Design of Small Molecules That Recognize RNA: Development of Aminoglycosides as Potential Antitumor Agents That Target Oncogenic RNA Sequences**

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Aminoglycosides are a structurally diverse family of aminosugar and aminocyclitol derivatives that are currently the only well-characterized class of small molecules that selectively bind RNA,[1] including sites on prokaryotic 16S rRNA which is believed to be the locus of aminogylcoside antibiotic activity.[2] Of particular relevance to our own studies were the recent observations that aminoglycoside–RNA interactions could be designed and could potentially be used to modify gene expression.[3] These results suggest that methods for synthesizing libraries of aminoglycosides or aminoglycoside mimetics may have wide application for inhibiting oncogenic RNA transcripts. We have chosen the breakpoints of the Bcr–Abl and PAX3–FKHR translocations as model mRNA sequences for studying aminoglycoside–mRNA interactions (Figure 1).

Figure 1. Biotinylated 5′-monophosphorotioates mimicking the break points of the Bcr–Abl (A) and PAX3–FKHR gene translocations (B).

Bcr–Abl was the first oncogenic fusion protein to be discovered in the late 1980s. It is expressed in nearly all cases of chronic myelogenous leukemia, and in 25% of patients with acute lymphocytic leukemia.[4, 5] Wild-type Bcr and Abl are signal transduction proteins. Wild-type Abl is a tyrosine kinase apparently involved in response to DNA damage, cell cycle progression, and cellular adhesion. Bcr is a serine/threonine kinase which is believed to be involved in signal transduction.[6] The chimeric protein Bcr–Abl is a constitutively active signaling molecule which switches on many pathways by phosphorylating several different proteins, such as Ras,[7] resulting in uncontrolled cellular growth.

The other oncogenic fusion protein, PAX3–FKHR, was discovered in a usually fatal pediatric cancer, alveolar rhabdomyosarcoma.[8] In this translocation, the 5′-domain of one transcription factor, PAX3, is fused to the 3′-domain of another transcription factor, FKHR.[9] This generates a new chimeric transcription factor which contains the DNA-binding domain of PAX3 and the potent transcription activation domain of FKHR. The chimeric transcription factor is essentially hyperactive and leads to overexpression of many proteins.

As previously described, Bcr–Abl and PAX3–FKHR arise from chromosomal translocations.[4, 5, 9] Translocations such as these are ubiquitous in many leukemias and some solid tumors. Therefore, these sequences can serve as a model for the inhibition of many other oncogenes. Importantly, the breakpoints of gene fusions are unique sequences not found in the wild-type genes and therefore are a specific target sequence. Based on previous successes with antisense agents, it has been shown that inhibiting expression of these oncogenes inhibits the growth of the cancer.[10] Thus, it is reasonable that similar results can be achieved with an aminoglycoside-based approach, that is, by using aminoglycosides as anticancer agents.

Biotinylated Bcr–Abl and PAX3–FKHR (Figure 1) were prepared chemoenzymatically with a previously reported method.[11] DNA templates for Bcr–Abl and PAX3–FKHR were transcribed in vitro with T7-polymerase-catalyzed transcription in the presence of guanosine 5′-monophosphorothioate to yield the respective 5′-phosphothioate. A biotin–iodoacetamide linker was used to biotinylate the 5′-phosphothioate of the RNA. The biotinylated RNAs were purified by polyacrylamide gel electrophoresis (PAGE) and immobilized on a streptavidine-coated surface plasmon resonance (SPR) sensor chip.

In order to establish that aminoglycosides may be good candidates for binding Bcr–Abl and PAX3–FKHR RNAs, the K_d values of several commercially available aminoglycosides (Figure 2) were determined by using SPR spectroscopy.[11] The results are shown in Table 1. The binders showing the highest affinity were the 4,5- and 4,6-linked deoxystreptamines. The order of binding was as follows: neomycin B > paromomycin = tobramycin > kanamycin = gentamicin.
These 4,5- and 4,6-linked aminoglycosides bind Bcr–Abl and PAX3-FKHR RNAs in the low micromolar range. Neamine, the simplest aminoglycoside, showed a K_d value of 25 μM for binding to Bcr–Abl and PAX3-FKHR RNAs. It was somewhat surprising that ribostamycin, neamine 5-O-linked with ribose, showed a fourfold lower binding affinity to the RNAs than neamine. This result suggests that the aminoglycoside–RNA binding was sensitive to modification at the 5-position of neamine and that it involved more than a simple electrostatic interaction.

