Decades of widespread clinical use of the bacterial ribosome A-site-targeting aminoglycosides (AGs) enhanced the evolution of resistance to these antibiotics and reduced their clinical efficacy. Three modes of action lead to bacterial resistance to AGs: reduction in the intracellular concentration of the antibiotics by efflux pump proteins or through reduced membrane permeability; structural modifications of the 16S ribosomal RNA that lead to reduced target affinity; and deactivation by AG-modifying enzymes (AMEs). AMEs are divided into three families: AG nucleotidyltransferases (ANTs), AG phosphotransferases (APHs), and AG acetyltransferases (AACs).

In many cases, AG-resistant bacteria have evolved combinations of resistance mechanisms, a fact that greatly increases the challenge of regaining their clinical efficacy through semisynthetic modifications. In recent years, several studies demonstrated the potential of exploiting AGs for the development of cationic amphiphilic antimicrobial agents by converting part or all of their pseudo-oligosaccharide alcohols into alkyl or aryl ethers. Some of these amphiphilic analogues demonstrated improved activities against several bacterial strains with resistance to the parent AG antibiotics. In addition to AG-based amphiphiles, several families of cationic amphiphiles including cationic steroids (ceragenins) as well as cationic antimicrobial peptides and peptidomimetic compounds have been developed and were found to possess potent antimicrobial activity. Unlike most mammalian cell membranes, bacterial membranes are rich in negatively-charged lipids, such as cardiolipins and phosphatidylglycerol, which attract cationic amphiphiles through ionic interactions, a fact that may be utilized for selective targeting of bacterial membranes.

Herein we report the design, synthesis, and antibacterial activity of 18 cationic amphiphiles (4a–r) derived from tobramycin (TOB; Scheme 1A), which is a clinically important AG antibiotic that is becoming increasingly compromised by bacterial resistance. We also provide evidence for the mode of action of these derivatives and for the structural requirements for targeting bacterial membranes compared to targeting membranes of red blood cells (RBCs).

We chose to modify the 6′ thioether TOB derivatives focused on two groups of lyophilic substituents: 1) aliphatic moieties including linear alkyl chains ranging from 6 to 22 carbon atoms in length as well as branched and cyclic alkyls, and 2) substituted aryl rings. The five amino groups of TOB were protected by Boc groups and the 6′ thioether TOB derivatives were selectively converted to the corresponding O-trisyl leaving group to provide compound 2 as reported (Scheme 1A). Compound 2 was then reacted with each of the 18 aliphatic and aromatic thiols resulting in the Boc-protected compounds 3a–r in yields ranging from 57 to 94%. Removal of all Boc protecting groups in neat TFA gave the TFA salts of the 6′-thioether TOB derivatives 4a–r with no need for further purification in yields ranging from 74 to 98%.

Compounds 4a–r were screened for their antibacterial activity against 21 Gram-positive and Gram-negative bacterial strains, and their minimum inhibitory concentrations (MICs) were determined (Table 1). Amongst the Gram-positive bacteria were pathogenic strains such as methicillin-
resistant Staphylococcus aureus (MRSA; strain C) and vancomycin-resistant Enterococcus (VRE; strain J) that displayed high levels of resistance to TOB (MIC $\geq$ 150 $\mu$g mL$^{-1}$). Amongst the Gram-negative were three strains of E. coli BL21 (DE3) that we cloned with AMEs: the bifunctional AAC(6’)/APH(2’), AAC(3)-IV, and the multi-acetylating Eis, an AAC that confers high levels of resistance to AGs in extensively drug-resistant (XDR) strains of Mycobacterium tuberculosis (Mtib)$^{[9]}$ (strains O, P, and Q, respectively). These three strains had significant to high levels of resistance to TOB (MICs $> 150$, 150, and 18.8 $\mu$g mL$^{-1}$, respectively).

