Facially Amphiphilic Cholic Acid–Lysine Conjugates as Promising Antimicrobials

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ABSTRACT: The emergence of multidrug-resistant microbes is a significant health concern posing a constant need for new antimicrobials. Membrane-targeting antibiotics are promising candidates with reduced ability of microbes to develop resistance. In the present investigation, the principal reason behind choosing cholic acid as the crucial scaffold lies in the fact that it has a facially amphiphilic nature, which provides ample opportunity to refine the amphiphilicity by linking the amino acid lysine. A total of 16 novel amphipathic cholic acid derivatives were synthesized by sequentially linking lysine to C3-β-amino cholic acid methyl ester to maintain the hydrophobic/hydrophilic balance, which could be the essential requirement for the antimicrobial activity. Among the synthesized conjugates, a series with fluorenyl-9-methoxycarbonyl moiety attached to cholic acid via lysine linker showed promising antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*. A pronounced effect of increase in lysine residues was noted on the observed activity. The lead compounds were found to be active against drug-resistant bacterial and fungal clinical isolates and also improved the efficacy of antifungal agents amphotericin B and voriconazole. Membrane-permeability studies demonstrated the ability of these compounds to induce membrane damage in the tested microbes. The active conjugates did not show any hemolytic activity and were also found to be nontoxic to the normal cells as well as the examined cancer cell lines. The observed antimicrobial activity was attributed to the facial amphiphilic conformations, hydrophobic/hydrophilic balance, and the overall charge on the molecules.

INTRODUCTION

Antimicrobial resistance is emerging as a significant public health concern worldwide, with several common microbial pathogens associated with hospital- and/or community-acquired infections being increasingly reported to be drug resistant. These include methicillin-resistant *Staphylococcus aureus*; third-generation cephalosporin, fluoroquinolone- or carbapenem-resistant *Enterobacteriaceae* (including *Escherichia coli*); multidrug-resistant *Acinetobacter* and *Pseudomonas aeruginosa*; and fluconazole-resistant *Candida*. Recently, *Candida auris* has also emerged as an important antifungal-resistant nosocomial pathogen implicated in a variety of diseases across five continents. Infections caused by such pathogens often lead to higher medical costs and longer periods of treatment and end up with greater morbidity and mortality rates. As a result, there is an immediate need to develop new classes of potent antimicrobials with a novel mechanism of action and a potential to combat the infections caused by drug-resistant microbes. Membrane-targeting antibiotics have emerged as promising candidates in this regard because of the reduced ability of microbes to develop resistance against them. In search of membrane-targeting antibiotics, several amphiphilic molecules have been reported as effective antimicrobials. Cationic antimicrobial peptides are...
the promising candidates, which are naturally occurring amphiphilic molecules produced by the host defense system as a part of the innate immune response. These peptides display wide structural heterogeneity due to variable amino acid sequences and are effective against a broad spectrum of infections, ranging from Gram-positive to Gram-negative bacteria and fungal to protozoal and viral infections. The structural integrity of antimicrobial peptides allows them to adopt different amphiphilic conformations responsible for the antimicrobial activity.10,11 but limited emphasis has been placed to investigate the exact role of different amphiphilic conformations for the variable antimicrobial activity.12,13

Squalamine 1 (Figure 1), initially isolated from a dogfish shark, is another type of cationic amphiphilic steroid polyamine conjugate with a unique chemical structure where a polyamine spermidine is attached to a hydrophobic steroid scaffold.14 Squalamine is exceptionally attractive because of its broad-spectrum antimicrobial activity against plethora of Gram-negative and Gram-positive bacteria and has potential for the development of a new class of antimicrobial agents. The fact that insufficient amounts of squalamine were available for mechanistic studies coupled with the challenges involved in its synthesis because of its structural complexity15 prompted several research groups to synthesize squalamine analogues such as 2 by placing multiple sulfate groups and various polyamines on a different steroid scaffold. Many of the squalamine analogues showed prominent antibacterial activity comparable to that of the parent compound squalamine.16 To mimic the antimicrobial activity of squalamine and cationic antimicrobial peptides, Savage designed novel cholic acid-based cationic steroid antibiotics (CSA) such as ceragenin 3 (Figure 1).17 Cholic acid having hydrophobic steroid skeleton and presence of three hydroxyl groups at C3, C7, and C12 positions along with a carboxylic acid functionality at the C24 position was selected because of its inherent amphipathic nature, which was altered by incorporating multiple amino functionalities.18 Conjugates of cholic acid with different bioactive compounds are known to act against a plethora of microbes.19−21 Bile acid-based peptide conjugates such as 4−6 are also gaining considerable importance as antimicrobial agents because these structures provide ample opportunity to alter the amphiphilicity by varying amino acid residues.22,25−27

