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Amphiphilic tobramycin-lysine conjugates sensitize multidrug resistant Gram-negative bacteria to rifampicin and minocycline

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Abstract

Chromosomally-encoded low membrane permeability and highly efficient efflux systems are major mechanisms by which *Pseudomonas aeruginosa* evades antibiotic actions. Our previous reports have shown that amphiphilic tobramycin-fluoroquinolone hybrids can enhance efficacy of fluoroquinolone antibiotics against multidrug-resistant (MDR) *P. aeruginosa* isolates. Herein, we report on a novel class of tobramycin-lysine conjugates containing an optimized amphiphilic tobramycin-C12 tether which sensitize Gram-negative bacteria to legacy antibiotics. Combination studies indicate the ability of these conjugates to synergize rifampicin and minocycline against MDR and extensively-drug resistant (XDR) *P. aeruginosa* isolates and enhance efficacy of both antibiotics in the *Galleria mellonella* larvae *in vivo* infection model. Mode of action studies indicate that the amphiphilic tobramycin-lysine adjuvants enhance outer membrane cell penetration and effect the proton motive force which energize efflux pumps. Overall, this study provides a strategy for generating effective antibiotic adjuvants that overcome resistance of rifampicin and minocycline in MDR and XDR Gram-negative bacteria including *P. aeruginosa*.

INTRODUCTION

The global health crisis caused by the resurgence of multidrug resistant bacteria strains, once believed to have been defeated, have called for an urgent need to rethink the principle of antibacterial drug discovery, and the judicious deployment of our current arsenal.[1] An FDA incentive of accelerated development and review process for breakthrough therapies[2] encourages the need to find alternative or better use of currently approved drugs instead of developing entirely new scaffolds. The Generating Antibiotics Incentives Now (GAIN) Acts of 2012 that seek to extend the exclusivity of new antibiotics[3] further stimulates the need to consolidate on the valuable knowledge of existing antibacterial scaffolds as a way out of the current attrition of drug candidates. Among the most recalcitrant bacteria, typified by the ESKAPE pathogens,[4] *Pseudomonas aeruginosa*, an opportunistic pathogen that commonly affects immunocompromised patients, is particularly infamous for its highly sophisticated intrinsic and acquired resistance machineries.[5,6] Compared to other bacteria, *P. aeruginosa* displays low membrane permeability which limits the penetration of most antibiotics into the cell and a highly efficient membrane-associated efflux system of broad substrate specificity that significantly reduces bioaccumulation of drugs within its cytosol.[7, 8] The reduced intracellular concentration further promotes the activation of secondary adaptive resistance mechanisms (such as overexpression of efflux pump proteins and a variety of sensor kinases) that renders it completely refractory to treatment.[9] In addition, resistance often emerges when antibiotics are administered as monotherapy,[10,11] thus combination therapy being the preferred choice in the treatment of complicated infections.[12,13] Although debatable, the argument for combination drug treatment is premised on the large-scale genetic interaction networks between targets.[14] The use of two or more antibiotics that impact multiple targets simultaneously, or adjuvants that aid the action of legacy antibiotics can indeed extend the antimicrobial space as well as mitigate the development of antibiotic resistance.[11] Unfortunately, rational combination regimens and correlative synergistic mechanisms have remained largely unexplored and clinical benefits are yet to be demonstrated. Since the outer membrane of *P. aeruginosa* remains a major impediment to the influx of antibiotics, we and others have been investigating the effects of adjuvants that perturb this 'impermeable' layer, and consequently synergize

the activities of other antibiotics. For instance, Evotec AG and Spero Therapeutics are currently developing a polymyxin-based antimicrobial peptide SPR-741 as a potentiator to overcome outer membrane impermeability of legacy antibiotics against Gram-negative pathogens in clinical trials[15]. Our group has recently shown that tobramycin-fluoroquinolone hybrids interact with both the outer- and inner-membranes of *P. aeruginosa* resulting in enhanced cell penetration and reduced efflux by dissipating the proton motive force (PMF) that drive efflux pumps in *P. aeruginosa*.[16, 17] Mode of action studies indicate that the function of the tobramycin moiety in these hybrids is limited to a membrane-destabilizing effect of the outer membrane that results in self-promoted uptake of the hybrid and/or the antibiotic. In contrast, the function of the fluoroquinolone moiety in the hybrid is less clear but the adjuvant- and antibacterial properties appear to be correlated to the hydrophobic nature and membrane destabilizing effect of the fluoroquinolone group.[16,17] Amphiphilic aminoglycosides (AAGs) that combine aminoglycosides such as tobramycin with alkyl or other hydrophobic groups have also been previously reported to show improved activity and different modes of action in killing pathogens, compared to their constituent parent antibiotics.[18] For example, some amphiphilic tobramycin derivatives were demonstrated to primarily target the bacterial membrane,[19-21] as well as possess immumodulatory properties that closely resemble that of the natural host defense peptides.[22] To put all these findings into context, we hypothesized that appending a lipophilic membrane-active component to a tobramycin vector will confer an adjuvant-like property that can revive the efficacies of clinical antibiotics against resistant pathogens in a similar fashion as previously reported for tobramycin-fluoroquinolone hybrid antibiotics.

To preserve the amphiphilic nature of the membrane-active tobramycin derivatives, it is imperative to carefully evaluate the hydrophobic nature of the moiety to be attached. Recently, Haldar and co-workers described a series of ultrashort antibacterial lysine-based peptoid mimics that contain facial segregation of positively-charged L-lysine, hydrophobic aromatic core, and alkyl chain.[23] These molecules were reported to facilitate the attraction of compounds to bacterial surfaces and permeate cell membrane, and displayed some promising antimicrobial properties.[23] We reasoned that the inherent amphipathic nature of these peptoids, combined with their antibacterial properties could be amplified by linking to a tobramycin-

based vector as previously reported for tobramycin-fluoroquinolone hybrid antibiotics.[16,17] In the current study, we designed a series of tobramycin analogs **1 – 3** by conjoining an amphiphilic peptoid mimic **4** to the *C-5* position of tobramycin, with varied alkyl tether, to investigate the adjuvant property of the resultant conjugates in combination with commonly used antibiotics (Figure 1). The synergistic effects of the tobramycin-lysine conjugates in combination with various classes of antibiotics, against *P. aeruginosa* (wild-type and clinical isolates) were determined using checkerboard study. The emergence of bacterial resistance was compared between single agent and combination therapy for 25 bacterial passages, and *Galleria mellonella* infection worm model was further used to assess *in vivo* synergistic benefits of the optimal drug combination that protects *P. aeruginosa*-challenged larvae. Hemolysis and cytotoxicity assays were carried out to ascertain toxicity against mammalian cells, and prokaryotic membrane-compound interactions were studied to gain mechanistic insights on possible modes of action.

RESULTS

Synthesis. The reference compound ultrashort peptoid mimic **4** was synthesized by reductive amination of aromatic aldehyde **5** with dodecylamine **6** generating secondary amine **7** which after coupling to di-Boc-protected lysine produced protected lysine-based peptoid **8**. Deprotection of the Boc-protecting groups with TFA afforded ultrashort lysine peptoid **4** as previously described (Scheme 1).[23] The synthesis of tobramycin-lysine conjugates **1 – 3** was achieved by preparing amphiphilic tobramycin derivatives (5 steps), followed by a single-step reductive amination conjoining and a final deprotection (Scheme 2). Preparation of the tobramycin derivatives commenced by protecting the amines on commercially available tobramycin with Boc anhydride, followed by silylation of the hydroxyl groups with a bulky protecting group such as TBDMSCl to give **9**. This is to ensure the more hindered *C-5* position of the Boc- and TBDMS-protected tobramycin intermediate, the desired point of alkylation, is unprotected. This is the preferred position because *C-5*-modified tobramycin derivatives retained antibacterial activity[24] and superior adjuvant properties against Gram-negative bacteria like *P. aeruginosa*.[16, 17] The *C-5* hydroxyl group of **9** was subsequently alkylated with 1,*n*-dibromoalkane (n = 4,

8, 12), under phase-transfer catalytic conditions, to afford alkylated tobramycin analogs **10a–c**. The terminal bromo-group of intermediate **10a–c** was then displaced by an azido nucleophile under anhydrous conditions to give **11a–c**, followed by reduction to free amine **12a–c** via catalytic hydrogenation. This free amine was successively reacted with commercially-available 10-chloro-9-anthracenaldehyde via reductive amination, to afford intermediate **13a–c** with secondary amine, which was then coupled to di-Boc-protected Lys to produce protected amphiphilic tobramycin amides **14a–c**. The final deprotection step involved the removal of Boc and TBDMS protecting groups using MeOH:HCl (3:2, v/v) to afford the desired target compounds **1 – 3**. The final compounds were characterized by NMR, mass spectrometry, and reverse-phase HPLC, with > 95 % purity (Supporting information).

Antimicrobial Activity. The antimicrobial activities of reference peptoid mimic (compound **4**) and tobramycin hybrids **1 – 3** against a panel of Gram-negative and Gram-positive bacteria are presented as the minimum inhibitory concentration (MIC) in Table 1. Reference peptoid mimic **4** with a C12 hydrophobe displayed weaker antimicrobial activity compared to the reported C10 peptoid,[23] with MIC ≥ 8 μg/mL against all the strains tested in this study. For tobramycin-lysine conjugates, there was a positive correlation between antimicrobial activity and the length of the carbon chain tether. Compound **3** with C12 tether was the most potent analog of all the hybrids, and displays moderate activity against Gram-positive bacteria (MIC of 2 – 32 μg/mL) but a relatively weak activity against Gram-negative bacteria (MIC ≥ 16 μg/mL). Further, the anti-pseudomonal activities of all compounds were evaluated against wild-type and seven clinical isolates of *P. aeruginosa*, including MDR, XDR, and colistin-resistant strains (Table 2). A similar trend of longer carbon chain displaying better activity was observed for drug-resistant *P. aeruginosa* as well, suggesting C12 as the optimal tether length of all analogues tested. In addition, we observed comparable activity of **3** against wild-type and drug-resistant strains (MIC ranging from 8 to 64 μg/mL) indicating that compound **3** is not greatly affected by resistance. Again, compound **4** did not show potent activity against any of the clinical isolates tested, with MIC ≥ 64 μg/mL. The MIC of tobramycin increased from 0.25 μg/mL in wild-type *P. aeruginosa* to > 8 μg/mL in drug-resistant strains, particularly in P262 (MIC = 512 μg/mL). Similar results were also observed for other tested antibiotics,

with the exception of colistin. It is however interesting to note that compound **3** displayed better activity against P262 (MIC = 32 μg/mL) than tobramycin, moxifloxacin, minocycline, rifampicin, chloramphenicol, erythromycin, and trimethoprim (Table 2).

Combination Study of Hybrids with Antibiotics. To assess the adjuvant properties of the hybrids, checkerboard studies were performed to determine the synergistic effects of the most active hybrid **3** with 14 different antibiotics (cutting across all classes) against wild type *P. aeruginosa* PAO1. The fractional inhibitory concentration (FIC) index, a numerical quantification of the interactions between antibiotics, was calculated as previously described.[16] FIC indices of ≤ 0.5, 0.5 - 4, and > 4 indicate synergy, no interaction, and antagonism respectively.[25] Compound **3** showed strong synergy with most antibiotics tested against PAO1 except ceftazidime, colistin, meropenem and the aminoglycosides gentamicin, kanamycin A and amikacin. The strongest potentiation was seen with novobiocin (FIC index = 0.071), minocycline and rifampicin (FIC index = 0.094 for both) as shown in Table 3. Synergism with minocycline and rifampicin was also observed with **2**, but not with **1**, **4**, and tobramycin (Table S1). As shown in Figure 2, the absolute MICs (the MIC of antibiotics in the presence of adjuvants at 4 μg/mL) of minocycline or rifampicin in combination therapy with hybrids was dramatically lower than monotherapy, in particular with compound **3** where MICs of minocycline and rifampicin were reduced from 8 μg/mL and 16 μg/mL in monotherapy to 0.25 μg/mL (32-fold potentiation) and 0.0625 μg/mL (256-fold potentiation), respectively. Thus, combinations of **3** with minocycline and rifampicin were selected for further synergy studies against a panel of *P. aeruginosa* clinical isolates (Table 4). Compound **3** demonstrated strong synergy with minocycline and rifampicin across the clinical isolates panel (FIC indices of 0.039 to 0.281), with the exception of rifampicin against *P. aeruginosa* 91433 (FIC index = 0.5). Since the breakpoints for minocycline and rifampicin against *P. aeruginosa* are not available (as they are not conventional drugs for treating *P. aeruginosa* infections), the susceptible or intermediate resistant breakpoints of minocycline against *Acinetobacter* spp. and that of rifampicin against *Enterococcus* spp. reported by CLSI,[26] were considered as interpretive MIC standards for this study. The susceptible breakpoints of minocycline (MIC ≤ 4 μg/mL) against *Acinetobacter* spp. were reached for all

minocycline-resistant, MDR, or XDR *P. aeruginosa* isolates at 4 μg/mL of **3**. For rifampicin, susceptible (MIC ≤ 1 μg/mL) or intermediate resistant (MIC = 2 μg/mL) breakpoints against *Enterococcus* spp. were reached in 5/7 rifampicin-resistant, MDR, or XDR *P. aeruginosa* isolates, with the exception of strain P262 and #91433.

To gain insights into the membrane effects and relevant synergistic mechanism of **3**, colistin, a membrane-active antibiotic, was tested in combination with five antibiotics against PAO1. Our results indicated that colistin was also able to potentiate the activity of rifampicin and novobiocin (Figure 3), but to a lesser extent than compound **3**. To investigate the relevance of efflux pumps on the observed adjuvant properties of **3**, combination studies of minocycline and rifampicin each with compound **3** were carried out in efflux pump-mutated strains, PAO200 and PAO750. PAO200 is a MexAB-OprM deletion strain while PAO750 is an efflux-sensitive strain that lacks five different clinically-relevant RND pumps (MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexJK, and MexXY) and the outer membrane protein OpmH. Some of these pumps are homologues of broad substrate specificities that expel different classes of antimicrobial agents and confer resistance on *P. aeruginosa*. For instance, the tripartite protein system MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM allow the translocation of a wide variety of substrates such as quinolones, chloramphenicol, trimethoprim, imipenem, and tetracyclines out of the cell.[27] As shown in Table 5, synergism was observed with rifampicin in both PAO200 (FIC index = 0.129) and PAO750 (FIC index = 0.156), but not with minocycline (FIC index > 0.5). The ability to potentiate minocycline in PAO1 but not in efflux-deficient strains corroborates the hypothesis that tobramycin-lysine conjugate compromises the efficiency of efflux proteins. Rifampicin, which is not a substrate for these pumps was however potentiated, suggesting a second mode of action consistent with membrane permeabilization.

To study the spectrum of activity of **3** in combination with minocycline and with rifampicin, we examined *in vitro* potency against other clinical isolates of highly pathogenic Gram-negative bacteria, including *Acinetobacter baumannii*, *Enterobacter cloacae*, and *Klebsiella pneumoniae*. Table 6 summarizes these results and indicates strong synergy of **3** with minocycline in other Gram-negative

species except AB031 and *K. pneumoniae* 110193 where the effect of **3** on minocycline is at best marginally additive. Surprisingly, combination of **3** with rifampicin displayed potent synergy in all tested isolates, with FIC indices < 0.25. The synergistic MIC of rifampicin in combination with 4 μg/mL of **3** against rifampicin-resistant *E. cloacae* 117029 was 0.063 μg/mL, which is 16-fold lower than rifampicin-susceptible breakpoints of ≤ 1 μg/mL.

Time-kill Curve. The kinetics of *P. aeruginosa* PAO1 killing as a function of time, using mono- and combination-therapy of minocycline, rifampicin, and compound **3**, are shown in Figures 4 and S24. Minocycline alone was not bactericidal even at 4 × MIC after 6 h, while rifampicin as a monotherapy was bactericidal at 4 × MIC after 2 h of drug exposure (Figure S24). Compound **3** showed bactericidal activity at 1 × MIC after 2 h, and more rapid killing was observed at 2 × MIC and 4 × MIC for only 30 and 10 mins antimicrobial exposure respectively (Figure S24). Minocycline, rifampicin, or **3** at sub-inhibitory concentrations were unable to suppress bacteria growth in monotherapy, even after 6 h exposure (Figure 4). However, upon combination with sub-MIC (1/8 to 1/2 × MIC) of **3**, *in vitro* bactericidal activities of minocycline and rifampicin were both enhanced, yielding synergistic killing at sub-MIC concentration (1/8 × MIC) after 90 mins of incubation (Figure 4).

