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## IN THE BEGINNING THERE WAS STREPTOMYCIN

JULIAN DAVIES

*Department of Microbiology and Immunology<sup>1</sup>, Life Sciences Institute<sup>2</sup>, University of  
British Columbia, Vancouver BC V6T 1Z3, Canada*

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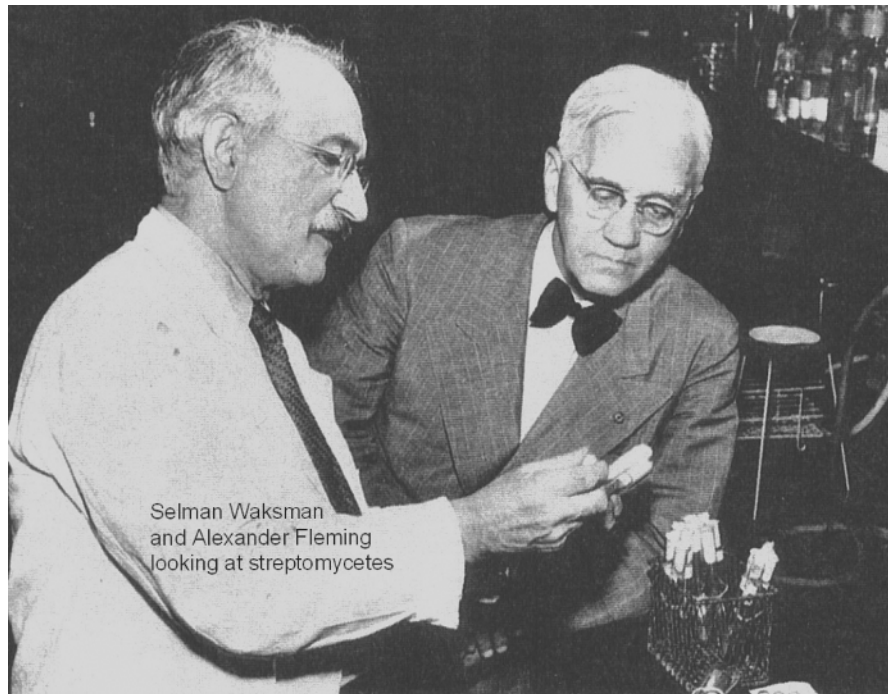
### 1.1. INTRODUCTION

Although streptomycin was not the first antibiotic (penicillin, a fungal product, had been isolated some years earlier), its discovery was a landmark in antibiotic history. It was the first effective therapeutic for tuberculosis, a disease that had terrorized humans for centuries and a cause of human morbidity and mortality unmatched by wars or any other pestilence. Streptomycin was the first aminoglycoside to be identified and characterized and is noteworthy in being the first useful antibiotic isolated from a bacterial source.<sup>1</sup> At the present time, the use

I dedicate this article to the memory of Kenneth Rinehart (1929–2005), who contributed much to structural studies and developed the mutasynthetic approach to novel aminoglycosides.

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*Aminoglycoside Antibiotics: From Chemical Biology to Drug Discovery, Second Edition*, by Dev P. Arya  
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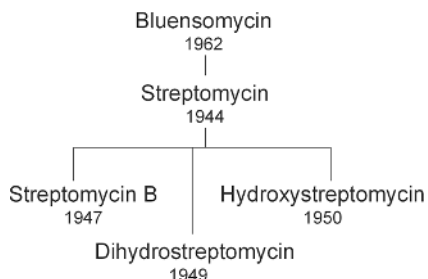


**Figure 1.1.** The founders of the antibiotic era: Selman Waksman (discoverer of streptomycin) and Alexander Fleming (discoverer of penicillin).