In the present investigation, we synthesized novel cholic acid−lysine based conjugates as cationic amphiphiles. The amphiphilicity of the molecules and the overall charge was fine-tuned by coupling mono-, di-, tri-, and tetra-lysine units at the C3 β-position of the cholic acid scaffold. A total of 16 cholic acid−lysine conjugates were synthesized and tested for their antimicrobial activity against representative Gram-positive bacteria, Gram-negative bacteria, and Candida spp. The lead compounds were further evaluated against drug-resistant clinical isolates, and their interaction with a range of known antimicrobial drugs was also determined. Membrane permeabilization studies were carried out to know the possible mechanism of action by these compounds. Cytotoxicity as well as hemolytic activity of the lead compounds was also evaluated to understand the selective toxicity of the compounds against the microorganisms. The biological assays validated the importance of the free amino group at the ε-position of lysine and 9-fluorenylmethoxycarbonyl (Fmoc)-protecting group at the α-amino group of lysine for the antimicrobial activity.

■ RESULT AND DISCUSSION

Based on the antimicrobial properties of cationic steroid antibiotics, we planned to introduce lysine residues to the cholic acid scaffold to obtain a library of facially amphiphilic cationic steroid antibiotics. To achieve the synthesis of the desired cholic acid−lysine conjugates, amino functionality was introduced on the cholic acid scaffold by converting the 3α-hydroxyl group of cholic acid to the 3β-aminogroup in four steps using the previously reported procedure25 with the exception that the intermediate azido functionality was reduced to an amino group via the Staudinger reaction.28 Commercially available Fmoc−Lys(Boc)−OH was coupled to C3 β-aminofunctionality of intermediate 7 in DMF via solution-phase peptide coupling protocol using EDCI, HOBt as a coupling reagent, and triethylamine as a base to synthesize fully protected cholic acid−monolysine conjugate 8 (Scheme 1). Selective deprotection of Fmoc at the α-position of lysine was achieved by 20% piperidine in DMF to yield intermediate 9, which on further deprotection of the tert-butylxycarbonyl
protecting group (Boc) at the ε-position using HCl/dioxane resulted in the formation of the desired cholic acid–lysine conjugate 10.

The synthesized conjugate 10 was screened for antimicrobial activity against bacterial strains viz. *S. aureus* (Gram-positive bacterium), *E. coli* (Gram-negative bacterium) and fungal strain *Candida albicans* by the disk diffusion method. The monolysine conjugate 10 was found to be inactive against bacteria and *C. albicans* when tested at a concentration of 30 μg, whereas a slight but insignificant activity was observed against *S. aureus* and *E. coli* at 100 μg concentration (Table S1) with a zone of inhibition (ZOI) of 7 mm. Amongst the intermediates (7−9), only the Boc-protected cholic acid–lysine conjugate 9 showed antibacterial activity against *S. aureus* at a 30 μg concentration with a ZOI of 9 mm. It exhibited an improved effect against *S. aureus*, but unlike conjugate 10, compound 9 inhibited *E. coli* at a higher concentration (100 μg) (Table S1).

Having observed a moderate activity of the intermediate 9, another cholic acid–lysine intermediate 11 with Fmoc functionality was prepared from compound 8 by selective deprotection of the Boc group at the ε-position using HCl in dioxane. Interestingly, compound 11 retained the activity against *S. aureus* with the ZOI of 8 mm at a 30 μg concentration, albeit to a lesser extent than the intermediate 9, and was inactive against *E. coli* (Table S1). None of these compounds demonstrated any inhibitory effect against *C. albicans* up to 100 μg by the disk diffusion method. Thus, incorporation of one lysine moiety to a C3-β amino-functionalized cholic acid scaffold resulted in compounds with moderate antimicrobial activity, which prompted us to synthesize additional cholic acid–lysine-based cationic amphiphiles. Accordingly, the intermediate 9 was coupled with Fmoc–Lys(Boc)–OH to yield a fully protected dipeptide 12 (Scheme 2).