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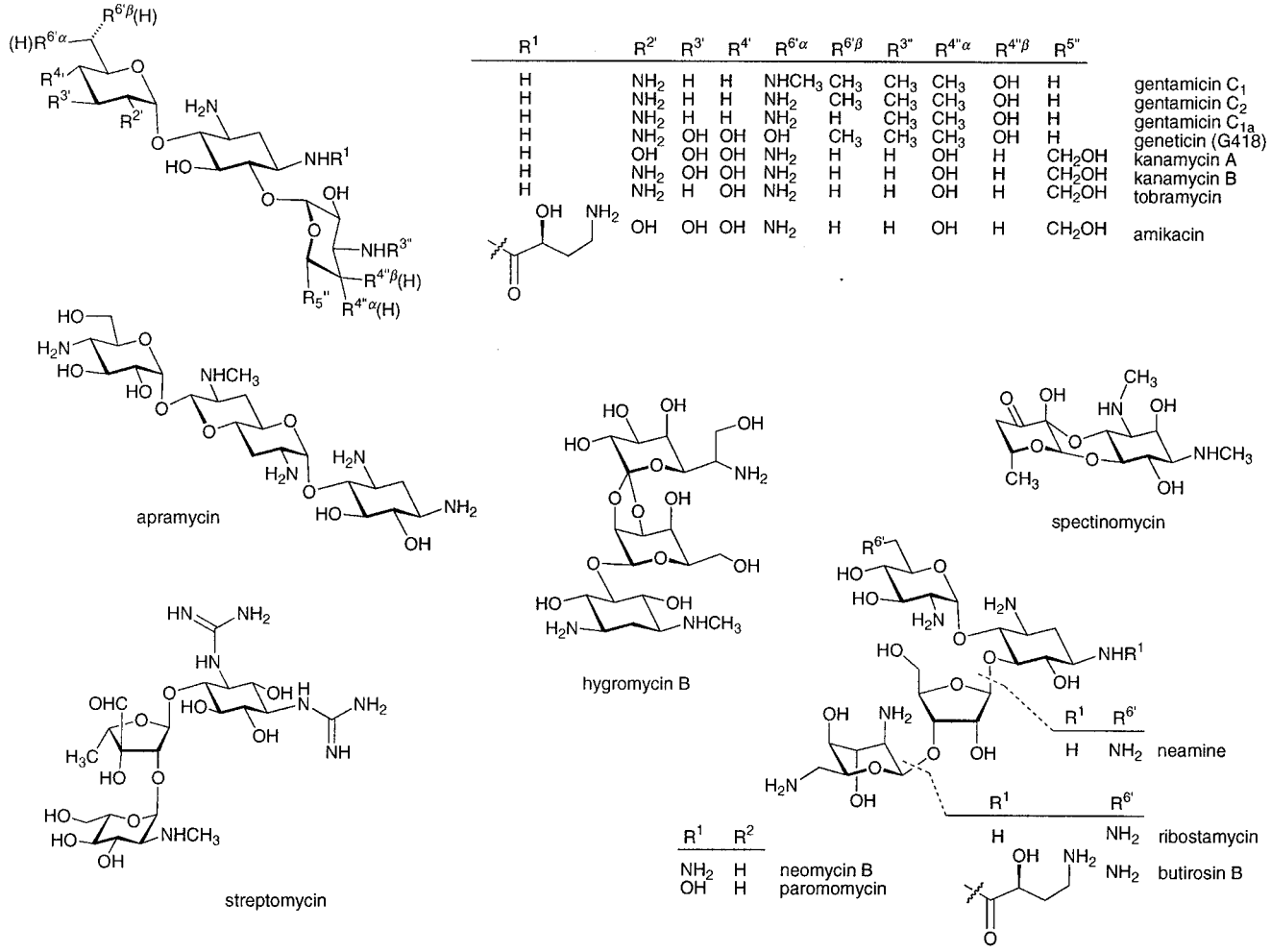


Figure 2. The structures of various commercially available aminoglycosides.