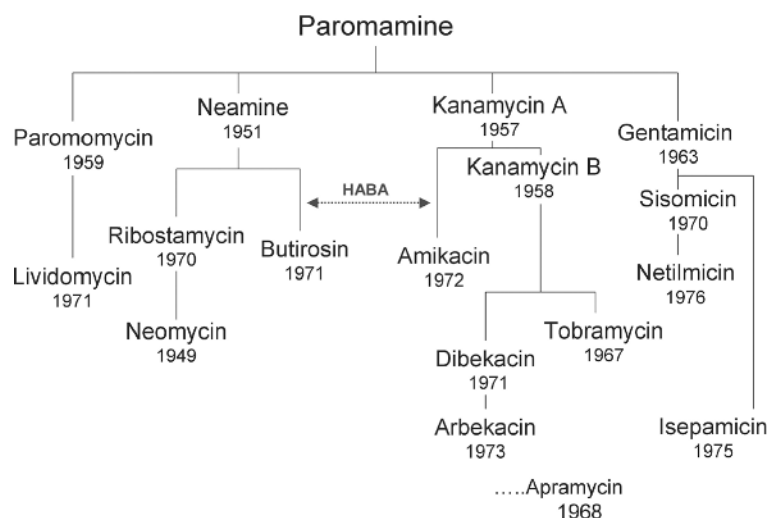
of streptomycin in infectious disease therapy has largely been replaced by less toxic and equally effective compounds, but it still has significant applications as a second-line treatment for TB and occasionally for the treatment of nosocomial multidrug-resistant gram-positive infections. Streptomycin's preeminent place in the history of antibiotics is assured!

Selman Waksman's commitment to the isolation and screening of soil bacteria in the search for bioactive small molecules, especially potential antibiotics, was validated by the discovery of streptomycin.<sup>2</sup> This led to the creation of the modern biopharmaceutical industry and the subsequent isolation of tens of thousands of bioactive small molecules from soil bacteria and other environments. A proportion of these compounds have become highly successful therapeutics, not only for all types of infectious diseases, but also in the treatment of many other human and animal ailments and as anticancer, immuno-modulatory, and cardiovascular agents.<sup>3</sup> Waksman and Fleming could be considered the fathers of chemical biology (Figure 1.1).

Following on the discovery of streptomycin and its streptamine-based relatives (Figure 1.2), a new generation of the aminoglycosides derived from 2-deoxystreptamine (DOS) was not long in coming (Figure 1.3). For a variety of reasons, many of these compounds have not been employed as human therapeutics; for



**Figure 1.2.** The structural relationships of the streptamine family of antibiotics.



**Figure 1.3.** The structural relationships of the 2-deoxystreptamine family of antibiotics.

example, neomycin has rarely been used in the clinic because of its extreme toxicity. Surprisingly, paromomycin, a naturally occurring 6'-desaminoderivative of neomycin, is receiving increasing interest in the treatment of a variety of tropical diseases, including leishmaniasis and certain types of fungal infection.<sup>4</sup> This serves to illustrate that the aminoglycosides (and the related aminocyclitols, such as spectinomycin) have a broad range of biological activities and have found use in a wide variety of applications as indicated in Table 1.1. In addition to these compounds, there is a large group of atypical aminoglycosides, compounds that are of diverse microbial origin, structure, and biological activity (Table 1.2).

Many applications of the aminoglycosides have been of historical significance in genetics and microbiology. For example, mutations to streptomycin resistance were employed as counterselective genetic markers in the historic experiments of William Hayes that demonstrated the existence of bacterial conjugation and the requirement of donor (Hfr or F+) and receptor (F-) species.<sup>5</sup>

**TABLE 1.1. Some of the Myriad Properties and Applications of the Aminoglycosides**

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Protein synthesis inhibition—prokaryotes
Protein synthesis inhibition—eukaryotes
Mistranslation on ribosomes
Nonsense mutation suppression
DNA translation
Phenotypic suppression
Membrane leakiness
Nucleic acid binding/precipitation
Probing ribosome structure
Allosteric activation of enzyme activity
Ribozyme/intron binding, inhibition, activation
Antibacterial
Antiprotozoal
Antiviral
Genetic markers for ribosome function
Broad-spectrum selective agents for gene transfer
Promoter-reporters (resistance genes)

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**TABLE 1.2. Other Classes of Aminoglycoside-Aminocyclitol Antibiotics**

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Ashimycin
Astromicin/Istamycin
Boholmycin
Kasugamycin
Myomycin
Spectinomycin
Trehalosamine
Validamycin

