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Current Opinion in Biotechnology

Biosynthetic pathways of aminoglycosides and their engineering

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Despite decades long clinical usage, aminoglycosides still remain a valuable pharmaceutical source for fighting Gram-negative bacterial pathogens, and their newly identified bioactivities are also renewing interest in this old class of antibiotics. As Nature's gift, some aminoglycosides possess natural defensive structural elements that can circumvent drug resistance mechanisms. Thus, a detailed understanding of aminoglycoside biosynthesis will enable us to apply Nature's biosynthetic strategy towards expanding structural diversity in order to produce novel and more robust aminoglycoside analogs. The engineered biosynthesis of novel aminoglycosides is required not only to develop effective therapeutics against the emerging 'superbugs' but also to reinvigorate antibiotic lead discovery in readiness for the emerging post-antibiotic era.

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Current Opinion in Biotechnology 2017, **48**:33–41

This review comes from a themed issue on **Pharmaceutical biotechnology**

Edited by **Chu-Young Kim** and **Tiangang Liu**

http://dx.doi.org/10.1016/j.copbio.2017.03.019

Introduction

Aminoglycosides (AGs) are one of the oldest classes of essential antibiotic agents. They possess two or three uncommon amino-sugars, which are attached by glycosidic bonds to the amino-substituted cyclohexane scaffold aminocyclitol (Figure 1). The first discovered AG streptomycin contains streptamine as the core aminocyclitol, whereas other pharmaceutically valuable AGs have 2-deoxystreptamine (2DOS) as the core [1–5]. The 2DOS-containing AGs are appended with amino-sugars at the C4 and C5 or C4 and C6 positions, thus yielding 4,5-disubstituted AGs (*e.g.*, butirosin and neomycin), or 4,6-disubstituted AGs (*e.g.*, kanamycin, tobramycin and gentamicin) (see Figure 1).

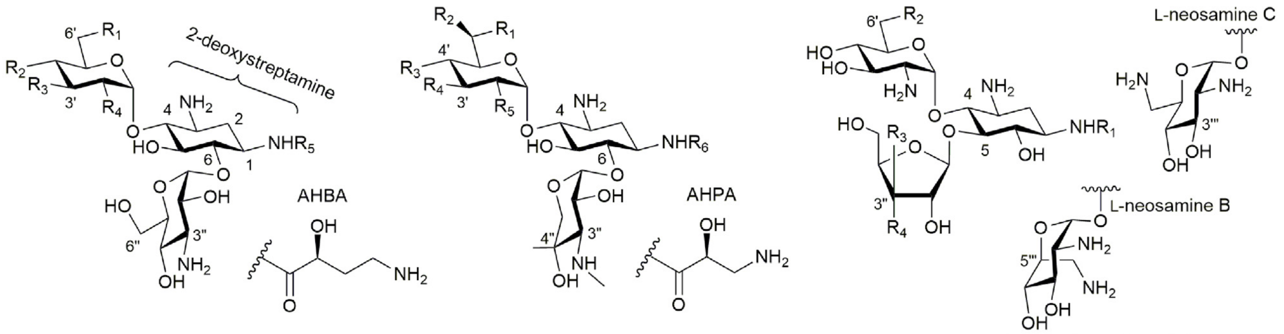
The AGs interfere with protein biosynthesis by acting on the smaller 30S subunit of the bacterial ribosome, causing bactericidal effects against the pathogens [6–8]. Moreover, their potential for the treatment of human immunodeficiency virus infection and human genetic diseases have also been recently demonstrated [9–12]. Therefore, the structural modification of AG scaffolds with diverse chemical motifs to improve or alter their activities along with the reduced toxicity can greatly expand their utility [13–15].

Widespread bacterial resistance to the AGs has limited their clinical use [6,7]. Deactivation of AGs by AG-modifying enzymes (AMEs) is the most common resistance mechanism and causes severe clinical problems [16]. Fortunately, some 2DOS–containing AGs possess natural defensive structural features active against these AMEs, including the *N*-aminoacyl moiety in butirosin (*e.g.*, 4-amino-2-hydroxybutyrate (AHBA) substituent on the C1-amine of the 2DOS core) and the 3′,4′- or 3′-deoxygenation in gentamicin or tobramycin, respectively. These naturally occurring AG modifications have thus been applied to generate second-generation semi-synthetic AGs (*e.g.*, amikacin, dibekacin and arbekacin) (see Figure 1). Despite the historical success of these conventional chemical modification approaches [17,18], biotechnological approaches such as combinatorial biosynthesis or pathway engineering can be attractive alternatives to invent structurally diverse AGs [2,19–22]. Herein, we discuss the up-to-date findings on 2DOS-containing AG biosynthetic pathways which provide the knowledge to guide rational biosynthetic efforts aimed at producing novel AG analogs, recent examples of their pathway engineering, and some pivotal advances in the development of semi-synthetic AG leads. The potential of pathway engineering for the revitalization of this old class of antibiotics are also addressed.