Resistance Study. The ability of drug combinations to suppress resistance development was determined using wild-type *P. aeruginosa* PAO1. This assay was validated by demonstrating that the MIC of colistin and tobramycin increased by 1024- and 256-fold respectively over 25 serial passages, while that of minocycline increased by 16-fold (Figure 5). Upon combination with compound **3,** the emergence of resistance in minocycline was suppressed by 4-fold while rifampicin did not promote resistance either as monotherapy or in combination with **3** (Figure 5).

Mode of Action Studies. *Outer Membrane Permeabilization*. Since the negatively-charged outer membrane of *P. aeruginosa* serves as the first barrier that prevents the uptake of antibiotics,[7, 8] the ability of the amphiphilic cationic compounds to perturb this lipid bilayer was investigated in PAO1 using carboxyfluorescein diacetate succinimidyl ester (CFDASE), a cell permeable dye.[28] We reasoned that the amphipathic nature of the hybrids might confer membrane effects similar to those of the host defense

peptides on them. The increased fluorescence induced by the compounds was calculated by subtracting the fluorescence of negative control that was treated similarly but in the absence of drug, while 1% Triton X-100 that exhibited the highest fluorescence compared to other treatments served as the positive control (Figure 6). At the concentration tested (32 μg/mL), the outer membrane permeabilization induced by **3** was slightly lower than that of colistin. In contrast, reference compound **4** displayed the weakest membrane permeabilizing ability with the lowest fluorescence increase.

Cytoplasmic Membrane Depolarization. Compounds that perturb the bacterial outer membrane can potentially be trapped within the periplasmic space where they could interfere with the respiratory chain on the cytoplasmic membrane to induce death. Indeed, this has been proposed as one of the mechanisms by which polymyxin exerts its antibacterial effects, among many others.[29] Depolarization of cytoplasmic membrane can lead to loss of membrane potential, an important electrochemical gradient used by the bacteria to maintain an active and functional efflux system.[30] To investigate the effect of **3** on bacterial cytoplasmic membrane, diSC_3-5, a membrane potential-dependent probe, was used to study the differential in fluorescence caused by membrane depolarization.[19] A dose-dependent fluorescence increasing was observed for all the tested antimicrobials in PAO1 (Figure S25). At 32 μg/mL, colistin was observed to depolarize the cytoplasmic membrane faster than other compounds in the first 300 secs, with an accompanying decrease in fluorescence thereafter (Figure 7). Although reference compound **4** displayed similar properties as colistin, the decline in fluorescence seems to be specific for colistin. Compound **3** however displayed the highest fluorescence up till 1200 secs, while only weak membrane depolarization ability was observed for tobramycin at 32 μg/mL, a 128-fold higher value than its MIC.

Swimming Motility Assay. Swimming motility is a flagellum-dependent bacterial movement that is governed by the respiratory chain on the cytoplasmic membrane. When cytoplasmic membrane potential or proton motive force (PMF) is disrupted, the electron transfer across respiratory chain is inhibited, resulting in a reduction of ATP synthesis which is essential for flagellar function.[31] Previous studies have implicated that amphiphilic tobramycin-moxifloxacin hybrids can perturb the PMF resulting in reduced or inefficient efflux.[17] We therefore studied the effect of the hybrids **3** and **4** on the swimming motility of

PAO1 by monitoring its movement on low-viscosity swim plates (0.3 % agar, w/v). Surprisingly, **3** and **4** were observed to significantly constrict the swimming bacteria diameter (at 4 μg/mL) relative to the untreated control (Figure 8). Meanwhile, these compounds repressed bacterial swimming motility in a dose-dependent manner, and the observed effects were superior to colistin at sub-inhibitory concentrations, while tobramycin was unable to inhibit bacterial motility with similar bacterial swimming diameters to untreated control (Figure S26).

Hemolytic Activity and Cytotoxicity. A potential problem usually associated with membrane-active agents is their toxicity towards eukaryotic cells. The hemolytic properties of the hybrid molecules were first examined using freshly collected pig erythrocytes. All hybrids demonstrated lower hemolytic activities (< 20 %) relative to **4**, which is highly toxic with 87 % hemolysis at the highest measured concentration of 512 μg/mL (Figure 9A). As to the structure-activity relationships (SAR) between the hybrids, a slight increase in hemolytic activity with increase in carbon length was evident. Compound **3** was also tested against human epithelial prostate (DU145) and breast (JIMT-1) cancer cell lines, with greater than 50 % viability at 20 μM (25.2 μg/mL), a six times higher concentration than effective synergistic concentration (4 μg/mL) in combination therapy (Figure 9B).

***In Vivo* Efficacy.** To gain insights into the potential clinical benefits of compound **3**, an *in vivo* efficacy evaluation using *Galleria mellonella* infection model was initiated. The maximum tolerable dose was first determined by injecting drugs alone at high concentrations (100 or 200 mg/kg), and the survival rates scored for 4 days. As shown in Figure S27, 100 % survival was observed after 4 days in the group that had been injected with 200 mg/kg of **3**, indicating the relative safety of the compound to the worms at this dose. Next, the ability of the drug or drug combinations to protect larvae from MDR *P. aeruginosa* P262 infection was determined at single doses of 75 mg/kg in drug monotherapy or 12.5 + 12.5, 25 + 25, 37.5 + 37.5, or 75 + 75 mg/kg in drug combinations (Figure 10). 100 % mortality was observed in the monotherapy of minocycline, rifampicin, and **3** at 75 mg/kg after 24 h, and in combination at lower doses. However, combinations of minocycline or rifampicin with **3** at a high dose of 75 + 75 mg/kg both resulted in 77 % survival after 24 h, demonstrating the ability of this compound to offer protection against

infection at very tolerable dose. Interestingly, it appears that combination therapy of rifampicin and **3** at low dosage appears to be superior when compared to combinations of minocycline and **3.** This is rather surprising as the *in vitro* studies suggest a lower MIC for minocycline when compared to rifampicin. This discrepancy may be related to the different pharmacokinetics of minocycline and rifampicin in the larvae or the difference between bacteriostatic minocycline and bactericidal rifampicin (Figure S24).

DISCUSSION

Bacterial resistance can frequently emerge in antibiotic monotherapy due to the selective pressure that naturally separates out the resistant phenotypes.[10, 11] Combination of two or more antimicrobials that can impact multiple targets simultaneously is believed to be capable of suppressing drug resistance, as well as broaden the spectrum of activity of a treatment course than single agents.[32, 33] In the last two years the FDA has approved two new combination drugs Avycaz (ceftazidime + avibactam) and Zerbaxa (ceftolozane + tazobactam to combat MDR Gram-negative infections. Ceftazidime/avibactam contains an older 3rd generation cephalosporin ceftazidime, with avibactam a synthetic non-β-lactam, β-lactamase inhibitor that inhibits the activities of Ambler class A and C β-lactamases and some Ambler Class D enzymes.[34] Limited data suggest that the addition of avibactam does not improve the activity of ceftazidime versus *Pseudomonas aeruginosa.* Ceftolozane is a novel cephalosporin with a chemical structure similar to that of ceftazidime, with the exception of a modified side-chain at the 3-position of the cephem nucleus, which confers potent antipseudomonal activity.[35] The addition of tazobactam extends the activity of ceftolozane to include most ESBL producers but not *P. aeruginosa.* Nevertheless, effective drug combinations often lead to inconclusive benefits of combination therapy over monotherapy during meta-analysis.[36] Recent reports about the potentials of amphiphilic tobramycin analogues to permeabilize cell membrane,[19,20] and our previous studies that demonstrated the intrinsic ability of tobramycin-fluoroquinolone hybrids to potentiate the antimicrobial activity of several classes of antibiotics against clinical *P. aeruginosa* isolates,[16, 17] encouraged a further optimization of this promising scaffold for use as adjuvants. In the current study, we prepared new amphiphilic tobramycin hybrids by taking advantage of

the membrane-active peptoid **4** as a modulator. The antimicrobial properties of these derivatives alone were assessed and demonstrated to be weaker compared to the parental tobramycin molecule (Table 1). Although tobramycin is believed to induce pleiotropic mechanisms of action,[37] the most acceptable hypothesis suggests tobramycin permeates the outer membrane via a self-promoted uptake mechanism[38] and acts by impairing bacterial protein synthesis through irreversible binding to the 30S ribosomal subunit.[39] The differing activity between tobramycin and the newly synthesized hybrid molecules suggests that the protein translation inhibitory effect is compromised by attachment of hydrophobic moieties to tobramycin as previously shown.[16, 17] Furthermore, activity trend between the hybrids revealed a correlation between antimicrobial potency and carbon chain length. The longer the carbon tether, the better the antimicrobial efficacy of the compound. This is however not surprising as studies have shown that high hydrophobicity facilitates penetration of membrane-active compounds across the bacterial membrane.[40, 41] The physicochemical properties necessary to navigate a complex membrane topology, especially as represented in *P. aeruginosa*, is perhaps the principal reason for the varied activity of the hybrid molecules. Although ultrashort and amphiphilic lysine-based peptoid mimics were previously reported to have promising activities against *P. aeruginosa* MTCC 424,[23] our evaluation of compound **4** (different alkyl chain) against a panel of organisms revealed otherwise (Table 1). This may be due to the slight change in the length of the alkyl chain (C10 to C12), and perhaps, the different bacterial strains tested. The amphiphilic nature of **4**, the reported properties of tobramycin hybrids,[16] and the differential activity of the new molecules based on carbon chain length, gave a clue on possible membrane effect of these compounds. Thus, we investigated antimicrobial activities of the amphiphilic conjugates in combination with other antibiotics, particularly against *P. aeruginosa*, the major nosocomial pathogen and leading cause of infection in cystic fibrosis patients. Although most of the clinical isolates investigated in this study were resistant to tobramycin and other antibiotics (with the exception of colistin), the MIC values of the conjugates against these strains were similar to that of wild-type *P. aeruginosa* PAO1 (Table 2), suggesting that the targeting site and/or mechanism of action of the conjugates was different than that of tobramycin.

The ability of **3** to perturb the membrane was verified by its synergistic effect with vancomycin, a drug that cannot pass through the outer membrane of *P. aeruginosa* due to its large size (M.W. = 1449.2). Synergism was also observed for other antibiotics with different modes of action against *P. aeruginosa*, the most prominent being novobiocin, minocycline and rifampicin. Importantly, combining **3** with minocycline, or with rifampicin, can revive the antimicrobial activities of these antibiotics against MDR and XDR *P. aeruginosa* isolates (Table 4). The uptake of tetracyclines, such as minocycline, is known to be driven by transmembrane chemical gradient (ΔpH) of PMF generated by the respiratory chain on the cytoplasmic membrane.[42] The other component of PMF is electrical potential (ΔΨ), which is known to drive aminoglycosides uptake.[43] Bacteria control ΔΨ and ΔpH exquisitely to maintain a constant value of PMF, and disruption of either component is compensated for by a counteracting increase in the other.[44] When a compound disrupts ΔΨ, antagonism effect will be observed in combination with aminoglycosides, while synergism will show in combination with tetracyclines due to the compensatory increase of ΔpH. Tetracyclines and aminoglycosides have therefore been used as two relevant antibiotics in combination studies with other drugs to identify compounds that affect membrane PMF and specifically dissipate either component of PMF.[31] In this study, compound **3** displayed different synergistic effects with minocycline (synergism) and aminoglycosides (no interaction), an observation that is consistent with dissipation of ΔΨ component of the PMF by **3**. However, the expected antagonistic effect of **3** with aminoglycosides was not observed, likely due to the membrane penetration induced by **3** that slightly affected aminoglycosides uptake into bacterial cells. The effect of **3** on ΔΨ was further corroborated by the increased diSC$_3$-5 fluorescence (Figure 7) and repression of swimming motility controlled by this parameter (Figure 8). Compounds that collapse the PMF are known to inhibit ATP synthesis and flagellar motility, preventing or reducing swimming activity.[45]

Membrane-associated efflux is another major mechanism that prevents bioaccumulation of drugs within the cytosol, thus preventing/reducing access of antibiotics to intracellular targets.[46] Efflux pump proteins localized in the cytoplasmic and outer membrane, and linked by a periplasmic membrane fusion protein (MFP), play a major role in intrinsic and acquired resistance of *P. aeruginosa*.[7-9] The associated

resistance is based on energy-dependent effluxes, which are usually driven by PMF.[30] We envisaged that the dissipation of ΔΨ in PMF will prevent electron transport across the respiratory chain, thus inhibiting ATP synthesis and ultimately affect efflux pump system. We therefore studied the effect of the conjugates on efflux pumps using *P. aeruginosa* efflux-deficient strains PAO200 and PAO750, and perhaps explain the observed synergistic mechanism more exquisitely. The efflux pump knock-out decreased the MIC of minocycline by 8-fold (Table 5), confirming that minocycline is a substrate for these efflux pumps, which is consistent with previous study.[47] The synergism observed when **3** was combined with minocycline against wild-type PAO1 was not observed in PAO200 and PAO750, with FIC indices > 0.5, indicating that the antimicrobial activity potentiation of minocycline by **3** is due to the inhibition of efflux pumps, particularly the RND pumps. To validate this hypothesis, the synergistic effects of **3** with other known *P. aeruginosa* efflux pump substrates were evaluated in PAO200 and PAO750, including chloramphenicol, erythromycin, trimethoprim, and moxifloxacin.[7, 48-50] The results showed weak synergy or additive effects of **3** in these combinations (Table S2), corroborating the efflux pump inhibitory activity of **3**. We posited that the dissipation of electrochemical gradient across the cytoplasmic membrane affected respiratory ATP production, thereby compromising efflux pump efficiency.

Surprisingly, rifampicin that is not substrate of the five efflux pumps investigated in this study (MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexJK, and MexXY) was similarly strongly synergized by **3** against PAO200 and PAO750 (Table 5). Rifampicin is known to kill bacteria by inhibiting RNA synthesis after binding to DNA-dependent RNA polymerase.[51] Both Gram-positive and Gram-negative bacteria are similarly sensitive to rifampicin, with the higher MICs reported in Gram-negative bacteria due to its low penetration across the outer membrane.[52] A combination study of rifampicin with colistin (a well-known membrane permeabilizer) has demonstrated that perturbation of *P. aeruginosa* outer membrane can indeed potentate the antimicrobial activity of rifampicin,[53] thus confirming the results of this study with **3** (Figure 3). Outer membrane perturbation is perhaps the reason why **3** was able to synergize rifampicin against PAO200 and PAO750 despite not being a substrate for the pumps. It is however clear that minocycline is more sensitive to PMF dissipation caused by compound **3** than simply

membrane penetration induced by colistin, as evident in the FIC indices > 0.5 shown in Table 5. These results suggest that compound **3** is not just able to penetrate *P. aeruginosa* cell membrane like colistin, but could also dissipate the cytoplasmic membrane, and compromise the efficient functioning of the efflux systems. Although reference compound **4** similarly displayed cytoplasmic membrane depolarization activity and suppressed bacterial swimming motility, it was to a lesser extent than **3** at the same concentration (Figures 7 and 8), and did not display any synergistic effect in combination with minocycline and with rifampicin (Table S1). The inability of **4** to potentiate minocycline like **3** despite its ability to partially depolarize the cytoplasmic membrane as well may be due to its weak outer membrane perturbation that prevents uptake of other antimicrobial agents. The simultaneous occurrence of both phenomenon is indeed critical to the adjuvant properties of tobramycin-lysine conjugates.

In contrast to the bactericidal nature of rifampicin, minocycline is known to be only bacteriostatic, which was evident in the time-kill assay with constant bacterial cells number at all concentrations tested (Figure S24), the observation that is consistent with previous studies.[54, 55] However, the increased killing efficiency of minocycline when used in combination with **3** is likely attributable to the effect of **3** on the membrane. Attempts to select for resistance with combination of **3** and minocycline during 25 serial passages resulted in a 4-fold increase in MIC, as opposed to minocycline and tobramycin alone that had 16- and 256-folds increase respectively (Figure 5). Indeed, it is more difficult for bacteria to develop resistance to simultaneously-acting drug combination, especially when one of the drug acts on membrane[56-58].

A major concern about membrane-acting and PMF-collapsing agents is their toxicities towards eukaryotic cells.[31] To verify the safety of these compounds, the toxicities of the conjugates were evaluated against pig erythrocytes and mammalian cancer cell lines. It was surprising to see a dramatic reduction of the hemolytic activity of **4** when joint to tobramycin (Figure 9A). This is probably caused by changes to the molecular amphipathy as previously seen for antimicrobial peptides.[59] Moreover, combination therapy would allow for reduced doses to be used, minimizing cytotoxicity, and **3** displayed negligible toxicity at its effective synergistic concentration (≤ 4 μg/mL). In the *in vivo* study, the high tolerance of *Galleria*

mellonella worms to **3** (100 % survival at 200 mg/kg after 96 h) further confirmed the safety of this compound. *Galleria mellonella* injection model has been commonly used in accessing the *in vivo* efficacy of antimicrobials against *P. aeruginosa* because it shares a high degree of structural and functional homology to the immune systems of vertebrates with both cellular and humoral defenses.[60] In contrast to monotherapy, single dose combination of **3** (75 mg/kg) plus minocycline (75 mg/kg) or **3** (75 mg/kg) plus rifampicin (75 mg/kg) effectively protected larvae from XDR *P. aeruginosa* P262 infection with more than 75 % survival after 24 h, indicating the therapeutic potential of amphiphilic tobramycin as an adjuvant to treat infection caused by XDR *P. aeruginosa*.

