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Lysine-Based Small Molecules That Disrupt Biofilms and Kill both Actively Growing Planktonic and Nondividing Stationary Phase Bacteria

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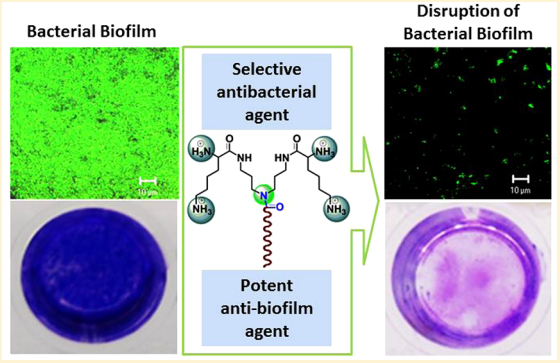
Supporting Information

ABSTRACT: The emergence of bacterial resistance is a major threat to global health. Alongside this issue, formation of bacterial biofilms is another cause of concern because most antibiotics are ineffective against these recalcitrant microbial communities. Ideal future antibacterial therapeutics should possess both antibacterial and anti-biofilm activities. In this study we engineered lysine-based small molecules, which showed not only commendable broad-spectrum antibacterial activity but also potent biofilm-disrupting properties. Synthesis of these lipophilic lysine−norspermidine conjugates was achieved in three simple reaction steps, and the resultant molecules displayed potent antibacterial activity against various Gram-positive (*Staphylococcus aureus, Enterococcus faecium*) and Gram-negative bacteria (*Escherichia coli*) including drug-resistant superbugs MRSA (methicillin-resistant *S. aureus*), VRE (vancomycin-resistant *E. faecium*), and β-lactam-resistant *Klebsiella pneumoniae*. An optimized compound in the series showed activity against planktonic bacteria in the concentration range of 3−10 μg/mL, and bactericidal activity against stationary phase *S. aureus* was observed within an hour. The compound also displayed about 120-fold selectivity toward both classes of bacteria (*S. aureus* and *E. coli*) over human erythrocytes. This rapidly bactericidal compound primarily acts on bacteria by causing significant membrane depolarization and K^+ leakage. Most importantly, the compound disrupted preformed biofilms of *S. aureus* and did not trigger bacterial resistance. Therefore, this class of compounds has high potential to be developed as future antibacterial drugs for treating infections caused by planktonic bacteria as well as bacterial biofilms.



KEYWORDS: *bacterial resistance, antibacterial, anti-biofilm, stationary phase, lysine*

The widespread emergence of bacterial resistance toward conventional antibiotics has raised serious concerns for the treatment of bacterial infections.[1−3] This situation has been further aggravated by the dwindling antibiotic pipeline.[4] Additionally, bacterial biofilms are a major challenge in infectious disease treatment due to their ability to facilitate an increased frequency of antibiotic resistance through horizontal gene transfer and their inherent resistance to antibiotic treatment.[5,6] In view of these challenges, there is a need for the development of alternative strategies for antibacterial therapeutics. Natural membrane active compounds (such as antimicrobial peptides (AMPs) and lipopeptide antibiotics) and their synthetic mimics, such as α-peptides,[7] β-peptides,[8] oligourea,[9] oligoacyl lysines,[10] arylamide foldamers,[11] aryl-alkyl-lysines,[12] antimicrobial polymers,[13−18] lipo-γ-AApeptides,[19] ultrashort synthetic lipopeptides,[20] alkylated lipopeptides[21] acylpolyamines,[22] cationic amphiphiles,[23−25] and other lipophilic membrane active compounds,[26−28] are being considered as future antibacterial agents. All of these membrane active molecules mainly act on bacteria either by causing lysis of the cell membrane or by perturbation of the membrane potential,[29,30] whereas most of the known antibiotics target specific cellular processes in bacteria.[31] Therefore, development of resistance against these membrane active molecules is difficult for bacteria, unlike against conventional antibiotics, which are rendered ineffective by elimination through efflux pumps, modification of target, and in some cases inactivation by enzymes.[32]