Table 1. Determination of approximate K_d values for naturally occurring aminoglycosides binding to Bcr2–Abl2 and PAX3–FKHR mRNAs.[a]

Compound	K_d [μM] (Bcr–Abl)	K_d [μM] (PAX3–FKHR)
neomycin	1.5	1.5
paromomycin	1.7	1.8
kanamycin B	2.5	5.0
tobramycin	3.6	1.8
gentamicin	3.0	6.0
apramycin	20	20
ribostamycin	80	90
kanamycin A	10	20
amikacin	10	15
neamine	17	20
butirosin B	15	25
geneticin (G418)	20	30
streptomycin	20	25
hygromycin B	>100	>100
spectinomycin	>100	>100

[a] Solution conditions: 150 mM NaCl, 10 mM HEPES (pH 7.4), 3.4 μM EDTA.

We then investigated the RNA-binding properties of synthetic aminoglycoside libraries that use neamine as a core RNA-binding motif. Neamine was chosen as a core structural element because the motif was found to be conserved in the naturally occurring aminoglycosides that bind the Bcr–Abl and the PAX3–FKHR RNAs with the highest affinity. Furthermore, neamine contains the β-hydroxyamine motif which is commonly found in aminoglycosides and essential for interaction with the phoshodiester group and the Hoogsteen face of guanine in RNA (Figure 3).[12]

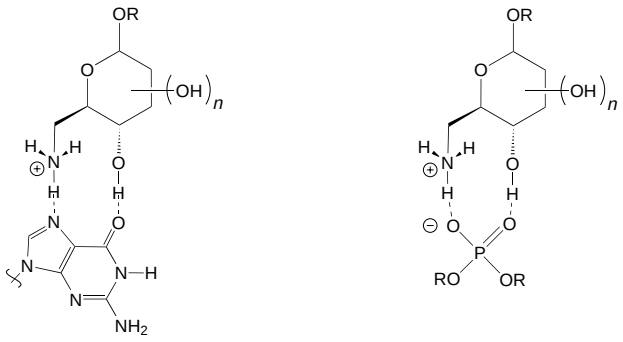
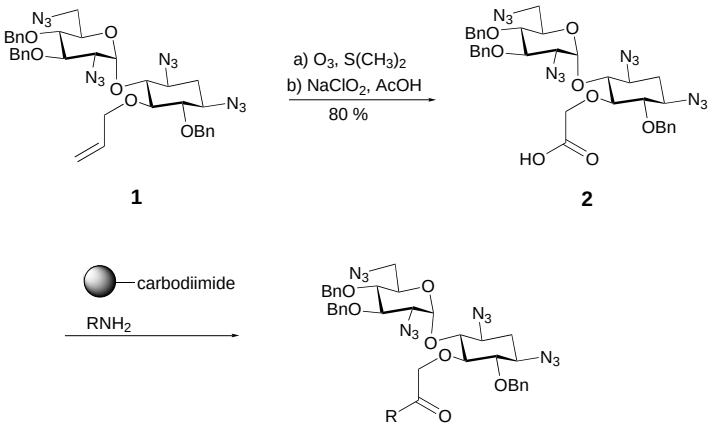


Figure 3. Proposed mode of complexation for hydroxyamines with phosphodiesters (right) and the Hoogsteen face of purines (left; guanine is shown as an example).

To exploit aminoglycosides as small-molecule inhibitors of translation, methods for preparing large libraries of aminoglycoside mimetics were necessary. The protected 5-*O*-carboxyethylneamine **2** was synthesized from a protected