CONCLUSIONS

In this study, we demonstrated that amphiphilic tobramycin-lysine conjugates preserve many of the known adjuvant properties of previously reported tobramycin-fluoroquinolone hybrids. From a medicinal chemistry point, linking a tobramycin C-12 vector to an amphiphilic lysine conjugate enhances the outer membrane destabilization effect of the amphiphilic lysine analog. As such our study suggests that a tobramycin-C12 tether at C-5 position in tobramycin serves as an effective vector to promote delivery of compounds through the outer membrane barrier of Gram-negative bacteria with an optimized effect on *P. aeruginosa*. However, the effect of the tobramycin-C12 tether appears to be not limited to the outer membrane but also involves the cytoplasmic membrane. For instance, we provide evidence that conjugates containing a tobramycin-C12 tether reduces the $\Delta\Psi$ component of the PMF located at the cytoplasmic membrane. This leads to decreased activity of the efflux associated pumps but at the same can lead to enhanced cytoplasmic uptake of agents which depend on the Δ pH component of the PMF like the tetracycline class of antibiotics. Overall, this study provides a promising strategy for generating effective antibiotic adjuvants that overcome drug resistance in MDR Gram-negative bacteria including *P. aeruginosa* by carefully designing amphiphilic tobramycin tethers. The discovery that compound **3** can

potentiate several classes of antibiotics against resistant pathogens is set to expand the antimicrobial space and optimize our usage of antibiotics in our current armamentarium.

EXPERIMENTAL SECTION

Chemical Synthesis. *General Information.* NMR spectra (^{1}H, ^{13}C, DEPT, COSY, HSQC and HMBC) were recorded on a Bruker Avance 500 spectrometer (500 MHz for ^{1}H NMR, 126 MHz for ^{13}C). All reactions were monitored by analytical thin-layer chromatography (TLC) on pre-coated silica gel plates 60 F254 (0.25 mm, Merck, Ontario, Canada), and the spots were visualized by ultraviolet light and/or by staining with ninhydrin solution in *n*-butanol. Mass spectrometry was carried by ESI analyses on a Varian 500 MS Ion Trap Mass Spectrometer, and MALDI-TOF on a Bruker Daltonics Ultraflex MALDI TOF/TOF Mass Spectrometer. Chromatographic separations were performed on a silica gel column by flash chromatography (Kiesel gel 40, 0.040-0.063 mm; Merck, Ontario, Canada). Yields were calculated after purification. When reactions were carried out under anhydrous conditions, the mixtures were maintained under nitrogen atmosphere. Analytical HPLC was performed on Hitachi LC system equipped with autosampler, using Superspher 100 RP-18 column and a detection wavelength of 260 nm. The purity of final compounds determined by HPLC analysis were > 95 %. Detailed experimental procedures of the intermediates Boc-Lys(Boc)-OH, **10a-c**, **11a-c** and **12a-c** were described in the Supporting Information. The following data are for the intermediates after reductive amination and the final compounds tested in the biological studies.

*General Procedure A: Deprotection of Boc and TBDMS Groups for the Synthesis of Tobramycin-lysine Conjugates (**1-3**).* Compounds **14a–c** (1 equiv) were treated with 40 % HCl in MeOH (~1.5 mL of solvent per 0.01 mmol of intermediates **14a–c**) at room temperature for 3 h. The reaction progress was monitored by TLC ($MeOH/CH_2Cl_2/NH_4OH$, 6:4:3). At the end of the reaction, the mixture was concentrated under reduced pressure to get the solid tobramycin conjugate as HCl salt. The crude was further purified via C-18 reverse-phase flash column chromatography (eluted with deionized water) to afford analytically pure compounds as yellow solid.

*General Procedure B: Reductive Amination for the Synthesis of 5-O-(alkylated-10-aminomethyl-9-chloroanthracene)-1,3,2',6',3''-penta-N-(tert-butoxycarbonyl)-4',2'',4'',6''-tetra-OTBDMS-tobramycin (**13a–c**)*. Compounds **12a–c** (1 equiv) and commercially available aromatic aldehyde **5** (1.2 equiv) were dissolved in dry CH_2Cl_2:MeOH (1:1, v/v) (~20.0 mL of solvent per mmol of intermediates **12a–c**) and stirred at room temperature overnight. The resulting clear solution was cooled to 0 °C, sodium borohydride (3 equiv) added and stirred at room temperature for 3 h. The solvents were then evaporated and the crude re-dispersed in diethyl ether followed by the addition of 2 N NaOH. The mixture was stirred at room temperature for 15 mins and the organic layer separated from aqueous phase, washed with water and brine, and dried over anhydrous Na_2SO_4. The mixture was concentrated and purified by flash column chromatography (eluted with CH_2Cl_2/MeOH from 300:1 to 30:1, v/v) to give the desired product as yellow solid.

*General Procedure C: Secondary Amide Coupling for the Synthesis of Compounds **14a–c***. Boc-Lys(Boc)-OH (1.5 equiv) dissolved in DMF (~30.0 mL of solvent per mmol of intermediates **13a–c**) was activated with DIPEA (3 equiv) and HBTU (1.5 equiv) at 0 °C for 15 mins and subsequently treated with **13a–c** (1 equiv). The mixture was stirred at 0 °C to room temperature overnight. The reaction progress was monitored by TLC (CH_2Cl_2/MeOH, 35:1), and at the end, the mixture was diluted with water and extracted with EtOAc. The organic layer was washed with water and brine, dried over anhydrous Na_2SO_4, and concentrated. The resulting residue was purified by flash column chromatography (eluted with CH_2Cl_2/MeOH from 300:1 to 30:1, v/v) to afford the desired compound as yellow solid.

*Compound **1***. Synthesized following general procedure A from **14a** (18.6 mg, 0.009 mmol). The crude material was purified by C-18 reverse-phase flash column chromatography using deionized water. The product was isolated as a yellow solid (6.3 mg, 78 %). ^{1}H NMR (500 MHz, Deuterium Oxide) δ 8.74 – 8.60 (m, 2H, anthracene), 8.33 – 8.24 (m, 2H, anthracene), 7.86 – 7.73 (m, 4H, anthracene), 5.99 (d, J = 15.4 Hz, 1H, N-CH^1H^2-anthracene), 5.30 (d, J = 15.3 Hz, 1H, N-CH$^1H^2$-anthracene), 5.14 (d, J = 2.5 Hz, 1H, H-1′), 4.95 (d, J = 3.4 Hz, 1H, H-1″), 4.43 (t, J = 6.3 Hz, 1H, α-C*H* of Lys), 4.29 – 4.24 (m, 1H, H-5′), 4.03 – 3.94 (m, 2H, H-4, H-4′), 3.83 – 3.77 (m, 2H, H-6, H-2″), 3.65 – 3.46 (m, 5H, H-1, H-3, H-2′,

H-4″, H-5″), 3.39 – 3.30 (m, 2H, H-6′), 3.21 – 3.12 (m, 3H, H-5, H-3″, H-6″), 3.03 – 2.95 (m, 1H, N-C*H*^{1}H^{2}CH$_2$), 2.90 – 2.64 (m, 5H, N-CH^{1}*H*^{2}CH$_2$, ε-C*H*$_2$ of Lys, O-C*H*$_2$ of linker), 2.59 – 2.54 (m, 1H, H-2), 2.39 – 2.32 (m, 1H, H-6″), 2.24 – 2.12 (m, 2H, H-3′), 1.97 – 1.82 (m, 3H, H-2, β-C*H*$_2$ of Lys), 1.64 – 1.54 (m, 2H, δ-C*H*$_2$ of Lys), 1.45 – 1.31 (m, 2H, γ-C*H*$_2$ of Lys), 1.27 – 1.10 (m, 3H, C*H*$_2$ of linker), 1.09 – 0.99 (m, 1H, C*H*$_2$ of linker). ^{13}C NMR (126 MHz, Deuterium Oxide) δ 169.34 (N*C*OCH), 131.18, 130.09, 128.13, 127.48, 127.40, 126.65, 125.62, 124.36, 100.79 (anomeric *C*H-1″), 93.15 (anomeric *C*H-1′), 82.41, 81.22, 77.20 (*C*H-4), 75.41 (*C*H-5′), 73.18, 72.80 (O-*C*H$_2$-linker), 68.44, 64.30, 63.42 (*C*H-6), 58.41 (*C*H$_2$-6″), 54.46, 50.61 (α-*C*H of Lys), 49.36, 48.60, 47.65, 45.32 (N-*C*H$_2$CH$_2$), 40.74 (N-*C*H$_2$-anthracene), 38.91 (ε-*C*H$_2$ of Lys), 38.60 (*C*H-6′), 30.90 (β-*C*H$_2$ of Lys), 28.34 (*C*H$_2$-3′), 28.18 (*C*H$_2$-2), 26.55 (δ-*C*H$_2$ of Lys), 25.36, 24.45, 21.16; MALDI-TOF-MS: *m/z* calc′d for $C_{43}H_{67}ClN_8O_{10}Na$: 913.457, found: 913.463 $[M+Na]^+$.

Compound ***2***. Synthesized following general procedure A from **14b** (28 mg, 0.013 mmol). The crude material was purified by C-18 reverse-phase flash column chromatography using deionized water. The product was isolated as a yellow solid (9.4 mg, 75 %). ^{1}H NMR (500 MHz, Deuterium Oxide) δ 8.49 – 8.44 (m, 2H, anthracene), 8.15 – 8.11 (m, 2H, anthracene), 7.71 – 7.62 (m, 4H, anthracene), 5.70 (d, *J* = 15.4 Hz, 1H, N-C*H*^{1}H^{2}-anthracene), 5.35 – 5.28 (m, 2H, N-CH^{1}*H*^{2}-anthracene, H-1′), 5.16 (d, *J* = 3.5 Hz, 1H, H-1″), 4.37 (t, *J* = 6.3 Hz, 1H, α-C*H* of Lys), 4.32 – 4.27 (m, 1H, H-5′), 4.15 (t, *J* = 9.8 Hz, 1H, H-4), 3.96 – 3.89 (m, 3H, H-6, H-4′, H-2″), 3.83 – 3.76 (m, 4H, H-5, O-C*H*$_1$H$_2$ of linker, H-4″, H-5″), 3.75 – 3.72 (m, 1H, H-6″), 3.67 – 3.55 (m, 5H, H-1, H-3, H-2′, H-3″, O-CH$_1$*H*$_2$ of linker), 3.53 – 3.49 (m, 1H, H-6″), 3.40 – 3.33 (m, 2H, H-6′), 2.98 – 2.90 (m, 1H, N-C*H*^{1}H^{2}CH$_2$), 2.86 – 2.78 (m, 2H, ε-C*H*$_2$ of Lys), 2.62 – 2.52 (m, 2H, H-2, N-CH^{1}*H*^{2}CH$_2$), 2.25 – 2.20 (m, 2H, H-3′), 2.02 – 1.96 (m, 1H, H-2), 1.92 – 1.82 (m, 2H, β-C*H*$_2$ of Lys), 1.64 – 1.57 (m, 2H, δ-C*H*$_2$ of Lys), 1.44 – 1.35 (m, 3H, γ-C*H*$_2$ of Lys, C*H*$_2$ of linker), 1.32 – 1.27 (m, 1H, C*H*$_2$ of linker), 1.11 – 1.04 (m, 1H, C*H*$_2$ of linker), 1.02 – 0.91 (m, 2H, C*H*$_2$ of linker), 0.83 – 0.63 (m, 4H, C*H*$_2$ of linker), 0.62 – 0.53 (m, 3H, C*H*$_2$ of linker). ^{13}C NMR (126 MHz, Deuterium Oxide) δ 169.14 (N*C*OCH), 131.04, 129.85, 127.86, 127.19, 127.09, 126.25, 125.25, 124.12, 101.39 (anomeric *C*H-1″), 92.71 (anomeric *C*H-1′), 82.04, 81.95, 76.97 (*C*H-4), 75.59 (*C*H-5′), 73.84 (O-

*C*H$_2$-linker), 73.18, 68.56, 64.70, 63.29, 59.12 (*C*H$_2$-6″), 54.72, 50.72 (α-*C*H of Lys), 49.76, 48.45, 47.37, 45.80 (N-*C*H$_2$CH$_2$), 40.80 (N-*C*H$_2$-anthracene), 38.94 (ε-*C*H$_2$ of Lys), 38.59 (*C*H-6′), 30.72 (β-*C*H$_2$ of Lys), 29.19, 28.20, 28.15, 28.08, 28.02, 27.25, 26.49 (δ-*C*H$_2$ of Lys), 25.15, 24.86, 21.24; MALDI-TOF-MS *m/z* calc′d for $C_{47}H_{75}ClN_8O_{10}Na$: 969.519, found: 969.523 $[M+Na]^+$.

*Compound **3***. Synthesized following general procedure A from **14c** (125 mg, 0.058 mmol). The crude material was purified by C-18 reverse-phase flash column chromatography using deionized water. The product was isolated as a yellow solid (42.3 mg, 73 %). ^{1}H NMR (500 MHz, Deuterium Oxide) δ 8.69 – 8.58 (m, 2H, anthracene), 8.30 – 8.24 (m, 2H, anthracene), 7.79 – 7.70 (m, 4H, anthracene), 5.87 (d, *J* = 15.4 Hz, 1H, N-C*H*^{1}H^2-anthracene), 5.47 (d, *J* = 15.3 Hz, 1H, N-CH^1*H*^{2}-anthracene), 5.43 (d, *J* = 2.6 Hz, 1H, H-1′), 5.22 (d, *J* = 3.6 Hz, 1H, H-1″), 4.40 (t, *J* = 6.3 Hz, 1H, α-C*H* of Lys), 4.32 – 4.28 (m, 1H, H-5′), 4.21 (t, *J* = 9.8 Hz, 1H, H-4), 3.99 – 3.74 (m, 11H, H-5, H-6, H-2′, H-4′, H-2″, H-4″, H-5″, H-6″, O-C*H*$_2$ of linker), 3.67 – 3.57 (m, 3H, H-1, H-3, H-3″), 3.45 – 3.32 (m, 2H, H-6′), 3.03 – 2.96 (m, 1H, N-C*H*^{1}H^{2}CH$_2$), 2.89 – 2.79 (m, 2H, ε-C*H*$_2$ of Lys), 2.78 – 2.71 (m, 1H, N-CH^1*H*^{2}CH$_2$), 2.58 – 2.54 (m, 1H, H-2), 2.32 – 2.23 (m, 2H, H-3′), 2.03 – 1.86 (m, 3H, H-2, β-C*H*$_2$ of Lys), 1.75 – 1.66 (m, 2H, C*H*$_2$ of linker), 1.65 – 1.59 (m, 2H, δ-C*H*$_2$ of Lys), 1.45 – 1.29 (m, 6H, γ-C*H*$_2$ of Lys, C*H*$_2$ of linker), 1.25 – 1.18 (m, 2H, C*H*$_2$ of linker), 1.13 – 0.94 (m, 4H, C*H*$_2$ of linker), 0.91 – 0.83 (m, 2H, C*H*$_2$ of linker), 0.78 – 0.71 (m, 1H, C*H*$_2$ of linker), 0.68 – 0.59 (m, 3H, C*H*$_2$ of linker), 0.58 – 0.50 (m, 2H, C*H*$_2$ of linker). ^{13}C NMR (126 MHz, Deuterium Oxide) δ 169.21 (N*C*OCH), 131.30, 130.11, 128.10, 127.30, 127.18, 126.53, 125.43, 124.24, 101.39 (anomeric *C*H-1″), 92.73 (anomeric *C*H-1′), 81.93, 81.89, 76.82 (*C*H-4), 75.66 (*C*H-5′), 73.84 (O-*C*H$_2$-linker), 73.22, 68.54, 64.77, 63.24, 59.27 (*C*H$_2$-6″), 54.76, 50.73 (α-*C*H of Lys), 49.79, 48.44, 47.34, 46.04 (N-*C*H$_2$CH$_2$), 41.03 (N-*C*H$_2$-anthracene), 38.94 (ε-*C*H$_2$ of Lys), 38.55 (*C*H-6′), 30.74 (β-*C*H$_2$ of Lys), 29.57 (*C*H$_2$ of linker), 29.13, 29.00, 28.70, 28.56, 28.19, 28.16 (*C*H$_2$-3′), 27.98, 27.91 (*C*H$_2$-2), 27.32, 26.50 (δ-*C*H$_2$ of Lys), 25.40, 25.22, 21.28; MALDI-TOF-MS: *m/z* calc′d for $C_{51}H_{83}ClN_8O_{10}Na$: 1025.582, found: 1025.586 $[M+Na]^+$.