However, a vast majority of the above-mentioned membrane active agents are designed specifically for targeting planktonic bacteria, often overlooking the threat posed by bacterial biofilms. Biofilms are adherent communities of bacteria embedded within a self-produced extracellular matrix composed of exopolysaccharides, proteins, and sometimes extracellular DNA.[33,34] Bacteria behave as multicellular organisms inside the biofilm and develop strategies that prevent the entrance of antibiotics.[35,36] Additionally, they exist in an altered metabolic state (such as stationary phase) inside the biofilm as compared

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to their planktonic condition, which contributes to the development of around 100−1000-fold increased resistance toward standard antibiotics.[37,38] As a result, antibiotics that act against planktonic bacteria are often ineffective in treating biofilm-associated infections such as in the lungs of patients suffering from cystic fibrosis (CF), burn wound infections, catheter infections, bacterial endocarditis, and chronic wound infections.[6] Unfortunately, some classes of antibiotics such as aminoglycosides have even been found to induce biofilm formation in bacteria.[39] Thus, in view of the threat posed by bacterial biofilms, there has been significant interest in developing potent anti-biofilm agents.[40,41] The derivatives of spermidine and norspermidine have been reported to possess anti-biofilm properties,[42] but none of the compounds demonstrated significant antibacterial activity at or close to their corresponding minimum biofilm inhibitory concentration (MBIC) values, showing a perceivable effect on bacterial growth only at concentrations above (~40 times) their MBIC value. The mechanism of antibacterial activity at such a high concentration remains unexplored. D-Amino acids such as D-Tyr, D-Leu, D-Trp, and D-Met were also shown to be natural triggers for biofilm disassembly, although none of these possessed significant antibacterial activity.[43] Whereas several strategies have been designed to achieve such dual antibacterial and anti-biofilm activities, clinical success remains elusive.[44−48] Consequently, there remains a pressing need for the development of simpler and better antibacterial/anti-biofilm agents.

Herein, we report lipophilic lysine−norspermidine conjugates (Figure 1), which not only exhibit broad-spectrum

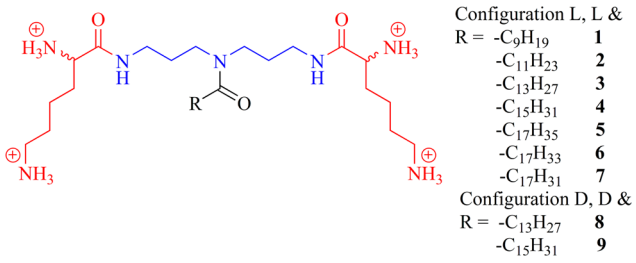

Figure 1. Structures of lipophilic lysine−norspermidine conjugates with trifluoroacetate counterions.

antibacterial activity but also act as anti-biofilm agents. In our design we incorporated a pendant lipophilic moiety and cationic amino acid lysine into norspermidine. We rationalized that the introduction of four positive charges and hydrogen bond-forming units into a norspermidine backbone would yield greater electrostatic and hydrogen-bonding interactions with the extracellular matrix components of the biofilm (such as exopolysaccharides and extracellular DNA). In addition to the aforementioned interactions, the lipophilic moiety was expected to further enhance the interaction with the bacterial membrane. Thus, we synthesized a set of compounds by varying the length and degree of unsaturation of the lipophilic group, as well as the stereochemistry (L/D) of the lysine residues to arrive at an optimized structure. The antibacterial activity of these compounds was evaluated against various actively growing planktonic bacteria, whereas toxicity was determined against both human erythrocytes (hRBCs) and mammalian cells (HeLa cell line). Compounds with D stereoisomers of lysine were synthesized to confer stability to the compounds in plasma. To check the potential longevity of these compounds as antibacterial agents, their ability to trigger resistance development was evaluated. Finally, the efficacy of the compound to kill stationary phase (metabolically inactive) cells and disrupt preformed biofilms of *Staphylococcus aureus* was studied.