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These experiments showed that conjugal gene transfer occurs with directional polarity and led to the subsequent characterization of sex factors that were ultimately shown to be extrachromosomal DNA elements, or plasmids. The finding by Ruth Sager that streptomycin interferes with chlorophyll production in *Chlamydomonas* and that high-level streptomycin resistance mutants exhibit cytoplasmic rather than Mendelian inheritance (due to alteration of the chloroplast genome)<sup>6</sup> provided evidence in support of the bacterial (endosymbiotic) origin of chloroplasts in *Chlamydomonas* and plants.<sup>7</sup> In the early 1970s, kanamycin resistance (encoded by a resistance plasmid) was used as a dominant selective genetic marker for heterologous gene transfer in the seminal recombinant DNA

studies of Herbert Boyer, Stanley Cohen, and their colleagues.<sup>8</sup> The later observation that kanamycin and certain other aminoglycosides have inhibitory activity against some types of eukaryotic cells led to their application in the genetic manipulation in higher organisms, including plants.<sup>9</sup> In particular, two antibiotics have been widely used for eukaryotic gene cloning: G-418 (Geneticin™)<sup>10</sup> and hygromycin B.<sup>11</sup> G-418, related to gentamicin, has become the preferred selective agent for mammalian cell studies, and large amounts of the compound are currently employed for this purpose. The neomycin phosphotransferase gene was the first bacterial gene approved in tests of human gene therapy in 1982<sup>12</sup> and remains the genetic marker of choice for all types of eukaryotic cloning.

## 1.2. MODE OF ACTION

The biochemical mode of action of the aminoglycosides as antibacterials has long been a topic of great interest. Early experiments carried out soon after the introduction of streptomycin suggested a variety of modes of action, but these conclusions were based largely on symptomatic analyses of antibiotic-treated bacterial cultures. One important experiment done in 1948 showed that streptomycin blocks enzyme induction in susceptible bacteria<sup>13</sup>; this was the closest that anyone came to identifying the mechanism of action at the time.

A series of genetic and biochemical studies in the late 1950s and early 1960s led to the definitive identification of protein synthesis as the primary target for the antibacterial action of streptomycin. Initially, Erdos and Ullmann employed the incorporation of radioactive amino acids to show that production of labeled protein by cell-free extracts of *Mycobacterium tuberculosis* is effectively blocked by streptomycin.<sup>14</sup> The results of these experiments were subsequently confirmed by others, using defined cell-free translation systems from other bacteria and with synthetic polynucleotides as messenger RNAs. A seminal paper by Spotts and Stanier proposed the ribosome as the probable target for streptomycin action and came up with a plausible biochemical mechanism for the phenomenon of streptomycin dependence.<sup>15</sup>

Within the next few years (1962–1965) a flurry of research activity in a number of laboratories confirmed this model, and *in vitro* translation studies employing hybrids of sensitive and resistant ribosome subunits showed that streptomycin acts by binding to the 30S ribosome subunit.<sup>16,17</sup> This led to the investigation of the effects of streptomycin and other aminoglycosides on coding fidelity during translation, providing evidence for the active role of the 30S subunit in protein synthesis and the important finding that streptomycin and other aminoglycosides induce errors in translation.<sup>18</sup> These studies provided the first evidence that the ribosome is not simply an inert support in the process of peptide bond formation but plays an active role in the selection of aminoacylated tRNAs by the ribosome-bound messenger RNA.

Current work on the three-dimensional structure of ribosome complexes has amply confirmed the dynamic role of the ribosome in translation and the mechanism by which this process is perturbed by the binding of aminoglycosides to specific sites on the 30S subunit.<sup>19</sup> There is now strong genetic and phenotypic evidence for translation misreading by aminoglycosides in living cells.<sup>20</sup> While it has been shown that a number of other translation inhibitors also provoke mistranslation, this may be a symptom of protein synthesis inhibition and not a direct effect on codon reading, as with the aminoglycoside antibiotics.<sup>21</sup> Surprisingly, in the presence of some aminoglycosides, DNA can be accurately read as a messenger on the ribosome and to generate polypeptides *in vitro*; it is not known if this occurs *in vivo*.<sup>22</sup> Parenthetically, the ability of aminoglycosides to cause mistranslation has itself been applied recently to an “indirect” form of gene therapy; the administration of gentamicin or related compounds to patients with hereditary diseases such as severe hemophilia or cystic fibrosis can result in partial suppression of the disease.<sup>23,24</sup> Aminoglycoside-induced read-through of nonsense mutations leads to the production of small amounts of the missing protein and prevents nonsense-mediated decay of messenger RNA.

