, K
2.2.3	via triazole links	NEO	5''	A
		TOB	6''	A
2.2.4	via amide links	KANA	6''	A
		NEO	5''	A
		TOB	6''	A
2.2.5	via carbamate links	AMK	(5, 2', 3', 4', 2'', 4'', 6'')	A
		KANA	(5, 2', 3', 4', 2'', 4'', 6'')	A
		NEA	(5, 6, 3', 4')	A
		NEO	(6, 3', 4', 2'', 5'', 3''', 4''')	A
2.3	acylation	ABK	4'', 6''	A
		AMK	(6', 4''')	A
		KANA	1, 6', (1, 3, 6', 3''')	A, D
		NEA	5	A
		NEO	2''', 6''', (1, 3, 2', 6', 2''', 6''')	D, I

Review section	Modification(s)	AG(s)	Position(s) modified	App(s) ^a
		NET	6'	A
		PAR	2''', 6''', (1,3,2', 2''', 6''')	D, I
		SIS	1, 6'	A, I
		TOB	1, 6',	A
2.4	AG conjugates			
2.4.1	AG bisubstrates	KANA	6'	B
		NEA	6'	B, I
		PRM	6'	I
		RIB	6'	B
2.4.2	AG-drug conjugates	ISP	6'	I
		NEA	6'	A
		NEO	5'', 6'''	A, D, E
		TOB	6''	A, B
2.4.3	AG-SM ^b conjugates			
2.4.3a	<i>via</i> thiocarbamate links	KANA	6''	E
		NEO	5''	E, F
2.4.3b	<i>via</i> triazole links	NEA	6'	N
		NEO	5''	E, F, G, L, M, N
		PAR	6'''	N
2.4.3c	<i>via</i> amide links	KANA	6''	N
		NEA	5, 4', 6', (5, 4')	E
		NEO	5'', (6', 6''')	E, F, L, M, N
		PAR	6'''	D, N
		RIB	5''	E
		TOB	6', 6''	E, O
2.4.3d	<i>via</i> nucleophilic substitution	KANA	6''	E
		NEA	3'	B
		NEO	4, 5''	E, F, G
		PAR	6', 5''	E
		TOB	6''	E, G
2.4.4	AG-peptide conjugates	GEN	(1,3,2')	E
		KANA	6'', (1,3,2', 6'')	A
		NEA	5, 6', (1,3,2', 6')	E
		NEO	5'', (1,3,2', 6', 2''', 6''')	A, D, E
		NET	6'	E
		PAR	6''', (1, 3, 2', 2''', 6''')	I
		TOB	6''	A
2.5	AG dimers	KANA	6''	A, E
		NEA	1, 4, 5, 4'	A, E, I

Review section	Modification(s)	AG(s)	Position(s) modified	App(s) ^a
		NEB	4, 6	A
		NEO	6', 5'', 6'''	A, B, D, E, F, G
		PAR	5'', 6'''	A, E, G
		PRM	3'	D, H
		SIS	1, 6'	I
		TOB	6', 6''	E
2.6	restricted AGs	KANA	(5, 2')	A
		NEA	(3, 6')	I
		NEO	(2', 5'')	B, E
		PAR	(2', 5''), (6, 6''')	B, E
2.7	glycodiversification	G418	5	H
		KANA	6	A
		KANB	4, 6	A, C, I
		NEA	5, 6, 3'	B, C, E, H, I
		NEO	5''	A, B
		PAR	6'	A, D
		PRM	5, 6, 3'	A, E
		RIB	1'', 3''	E
		TOB	6	E
2.8	chemoenzymatic	APR	3	J
		KANA	1, (1, 3, 6', 3'')	I
		KANB	(1, 3, 6', 3'')	F, I
		NEA	(1, 3, 2', 6')	F, I
		NEO	1, (3, 6'), (1, 3, 2', 6', 2''', 6''')	B, I, F

^a Abbreviations for applications: A = antibacterial, B = inhibition of AMEs, C = antifungal, D = ribosome binding, E = RNA binding, F = DNA binding, G = cellular uptake, H = PTC disorders, I = synthetic methodology, J = microarray, K = immunomodulatory properties, L = anticancer, M = bioactive cargo transporter, N = transfection efficiency, and O = biofilm inhibition.

^b SM = small molecule.

Note: when numbers are presented into parentheses it indicates that all of these positions are modified simultaneously on the corresponding AG.