RESULTS AND DISCUSSION

Synthesis. The lipophilic lysine−norspermidine conjugates were prepared in three simple synthetic steps as outlined in Scheme 1. The first step involved selective amide coupling of the primary amine groups of norspermidine with Boc-L/D-Lys(Boc)−OH. Then a lipophilic group was introduced at the secondary amine functionality through another amide coupling reaction with various aliphatic acids. In the final step, deprotection of Boc groups with trifluoroacetic acid (TFA) yielded the final compounds (**1**−**9**). Compounds **1**−**5** were synthesized by varying the length of the lipophilic chain from a decanoyl to an octadecanoyl group, whereas compounds **6** and **7** contained unsaturaturated alkyl chains, oleyl and linoleyl, respectively. All of the above-mentioned compounds (**1**−**7**) employed L-lysine. Compounds **8** and **9** were the D-lysine containing isomers of compounds **3** (tetradecanoyl analogue) and **4** (hexadecanoyl analogue), respectively. The control compound **10**, which lacked an aliphatic group (Scheme 1), was prepared by directly adding TFA to the product of the first step. The purity (>95%) of all the final compounds was determined by HPLC, and all final compounds were characterized by IR, ^{1}H NMR, ^{13}C NMR, and HR-MS (Supporting Information Figures S1−S36).

Antibacterial Activity. The antibacterial potency of these compounds was evaluated against both Gram-positive (*Staphylococcus aureus* and *Enterococcus faecium*) and Gram-negative (*Escherichia coli*) bacteria including drug-resistant superbugs such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecium* (VRE), and β-lactam-resistant *Klebsiella pneumoniae*. The activity was reported as their minimum inhibitory concentration (MIC) and is summarized in Table 1.

In general, the lipophilic lysine−norspermidine conjugates displayed potent antibacterial activity against both Gram-positive and Gram-negative bacteria unlike the previously reported phenylalanine-based norspermidine derivatives, which were active mainly against Gram-positive bacteria.[27] Compound **1**, consisting of the shortest aliphatic chain (decanoyl chain) in the series, was found to be moderately active against both Gram-positive and Gram-negative bacteria with MIC values of 53 and 40 μg/mL against *S. aureus* and *E. coli*, respectively. An improvement in activity was observed by increasing the long chain to dodecanoyl, as in compound **2** (MIC = 38 and 14 μg/mL against *S. aureus* and *E. coli*, respectively). Further increase in lipophilicity yielded compound **3** (tetradecanoyl analogue), which displayed improved antibacterial activity with MIC values of 8 and 6 μg/mL against *S. aureus* and *E. coli*, respectively. Increasing the long chain to the hexadecanoyl group yielded compound **4**, which showed little improvement in antibacterial activity over compound **3**, displaying MIC values of 5 and 5.8 μg/mL against *S. aureus* and *E. coli*, respectively. However, any further increase in lipophilicity compromised the activity as was observed for compound **5** (octadecanoyl analogue). Hence, a parabolic pattern of chain length-dependent antibacterial activity was observed in this structure−activity relationship (SAR) study, which is indicative of the fact that the optimum lipophilicity lies somewhere between those of the tetradecanoyl and hexadecanoyl groups (Supporting Information Figure S37).

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Scheme 1. Synthesis of Lipophilic (1−9) and Non-lipophilic (10) Lysine−Norspermidine Conjugates