![Scheme 1. Synthesis of Monolysine-Derived Cholic Acid Conjugates](https://dx.doi.org/10.1021/acsomega.9b03425)

The selective as well as complete deprotection of N-ε-Boc and N-α-Fmoc functionalities of the dilysine conjugate as described earlier furnished the compounds 13, 14, and 15 (Scheme 2). Compound 15, alike compound 10, displayed marginal antibacterial activity against *S. aureus* with a ZOI of 8 mm at a 100 μg concentration (Table S1). Whereas, compound 14 was observed to be inactive, unlike compound 9. The N-α-Fmoc-protected dilysine cholic acid conjugate 13 exhibited improved antibacterial activity against *S. aureus* and *E. coli* with a ZOI of 9 mm at a 30 μg concentration as compared to the monolysine derivative 11 and was also found to be active against *C. albicans* with a ZOI of 7 mm at a 30 μg concentration (Table S1). Thus, the introduction of one more lysine moiety resulted in the enhancement of antimicrobial activity. The observed Structure–Activity Relationship (SAR)
was further extended by coupling another Fmoc−Lys(Boc)−OH to furnish the fully protected trilysine conjugate 16, which on further selective and global deprotection of amino protecting groups yielded compounds 17, 18, and 19 (Scheme 3).

In accordance with the previous data obtained for dilysine cholic acid conjugates, compounds 16, 18, and 19 did not display any promising activity against the bacterial and fungal strains tested. On the other hand, the Fmoc-bearing conjugate 17 displayed significant antimicrobial activity, similar to compound 13 (Table S1) with ZOIs of 9, 10, and 7 mm.
against *S. aureus*, *E. coli*, and *C. albicans*, respectively, at a 30 μg concentration. Therefore, to our curiosity and to further extend the SAR studies, the addition of one more lysine moiety was planned and executed, which furnished fully protected tetralysine conjugate 20 as well as the related *N*-α-Fmoc and *N*-ε-Boc derivatives 21, 22, and the desired cholic acid tetralysine conjugate 23 (Scheme 4).

Amphiphilic tetralysine cholic acid derivatives 20 and 22 similar to dilysine and trilysine conjugates were found to be devoid of any antimicrobial activity. The tetralysine conjugate 23 exhibited subtle antimicrobial activity only against *S. aureus* at a higher concentration tested (ZOI of 10 mm at 100 μg; Table S1). As expected, the Fmoc-bearing tetralysine conjugate 21 demonstrated antibacterial activity similar to dilysine and trilysine conjugates 13 and 17, respectively, with a ZOI of 9 mm at a 30 μg concentration, but the antifungal activity of compound 21 was diminished (Table S1).

Based on the preliminary results obtained with the disk diffusion assay, compounds 9 (against *S. aureus* only), 13, 17, and 21 were selected as the lead active compounds. Measuring
a zone of inhibition using the disk diffusion assay is often employed as a useful preliminary method to identify active compounds. The magnitude of the zone obtained in the disc diffusion assay depends upon many variables, including the solubility and rate of diffusion of the tested compounds through agar. To confirm the preliminary results, the minimum inhibitory concentrations (MICs) of the compounds 9 (against S. aureus only), 13, 17, and 21 were then evaluated using the broth dilution method (Table 1).

Among the selected derivatives, the Fmoc-bearing cholic acid−lysine conjugates 13 and 17 were observed to be the promising broad-spectrum antimicrobials with MIC values of 4, 2, and 8 μg/mL against S. aureus, E. coli, and C. albicans, respectively. The scanning electron micrographs of E. coli ATCC 25922 treated with 2 μg/mL of compound 13 demonstrated morphological alterations, including membrane blebbing, loss of cell architecture, alterations in cell size, and cellular damage in the treated cells compared with the untreated controls (Figure 2).

Exposure to the MIC levels of 13 and 17 was found to cause a considerable amount of cell lysis in S. aureus, E. coli, and C. albicans, as determined by the glucose-6-phosphate dehydrogenase assay. Significantly greater levels of this intracellular enzyme were detected in the extracellular milieu of the treated samples compared with the untreated controls (p < 0.05; Table 2). Membrane permeability studies using the hydrophobic probe N-phenyl-1-naphthylamine (NPN) further demonstrated the ability of these compounds to induce membrane damage. NPN is generally excluded from the cells with intact outer membranes. However, once the outer membrane integrity is disturbed, it can penetrate and exhibit fluorescence upon interaction with the phospholipid microenvironment. Treatment of E. coli ATCC 25922 with 13 and 17 led to a substantial increase in the fluorescence of NPN. The average NPN fluorescence in the treated samples relative to that in untreated controls was 2704.78 ± 293.37 and 6900.05 ± 579.94 AU for 13 and 17, respectively.