As is the case with most antibiotics, at subinhibitory concentrations the aminoglycosides induce significant changes in the transcription of some 5% of the genes in susceptible bacteria.<sup>25</sup> The mechanism responsible is not known but may be due to some form of coupling between translation and transcription not previously identified. We can assume that transcription modulation is associated with antibiotic activity in therapeutic use and may contribute to some of the side effects. On the other hand, at low concentrations in the environment, the aminoglycosides and other antibiotics may be acting as cell-signaling molecules.<sup>26</sup>

The use of streptomycin or spectinomycin resistance as a genetic marker was critical to the cloning and identification of the gene clusters encoding structural elements of the ribosome in bacteria. Once it had been demonstrated that resistance to streptomycin and spectinomycin is associated with amino acid changes in ribosomal proteins, bacteriophage P1 transduction studies showed that the associated genes are linked in clusters on the bacterial chromosome.<sup>27</sup> Masayasu Nomura and others then used disruption and reconstitution of ribosome particles from 16S rRNA and isolated R proteins to demonstrate the roles of the proteins RpsL (str) and RpsE (spc) in the determination of antibiotic resistance<sup>28</sup>; this confirmed the earlier genetic and phenotypic studies and ratified the role of R proteins in ribosome function. There followed a decade of argument as to the relative importance of ribosomal RNA *versus* ribosomal proteins in the structure and function of the particle, and a paradigm change occurred when it was shown by numerous sequence and functional studies that the two major rRNA molecules are the structural basis of ribosome function in translation. The fact that these RNA molecules are the targets for the binding and interaction of different antibiotics on the ribosome, resulting in interruption of the translation process, provides strong confirmation of their roles in translation. The spectacularly successful rRNA footprinting studies and X-ray structure analyses carried out by the groups of Noller,<sup>29</sup> Ramakrishnan,<sup>30</sup> and others have amply confirmed

this dominant role of rRNA and the consequences of antibiotic binding to the ribosome, initially with the aminoglycosides but subsequently with most ribosomal inhibitors. However, although the primordial template for peptide bond formation is likely to have been RNA alone, the involvement of both RNA and protein is essential in the dynamic role of the “modern” ribosome in translation; this is a topic of continuing interest.<sup>31</sup> To date, it is only in the case of streptomycin that three-dimensional structure analysis of the antibiotic/ribosome complex identifies an interaction of the drug with both R proteins and rRNA.<sup>19</sup> There is increasing evidence for the existence of nonribosomal functions of the protein components of the ribosome. Studies using antibiotics such as the aminoglycosides will undoubtedly continue to play important roles in developing this story.

During these years of exciting revelations concerning aminoglycoside activity and the ribosome, one question relative to the therapeutic use of aminoglycosides has remained unsolved. Unlike most antibiotic inhibitors of protein synthesis in bacteria that lead to bacteriostasis, the aminoglycosides are rapidly bactericidal. The ability of the aminoglycosides to kill bacterial pathogens is an important attribute in their therapeutic use. This action is somewhat surprising when we consider that most inhibitors of ribosome function act in a similar fashion to the aminoglycosides, by binding to target sequences within the 16S or 23S rRNAs (as described above). For example, the aminocyclitol spectinomycin is bactericidal in action. The difference between cidal and static action has been the topic of much discussion and many publications; this work has been largely physiological in nature, and a satisfactory biochemical explanation for the lethal action of the aminoglycosides still eludes us.<sup>32</sup> The possibility that aminoglycosides (as distinct from other translation inhibitors) induce a process of programmed cell death (apoptosis) in bacteria<sup>33</sup> could provide an explanation.

### 1.3. RESISTANCE AND AMINOGLYCOSIDE EVOLUTION

Antibiotic resistance (both endogenous and acquired) is an important determining factor in the historical development of the aminoglycosides as therapeutic agents. After streptomycin was introduced for the treatment of tuberculosis, it was found that bacterial resistance to the drug often developed; this was shown to be due to spontaneous mutants arising during the course of therapy with the antibiotic, although the biochemical mechanism was not known at the time. Kanamycin, the first useful DOS aminoglycoside, was isolated in Japan in 1957 and rapidly became an antibiotic of choice in that country. However, the appearance of strains resistant to both streptomycin and kanamycin increasingly interfered with their therapeutic use; in addition, hospital infections of *Pseudomonas aeruginosa*, a bacterium that is naturally less susceptible to antibiotics, were on the rise. A major breakthrough came with the discovery of a novel class of 2-DOS compounds, the gentamicins.<sup>34</sup> These are extremely effective antibiotics with good activity against the pseudomonads and other problem pathogens, such as *Proteus* and *Serratia* species, that were being increasingly encountered as nosocomial infections.