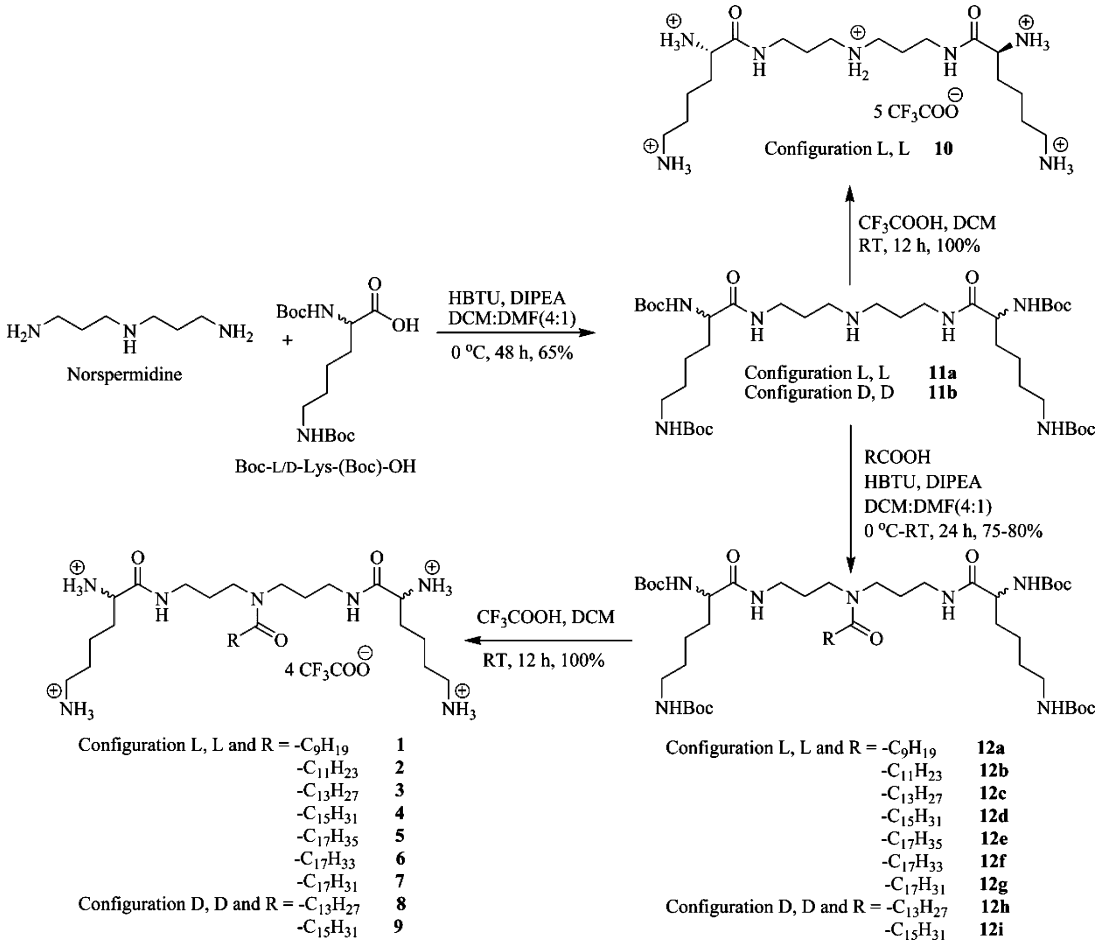

Table 1. Antibacterial and Hemolytic Activity of the Compounds

	minimum inhibitory concentration (μg/mL)						
	drug-sensitive bacteria			drug-resistant bacteria			
compound	*S. aureus*	*E. faecium*	*E. coli*	MRSA[a]	VRE[b]	*K. pneumoniae*[c]	HC_{50} (μg/mL)
1	53	60	40	65	58	98	>1000
2	38	33	14	46	31	41	>1000
3	8	2	6	5.8	1.6	10	588
4	5	1.2	5.8	5	1	7	264
5	8.5	5	9	16	2.4	8.3	108
6	5.6	7.6	8.6	16	5.6	11	950
7	14.5	22	65	81	13	>250	>1000
8	6	4	5.7	6	3	9.5	730
9	5.6	2.9	5	4.5	2.4	3.4	283
10	>250	>250	>250	>250	>250	>250	>1000
BAC-12	0.8	3	9	3	3.5	11	60
DTAB	3	20	20	31	32	>50	218
CTAB	0.4	0.5	1.5	0.5	0.6	4.6	30
DDAB-10	0.4	0.5	1.2	0.6	0.8	3	27
colistin	25	>250	0.5	68	>250	1.5	ND[d]
vancomycin	0.8	0.8	ND	0.8	>1000	ND	ND

[a]Methicillin-resistant *S. aureus*. [b]Vancomycin-resistant *E. faecium*. [c]β-Lactam-resistant *K. pneumoniae*. [d]ND, not determined.