*Lys-N-dodecyl-10-aminomethyl-9-chloroanthracene Trifluoroacetate (**4**).* The Boc-Lys(Boc)-N-alkyl-aromatic compound **8** (22.8 mg, 0.031 mmol) was dissolved in CH_2Cl_2:TFA (2:1, v/v) (3 mL) and stirred

at room temperature for 1 h. The reaction was monitored by TLC (CH_2Cl_2/NH_4OH/MeOH, 5:1:1). At the end of the reaction, the mixture was evaporated to dryness, and purified by C-18 reverse-phase flash column chromatography (eluted with deionized water) to get analytically pure compound **4** as a yellow solid (10.3 mg, 62 %). ^{1}H NMR (500 MHz, Methanol-d_4) δ 8.61 – 8.57 (m, 2H, anthracene), 8.43 – 8.38 (m, 2H, anthracene), 7.69 – 7.61 (m, 4H, anthracene), 6.10 (d, J = 15.3 Hz, 1H, N-CH^1H^2-anthracene), 5.45 (d, J = 15.3 Hz, 1H, N-CH$^1H^2$-anthracene), 4.28 (t, J = 6.2 Hz, 1H, α-CH of Lys), 3.02 – 2.96 (m, 1H, N-CH^1H^{2}CH$_2$), 2.78 – 2.69 (m, 2H, ε-CH_2 of Lys), 2.68 – 2.60 (m, 1H, N-CH$^1H^2$CH$_2$), 1.86 – 1.76 (m, 2H, β-CH_2 of Lys), 1.59 – 1.52 (m, 2H, δ-CH_2 of Lys), 1.48 – 1.39 (m, 2H, γ-CH_2 of Lys), 1.34 – 1.20 (m, 10H, N-CH$_2$(CH_2)$_{10}$CH$_3$), 1.17 – 1.11 (m, 2H, N-CH$_2$(CH_2)$_{10}$CH$_3$), 1.09 – 1.03 (m, 2H, N-CH$_2$(CH_2)$_{10}$CH$_3$), 0.97 – 0.77 (m, 9H, N-CH$_2$(CH_2)$_{10}$CH_3). ^{13}C NMR (126 MHz, Methanol-d_4) δ 168.34 (N*C*OCH), 131.61, 130.03, 128.40, 127.33, 126.75, 126.61, 125.23, 124.19, 50.46 (α-*C*H of Lys), 45.08 (N-*C*H$_2$CH$_2$), 39.48 (N-*C*H$_2$-anthracene), 38.70 (ε-*C*H$_2$ of Lys), 31.64, 30.92 (β-*C*H$_2$ of Lys), 29.27, 29.16, 29.06, 29.02, 28.82, 28.56, 28.37, 26.76 (δ-*C*H$_2$ of Lys), 25.95, 22.31, 21.25, 13.01 (CH$_2$*C*H$_3$); MALDI-TOF-MS: *m/z* calc′d for $C_{33}H_{49}ClN_3O$: 538.356, found: 538.358 $[M+H]^+$.

N-dodecyl-10-aminomethyl-9-chloroanthracene Hydrochloride (7). Dodecylamine **6** (11.9 mg, 0.065 mmol) and aromatic aldehyde **5** (19 mg, 0.078 mmol) were dissolved in dry CH_2Cl_2:MeOH (1:1, v/v) (3 mL) and stirred at room temperature overnight. The mixture was cooled to 0 °C, and exposed to sodium borohydride (7 mg, 0.195 mmol) at room temperature overnight. The reaction mixture was subsequently concentrated, re-dispersed in diethyl ether, and treated with 2 N NaOH (3 mL) at room temperature for additional 15 mins. The organic layer was separated from the aqueous phase, washed with water and brine, dried over anhydrous Na_2SO_4, concentrated, and purified by flash column chromatography (eluted with CH_2Cl_2/MeOH from 300:1 to 30:1, v/v) to afford the desired compound as a yellow solid (19 mg, 70 %). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.60 – 8.55 (m, 2H, anthracene), 8.40 – 8.35 (m, 2H, anthracene), 7.64 – 7.54 (m, 4H, anthracene), 4.70 (s, 2H, NH-CH_2-anthracene), 2.91 – 2.84 (m, 2H, NH-CH_2CH$_2$), 1.62 – 1.57 (m, 2H, NH-CH$_2$CH_2), 1.37 - 1.24 (m, 18H, NH-CH$_2$CH$_2$(CH_2)$_9$CH$_3$), 0.89 (t, J = 6.8 Hz, 3H, CH$_2$CH_3). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 131.67, 130.70, 129.00, 128.66, 126.31,

126.21, 125.54, 124.53 (anthracene), 50.71 (NH-*C*H_2CH$_2$), 45.93 (NH-*C*H_2-anthracene), 32.00, 30.17 (NH-CH$_2$*C*H$_2$), 29.75, 29.72, 29.70, 29.64, 29.44, 27.45, 22.77, 14.20 (CH$_2$*C*H$_3$); ESI-MS: *m/z* calc′d for $C_{27}H_{37}ClN$: 410.2, found: 410.1 $[M+H]^+$.

*Boc-Lys(Boc)-N-dodecyl-10-aminomethyl-9-chloroanthracene (**8**)*. Boc-Lys(Boc)-OH (24 mg, 0.068 mmol) dissolved in DMF:$CHCl_3$ (5:2, v/v) (3.5 mL) was activated with DIPEA (18 mg, 0.135 mmol) and HBTU (26 mg, 0.068 mmol) at 0 °C for 15 mins, and treated with **7** (19 mg, 0.045 mmol). The mixture was stirred at 0 °C to room temperature overnight, concentrated under reduced pressure, and the resulting solution diluted in ethyl acetate. The mixture was washed with 0.5 M $KHSO_4$, water, and brine successively, and dried over anhydrous Na_2SO_4. The organic layer was concentrated and purified by flash column chromatography (eluted with CH_2Cl_2/MeOH from 300:1 to 100:1, v/v) to afford the desired compound as a yellow solid (30 mg, 90 %). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.64 – 8.54 (m, 2H, anthracene), 8.30 – 8.22 (m, 2H, anthracene), 7.64 – 7.51 (m, 4H, anthracene), 6.05 (d, *J* = 15.3 Hz, 1H, N-C*H*^{1}H^2-anthracene), 5.28 (d, *J* = 15.2 Hz, 1H, N-CH^1*H*^{2}-anthracene), 4.57 – 4.51 (m, 1H, α-C*H* of Lys), 3.12 – 2.95 (m, 2H, ε-C*H*$_2$ of Lys), 2.94 – 2.86 (m, 1H, N-C*H*^{1}H^{2}CH$_2$), 2.77 – 2.66 (m, 1H, N-CH^1*H*^{2}CH$_2$), 1.63 – 1.56 (m, 2H, β-*C*H$_2$ of Lys), 1.51 – 1.34 (m, 23H), 1.33 – 1.18 (m, 9H), 1.18 – 1.12 (m, 2H), 1.11 – 1.05 (m, 2H), 1.04 – 0.98 (m, 2H), 0.97 – 0.83 (m, 7H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 172.45 (N*C*OCH), 155.91, 155.49 (2 *C*OOC), 131.66, 130.50, 128.51, 127.57, 126.74, 126.56, 125.78, 124.22, 50.28 (α-*C*H of Lys), 45.28 (N-*C*H$_2$CH$_2$), 40.29 (ε-*C*H$_2$ of Lys), 39.54 (N-*C*H$_2$-anthracene), 33.77 (β-*C*H$_2$ of Lys), 31.90, 29.57, 29.51, 29.48, 29.39, 29.32, 29.25, 29.22, 28.84, 28.40 - 28.38 (*C*H$_3$), 26.54, 22.67, 14.11 (CH$_2$*C*H$_3$); ESI-MS: *m/z* calc′d for $C_{43}H_{64}ClN_3O_5Na$: 760.443, found: 760.485 $[M+Na]^+$.

*5-O-(4-butyl-10-Aminomethyl-9-chloroanthracene)-1,3,2′,6′,3″-penta-N-(tert-butoxycarbonyl)-4′,2″,4″,6″-tetra-OTBDMS-tobramycin (**13a**)*. Synthesized following general procedure B from **12a** (33 mg, 0.022 mmol), aromatic aldehyde **5** (6 mg, 0.026 mmol) and sodium borohydride (2.5 mg, 0.066 mmol). The resulting residue was purified by flash column chromatography using CH_2Cl_2/MeOH (300:1 to 30:1, v/v) to afford the desired compound as a yellow solid (21 mg, 55 %). ^{1}H NMR (500 MHz,

Chloroform-*d*) δ 8.58 – 8.53 (m, 2H, anthracene), 8.41 – 8.36 (m, 2H, anthracene), 7.62 – 7.55 (m, 4H, anthracene), 5.29 – 5.13 (m, 2H, H-1′, H-1″), 4.71 (s, 2H, NH-CH_2-anthracene), 4.30 – 4.06 (m, 3H), 3.85 – 3.17 (m, 15H), 2.84 (t, J = 7.1 Hz, 2H, NH-CH_2CH$_2$), 2.51 – 2.40 (m, 1H), 2.03 – 1.96 (m, 1H), 1.66 – 1.55 (m, 4H, CH_2 of linker), 1.55 – 1.51 (m, 1H), 1.48 – 1.29 (m, 45H), 1.10 – 1.01 (m, 1H), 0.94 – 0.81 (m, 36H, Si-CCH_3), 0.14 – -0.05 (m, 24H, Si-CH_3). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 155.64, 155.47, 154.78, 154.56, 154.27 (5 *C*OOC), 131.73, 130.81, 128.94, 128.65, 126.35, 126.28, 125.45, 124.69, 97.77 (anomeric *C*H-1″), 96.36 (anomeric *C*H-1′), 85.81, 79.91, 79.39, 79.27, 79.19, 78.71, 75.25, 73.17, 72.60, 71.49, 67.99, 66.93, 63.16, 57.26, 50.89 (NH-*C*H$_2$CH$_2$), 50.54, 48.89, 48.32, 45.81 (NH-*C*H$_2$-anthracene), 41.62, 35.98, 35.73, 28.62 - 28.22 (O-C*C*H$_3$), 26.72, 26.10 - 25.78 (Si-C*C*H$_3$), 18.44, 18.29, 18.06, 17.91 (4 Si-*C*CH$_3$), -3.45, -3.80, -4.22, -4.87, -4.92, -5.06, -5.19, -5.22 (8 Si-*C*H$_3$); ESI-MS: *m/z* calc′d for $C_{86}H_{152}ClN_6O_{19}Si_4$: 1721.9, found: 1721.6 $[M+H]^+$.

*5-O-(8-octyl-10-aminomethyl-9-chloroanthracene)-1,3,2',6',3''-penta-N-(tert-butoxycarbonyl)-4',2'',4'',6''-tetra-OTBDMS-tobramycin (**13b**)*. Synthesized following general procedure B from **12b** (35 mg, 0.023 mmol), aromatic aldehyde **5** (7 mg, 0.028 mmol) and sodium borohydride (3 mg, 0.069 mmol). The resulting residue was purified by flash column chromatography using CH_2Cl_2/MeOH (300:1 to 30:1, v/v) to afford the desired compound as a yellow solid (28 mg, 70 %). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.62 – 8.52 (m, 2H, anthracene), 8.42 – 8.33 (m, 2H, anthracene), 7.67 – 7.50 (m, 4H, anthracene), 5.26 – 5.12 (m, 2H, H-1′, H-1″), 4.71 (s, 2H, NH-CH_2-anthracene), 4.31 – 4.21 (m, 1H), 4.21 – 4.13 (m, 1H), 4.12 – 4.04 (m, 1H), 3.83 – 3.18 (m, 15H), 2.87 (t, J = 7.4 Hz, 2H, NH-CH_2CH$_2$), 2.51 – 2.44 (m, 1H), 2.03 – 1.98 (m, 1H), 1.61 – 1.56 (m, 2H, CH_2 of linker), 1.55 – 1.51 (m, 1H), 1.51 – 1.37 (m, 47H), 1.35 – 1.24 (m, 8H), 1.09 – 1.00 (m, 1H), 0.97 – 0.82 (m, 36H, Si-CCH_3), 0.19 – -0.00 (m, 24H, Si-CH_3). ^{13}C NMR (126 MHz, $CDCl_3$) δ 155.69, 155.53, 154.73, 154.56, 154.18 (5 *C*OOC), 131.19, 130.76, 129.16, 128.66, 126.37, 125.55, 124.51, 97.86 (anomeric *C*H-1″), 96.49 (anomeric *C*H-1′), 85.75, 79.91, 79.38, 79.22, 79.08, 78.88, 75.29, 73.33, 72.66, 71.55, 68.00, 66.86, 63.12, 57.25, 50.64 (NH-*C*H$_2$CH$_2$), 50.51, 48.91, 48.34, 45.79 (NH-*C*H$_2$-anthracene), 41.67, 35.94, 35.67, 30.65, 30.04 (*C*H$_2$ of linker), 29.62, 28.63 - 28.40 (O-C*C*H$_3$), 27.50, 26.18 - 25.78 (Si-C*C*H$_3$), 18.48, 18.32, 18.09, 17.91 (4 Si-*C*CH$_3$), -3.42, -3.79, -

4.20, -4.88, -4.94, -5.07, -5.17, -5.22 (8 Si-*C*H$_3$); ESI-MS: *m/z* calc′d for $C_{90}H_{160}ClN_6O_{19}Si_4$: 1778.0, found: 1778.0 $[M+H]^+$.

*5-O-(12-dodecyl-10-aminomethyl-9-chloroanthracene)-1,3,2',6',3''-penta-N-(tert-butoxycarbonyl)-4',2'',4'',6''-tetra-OTBDMS-tobramycin (**13c**)*. Synthesized following general procedure B from **12c** (116 mg, 0.072 mmol), aromatic aldehyde **5** (21 mg, 0.086 mmol) and sodium borohydride (8 mg, 0.216 mmol). The resulting residue was purified by flash column chromatography using CH_2Cl_2/MeOH (300:1 to 30:1, v/v) to afford the desired compound as a yellow solid (112 mg, 85 %). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.61 – 8.51 (m, 2H, anthracene), 8.41 – 8.32 (m, 2H, anthracene), 7.65 – 7.51 (m, 4H, anthracene), 5.25 – 5.12 (m, 2H, H-1′, H-1″), 4.70 (s, 2H, NH-C*H*$_2$-anthracene), 4.32 – 4.22 (m, 1H), 4.21 – 4.13 (m, 1H), 4.11 – 4.04 (m, 1H), 3.82 – 3.19 (m, 15H), 2.86 (t, *J* = 7.3 Hz, 2H, NH-C*H*$_2$CH$_2$), 2.51 – 2.43 (m, 1H), 2.04 – 1.98 (m, 1H), 1.61 – 1.55 (m, 2H, C*H*$_2$ of linker), 1.55-1.51 (m, 1H), 1.50 – 1.36 (m, 47H), 1.34 – 1.22 (m, 16H), 1.11 - 1.02 (m, 1H), 0.97 – 0.82 (m, 36H, Si-CC*H*$_3$), 0.19 – -0.02 (m, 24H, Si-C*H*$_3$). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 155.67, 155.51, 154.72, 154.55, 154.19 (5 *C*OOC), 131.59, 130.71, 129.00, 128.65, 126.32, 126.25, 125.53, 124.51, 97.79 (anomeric *C*H-1″), 96.54 (anomeric *C*H-1′), 85.71, 79.88, 79.35, 79.19, 79.04, 78.80, 75.28, 73.40, 72.68, 71.53, 68.01, 66.81, 63.09, 57.27, 50.67 (NH-*C*H$_2$CH$_2$), 50.52, 48.95, 48.38, 45.90 (NH-*C*H$_2$-anthracene), 41.65, 35.90, 35.65, 30.63, 30.13, 30.05, 29.72, 29.70, 29.66, 29.61, 28.63 - 28.40 (O-C*C*H$_3$), 27.42, 26.19 - 25.78 (Si-C*C*H$_3$), 18.47, 18.33, 18.09, 17.90 (4 Si-*C*CH$_3$), -3.42, -3.80, -4.20, -4.88, -4.94, -5.07, -5.18, -5.23 (8 Si-*C*H3); ESI-MS: *m/z* calc′d for $C_{94}H_{168}ClN_6O_{19}Si_4$: 1833.3, found: 1833.1 $[M+H]^+$.