Gentamicin and related compounds lack the 3' OH group, and the absence eliminates the modification by phosphorylation at this site and confers activity against pathogens possessing aminoglycoside 3' OH phosphotransferases; gentamicin, being a mixture, contains one component with a modified 6' amino group and has reasonable potency against strains harboring plasmid-encoded 6' acetyltransferases that inactivate kanamycin. By this time it was known that resistance to antibiotics by enzymic modification could be acquired by plasmid transfer. Gentamicin was also effective for the treatment of staphylococcal and enterococcal infections, frequently being used in combination with a  $\beta$ -lactam antibiotic in these circumstances.

In spite of its nephrotoxicity, gentamicin was the treatment of choice for gram-negative nosocomial infections for many years, and its success led to the introduction of tobramycin, a related compound. However, novel antibiotic resistance mechanisms began to appear on the scene; of particular concern was the adenylation of the 2' OH of gentamicin and related compounds that appeared on the scene in 1971 and conferred high-level resistance to the newest generation of aminoglycosides.<sup>35,36</sup> The increasing, worldwide use of different aminoglycosides led to the appearance of many different types of resistant strains; the local use of specific classes of aminoglycoside often led to the selection of distinct local classes of resistance.<sup>37</sup>

Fortunately, the discovery of a novel DOS derivative in 1971 provided the next breakthrough. This compound, butirosin, related to ribostamycin and produced by a *Bacillus* species (not an actinomycete), inhibits a variety of aminoglycoside-resistant hospital pathogens, including those inactivating the drugs by 3' phosphorylation and 2' adenylation.<sup>38</sup> This property is due to the presence of a 4-hydroxy-2-aminobutyric acid (HABA) substituent on the 1-position of the DOS of butirosin. The latter antibiotic lacked good pharmaceutical properties, but synthetic insertion of a HABA or related group on the 1-position of the DOS of kanamycin (and subsequently of gentamicin-derived compounds) provided a novel series of potent semisynthetic aminoglycoside antibiotics with improved activity against a number of types of resistant strains. In particular, amikacin, (Figure 1.3), a kanamycin derivative with a broad spectrum of activity against resistant strains, has had considerable clinical and commercial success.<sup>39</sup> Since its discovery in 1976, no chemical modifications of substance have been reported, in spite of the fact that the spread of resistance has continued unabated and new resistance enzymes and efflux systems have appeared,<sup>40</sup> in particular a great variety of 6' acetyltransferases.<sup>41</sup> Effort has been channeled primarily to tinkering with the DOS core.<sup>42</sup>

A variety of bacterial genera have been shown to produce aminoglycoside-aminocyclitol antibiotics. These include *Streptomyces*, *Micromonospora*, *Bacillus*, and so on. Only those compounds emanating from *Streptomyces* are named "-mycins" (e.g., tobramycin) while others are "-micins" (gentamicin), "-osins," "-asins," or "-acins." The biosynthetic pathways for the aminoglycosides and the control of their expression are not well-studied. Streptomycin is the exception,

and Piepersberg's group has contributed significantly to this effort.<sup>43</sup> The intricacy of the biosynthesis is evident from the fact that upwards of 30 enzymatic steps are required for the formation of streptomycin from D-glucose.

The therapeutic use of aminoglycosides has diminished somewhat, but they are still important potent and widely used antibiotics in hospitals; most of the class are now generics. There is no question that a novel aminoglycoside derivative with demonstrated activity against the current generation of resistant pathogens, and preferably with reduced toxicity, would be a welcome addition for the treatment of infectious diseases. Attempts have been made to produce inhibitors of one or more of the aminoglycoside-modifying enzymes, and a number of different small molecule inhibitors have been described.<sup>44-47</sup> In principal, such inhibitors could be used in combination with an aminoglycoside for the treatment of resistant infections, much like the successful combination of a  $\beta$ -lactam antibiotic with a lactamase inhibitor. However, none of the inhibitors of aminoglycoside resistance enzymes have been employed in serious clinical trials. Given the increasing problems of antibiotic resistance in hospitals worldwide, it is surprising that this approach has not been pursued with more purpose.