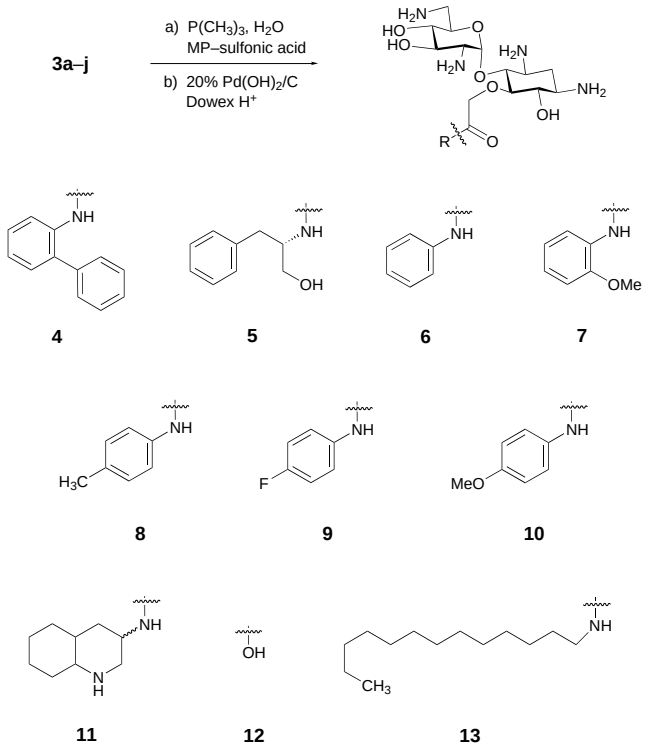
and allylated neamine (**1**) by a two-step oxidation procedure (Scheme 1). Attempts to prepare the acid **2** directly by oxidation of the allylated neamine **1** with $RhO_2/NaIO_4$ were only modestly effective. The allylated neamine **1** was easily oxidized to an intermediate aldehyde with ozone followed by reductive workup. The crude aldehyde was oxidized to the acid **2** with sodium chlorite in carbon tetrachloride/acetic acid (1:1). The carboxyethylneamine **2** was used as a core starting material for parallel synthesis.



3a R = 2-aminobiphenyl
3b R = (*S*)-2-amino-3-phenylpropanol
3c R = aniline
3d R = *o*-anisidine
3e R = *p*-toluidine
3f R = 4-fluoroaniline
3g R = *p*-anisidine
3h R = 3-aminoquinoline
3i R = 2-aminopyridine
3j R = tetradecylamine

Scheme 1. Parallel synthesis of a 5-*O*-alkylated neamine library prepared by the resin-bound activated ester. Bn = benzyl.

The carboxyethylneamine **2** was distributed into a parallel synthesizer (Quest 210) and activated with cyclohexylcarbodiimide bound to a macroporus polystyrene (MP) resin. A ratio of two equivalents of resin to one equivalent of acid and 0.8 equivalents of amine was used to synthesize a library of protected neamine amides. Various aromatic and aliphatic amines were coupled to the neamine core through the resin-bound activated ester to form neamine amides **3a–j** (Scheme 1). The amides **3a–j** were isolated by filtration and were >95% pure, as determined by NMR spectroscopy. The amides were deprotected by a two-step reduction. Simultaneous reduction of the azides and benzyl protecting groups was not feasible by hydrogenolysis owing to the formation of complex product mixtures. Therefore, the azides were reduced under classical Staudinger conditions. The resulting amines were captured from solution with a resin-bound sulfonic acid scavenger (MP-TsOH; Ts = toluene-4-sulfonyl). Impurities were removed by washing and the free amine was released from the resin by elution with 2 M NH_3 in methanol (Scheme 2). The amines were pure, as determined by TLC analysis, and were subjected to hydrogenolysis with 20% $Pd(OH)_2$ on carbon under an H_2 atmosphere. The reaction mixtures were filtered, concentrated, and purified by



Scheme 2. Catch-and-release deprotection/purification strategy for the synthesis of aminoglycoside libraries (e.g., **4–13**).

cation exchange chromatography to provide the aminoglycosides **4–13** (Scheme 2).

Several other aromatic and heteroaromatic analogues of neamine were prepared by reductive amination. Benzimidazole as well as several isomeric aminoquinolines were chosen because of their potential ability to intercalate or form hydrogen bonds with nucleotides. The allylated neamine **1** was oxidized to an aldehyde as previously described[13] (Scheme 3). The intermediate aldehyde was used for reductive aminations with several different heteroaromatic amines. The azide protecting groups were reduced with hydrazine and Raney nickel followed by debenzylation with 20% $Pd(OH)_2$ on carbon to provide aminoglycosides **14–19**. The aminoquinolines **14–18** were partially reduced; however, these mixtures were separable by ion exchange chromatography in most cases.