*Compound **14a***. Synthesized following general procedure C from **13a** (21 mg, 0.012 mmol), Boc-Lys(Boc)-OH (6 mg, 0.018 mmol), DIPEA (5 mg, 0.036 mmol) and HBTU (7 mg, 0.018 mmol). After column chromatography (eluted with CH_2Cl_2/MeOH from 300:1 to 30:1, v/v), the product was afforded as a yellow solid (18.6 mg, 74 %). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.66 – 8.55 (m, 2H, anthracene), 8.47 – 8.18 (m, 2H, anthracene), 7.71 – 7.54 (m, 4H, anthracene), 6.27 – 5.95 (m, 1H, N-C*H*^{1}H^2-anthracene), 5.36 – 5.23 (m, 1H, N-CH^1*H*^{2}-anthracene), 5.11 – 4.99 (m, 2H, H-1′, H-1″), 4.52 – 4.39 (m, 1H, α-C*H* of Lys), 4.26 – 3.95 (m, 2H), 3.83 – 3.73 (m, 1H), 3.70 – 3.10 (m, 15H), 3.08 – 2.95 (m, 2H, ε-

C*H*$_2$ of Lys), 2.95 – 2.68 (m, 2H, N-C*H*$_2$CH$_2$), 2.55 – 2.35 (m, 1H), 2.01 – 1.91 (m, 1H), 1.54 – 1.52 (m, 2H, β-C*H*$_2$ of Lys), 1.51 – 1.37 (m, 53H), 1.37 – 1.16 (m, 17H), 1.13 – 1.05 (m, 1H), 0.94 – 0.75 (m, 38H), 0.18 – -0.19 (m, 24H, Si-C*H*$_3$). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 172.15 (N*C*OCH), 155.88, 155.80, 155.65, 155.21, 154.82, 154.49, 154.35 (7 *C*OOC), 131.89, 131.77, 130.49, 128.54, 127.87, 127.32, 126.92, 126.65, 125.94, 125.79, 125.65, 124.67, 124.38, 123.70, 98.24 (anomeric *C*H-1″), 96.55 (anomeric *C*H-1′), 86.13, 79.71, 79.38, 79.29, 79.24, 79.18, 78.92, 78.82, 75.56, 72.61, 72.30, 71.76, 67.59, 66.90, 63.15, 57.11, 50.53, 50.30 (α-*C*H of Lys), 49.27, 48.44, 45.45 (N-*C*H$_2$CH$_2$), 41.47, 40.35 (ε-*C*H$_2$ of Lys), 39.55 (N-*C*H$_2$-anthracene), 35.77, 35.57, 33.2 (β-*C*H$_2$ of Lys), 29.67, 29.56, 28.72 - 28.30 (O-C*C*H$_3$), 27.34, 26.12 - 25.78 (Si-C*C*H$_3$), 22.45, 18.38, 18.31, 18.08, 17.92 (4 Si-*C*CH$_3$), -3.35, -3.74, -4.25, -4.87, -5.03, -5.16, -5.22, -5.35 (8 Si-*C*H$_3$); MALDI-TOF-MS: *m/z* calc′d for $C_{102}H_{179}ClN_8O_{24}Si_4Na$: 2071.173, found: 2071.203 $[M+Na]^+$

*Compound **14b***. Synthesized following general procedure C from **13b** (28 mg, 0.016 mmol), Boc-Lys(Boc)-OH (8 mg, 0.024 mmol), DIPEA (6 mg, 0.048 mmol) and HBTU (9 mg, 0.024 mmol). After column chromatography (eluted with CH_2Cl_2/MeOH from 300:1 to 30:1, v/v), the product was afforded as a yellow solid (28 mg, 85 %). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.66 – 8.56 (m, 2H, anthracene), 8.33 – 8.24 (m, 2H, anthracene), 7.71 – 7.50 (m, 4H, anthracene), 6.10 (d, *J* = 15.2 Hz, 1H, N-C*H*^{1}H^2-anthracene), 5.33 – 5.27 (m, 1H, N-CH^1*H*^{2}-anthracene), 5.25 – 5.19 (m, 1H, H-1′), 5.14 – 5.11 (m, 1H, H-1″), 4.56 – 4.51 (m, 1H, α-C*H* of Lys), 4.30 – 4.21 (m, 1H), 4.20 – 4.12 (m, 1H), 4.10 – 4.02 (m, 1H), 3.84 – 3.19 (m, 15H), 3.09 – 2.97 (m, 2H, ε-C*H*$_2$ of Lys), 2.96 – 2.86 (m, 1H, N-C*H*^{1}H^{2}CH$_2$), 2.74 – 2.64 (m, 1H, N-CH^1*H*^{2}CH$_2$), 2.54 – 2.42 (m, 1H), 2.04 – 1.97 (m, 1H), 1.58 – 1.55 (m, 2H, β-C*H*$_2$ of Lys), 1.54 – 1.52 (m, 1H), 1.51 - 1.38 (m, 65H), 1.38 – 1.31 (m, 4H, γ-C*H*$_2$ of Lys, δ-C*H*$_2$ of Lys), 1.31 – 1.21 (m, 2H, C*H*$_2$ of linker), 1.21 – 1.12 (m, 2H, C*H*$_2$ of linker), 1.12 – 1.00 (m, 5H), 1.00 – 0.79 (m, 38H), 0.22 – -0.09 (m, 24H, Si-C*H*$_3$). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 172.37 (N*C*OCH), 155.90, 155.74, 155.54, 155.42, 154.70, 154.56, 154.34 (7 *C*OOC), 131.69, 130.49, 128.54, 127.70, 127.16, 126.76, 126.61, 125.79, 124.27, 123.92, 97.91 (anomeric CH-1″), 96.44 (anomeric CH-1′), 85.83, 79.91, 79.45, 79.38, 79.24, 79.18, 78.93, 78.78, 75.34, 73.23, 72.65, 71.57, 67.95, 66.87, 63.16, 57.24, 50.48, 50.31 (α-

*C*H of Lys), 48.87, 48.32, 45.26 (N-*C*H_2*C*H_2), 41.69, 40.28 (ε-*C*H_2 of Lys), 39.36 (N-*C*H_2-anthracene), 35.92, 35.69, 33.69 (β-*C*H_2 of Lys), 30.73, 30.03, 29.47, 29.28, 28.63 - 28.39 (O-C*C*H_3), 26.95, 26.22 - 25.78 (Si-C*C*H_3), 22.52, 18.47, 18.32, 18.10, 17.91 (4 Si-*C*CH_3), -3.39, -3.77, -4.20, -4.87, -4.93, -5.08, -5.16, -5.20 (8 Si-*C*H_3); MALDI-TOF-MS: *m/z* calc′d for $C_{106}H_{187}ClN_8O_{24}Si_4Na$: 2127.206, found: 2127.219 $[M+Na]^+$.

*Compound **14c***. Synthesized following general procedure C from **13c** (112 mg, 0.061 mmol), Boc-Lys(Boc)-OH (31.9 mg, 0.092 mmol), DIPEA (24 mg, 0.183 mmol) and HBTU (35 mg, 0.092 mmol). After column chromatography (eluted with CH_2Cl_2/MeOH from 300:1 to 30:1, v/v), the product was afforded as a yellow solid (125 mg, 95 %). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.63 – 8.55 (m, 2H, anthracene), 8.31 – 8.24 (m, 2H, anthracene), 7.64 – 7.53 (m, 4H, anthracene), 6.06 (d, *J* = 15.3 Hz, 1H, N-C*H*^{1}H^2-anthracene), 5.29 (d, *J* = 15.2 Hz, 1H, N-CH^1*H*^{2}-anthracene), 5.24 – 5.19 (m, 1H, H-1′), 5.17 – 5.12 (m, 1H, H-1″), 4.56 – 4.52 (m, 1H, α-C*H* of Lys), 4.31 – 4.21 (m, 1H), 4.20 – 4.12 (m, 1H), 4.11 – 4.03 (m, 1H), 3.82 – 3.15 (m, 15H), 3.08 – 2.96 (m, 2H, ε-C*H*$_2$ of Lys), 2.95 – 2.85 (m, 1H, N-C*H*^{1}H^{2}CH_2), 2.76 – 2.67 (m, 1H, N-CH^1*H*^{2}CH_2), 2.53 – 2.40 (m, 1H), 2.02 – 1.97 (m, 1H), 1.60 – 1.55 (m, 2H, β-C*H*$_2$ of Lys), 1.54 – 1.52 (m, 1H), 1.50 – 1.39 (m, 65H), 1.38 – 1.32 (m, 4H, γ-C*H*$_2$ of Lys, δ-C*H*$_2$ of Lys), 1.30 – 1.23 (m, 4H, C*H*$_2$ of linker), 1.22 – 1.11 (m, 6H, C*H*$_2$ of linker), 1.09 – 0.98 (m, 5H), 0.97 – 0.81 (m, 40H), 0.21 – -0.03 (m, 24H, Si-C*H*$_3$). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 172.42 (N*C*OCH), 155.89, 155.68, 155.66, 155.46, 154.72, 154.55, 154.43 (7 *C*OOC), 131.67, 130.50, 128.52, 127.59, 127.14, 126.75, 126.57, 125.79, 124.23, 123.95, 97.82 (anomeric *C*H-1″), 96.51 (anomeric *C*H-1′), 85.74, 79.89, 79.76, 79.51, 79.36, 79.20, 78.94, 78.73, 75.29, 73.38, 72.68, 71.54, 68.03, 66.81, 63.09, 57.27, 50.51, 50.28 (α-*C*H of Lys), 48.94, 48.36, 45.28 (N-*C*H_2*C*H_2), 41.68, 40.27 (ε-*C*H_2 of Lys), 39.52 (N-*C*H_2-anthracene), 35.93, 35.64, 33.75 (β-*C*H_2 of Lys), 30.68, 30.13, 29.75, 29.66, 29.53, 29.41, 29.26, 28.95, 28.62 - 28.37 (O-C*C*H_3), 26.60, 26.25 - 25.77 (Si-C*C*H_3), 22.55, 18.47, 18.32, 18.09, 17.90 (4 Si-*C*CH_3), -3.42, -3.80, -4.21, -4.89, -4.95, -5.08, -5.19, -5.23 (8 Si-*C*H_3); MALDI-TOF-MS: *m/z* calc′d for $C_{110}H_{195}ClN_8O_{24}Si_4Na$: 2183.298, found: 2183.302 $[M+Na]^+$.

Biological Activity Assays. *Bacterial Isolates.* Bacterial isolates were obtained as part of the Canadian National Intensive Care Unit (CAN-ICU) study[61] and Canadian Ward Surveillance (CANWARD) studies[62, 63]. The CAN-ICU study included 19 medical centres across Canada with active ICUs. From September 2005 to June 2006, 4180 isolates represented in 2580 ICU patients were recovered from clinical specimens including blood, urine, wound/tissue, and respiratory specimens (one pathogen per cultured site per patient). Only "clinically significant" specimens from patients with a presumed infectious disease were collected. The isolates obtained were shipped to the reference laboratory (Health Sciences Centre, Winnipeg, Canada) on Amies charcoal swabs. Then isolates were sub-cultured onto appropriate medium and stocked in skim milk at -80 °C until subsequent MIC testing was carried out. The quality control strains including *Staphylococcus aureus* ATCC 29213, methicillin-resistant *S. aureus* (MRSA) ATCC 33592, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium* ATCC 27270, *Streptococcus pneumoniae* ATCC 49619, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Klebsiella pneumoniae* ATCC 13883 were acquired from the American Type Culture Collection (ATCC). The clinical strains, including methicillin-resistant *Staphylococcus epidermidis* (MRSE), CAN-ICU 61589 (cefazolin MIC > 32 μg/mL), gentamicin resistant *E. coli* CAN-ICU 61714, Amikacin-resistant *E. coli* CAN-ICU 63074 (MIC = 32 μg/mL), gentamicin resistant *P. aeruginosa* CAN-ICU 62584, *Strenotrophomonas maltophilia* CAN-ICU 62584, and *Acinetobacter baumannii* CAN-ICU 63169 were obtained from hospitals across Canada as a part of the CAN-ICU study. Methicillin-susceptible *S. epidermidis* (MSSE) CANWARD-2008 81388 was obtained from the 2008 CANWARD study, while gentamicin-resistant tobramycin-resistant ciprofloxacin-resistant [aminoglycoside modifying enzyme aac(3)-IIa present] *E. coli* CANWARD-2011 97615, and gentamicin-resistant tobramycin-resistant *P. aeruginosa* CANWARD-2011 96846 were obtained from the 2011 CANWARD study. In addition, *P. aeruginosa* PAO1, *P. aeruginosa* P259-96918, *P. aeruginosa* P262-101856, *P. aeruginosa* P264-104354, colistin-resistant *P. aeruginosa* 91433, colistin-resistant *P. aeruginosa* 101243, *A. baumannii* AB027, *A. baumannii* AB030, *A. baumannii* AB031, *A. baumannii* 110193, *Enterobacter cloacae* 117029, and *Klebsiella pneumonia* 116381 were kindly provided by Dr. George G. Zhanel. The

efflux pump-mutated strains, *P. aeruginosa* PAO200 and *P. aeruginosa* PAO750, were provided by Dr. Ayush Kumar from University of Manitoba in Canada.

Multi-drug resistance in *P. aeruginosa* was defined as concomitant resistance to 3 or more chemically unrelated antimicrobial classes, while extensively drug resistant was defined as concomitant resistance to 5 or more chemically unrelated antimicrobial classes.

Antimicrobial Activity Assay. The antimicrobial activity of the compounds against a panel of bacteria was evaluated by microliter dilution method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines. The overnight bacterial culture was diluted in saline to 0.5 McFarland turbidity, and then 1:50 diluted in Mueller-Hinton broth (MHB) for inoculation. The antimicrobial agents were 2-fold serially diluted in MHB in 96-well plate and incubated with equal volumes of inoculum for 18 h at 37 °C. The lowest concentration that prevented visible bacterial growth was taken as the MIC for each antimicrobial agent. The broth with or without bacterial cells was employed as positive or negative controls, respectively.

Combination Studies with Different Antibiotics. FIC index was determined by setting up standard checkerboard assay in 96-well plate as previously described.[64] Each antibiotic to be tested was serially diluted along the abscissa in MHB, while adjuvant was diluted along the ordinate to create a 10 × 7 matrix. The bacterial culture was prepared in MHB by 1:50 dilution from the 0.5 McFarland turbidity culture in saline. The inoculum was added to each well of the plate and incubated for 18 h at 37 °C. After the incubation, plates were read on EMax® Plus microplate reader (Molecular Devices, Sunnyvale, CA, USA). MIC was recorded as wells with the lowest concentration of drugs with no bacterial growth. The FIC for each antibiotic was calculated as the concentration of the antibiotic for a well showing no growth in the presence of adjuvant divided by the MIC for that antibiotic alone. The FIC for each adjuvant was calculated as the concentration of the adjuvant for a well showing no growth in the presence of antibiotic divided by the MIC for that adjuvant alone. The FIC index is the sum of the two FICs. Chemical-

chemical interactions with an FIC index $\leqslant$ 0.5 were deemed synergistic; 0.5 - 4, no interaction; and $\geqslant$ 4, antagonism.

Time-kill Curve Assay. The kinetics of bacterial killing was measured using *P. aeruginosa* PAO1, as previously described.[59] Overnight bacterial culture was diluted in saline to 0.5 McFarland turbidity and then 1:50 diluted in Luria-Bertani (LB) broth. The cell suspension was incubated with minocycline, rifampicin, or hybrid **3** diluted in PBS (pH 7.2) alone at desired concentrations (1/2 ×, 1 ×, 2 ×, 4 × MIC). For synergistic time-kill, the combination of compound **3** with minocycline or rifampicin at various concentrations, 1/8 + 1/8, 1/8 + 1/4, 1/4 + 1/4, 1/2 + 1/4, and 1/2 + 1/2 × MIC, were determined. Samples were incubated at 37 °C for 6 h. At specific intervals (0, 10, 30, 60, 90, 120, 240, and 360 mins), aliquots (50 μL) were removed from the samples, serially diluted in PBS and plated on LB agar plates. Bacterial colonies were formed and counted after 20 h of incubation at 37 °C.