#### 1.4. TOXICITY

As previously mentioned, another drawback limiting an expanded therapeutic use of the aminoglycosides is their toxicity, which varies in form and intensity with the different types of molecules; the main toxic responses are ototoxicity and renal toxicity.<sup>48</sup> Streptomycin and other aminoglycosides target sensory hair cells of the inner ear and can lead to hair-cell degeneration and permanent loss; this occurs by an as yet undetermined mechanism and leads to irreparable hearing loss in up to 5% of patients on extended treatment with aminoglycosides.<sup>49</sup> A variety of dosing regimens have been employed and shown to reduce the incidence of toxicity.<sup>50</sup> On the positive side, significant advances in understanding of the general mechanisms of drug-induced ototoxicity in recent years have provided important information on the genetic and structural elements of hearing loss in humans; it would appear that mutations affecting mitochondrial rRNA predispose to aminoglycoside ototoxicity.<sup>51</sup>

From a therapeutic point of view, however, relatively little effort has been put into attempts to redesign aminoglycoside structure to reduce toxic responses, probably because good *in vitro* testing models have not been available. The largely random analyses of structure–activity relationships between the inhibitory and toxicity responses of the aminoglycosides have provided few significant insights into the problem. One has the impression that, because the two responses are so closely related in structure–activity terms, a less toxic, equipotent aminoglycoside is unattainable! An interesting series of experiments on the relationship between activity against eukaryotic cells and the role of the various functional groups of the DOS aminoglycosides has provided some valuable clues concerning antibiotic/ribosome/rRNA interactions,<sup>52,53</sup> but this work has not yet been exploited

with reference to toxic responses during aminoglycoside therapy. Obviously, such information would be of great value in the design of new aminoglycosides for use as antimicrobials or in other therapeutic applications. To date, the development of semisynthetic aminoglycosides has been largely driven by the goal of finding compounds active against evolving resistant or recalcitrant bacterial pathogens.

### 1.5. CONCLUSIONS AND COMMENTS

The aminoglycosides have wide-ranging properties and are known to enhance or interfere with many cellular processes; (Table 1.1) significant and diverse research on potential medical and industrial applications is ongoing (see relevant chapters in this volume). The fact that this class of compounds interacts specifically with different types of nucleic acids has long been known and explored extensively. For example, the aminoglycosides interact with ribozymes and other forms of catalytic RNA, and the possibility of using small molecule effectors to modulate RNA reactions has been the subject of many investigations.<sup>54,55</sup> Much effort has gone into work on aminoglycoside-based inhibitors of the replication and function of viral RNA genomes.<sup>56,57</sup>

It has been suggested that bioactive small molecules, including the aminoglycosides, may have acted as naturally occurring allosteric effectors of catalytic RNAs during the “RNA world” stage of chemical evolution;<sup>58</sup> they may have been among the original riboswitches.<sup>59</sup> Aminoglycosides (and related compounds) could equally well have been involved as effector molecules in primordial DNA-based reactions; as has been mentioned, in the presence of aminoglycosides, DNA directs polypeptide synthesis on ribosomes.<sup>22</sup>

Finally, it should be clear to the reader that the aminoglycosides are a biologically and chemically diverse class of molecules with great therapeutic potential. Their exploitation as molecular tools in chemical biology applications is of continuing interest. The structures of these relatively simple natural products can be manipulated synthetically and, in principle, by methods of combinatorial biology. The latter molecular genetic-based approach has been successfully applied to achieve novel structural modification of the polyketides<sup>60</sup> and nonribosomal peptides.<sup>61</sup> Since the biosynthetic gene clusters of a number of aminoglycosides have recently been cloned and sequenced,<sup>62</sup> the stage is set for the use of molecular genetic approaches to develop aminoglycoside biology in greater depth. This approach holds great promise for the discovery and development of novel molecules that are not readily available by chemical synthesis. The aminoglycosides are still very much alive!

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