The analogues with linear aliphatic chains 4a-h exhibited a parabolic pattern of chain-length-dependent antibacterial activity (Figure S45A in the Supporting Information). Compared to the antibiotic TOB, both the C$_6$ and C$_8$ chain analogues 4a and 4b demonstrated a dramatic loss of antibacterial activity, the C$_{10}$ chain derivative 4c regained some activity, and the C$_{12}$ chain analogue 4d demonstrated potent antibacterial activity against several of the strains with resistance to TOB. The greatest improvement in antibacterial activity was observed for the C$_{14}$ and C$_{16}$ chain derivatives 4e and 4f, with the most significant effect for 4e, which showed marked activity against all of the 21 tested strains. The MIC values of 4e ranged between 0.3 to 18.8 $\mu$g mL$^{-1}$ against 19 of the 21 tested strains: the exceptions were E. faecalis (K) and S. enterica (U) where only a limited improvement in the antibacterial activity of 4e as compared to TOB was observed (MIC of 4e: 75 and 37.5 $\mu$g mL$^{-1}$, respectively, and for TOB: 150 $\mu$g mL$^{-1}$). The antibacterial activity dropped again for the C$_{18}$ and the C$_{22}$ chain analogues 4g and 4h. With few exceptions, a general drop in the antibacterial activity was observed for the 6’ aromatic thioethers (4k-r). The more substitution around the aryl ring, the more significant was the loss of antibacterial activity against the tested strains. For example, of the aromatic thioether analogues, 4k with the thiophenyl ring, and 4l with the 4-methyl-thiophenyl ring, demonstrated the best overall antibacterial activities against the tested strains. However, a drop in antibacterial activity was observed for the 2.6-dimethyl-thiophenyl derivative 4m, and a more significant drop was observed for the 2,4,6-trimethyl-thiophenyl analogue 4n. Since thioethers may be susceptible to cellular mediated S-oxidation,$^{[9]}$ we oxidized two of the most potent thioethers (4d-e) to diastereomeric mixtures (ca. 4:1 ratio) of the corresponding sulfoxides (5d-e) and to the sulfones (6d-e; Scheme 1B). The effect of S-oxidation on the antibacterial activity varied amongst the tested bacterial strains. A reduction in the antibacterial activity of both sulfoxide and sulfone analogues as compared to the parent thioethers was observed for the E. coli BL21 (DE3) strains M-Q and for B. subtilis 168 with AAC(6’)/APH(2’)-pRB374 (strain G). In contrast, when tested against E. faecalis (K) and L. monocytogenes (L), all four S-oxidized analogues demonstrated improved antibacterial activities compared to those of the thioethers 4d-e. For most of the tested strains, S-oxidation did not have a dramatic effect on the antibacterial activity with MIC values identical or one double dilution higher than those of the thioethers 4d-e. In most of the tested strains, MIC values were identical for the sulfoxides (5d-e) and the corresponding sulfones (6d-e), thus indicating that the level of S-oxidation has little to no effect on antibacterial activity.

To uncover the reasons for the broad spectrum and improved antimicrobial activity of some of the thioether analogues, we performed several biological tests. The effect of compound 4e on the translation of a luciferase reporter gene was measured in E. coli cell lysates. In lysates of Mycobacterium smegmatis and E. coli, previous studies reported that the antibiotic TOB inhibited luciferase translation at IC$_{50}$ values of approximately 20 nm.$^{[10]}$ In our E. coli cell lysate, TOB potently inhibited translation (IC$_{50}$ = (8.9 ± 1.9) nm), whereas 4e did not reach an IC$_{50}$ value even at 147 nm (measured using the free base forms of TOB and 4e), thus suggesting that this compound does not target the bacterial ribosome as its major mode of antibacterial activity.

Furthermore, time-of-kill assays performed on S. mutans UA159 (E) and S. pyogenes (D) revealed that 4e rapidly conferred bacterial cell death as compared to TOB (Figure S45B in the Supporting Information). At MIC values of 2.3 $\mu$g mL$^{-1}$ for 4e on both strains (75 $\mu$g mL$^{-1}$ (S. mutans) or
<table>
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**Note:** MIC values are given in μg/mL and represent the minimum concentration required to inhibit bacterial growth.

**References:**

1. Angew. Chem. 2012, 124, 5750–5754

**Table 1: Antibacterial activity: MIC values (μg/mL) of the thioether TOB derivatives 4a–7 and 6d–e compared to the parent drug TOB.**
(C16 chain) caused (26.3 ± 1.9)% hemolysis of rat RBCs and
(8.0 ± 0.8)% hemolysis of human RBCs. At 18.8 μg mL⁻¹
compound 4h (C22 chain) caused (4.4 ± 0.5)% hemolysis of
rat RBCs and no measurable hemolysis of human RBCs.
Although compound 4f with the C16 chain was one of the
most active TOB analogues against the tested bacterial
strains, it readily disrupted RBC membranes as well. In
contrast, compound 4d (C12 chain) demonstrated potent
antimicrobial activity against several of the TOB-resistant
bacterial strains and caused little to no measurable hemolysis
at the tested concentrations. The hemolysis assay demon-
strated that the length of the aliphatic chain plays a key role in
selective targeting of the bacterial membranes versus those of
RBCs.