Resistance Development Assay. Wild-type *P. aeruginosa* PAO1 was used to study resistance development against antibiotics by sequential passaging method as previously described.[65] Briefly, MIC testing was first conducted for all drugs or drug combinations to be tested, as described above. After 18 h incubation, the bacterial cells growing in the well of half-MIC concentration were harvested and diluted to 0.5 McFarland in saline followed by 1:50 dilution in fresh MHB broth. The inoculum was subjected to next passage MIC testing, and the process repeated for 25 passages. The fold change in MIC was plotted against the number of passages.

Outer Membrane Permeability Assay. The CFDASE dye was used to determine the outer membrane permeability of drugs against *P. aeruginosa* PAO1, following established protocols.[28] Logarithmic phase *P. aeruginosa* was harvested by centrifugation and washed twice with PBS. The bacterial cells were resuspended in the same buffer to OD_{600} of 0.5, followed by staining with CFDASE at 100 μM for 30 mins at 37°C. The unbound dye was then removed by washing the cells with excess buffer, and the cells were again resuspended to the initial volume. The bacterial suspension was treated with drugs at 37 °C for 30 mins at desired concentration and the supernatant obtained by centrifugation was transferred to 96-well

black plate for measuring the fluorescence at an excitation wavelength of 488 nm and an emission wavelength of 520 nm using a microplate reader FlexStation 3 (Molecular Devices, Sunnyvale, CA, USA).

Cytoplasmic Membrane Depolarization Assay. To assess the effect of the compounds on cytoplasmic membrane potential, diSC_3-5, the membrane-potential-sensitive fluorescent dye was utilized to determine the membrane depolarization of *P. aeruginosa* PAO1 as previously described.[19] Overnight growth *P. aeruginosa* PAO1 was diluted in fresh LB broth and cultured to the mid-log phase. The bacterial cells were harvested and washed three times with 5 mM sodium HEPES buffer, pH 7.4, containing 20 mM glucose, and resuspended to OD_{600} of 0.05 in the same buffer. The cell suspension was incubated with 0.2 mM EDTA and 0.4 μM diSC_3-5 in the dark for 2 h at 37 °C under constant shaking (150 rpm). 100 mM KCl was then added to equilibrate the cytoplasmic and external K^+, and incubated for additional 30 mins. The depolarization assay was carried out in 96-well black plate by adding the antimicrobial agents to 100 μL of the above cell suspension to desired concentration. Fluorescence was monitored using a FlexStation 3 (Molecular Devices, Sunnyvale, CA, USA) microplate reader at an excitation wavelength of 622 nm and an emission wavelength of 670 nm.

Motility Assay. Cell motility assay was performed on 0.3 % (w/v) agar media supplemented with tryptone (5 g/L) and NaCl (2.5 g/L).[66] Antimicrobial agents were added to 25 mL medium to the desired concentration and poured on 100 × 15 mm petri dishes followed by 2 h drying. Overnight *P. aeruginosa* PAO1 culture was diluted in 0.85 % saline to 1.0 McFarland and point inoculated into the center of the motility agar plates. Plates were incubated at 37 ℃ for 20 h. The images presented were taken using a FluroChem®Q (Cell biosciences).

Quantification of Hemolytic Activity. The hemolytic activity of the compounds was determined as the amount of hemoglobin released by lysing pig erythrocytes.[67] Fresh pig blood (provided by Dr. Charles M. Nyachoti from University of Manitoba) drawn from pig antecubital were centrifuged at 1,000 × g for 5 mins at 4 °C, washed with PBS thrice, and resuspended in the same buffer. Compounds were 2-fold serially diluted in PBS in 96-well plate and mixed with equal volumes of erythrocyte solution. After 1 h

incubation at 37 °C, intact erythrocytes were pelleted by centrifuging at 1,000 × g for 5 mins at 4 °C, and the supernatant was transferred to a new 96-well plate. The hemoglobin release was monitored at 570 nm using an EMax® Plus microplate reader (Molecular Devices, Sunnyvale, CA, USA). Blood cells in PBS and 0.1% Triton X-100 were employed as negative and positive controls respectively.

Cytotoxicity Assay. DU145 (ATCC, Manassas, VA, USA) and JIMT-1 (DSMZ, Braunschweig, Germany) were cultured and maintained in Dulbecco's modified Eagle's References medium supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 10 % (v/v) fetal bovine serum (FBS) at 37 °C under a humidified atmosphere of 5 % CO_2 and 95 % air. The methanethiosulfonate (MTS) cell viability assay was employed to measure the cytotoxicity of compound **3** as previously described.[16] Briefly, the cells were seeded in 96-well plate with a final concentration of 7500-9000 cells per well and incubated for 24 h. Then the cells were treated with test compound at final concentrations of 2.5 to 30 μM and incubated for an additional 48 h at the same condition. MTS reagent (20 %, v/v) was further added to each well and the plates were incubated for 4 h on a Nutating mixer in the incubator. The optical density was measured using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA, USA) at 490 nm. Only medium without cells were served as blank and the blank values were subtracted from each sample value. The cell viability relative to the control with vehicle was calculated.

Galleria mellonella Model of *P. aeruginosa* Infection. *Galleria mellonella* waxworms were obtained from The Worm Lady® Live Feeder Insects (http://thewormlady.ca/). Worms (average weight at 250 mg) were used within 7 days of delivery to determine the survival rate after bacteria or antimicrobials injection using previously described methods.[68] The tolerability study was performed by only injecting antimicrobial agent into the worms at 100 and 200 mg/kg without bacteria. The worms (ten larvae in each group) were incubated at 37 °C and monitored for 96 h for survival. For therapeutic study, overnight XDR *P. aeruginosa* P262 culture was diluted in PBS to a final concentration of 1.0×10^3 CFU/mL. 15 larvae per group were infected with 10 μL bacterial suspensions. After 2 h bacterial challenge, worms in monotherapy experimental groups received a 10 μL injection of minocycline, rifampicin, or compound **3** individually at 75 mg/kg. For combination groups, **3** plus minocycline and **3** plus rifampicin were injected

to give final dosages of 12.5 + 12.5, 25 + 25, 37.5 + 37.5, and 75 + 75 mg/kg respectively. Only vehicle (PBS) without antimicrobials was injected as control group. The larvae were monitored for 24 h at 37 °C in petri dishes lined with filter paper and scored for survivability. Larvae considered dead if they do not respond to touch.

ASSOCIATED CONTENT

Supporting Information.

Additional figures, tables, NMR, and HPLC spectra (PDF).

Molecular formula strings (CSV).

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Y.L., X.Y. carried out organic synthesis; R.D. helped with compound characterization. Y.L., S.G. and G.G.Z. were involved biological and mechanistic studies; B.K.G and Y.L. carried out the *in vivo* experiments; T.I. and F.S. critically revised the manuscript; F.S. and A.S. supervised the project; Y.L. wrote the main manuscript with contributions from all authors.

Competing Finical Interests: The authors declare that a provisional patent USSN62/484,995 has been filed on the content of this paper.

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ABBREVIATIONS USED

AAG, amphiphilic aminoglycosides; FIC, fractional inhibitory concentration; MDR, multidrug-resistant; MIC, minimal inhibitory concentration; PMF, proton motive force; XDR, extensively drug-resistant.

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Table 1. MIC of Compounds against a Panel of Gram-positive and Gram-negative Bacteria

Organisms	MIC[a] (μg/mL)				
	Tobramycin	**4**	Amphiphilic tobramycin-lysine conjugates		
			1	**2**	**3**
Gram-positive bacteria					
S.aureus ATCC 29213	≤0.25[b]	32	64	16	8
MRSA ATCC 33592	≤0.25[b]	64	64	16	8
MSSE CANWARD-2008 81388	≤0.25[b]	8	16	8	2
MRSE CAN-ICU 61589 (CAZ >32)	1[b]	8	32	8	4
E. faecalis ATCC 29212	8[b]	32	>128	64	16
E. faecium ATCC 27270	8[b]	8	>128	16	8
S. pneumoniae ATCC 49619	2[b]	32	>128	128	32
Gram-negative bacteria					
E.coli ATCC 25922	0.5[b]	64	32	16	32
E.coli CAN-ICU 61714 (GEN-R)	8[b]	128	64	32	32
E.coli CAN-ICU 63074 (AMK 32)	8[b]	64	64	128	16
E.coli CANWARD-2011 97615 (GEN-R, TOB-R, CIP-R) aac(3')iia	128[b]	64	64	32	32
P. aeruginosa ATCC 27853	0.5[b]	>128	>128	>128	32
P. aeruginosa CAN-ICU 62308 (GEN-R)	16[b]	128	64	16	16
P. aeruginosa CANWARD-2011 96846 (GEN-R, TOB-R)	256[b]	>128	>128	64	32
S. maltophilia CAN-ICU 62584	>512[b]	>128	>128	>128	>128
A. baumannii CAN-ICU 63169	32[b]	128	>128	>128	128
K. pneumoniae ATCC 13883	≤0.25[b]	>128	128	>128	128

[a] Minimum inhibitory concentration (MIC) was determined as the lowest concentration of compound that inhibited bacteria growth. [b] MIC data of tobramycin as previously reported.[16]

MRSA: Methicillin-resistant *S. aureus*; MSSE: Methicillin-susceptible *S. epidermidis*; MRSE: Methicillin-resistant *S. epidermidis*; CANWARD: Canadian Ward surveillance; CAN-ICU: Canadian National Intensive Care Unit surveillance; CAZ: Ceftazidime; GEN-R: Gentamicin-resistant; AMK: Amikacin; TOB-R: Tobramycin-resistant; CIP-R: Ciprofloxacin-resistant.

Table 2. MIC of Compounds 1 – 4 and Antibiotics against Wild-type and Clinical Isolate *P. aeruginosa*.

P. aeruginosa strains	MIC (μg/mL)													
	TOB	**4**	Amphiphilic tobramycin-lysine conjugates			Antibiotics								
			1	**2**	**3**	MOX	CIP	MIN	RMP	CAZ	CAM	ERY	TMP	COL
PAO1	0.25	256	>512	128	32	1	ND	8	16	2	64	256	256	1
100036	64	256	>512	512	32	128	64	32	16	8	>512	256	256	2
101885	0.25	128	256	64	16	64	32	32	16	8	512	256	>512	0.5
P259-96918	128	64	>512	512	64	512	128	32	16	512	512	256	512	0.5
P262-101856	512	128	>512	128	32	128	32	256	1024	16	1024	1024	>1024	2
P264-104354	128	128	>512	256	32	128	32	64	16	64	1024	256	256	4
91433	8	256	32	8	8	8	2	64	16	256	16	512	512	4
101243	256	64	256	32	16	4	2	4	8	64	4	1024	1024	>1024

TOB = Tobramycin; MOX = Moxifloxacin; CIP = Ciprofloxacin; MIN = Minocycline; RMP = Rifampicin; CAZ = Ceftazidime; CAM = Chloramphenicol; ERY = Erythromycin; TMP = Trimethoprim; COL = Colistin. ND = Not determined.

Table 3. Combination Study of Compound 3 with Antibiotics against Wild-type *P. aeruginosa* PAO1.

Antibiotics	MIC alone (μg/mL)	Synergistic MIC (μg/mL)	FIC $_{antibiotic}$	Hybrid	MIC alone (μg/mL)	Synergistic MIC (μg/mL)	FIC $_{hybrid}$	FIC index
Moxifloxacin	1	0.25	0.25	**3**	32	2	0.063	0.313
Novobiocin	1024	8	0.008	**3**	32	2	0.063	0.071
Minocycline	8	0.5	0.063	**3**	32	1	0.031	0.094
Rifampicin	16	1	0.063	**3**	32	1	0.031	0.094
Ceftazidime	2	1	0.5	**3**	32	2	0.063	0.563
Chloramphenicol	64	2	0.031	**3**	32	4	0.125	0.156
Erythromycin	256	8	0.031	**3**	32	4	0.125	0.156
Trimethoprim	256	16	0.063	**3**	32	2	0.063	0.126
Colistin	1	1	1	**3**	32	1	0.031	1.031
Gentamicin	1	1	1	**3**	32	4	0.125	1.125
Kanamycin A	64	64	1	**3**	32	0.5	0.016	1.016
Amikacin	1	1	1	**3**	32	2	0.063	1.063
Meropenem	0.5	1	2	**3**	32	1	0.031	2.031
Vancomycin	>1024	128	<0.125	**3**	32	4	0.125	0.125 < 0.25

Table 4. Synergistic Effects of Compound 3 with Minocycline or Rifampicin against Clinical MDR or XDR *P. aeruginosa* Isolates.

P. aeruginosa strain	Antibiotics (MIC[a])	FIC $_{antibiotic}$	Hybrid (MIC[a])	FIC $_{hybrid}$	FIC index	Absolute MIC[b]	Potentiation[c]
100036	Minocycline (32)	0.063	**3** (32)	0.031	0.094	1	32-fold
100036	Rifampicin (16)	0.016	**3** (32)	0.063	0.079	0.125	128-fold
101885	Minocycline (32)	0.125	**3** (16)	0.031	0.156	2	16-fold
101885	Rifampicin (16)	0.063	**3** (16)	0.031	0.094	0.125	128-fold
P259-96918	Minocycline (32)	0.031	**3** (64)	0.016	0.047	0.5	64-fold
P259-96918	Rifampicin (16)	0.008	**3** (64)	0.031	0.039	0.063	256-fold
P262-101856	Minocycline (256)	0.008	**3** (32)	0.125	0.133	2	128-fold
P262-101856	Rifampicin (1024)	0.016	**3** (32)	0.125	0.141	16	64-fold
P264-104354	Minocycline (64)	0.016	**3** (32)	0.063	0.079	0.5	128-fold
P264-104354	Rifampicin (16)	0.031	**3** (32)	0.063	0.094	0.25	64-fold
91433[d]	Minocycline (64)	0.016	**3** (8)	0.25	0.266	0.5	128-fold
91433[d]	Rifampicin (16)	0.25	**3** (8)	0.25	0.5	2	8-fold
101243[d]	Minocycline (4)	0.25	**3** (16)	0.031	0.281	1	4-fold
101243[d]	Rifampicin (8)	0.063	**3** (16)	0.125	0.188	0.25	32-fold

[a] All MIC data presented in μg/mL. [b] Absolute MIC of antibiotic was determined in the presence of hybrid at 4 μg/mL. [c] Antibiotic activity potentiation at 4 μg/mL of hybrid. [d] Colistin-resistant strain.

Table 5. Synergistic Effects of Compound 3 with Minocycline and with Rifampicin against *P. aeruginosa* PAO1 and Efflux Pump Deficient *P. aeruginosa* PAO200 and PAO750 Strains.

P. aeruginosa strain	Antibiotic (MIC μg/mL)	Adjuvant (MIC μg/mL)	FIC index
PAO1	Minocycline (8)	**3** (32)	0.094
PAO1	Rifampicin (16)	**3** (32)	0.094
PAO200	Minocycline (1)	**3** (16)	0.531
PAO200	Rifampicin (8)	**3** (16)	0.129
PAO750	Minocycline (1)	**3** (8)	0.75
PAO750	Rifampicin (8)	**3** (8)	0.156

Table 6. Combination Study of Compound 3 with Minocycline or Rifampicin against MDR *Acinetobacter baumannii*, *Enterobacter cloacae* and *Klebsiella pneumoniae*.

Organisms[#]	Antibiotics	MIC alone (μg/mL)	Synergistic MIC (μg/mL)	FIC $_{antibiotic}$	Hybrids	MIC alone (μg/mL)	Synergistic MIC (μg/mL)	FIC $_{hybrid}$	FIC index
AB027	Minocycline	1	0.25	0.25	**3**	128	1	0.008	0.258
AB027	Rifampicin	2	0.031	0.016	**3**	128	8	0.063	0.079
AB030	Minocycline	4	0.5	0.125	**3**	64	16	0.25	0.375
AB030	Rifampicin	>256	32	<0.125	**3**	64	8	0.125	0.125 < 0.25
AB031	Minocycline	2	1	0.5	**3**	16	2	0.125	0.625
AB031	Rifampicin	2	0.125	0.06	**3**	32	1	0.03	0.09
110193	Minocycline	2	1	0.5	**3**	16	2	0.13	0.63
110193	Rifampicin	2	0.063	0.032	**3**	16	1	0.063	0.095
117029	Minocycline	128	4	0.031	**3**	256	8	0.031	0.062
117029	Rifampicin	16	0.063	0.004	**3**	256	4	0.016	0.02
116381	Minocycline	128	32	0.25	**3**	256	16	0.063	0.313
116381	Rifampicin	1024	32	0.031	**3**	256	4	0.016	0.047

[#]AB027, [#]AB030, [#]AB031, and [#]110193 = *Acinetobacter baumannii*; [#]117029 = *Enterobacter cloacae*; [#]116381 = *Klebsiella pneumoniae*.