Both compounds 13 and 17 were found to be effective against drug-resistant clinical isolates of S. aureus and E. coli and were also active against drug-resistant C. auris, though to a lesser extent than the bacterial strains (Table 3).
Many antimicrobial peptides especially containing Fmoc-moiety are known to self-assemble into supramolecular architectures, which in turn are reported to be responsible for their antimicrobial properties.\(^{29}\) In the present investigation, the results also specifically highlight the critical role of N-α-Fmoc functionality at the N-terminal of the cholic acid–lysine-based peptide conjugates. Overall, the observed activity can be attributed to the altered hydrophobic/hydrophilic balance, overall amphiphilicity, and/or possible self-assembly structures of the conjugates prepared by the attachment of 9-fluorenylmethoxycarbonyl functionality to a cholic acid scaffold via lysine linkage. The presence of a positively charged ε-amino group was also observed to be equally important for the observed activity as the increase in the number of amino acid residues with primary amine functionalities resulted in the improvement of the activity.

We further evaluated the synergistic effect of these compounds, if any, with the known antimicrobial drugs. In this context, a range of antibacterial agents belonging to different classes (cefotaxime, amikacin, ciprofloxacin, and vancomycin) were tested in combination with compounds 13 and 17 by fractional inhibitory concentration assays. The interaction was, however, found to be indifferent (\(\sum FIC = 1.01\)), and the MICs of antibiotics in combination with compounds were the same as those obtained with antibiotics alone against \(S.\) \(aureus\) and \(E.\) \(coli\). In contrast, a combination of these compounds with antifungal drugs demonstrated a substantially improved efficacy against \(C.\) \(albicans\) compared with the drugs or the compounds alone. A synergistic effect was noted with the polyene antifungal agent amphotericin B, and the \(\sum FICs\) were 0.12 and 0.25 with compounds 13 and 17, respectively. These compounds also displayed an additive effect with the triazole drug voriconazole (\(\sum FICs = 0.75\) and 0.63, respectively). This may be attributed to the fact that the synthesized cholic acid–lysine conjugates and the antifungal drugs tested target the same cellular site, that is, the cell membrane. While amphotericin B binds to the membrane ergosterol and leads to the formation of pores along with leakage of the intracellular content, voriconazole blocks ergosterol biosynthesis by inhibiting the enzyme lanosterol 14α-demethylase.\(^{30}\) The synthesized cholic acid–lysine conjugates are likely to act on the microbial cell membrane, possibly via the carpet model.\(^{31}\)

The in-vitro cell viability assay of the selected synthesized compounds was carried out using the MTT reduction assay. Raw 264.7 murine macrophages, human embryonic kidney 293 (HEK 293), and lung adenocarcinoma A549 cell cultures were used for cell viability assay. It is evident from Figure 3 that most of the synthesized compound did not show any considerable cytotoxicity against any of the cell lines that were tested, and cells were significantly viable even after 24 h treatment with different test compounds. As shown in Figure 3A, for Raw 264.7 murine macrophages, compounds 9 and 14 were found to be slightly toxic at a higher concentration (200 \(\mu M\)) with \(-30\) and \(32\)\% decrease in cell viability, respectively, in which more than \(90\)\% of the cell were viable at the lowest concentration (5 \(\mu M\)) for both the compounds. For HEK293 cells (Figure 3B), only compounds 9 and 11 were found to be toxic at a higher concentration (200 \(\mu M\)) with \(-32\) and \(28\)\% decrease in cell viability, respectively, whereas almost \(92\)\% of

![Figure 3. Cell viability assay and hemolytic assay: MTT reduction assay using (A) Raw 264.7 murine macrophages, (B) human embryonic kidney 293 (HEK 293), (C) lung adenocarcinoma A549 cells with different concentrations of test compounds and untreated cells as the positive control, and (D) hemolytic assay results for the test compounds.](https://dx.doi.org/10.1021/acsomega.9b03425)
the cells were viable at the lowest concentration (5 μM) tested for both compounds 9 and 11. Interestingly, out of the three cell lines used, the test compounds were least toxic to lung adenocarcinoma A549 cells (Figure 3C). Compounds 14 and 22 were slightly toxic at a higher concentration (200 μM) and caused ~28 and 24% decrease in cell viability, respectively. More than 92% of lung adenocarcinoma A549 cells were viable at the lowest concentration (5 μM) tested for both the compounds.