Also included in the study were the aminoglycoside mimetics **20–26** (Figure 4). These congeners contain various polyamines varied at the 5-*O*-position of neamine. They can be expected to enhance aminoglycoside–RNA binding through hydrogen bonding or electrostatic interactions with the negatively charged nucleotides. These analogues were prepared as previously reported.[13]

The compounds with the highest binding affinity were the polyamine analogues **20–26** and the 6-aminoquinoline **14** which consisted of a 5:4:1 phenylene diamine/pyridine/quinoline mixture (Table 2). Aminoglycoside binding and selectivity was also modulated when the aromatic groups at the 5-*O*-position of neamine were varied for compounds **4–13**. The *p*-anisidine analogue **10** showed a fivefold preference for the Bcr–Abl transcript compared to PAX3–FKHR RNA. Sev-

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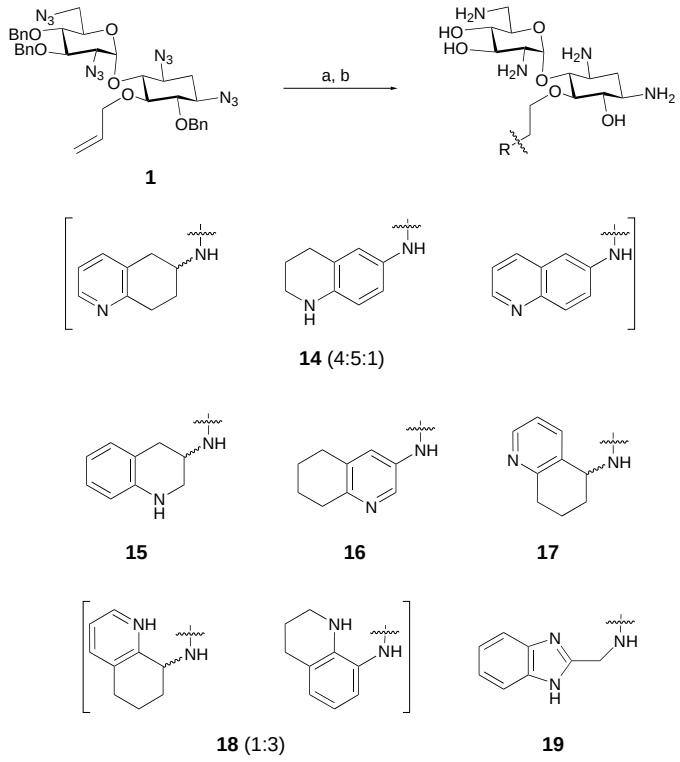

Scheme 3. Parallel synthesis of a 5-*O*-alkylated neamine library (**14**–**19**) prepared by reductive amination. a) 1. O_3, $S(CH_3)_2$; 2. amine, AcOH, $NaCNBH_3$, 51–75%; b) 1. Raney nickel, N_2H_4, EtOH; 2. H_2 (1 atm), 20% $Pd(OH)_2$/C, AcOH/H_2O, 23–54%.