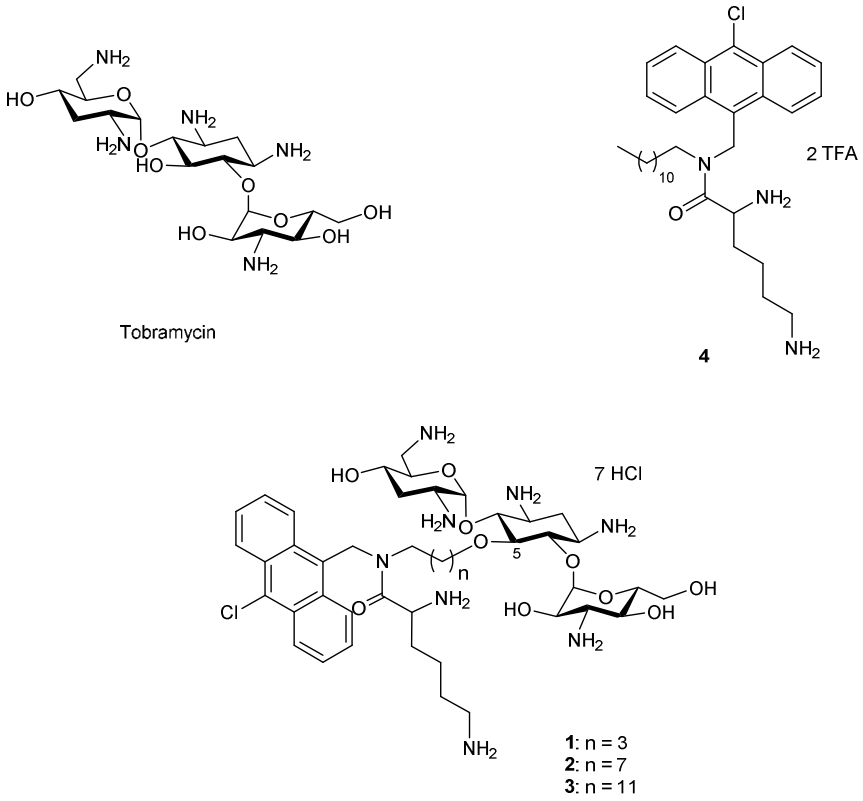


Figure 1. Structures of tobramycin, reference peptoid (**4**), and amphiphilic tobramycin-lysine conjugates with varied alkyl tether (**1 – 3**).

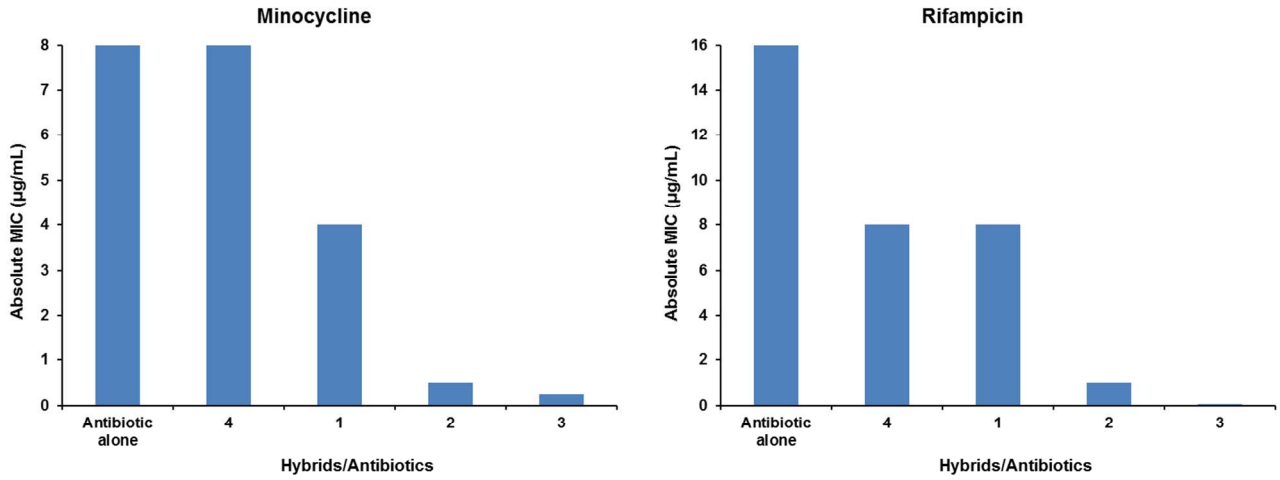


Figure 2. Absolute MIC of minocycline or rifampicin alone or in combination with 4 µg/mL of compound **1 – 4** against *P. aeruginosa* PAO1.

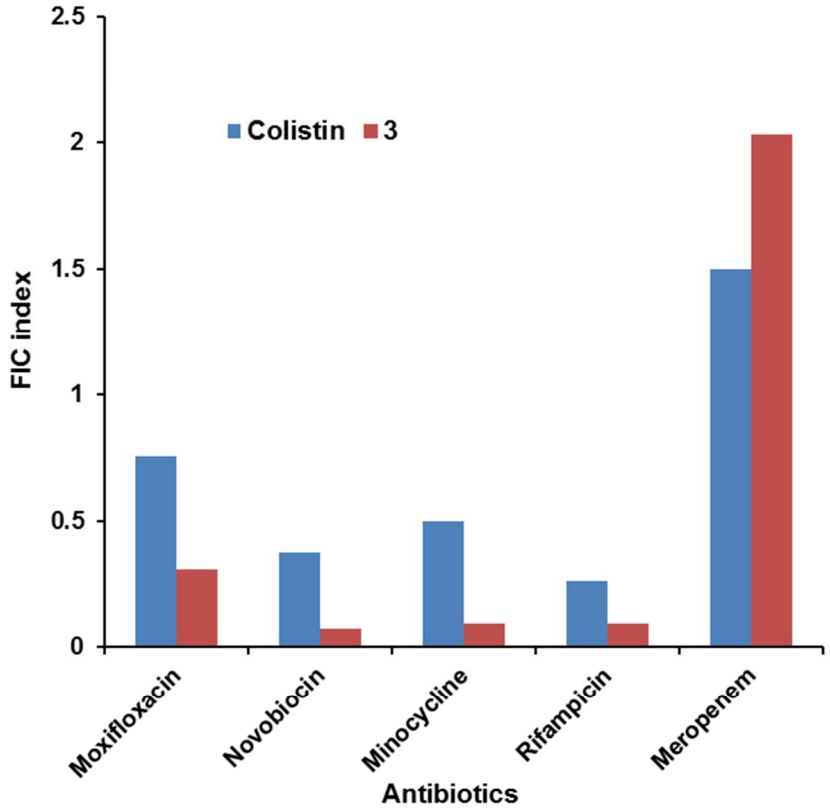


Figure 3. FIC index comparison of compound **3** and colistin in combination with antibiotics against *P. aeruginosa* PAO1.

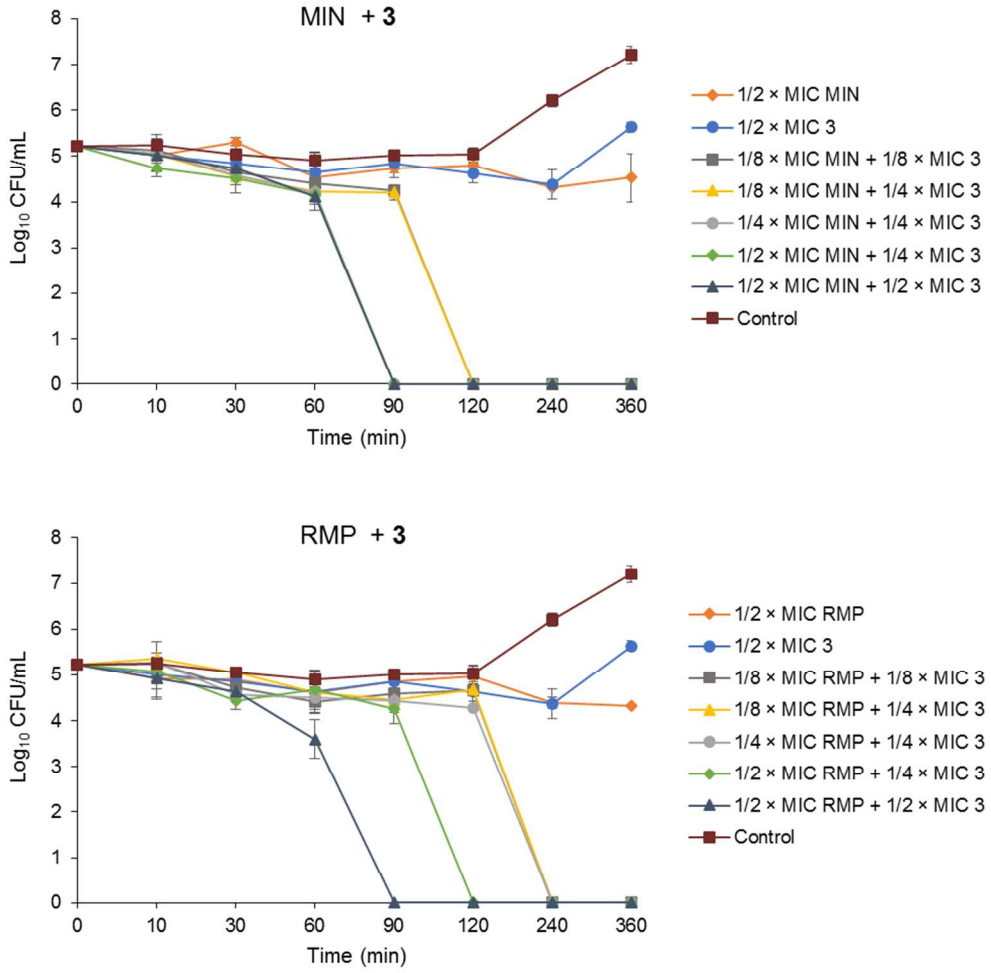


Figure 4. Time killing kinetics of minocycline (MIN) or rifampicin (RMP) alone at 1/2 × MIC (4 μg/mL for MIN and RMP for 8 μg/mL) or in combination with compound **3** against *P. aeruginosa* PAO1 at varied concentrations.

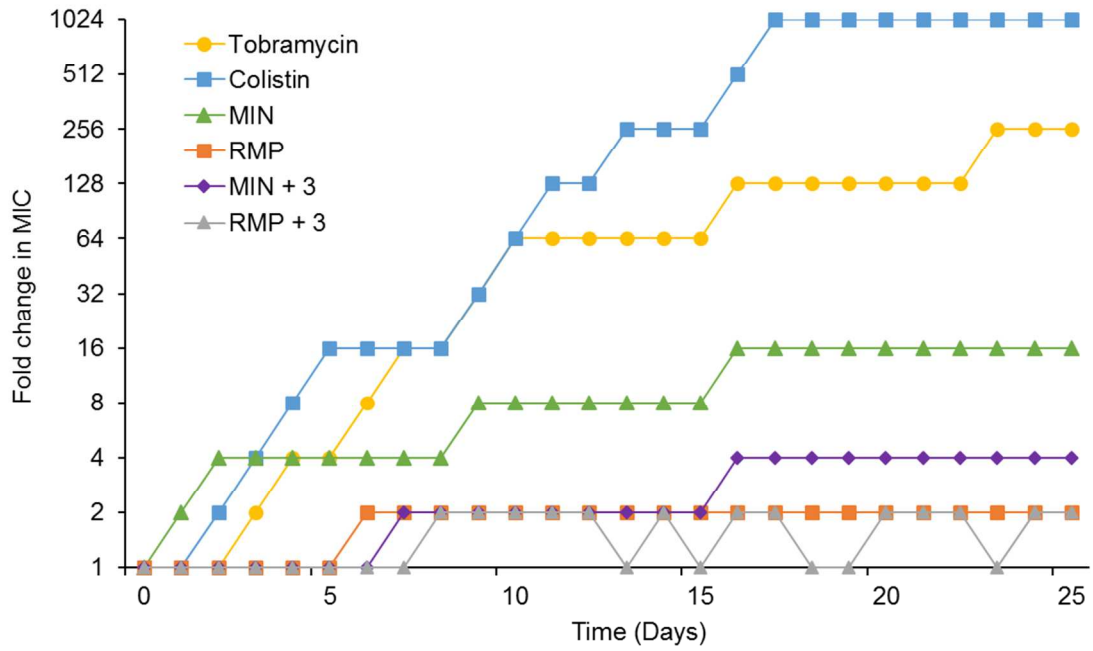


Figure 5. Emergence of bacterial resistance after treatment of *P. aeruginosa* PAO1 with antimicrobials for 25 passages at sub-MIC concentration was determined in monotherapy of tobramycin, colistin, minocycline (MIN), and rifampicin (RMP) or in combination therapy of MIN plus compound **3** or RMP plus compound **3**.

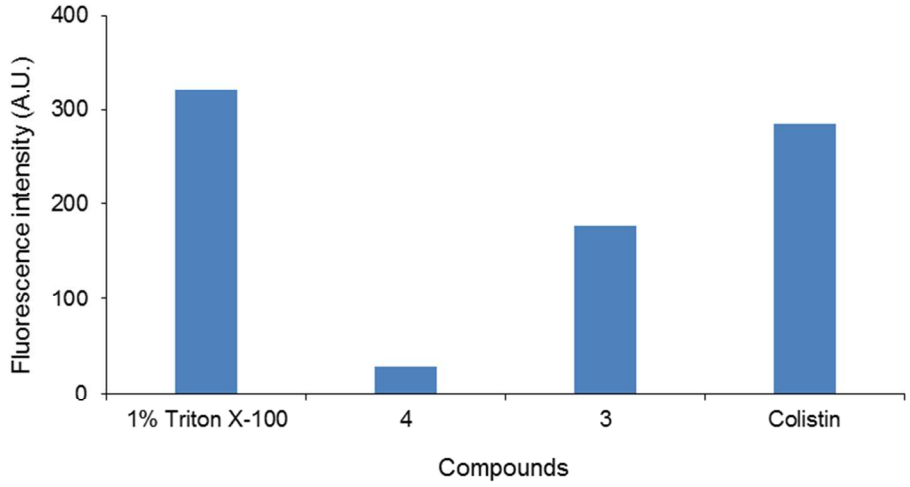


Figure 6. Outer membrane permeabilization of *P. aeruginosa* PAO1 by colistin, compound **3**, or **4** at 32 μg/mL was determined using fluorescence dye CFDA at an excitation wavelength of 488 nm and an emission wavelength of 520 nm. 1% Triton X-100 was served as positive control.

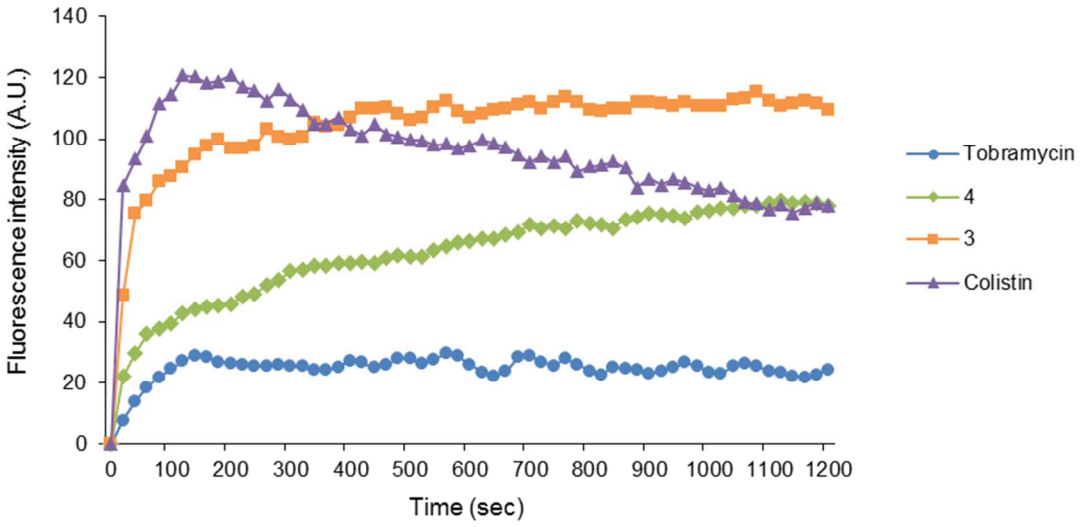


Figure 7. Cytoplasmic membrane depolarization of *P. aeruginosa* PAO1 treated with tobramycin, colistin, compound **3**, or **4** at 32 μg/mL was measured using the membrane potential-sensitive dye $diSC_3$-5. The fluorescent intensity was monitored at an excitation wavelength of 622 nm and an emission wavelength of 670 nm over a 1200 seconds period.