The hemolytic activity of test compounds at the 100 and 200 μM concentrations was evaluated where Triton X-100 (1% v/v) and PBS were used as positive and negative controls, respectively (Figure 3D). The hemolytic assay is another good method for cytotoxicity evaluation of test compounds, which is characterized by erythrocyte rupture with the release of hemoglobin. The biocompatibility of therapeutic compounds is of utmost importance during drug discovery and development. The compounds with % hemolysis less than 10 are considered to be nonhemolytic while the ones with % hemolysis more than 25 are assumed to be toxic.29 In the current study, compounds 9 and 14 were found to be hemolytic at both tested concentrations (Figure 3D), whereas the compound 18 showed 7.2 and 13.7% hemolysis at 100 and 200 μM, respectively. Interestingly, the monolysine (9), dilsyline (14), and trilsyline (18) conjugates of cholic acid with N-e-Boc functionalities and the free α-amine group at the N-terminal demonstrated hemolytic activities. All the other compounds including the active lead compounds 13 and 17 showed less than 10% hemolysis at a very high concentration of 200 μM, confirming the selective toxicity of these lead compounds against the deadly microorganisms.

Very recently, we observed a selective antibacterial activity of dimeric cholic acid-based amphiphiles linked via amino or polyamino functionalities,20 whereas the present investigation demonstrated a broad-spectrum selective antimicrobial activity of the cholic acid derivatives linked with fluorenyl-9-methoxycarbonyl moiety via dilsyline and trilsyline linkers. Although the preliminary membrane permeability studies demonstrated the ability of these compounds to induce cell lysis and membrane damage in the microbes, further mechanistic investigations are needed to confirm the exact mechanism of action of these compounds.

■ CONCLUSIONS

A library of cholic acid–lysine-based cationic amphiphiles was designed and synthesized. Among all the synthesized compounds, two compounds 13 and 17 where cholic acid is linked to fluorenyl-9-methoxycarbonyl moiety by the lysine linker displayed broad-spectrum antimicrobial activity against S. aureus, E. coli, and C. albicans with MIC values of 4, 2, and 8 μg/mL, respectively. The lead compounds 13 and 17 were active against resistant strains of S. aureus, E. coli, and C. auris as well. A synergistic and additive effect was also exhibited by these two compounds with a polye cationic Amphotericin B and triazole drug voriconazole, respectively. The selective toxicity of the compounds against the microorganisms was also determined using the cell viability assay (using Raw 264.7 murine macrophages, human embryonic kidney 293 (HEK 293), and lung adenocarcinoma A549 cells) as well as the hemolytic assay. Our results thus demonstrate the therapeutic potential of these cationic amphiphiles for use either alone or in combination with the currently available antimicrobial agents against infections caused by multidrug-resistant microbes. A considerable amount of cell lysis by the lead compounds at MIC determined by the glucose-6-phosphate dehydrogenase assay in addition to the membrane permeability studies using the hydrophobic probe N-phenyl-1-naphthylamine (NPN) further demonstrated the ability of these compounds to induce membrane damage. Their precise mechanism of action and structural requirements for the biological activity are under further investigation.

■ EXPERIMENTAL SECTION

General Procedure. Routine solvents, EDCl, HOBr, Et3N were purchased from commercial suppliers. L-amino acid i.e. Fmoc-Lys(Boc)-OH, cholic acid and HPLC solvents (water and acetonitrile) were purchased from Sigma Aldrich. The bulk solvents such as ethylacetate, DCM, and MeOH were distilled before use. The organic solvents were dried wherever necessary. HCl/dioxane was obtained by passing HCl gas in dioxane for 2 h. The reactions were performed under a nitrogen atmosphere. All the reactions were monitored by thin-layer chromatography (TLC) carried out on Merck silica gel aluminum sheets and visualized under UV light and with different stains such as ethanolic solutions of phosphomolybdic acid and ninhydrin. Column chromatography was carried out using a 230–400 mesh silica gel or neutral alumina as per the requirement. 1H and 13C NMR of samples were recorded on a Bruker 300 MHz, Bruker Advance II 400 MHz, JEOL JNM ECS400, or JEOL 500 MHz spectrometer with DMSO-d6 and CDC13 as solvents and TMS as the internal standard. The splitting patterns in 1H NMR are designated as s, singlet; d, doublet; t, triplet; m, multiplet. The chemical shift values are reported in units of δ. The mass spectra were recorded on a Waters Q-TOF or Thermo LTQ- XL mass spectrometer. HPLC analysis was performed on a Waters HPLC system attached to a waters S15 HPLC pump linked with a Waters 2996 photodiode array detector-equipped C18 column, 4.6 × 250 mm (5 μm) with UV detection at 254 nm. An HPLC gradient 25 minute-run program was set using the solvents water (Solvent A) and acetonitrile (Solvent B) at 25 °C at a flow rate of 1 mL/min.