State-of-the-art research in aminoglycoside biosynthetic pathways

The AHBA side chain of 4,5-disubstituted butirosin is one of the critical structural traits utilized to circumvent the resistance mechanisms of AMEs, hence giving birth to semi-synthetic AGs [23]. The function of seven genes

Figure 1



	R_1	R_2	R_3	R_4	R_5
kanamycin A	NH_2	OH	OH	OH	H
kanamycin B	NH_2	OH	OH	NH_2	H
tobramycin	NH_2	OH	H	NH_2	H
amikacin	NH_2	OH	OH	OH	AHBA
dibekacin	NH_2	H	H	NH_2	H
arbekacin	NH_2	H	H	NH_2	AHBA

	R_1	R_2	R_3	R_4	R_5	R_6
gentamicin C1	$NHCH_3$	CH_3	H	H	NH_2	H
gentamicin C2	NH_2	CH_3	H	H	NH_2	H
gentamicin C2b	$NHCH_3$	H	H	H	NH_2	H
gentamicin C1a	NH_2	H	H	H	NH_2	H
isepamicin	NH_2	H	OH	OH	OH	AHPA

	R_1	R_2	R_3	R4
ribostamycin	H	NH_2	H	OH
xylostasin	H	NH_2	OH	H
butirosin B	AHBA	NH_2	H	OH
butirosin A	AHBA	NH_2	OH	H
neomycin C	H	NH_2	H	L-neosamine C
neomycin B	H	NH_2	H	L-neosamine B

Natural and semi-synthetic 2-deoxystreptamine-containing aminoglycosides. Greenish insets depict chemical features of the semi-synthetic aminoglycosides.

in the butirosin cluster involved in the biosynthesis and installation of the AHBA moiety onto butirosin have been elucidated (Figure 2a) [24,25]. These findings allowed for the incorporation of the AHBA biosynthetic machinery into the different AG biosynthetic pathways to generate novel AG analogs including 1-*N*-AHBA-neomycin B and 1-*N*-AHBA-kanamycin X [26••,27••]. Recently, it was reported that BtrE oxidizes the C-3″ of ribostamycin in the presence of nicotinamide adenine dinucleotide (NAD) to give 3″-oxoribostamycin, which is subsequently reduced by BtrF to xylostasin, completing the last step in the butirosin biosynthetic pathway [28] (Figure 2a). A radical *S*-adenosyl-L-methionine (SAM)-dependent epimerase, NeoN, was recently characterized for the epimerization of neomycin C to neomycin B [29].

The entire kanamycin biosynthetic pathway was revealed through the heterologous expression of diverse combinations of putative biosynthetic genes in a non-AG-producing *Streptomyces venezuelae* host [27••]. Interestingly, it was realized that the parallel biosynthetic pathway for kanamycins A/X and B/C was mainly governed by the glycosyltransferase (GT) KanF (also known as KanM1) which transfers both glucose and *N*-acetylglucosamine onto 2DOS (Figure 2b). On the other hand, it was reported that two unique enzymes including an α-ketoglutarate-dependent dioxygenase KanJ and a NADH-dependent reductase KanK are responsible for the deamination of kanamycin B to kanamycin A [30] (see Figure 2b). However, in order to fill up the divergence between the above *in vivo* and *in vitro* studies, further evidence on the major biosynthetic flux in the native kanamycin producer is needed.

The 3′-deoxygenation is another clinically important structural feature of natural AGs and prevents modification by the common AME AG-3′-phosphotransferase (or APH (3′)) that phosphorylates the C-3′ hydroxy group of AGs [18]. Because tobramycin is a 3′-deoxykanamycin B, the deoxygenation of paromamine (or kanamycin B) is involved in its biosynthesis (Figure 2c). *In silico* analyses suggested that the putative radical SAM enzyme AprD4 and the oxidoreductase AprD3 located in the apramycin biosynthesis gene cluster are candidates for catalyzing the deoxygenation in tobramycin/apramycin biosynthesis. The involvement of AprD4 in the C3′-deoxygenation was confirmed by gene inactivation in *Streptomyces tenebrarius* [31]. Conversion of neamine to nebramine (3′-deoxyneamine) with the cell-free extract of *S. venezuelae* harboring the *aprD3* and *aprD4* genes has also been reported [27••]. Nevertheless, the catalytic reaction mechanism for this C3′-deoxygenation process has long remained unknown. Recently, the detailed catalytic mechanism of both recombinant AprD4 and AprD3 was characterized *in vitro*. AprD4 is the first example of a radical SAM diol-dehydratase catalyzing the radical-mediated dehydration of paromamine and, along with the NADPH-dependent dehydrogenase AprD3, is responsible for C3′-deoxygenation of the pseudo-disaccharide substrate [32] (see Figure 2c). In a series of genetic and biochemical studies [33], it was found that AprD3/AprD4 pair acts on different pseudo-disaccharide substrates including paromamine and neamine but does not catalyze the deoxygenation of 3′-oxyapramycin, suggesting that 3′-oxyapramycin and apramycin are partitioned into two parallel pathways at an early biosynthetic stage. Furthermore, the catalytic versatility of the C-6′