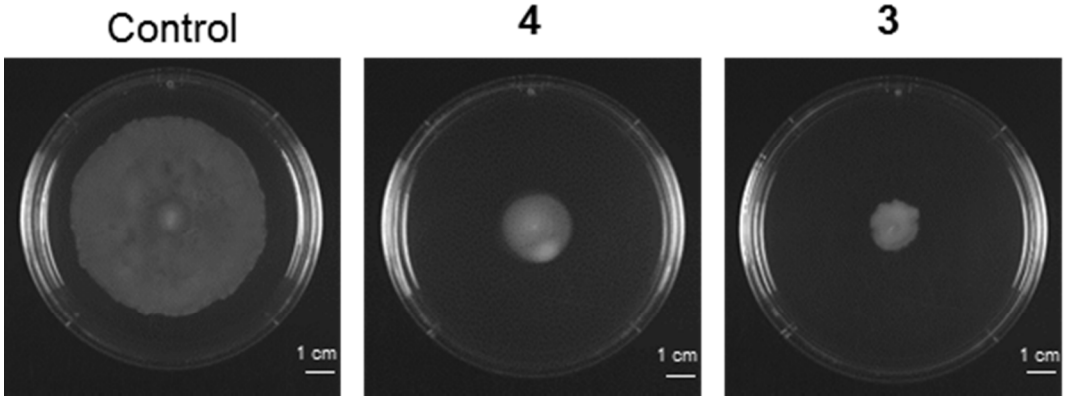


Figure 8. Swimming motility of *P. aeruginosa* PAO1 treated with compound **3** or **4** at 4 μg/mL.

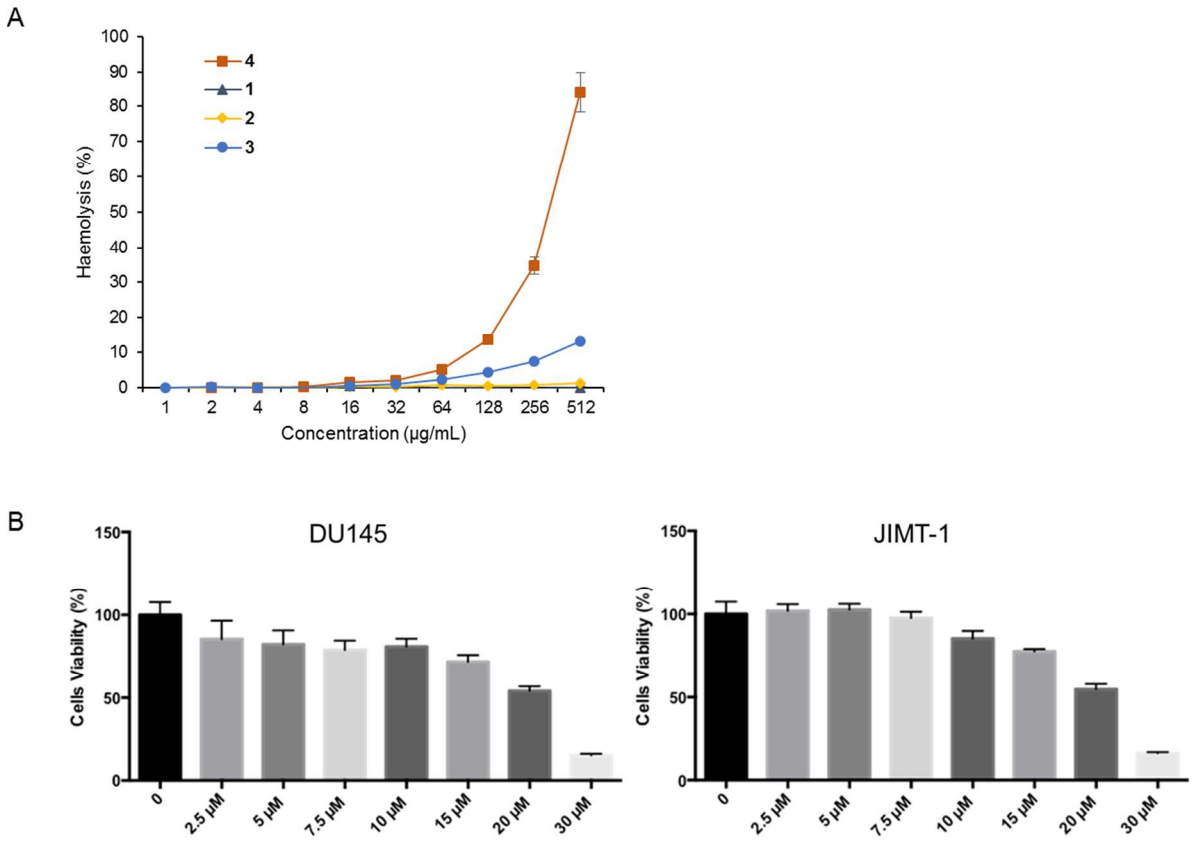


Figure 9. (A) Hemolytic activity of compounds **1 – 4** was evaluated against pig red blood cells. 0.1% Triton X-100 was employed as positive control to calculate the percentage of hemolysis. (B) Cytotoxicity of compound **3** was demonstrated against DU145 and JIMT-1 cell lines by MTS assay.

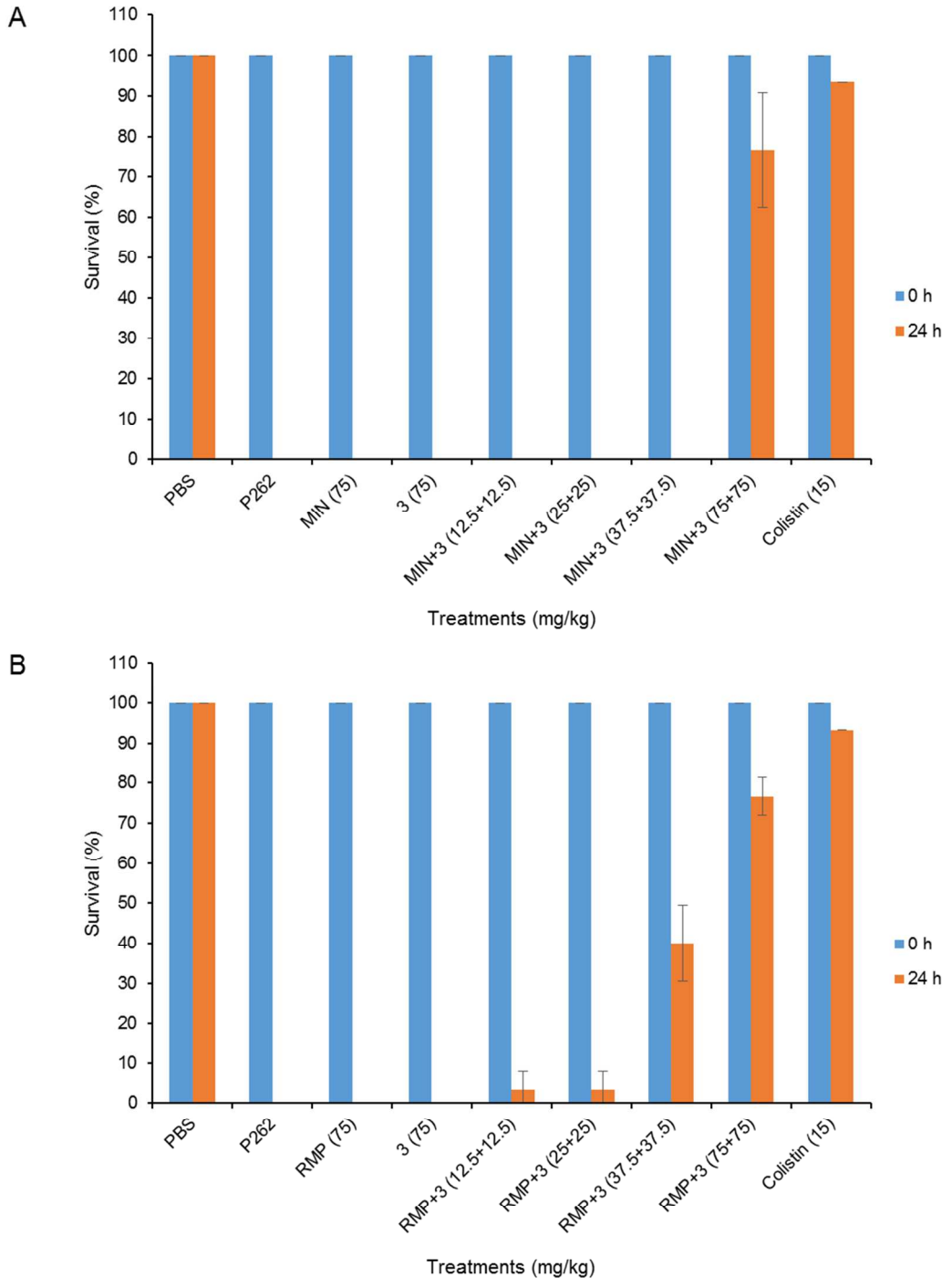
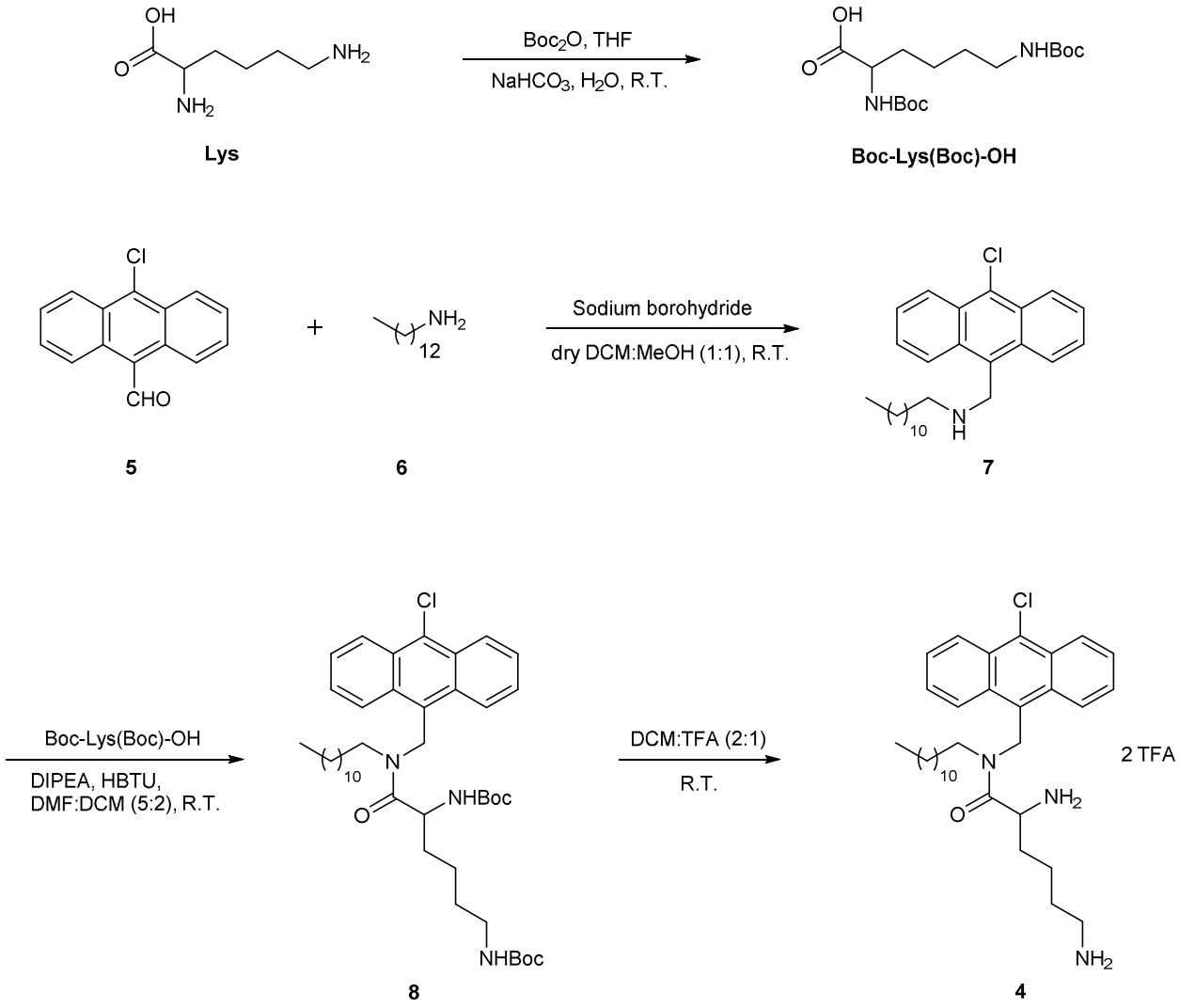
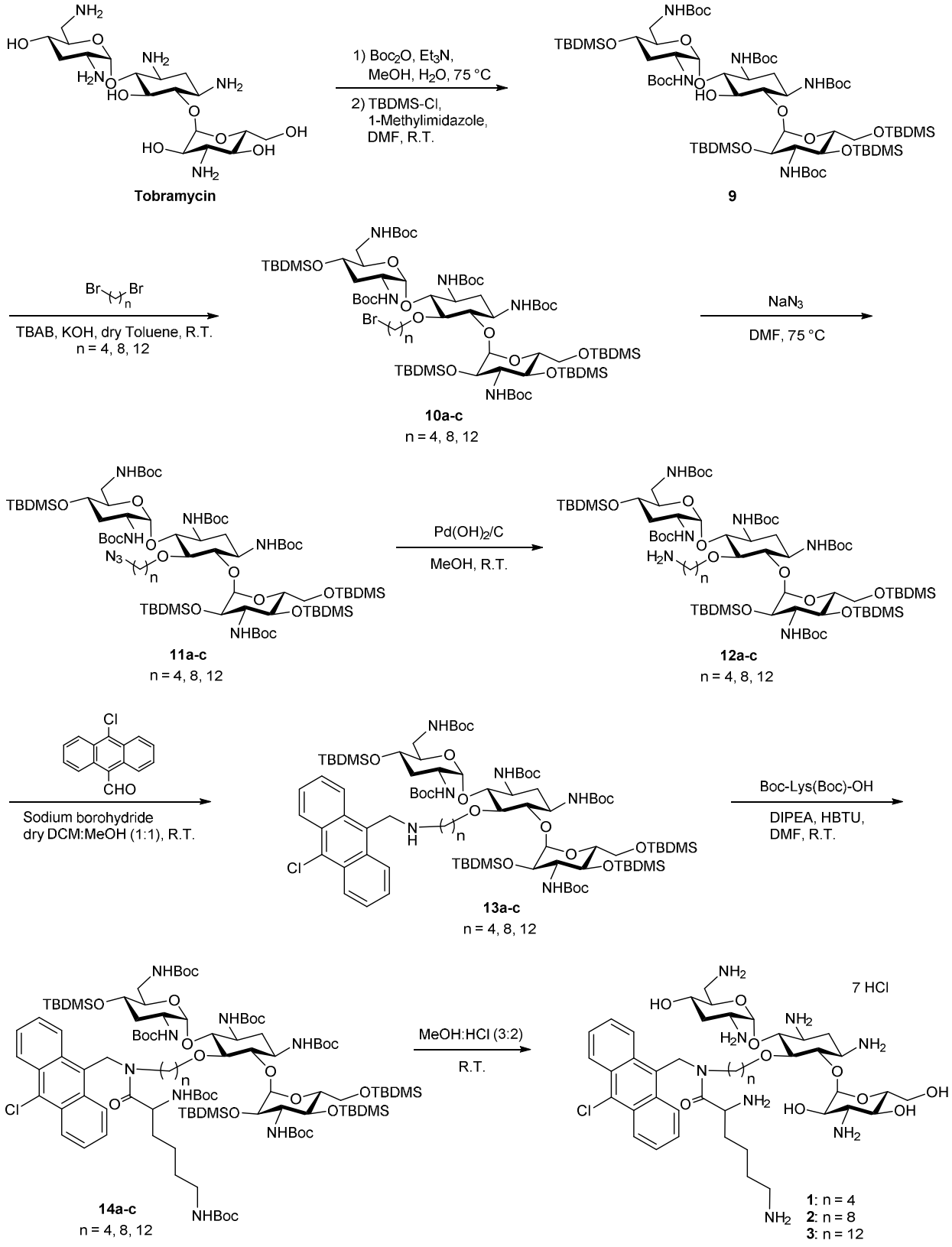


Figure 10. Evaluation of monotherapy and combination therapy in protecting *Galleria mellonella* larvae from XDR *P. aeruginosa* #P262 infection. MIN = minocycline; RMP = rifampicin.

Scheme 1. Preparation of Amphiphilic Peptoid 4.



Scheme 2. Preparation of Amphiphilic Tobramycin-lysine Conjugates 1-3.



Graphical Abstract