Methyl (R)-4-(((35S,55R,7R,8R,9S,10S,12R,13S,14S,15S)-3-((9H-Fluoren-9 yl)methoxy)carbonylamino)-6-((tert-butoxycarbonyl)amino)hexanamido)-7,12-dihydroxy-13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanoate (8). To a solution of cholic acid derivative 7 (500 mg, 1.2 mmol) in dry DMF (10 mL), Fmoc–Lys(Boc)—OH (500 mg, 1.1 mmol), HOBt (80 mg, 0.6 mmol), and triethylamine (335 μL, 2.4 mmol) were added at 0 °C under a nitrogen atmosphere. After 10 min of stirring at 0 °C, EDCl (455 mg, 2.4 mmol) was added to the reaction mixture. The reaction mixture was allowed to warm to room temperature and stirred for 6 h. Completion of the reaction was monitored by TLC. After completion of the reaction, the solvent was removed under reduced pressure. The residue was dissolved in 50 mL of ethylacetate, further washed with water and aqueous sodium chloride, and dried over sodium sulfate. The solvent was removed under vacuum and the residue was purified by flash column chromatography using 230–400 mesh silica gel using MeOH/DCM as eluents to obtain compound 8 (755 mg, 73% yield) as an amorphous white solid. mp: 120–123 °C; 1H NMR (300 MHz, CDCl3) δ 7.76 (d, J = 7.5 Hz, 2H), 7.57–7.60 (m, 2H), 7.40 (t, J = 7.4 Hz, 2H), 7.28–7.33 (m, 2H), 6.42 (m, 1H), 5.66 (m, 1H), 4.67 (m, 1H), 4.36–4.38 (m, 2H), 4.18–4.23 (m, 1H), 4.09 (m, 2H), 3.97 (m, 1H), 7.33 (m, 2H), 7.28
Amorphous white solid; mp: 75–77 °C; Yield: 94%; 1H NMR (500 MHz, DMSO-d$_6$) δ 8.07 (d, J = 8.1 Hz, 1H), 7.89–7.90 (m, 8H), 7.71–7.74 (m, 2H), 7.60 (d, J = 7.0 Hz, 1H), 7.54 (d, J = 8.1 Hz, 1H), 7.42 (t, J = 7.4 Hz, 2H), 7.33 (t, J = 7.2 Hz, 2H), 4.22–4.31 (m, 4H), 3.98–4.03 (m, 2H), 3.83 (m, 1H), 3.77 (m, 1H), 3.61 (m, 1H), 3.57 (s, 3H), 3.56 (m, 1H), 2.68–2.77 (m, 4H), 2.09–2.36 (m, 3H), 1.52–2.01 (m, 17H), 1.13–1.39 (m, 16H), 0.91 (d, J = 6.3 Hz, 3H), 0.86 (s, 3H), 0.58 (s, 3H).

13C NMR (126 MHz, DMSO-d$_6$) δ 173.8, 171.8, 170.7, 156.0, 143.83, 143.77, 140.7, 127.6, 127.0, 125.3, 120.1, 71.0, 70.1, 66.3, 66.2, 51.2, 46.6, 46.0, 45.8, 44.7, 43.1, 43.8, 38.5, 36.6, 34.7, 34.4, 30.7, 30.5, 30.4, 28.7, 27.2, 26.6, 25.4, 24.9, 24.2, 22.8, 22.7, 22.4, 22.2, 16.9, 12.3. (HRMS) m/z calculated for C$_{36}$H$_{64}$N$_2$O$_7$ [M + H]$: 900.5845, found: 900.5843.

Methyl (R)-4-(((3S,5S,7R,8R,9S,10S,12S,13R,14S,17R)-3-((S)-2-Amino-6-(tetrahydroisoquinolin-8-yl)hexadecahydro-1H-pyrido[1′,2′:3,4]pyrazin-5-yl)pentanoate Trihydrochloride (17).

Amorphous white solid; mp: 60–62 °C; Yield: 95%; 1H NMR (500 MHz, DMSO-d$_6$) δ 8.17 (d, J = 7.8 Hz, 1H), 7.97–8.04 (m, 10H), 7.90 (d, J = 7.5 Hz, 2H), 7.73 (m, 2H), 7.65 (d, J = 7.0 Hz, 1H), 7.53 (d, J = 7.9 Hz, 1H), 7.42 (t, J = 7.5 Hz, 2H), 7.33 (t, J = 7.4 Hz, 2H), 4.20–4.32 (m, 5H), 3.99–4.03 (m, 1H), 3.84 (m, 1H), 3.77 (m, 1H), 3.66–3.71 (m, 1H), 3.57 (m, 3H), 2.71–2.73 (m, 6H), 1.75–2.36 (m, 7H), 1.54–1.68 (m, 17H), 1.15–1.40 (m, 18H), 0.91 (d, J = 6.2 Hz, 3H), 0.86 (s, 3H), 0.58 (s, 3H).