Several amphiphilic AGs based on paromomycin were
recently shown to act as inhibitors of the AME APH(3)\(^{\text{a}}\).
Moreover, amphiphilic AGs can make their way into the
bacterial cell where they are exposed to enzymatic modifica-
tions by AMEs. Modifications by AMEs (O-phosphorylation,
O-adenylation, and N-acetylation) result in a reduction of the
overall positive charge of the parent AG. Hence, modifica-
tions by AMEs may reduce the affinity of amphiphilic AGs to
the negatively-charged bacterial membrane and hamper their
antimicrobial activity. Structural information obtained from
crystallographic studies of several AMEs indicated that the
AG binding pocket is rich in negatively-charged amino acid
residues, such as glutamic and aspartic acids, and that several
water molecules are required to stabilize the interactions
between AGs and the AMEs' binding pockets. We reasoned
that the replacement of the 6'-primary alcohol of TOB with hydrophobic residues would interfere with these
hydrophilic binding interactions and reduce the ability of the
enzymes to modify these molecules. To test this hypothesis,
the relative activities of seven different AME\(^{\text{b}}\) with
compounds 4a–r as substrates were compared to that with
TOB as substrate (Figure 3). While some of the modified
compounds served as better substrates for some AMEs, in
general a drop in the catalytic activity of the AMEs was
observed in most of the cases. Analogues 4d–f, which
demonstrated the most potent antimicrobial activities, were
also the poorest substrates for all of the tested AMEs.

Hence, bacterial strains that contain the tested AMEs will
have limited to no ability to inactivate analogues 4d–f
through chemical modifications that are catalyzed by these
enzymes. This inability is also true for the S-oxidized
compounds 5d–e and 6d–e, which behaved similarly to their
non-oxidized counterparts 4d–e (Figure S46 in the Support-
ing Information).

In conclusion, 18 novel 6'-thioether TOB analogues (4a–
r) and four S-oxidized compounds (5d–e and 6d–e) have been
synthesized and screened for antibacterial activity. The
analogues 4d–f, with linear C12, C16, and C18 chains, demon-
strated potent activity against bacterial strains with high levels of
resistance to TOB. We found evidence that the most potent
analogue 4e targets bacterial membranes and no longer
targets the ribosome as does the parent drug. Hemolysis tests
indicated that it is possible to improve the antibacterial
activity while reducing the undesired hemolytic effect by
altering the length of the aliphatic chain. Finally, thioethers

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**Figure 1.** Bright-field and epi-fluorescence microscopy. *B. subtilis* (PY79) cells carrying the gene for the yellow fluorescent protein (YFP) under an inducible isopropyl-β-d-thiogalactoside (IPTG) promoter were treated with TOB at 2.3 μg mL⁻¹ (2x MIC) and 9.4 μg mL⁻¹ (8x MIC) or with compound 4e at 4.7 μg mL⁻¹ (2x MIC) and 18.8 μg mL⁻¹ (8x MIC).

**Figure 2.** Hemolysis tests. Human RBCs and rat RBCs were incubated with TOB or with analogues 4b–h at concentrations of 18.8 μg mL⁻¹ or 75 μg mL⁻¹ for 1 h at 37°C.

6.0)% hemolysis of human RBCs. A significant drop in the
hemolytic activity was observed for compound 4h (C22 chain),
which caused hemolysis of (24.4 ± 5.8)% of rat RBCs and
(7.1 ± 0.1)% of human RBCs.

At 18.8 μg mL⁻¹, TOB and analogues 4b–d with the linear
C6, C10, and C12 chains caused no measurable hemolysis of
both rat and human RBCs, and compound 4e (C14 chain)
causd (19.5 ± 0.3)% hemolysis of rat RBCs and (14.3 ± 1.7)% hemolysis of human RBCs. Compound 4f (C16 chain)
demonstrated the maximal hemolytic effect of (40.4 ± 1.7)%
(rat RBCs) and (25.4 ± 2.1)% (human RBCs), while 4g

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