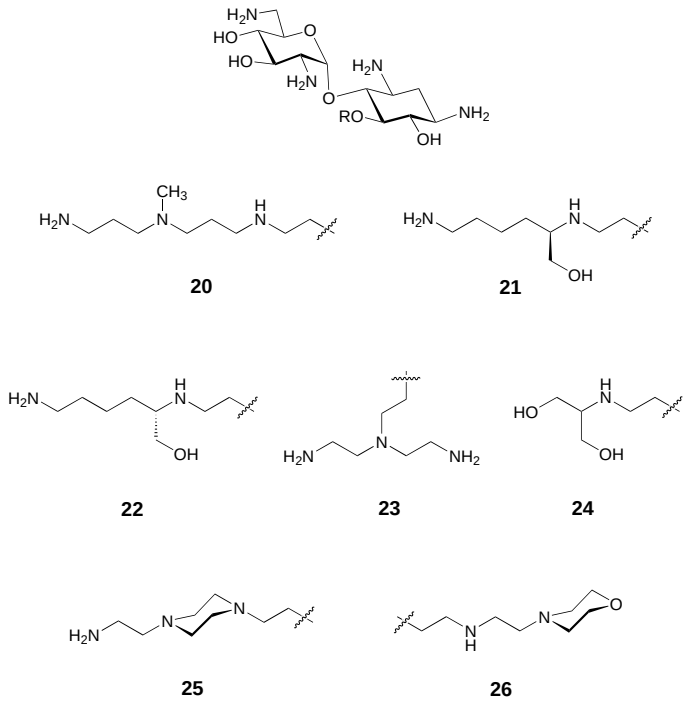

Figure 4. A 5-*O*-alkylated neamine library (**20**–**26**) based on polyamines.

eral of the aminoglycosides studied showed nanomolar binding affinities with RNA. The 6-aminoquinolines showed the highest preference for the Bcr–Abl transcript, eightfold over PAX3–FKHR. The additional binding for these compounds

Table 2. Determination of approximate K_d values for aminoglycoside mimetics binding to Bcr2–Abl2 and PAX3–FKHR mRNAs.[a]

Compd	K_d [μM] (Bcr2–Abl2)	K_d [μM] (PAX3–FKHR)
4	10	7.2
5	26	8.7
6	5.0	1.7
7	20	8.0
8	5.0	3.0
9	10	2.5
10	5.0	1.0
11	5.0	6.0
12	> 100	> 100
13	6.7	6.0
14	0.52	4.0
15	2.3	4.8
16	2.1	2.6
17	3.2	5.0
18	18	11
19	5.1	6.8
20	0.36	0.50
21	0.61	1.0
22	0.70	1.0
23	1.1	2.0
24	5.8	9.0
25	13	11
26	7.0	5.7

[a] Solution conditions: 150 mM NaCl, 10 mM HEPES (pH 7.4), 3.4 μM EDTA.

can be partially explained as arising from enhanced electrostatic interactions or, in the case of the aminoquinolines, through intercalation with RNA. However, the increase in binding was not the same for both RNA sequences studied. The greater selectivity exhibited by the 6-aminoquinolines for binding to Bcr–Abl may result from an interaction with a specific conformation or structural domain in that sequence. Notably, the GC content for both sequences is quite similar, 54% and 51% for Bcr–Abl and PAX3–FKHR, respectively, and the sequences are of similar length. Therefore, it is unlikely that the observed preference for aminoglycoside **14** binding Bcr–Abl results from a difference in the nucleotide composition of the RNA. It is likely, however, that the difference in binding selectivity for Bcr–Abl results from the high degree of secondary structure in Bcr–Abl, while the PAX3–FKHR sequence has significantly less secondary-structure content based on structure calculations.

These results demonstrate for the first time that aminoglycosides act as high-affinity small-molecule ligands for the oncogenic Bcr–Abl and PAX3–FKHR single-stranded mRNA breakpoints. Based on the affinities of these synthetic aminoglycosides for RNA, it is reasonable to believe that aminoglycosides can potentially act as regulators of gene expression. The future direction of this work should focus on validating the use of aminoglycosides as inhibitors of gene expression in cell culture and on generating larger libraries of aminoglycosides in order to find more highly selective ligands for target mRNAs.

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New Selenium-Based Safety-Catch Linkers: Solid-Phase Semisynthesis of Vancomycin**

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The generation of molecular diversity through solid-phase combinatorial chemistry has become an important component of the drug discovery process.[1] An enduring challenge in the design of solid-phase synthetic sequences is the selection of an appropriate linker[2] that is stable to the proposed chemistry, yet which can be readily and practically cleaved. Solid-phase synthesis of natural products offers a particularly good platform to evaluate linkers because of the multitude of functionality often present. Vancomycin[3] (**1**, Scheme 1), renowned for its activity against methicillin-resistant *Staphylococcus aurus* (MRSA), has been used for the past forty years to treat Gram-positive bacterial infections. The emergence of vancomycin-resistant *enterococci* strains (VRE) has raised serious health concerns and prompted a renewed vigor in the field of glycopetide antibiotics.[3, 4] Researchers from Eli Lilly have demonstrated that modificat

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