13C NMR (126 MHz, DMSO-d$_6$) δ 173.8, 172.0, 171.3, 170.7, 156.0, 143.8, 140.7, 127.6, 127.1, 125.3, 120.1, 71.0, 66.5, 65.8, 62.8, 54.2, 52.4, 51.2, 46.7, 46.0, 45.8, 44.7, 44.1, 38.5, 36.5, 35.0, 34.7, 34.4, 33.3, 31.6, 31.2, 31.0, 30.5, 28.7, 27.2, 26.4, 25.6, 24.3, 22.8, 22.4, 16.9, 12.3. (HRMS) m/z calculated for C$_{19}$H$_{28}$N$_2$O$_7$ [M + H]$: 514.6795, found: 514.6780.

Microbial Strains. One representative standard strain each belonging to Gram-positive bacteria (S. aureus ATCC 29213), Gram-negative bacteria (E. coli ATCC 25922), and Candida species (C. albicans ATCC 90028) was employed for screening the antimicrobial activity of the test compounds. S. aureus 1704, E. coli 4052, and C. auris AHD62 from our laboratory collection were taken as drug-resistant isolates. S. aureus 1704 is a methicillin-resistant isolate, which is also resistant to ciprofloxacin and chloramphenicol. E. coli 4052 is resistant to cefotaxime and ciprofloxacin. C. auris AHD62 is resistant to fluconazole and amphotericin B according to the current susceptibility recommendations of Center for Disease Control and Prevention (CDC), U.S.

Determination of Antimicrobial Activity. The antimicrobial activity of the test compounds was first evaluated by the disk diffusion assay at a concentration of 30 and 100 μg/disk according to the guidelines of the Clinical Laboratory Standards Institute (CLSI) using Mueller Hinton agar (MHA) for bacterial strains and MHA with 2% glucose and 0.5 μg methylene blue/mL for Candida species. The MICs of the lead compounds were then determined by the broth microdilution assay. For the antibacterial assay, two-fold dilution series of the compounds were prepared against S. aureus and the microtiter plates were incubated for 18 h at 37 °C. For the antifungal assay, 0.5–2.5 × 10$^5$ Candida cells/mL were exposed to the test compounds in RPMI-1640 for 24 h at 37
amphotericin B) were set up in parallel. MIC was determined as the lowest concentration that resulted in complete inhibition of microbial growth.

**Glucose-6-phosphate Dehydrogenase Assay.** The intracellular enzyme glucose-6-phosphate dehydrogenase was used as a marker to determine microbial cell lysis after exposure to the test compounds. Briefly, the bacterial or fungal strains were treated with the test compounds at MIC according to the broth antimicrobial assay described above, and 40 μL of the treated samples were incubated in a 250 mM glycine buffer (pH 7.4) containing 60 mM glucose 6-phosphate, 20 mM NADP, and 300 mM MgCl₂ in a reaction volume of 1.2 mL for 5 min at 25 °C. Untreated controls were set up in parallel. The production of NADPH was monitored as the change in absorbance at 340 nm. One enzyme unit (U) was defined as the enzyme activity catalyzing the formation of 1 mmol NADPH per min.

**NPN Permeability Assay.** E. coli ATCC 25922 was exposed to the test compounds at MIC for 4 h according to the broth antimicrobial assay described above. Thereafter, the cells were harvested by centrifugation, followed by addition of NPN at a final concentration of 10 μM. The fluorescence was measured using an excitation and emission wavelength of 340 and 420 nm, respectively, and expressed as AU per unit OD in the treated samples relative to the untreated controls.

**Synergism Testing.** The interaction of lead compounds with known antimicrobial agents was evaluated by FIC testing. Cefotaxime, amikacin, ciprofloxacin, and vancomycin vancomycin (for S. aureus only) belonging to β-lactam, aminoglycoside, fluoroquinolone, and glycopeptide classes were used as the antibiotic drugs. Amphotericin B and voriconazole from the polyene and triazole groups were employed as the antifungal agents. Broth microdilution assays containing two-fold grading concentrations of the test compounds, either alone or in combination with the antimicrobial drugs, were performed, and the FIC indices were calculated. The FIC of ≤0.5, >0.5 and ≤1, >1, and ≤4, and >4 were defined as synergism, additiveness, indifference, and antagonism, respectively.

**Cell Viability Studies.** The cytotoxicity studies of synthesized compounds were carried out using three different cell lines. Raw 264.7 murine macrophages cells were grown as a monolayer in DMEM high glucose media supplemented with sodium bicarbonate, L-glutamine, 10% FBS, and 1% pen-strap. The culture condition was maintained at 37 °C with 5% CO₂ in a CO₂ incubator. Raw 264.7 murine macrophages cells having 90% confluence with passage number 32 were used for in vitro cell viability experiment. Human embryonic kidney 293 (HEK 293) cells were grown as a monolayer in DMEM high-glucose media supplemented with sodium bicarbonate, sodium pyruvate, L-glutamine, 10% FBS, and 1% pen-strap. The culture condition was maintained at 37 °C with 5% CO₂ in a CO₂ incubator. Human embryonic kidney 293 (HEK 293) cells having 90% confluence with passage number 38 were used for in vitro cell viability experiment. Lung adenocarcinoma A549 cells were grown in an RPMI1640 medium supplemented with 10% FBS and 1% Pen-strep. The culture condition was maintained at 37 °C with 5% CO₂ in a CO₂ incubator. Cells having 90% confluence with passage number 35 were used for in vitro cell viability experiment.

The in vitro cell viability study was performed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay. For the assay, Raw 264.7 murine macrophages were scrapped with the help of a sterile scrapper, HEK293 and A549 cells were trypsinized, and all three types of cells were seeded separately in a 96-well plate at a cell density of 1–1.5 × 10⁵ cells/well in 100 μL in their respective complete media. The MTT reduction assay was carried out in triplicate for all concentrations of test compounds. After 24 h incubation at 37 °C with 5% CO₂ in a CO₂ incubator, cells were then treated with test compounds at varying concentrations (5, 10, 50, 100, and 200 μM) in complete media and incubated for 24 h. After 24 h, to the treatment mixture, 10 μL of MTT reagent (5 mg/mL) was added in each well and again incubated at 37 °C for 4 h. The absorbance was recorded at 590 nm after 4 h using a microplate reader. The untreated cells were taken as control with 100% viability that was used to compare the relative cell viability in the test wells.

**Hemolytic Assay.** Hemolytic activity of synthesized compounds was studied according to the literature methods. Freshly obtained 3 mL blood from healthy rat was collected in vacutainer heparin tubes. The blood was centrifuged at 1000 g for 10 min, the separated plasma was discarded, and cells were washed three times with sterile isotonic phosphate buffer saline (PBS, pH 7.4). The washed RBCs were resuspended in PBS and constant volume of 200 μL of suspended RBCs was used for the hemolytic assay. Each test compound was mixed separately with washed RBC at concentrations of 100 and 200 μM, respectively. The samples were incubated immediately after mixing for 30 min at 37 °C with intermittent shaking. After incubation, the samples were centrifuged at 1000 g for 10 min, and the supernatant was collected from each test mix into a 96-well plate. Triton X-100 (1% v/v) and PBS were used as positive control and negative control, respectively. The absorbance was measured at 540 nM using a microplate reader. The assay was repeated twice, and each time, it was performed in duplicate. The hemolysis for each sample was calculated using this formula: %Hemolysis = (A₅/A₅₀) × 100 where A₅ is the absorbance of the sample and A₅₀ is the absorbance of the control.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b03425.

Antimicrobial activity with disk diffusion assay, analytical data ¹H NMR, ¹³C NMR, HRMS, and HPLC spectra of the synthesized compounds (PDF)

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ABBREVIATIONS USED

MIC minimum inhibitory concentration;
Amp B amphotericin B
SEM scanning electron microscopy
FIC fractional inhibitory concentration
EDCI 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
HOBt 1-hydroxybenzotriazole
HPLC high-performance liquid chromatography
HRMS high-resolution mass spectrometry
TLC thin-layer chromatography
DMF N,N′-dimethylformamide
TMS tetramethylsilane
CLSI clinical laboratory standards institute
MHA Mueller Hinton agar
MBH Mueller Hinton broth
DMEM Dulbecco’s Modified Eagle Medium
FBS fetal bovine serum
HEK human embryonic kidney cells
RPMI1640 Roswell Park Memorial Institute
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NPN N-Phenyl-1-naphthylamine

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