These results suggested that compounds **3** and **4** were the most potent antibacterial agents among all of the lipophilic lysine−norspermidine conjugates. These two best compounds (**3** and **4**) also exhibited high activity against *E. faecium* with MIC values of 2 and 1.2 μg/mL, respectively, whereas the less lipophilic analogues **1** and **2** displayed lower activity with MIC values of 60 and 33 μg/mL, respectively. The highest long-chain analogue, compound **5**, was significantly active against this strain with an MIC value of 5 μg/mL. Most notable was the activity of these lipophilic lysine−norspermidine conjugates

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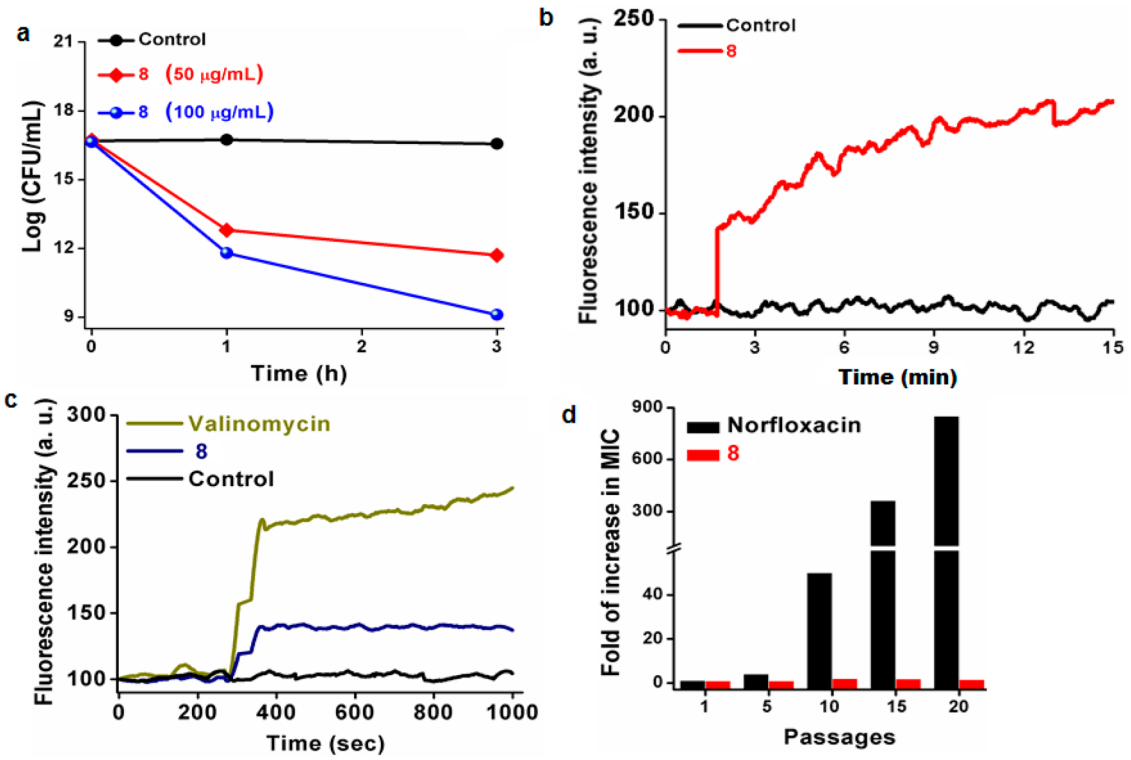

Figure 2. (a) Bactericidal kinetics of compound **8** against stationary phase *S. aureus*. (b) Membrane depolarization of *S. aureus* by compound **8** (12 × MIC). (c) K^+ leakage of *S. aureus* by compound **8** (12 × MIC). (d) Comparison of fold increase in MIC of norfloxacin and compound **8** against *S. aureus*.

against the drug-resistant bacteria MRSA, VRE, and *K. pneumoniae*.

As seen with the drug-sensitive bacteria, compounds **3** and **4** displayed the best MIC values against the drug-resistant strains as well. Compound **3** showed activity with MIC values of 5.8, 1.6, and 10 μg/mL against MRSA, VRE, and *K. pneumoniae*, respectively. Compound **4** exhibited slightly higher potency against these drug-resistant bacteria with MIC values of 5, 1, and 7 μg/mL, respectively. In an effort to elucidate the role of unsaturation in lipophilicity toward modulating antibacterial activity, we synthesized compounds **6** (consisting of an oleyl group, bearing a single unsaturation) and **7** (consisting of a linoleyl group, bearing a double unsaturation) by replacing the octadecanoyl group of compound **5**. Results showed that compound **6** exhibited similar or slightly diminished antibacterial activity compared to compound **5**, but any further increase in the unsaturation compromised antibacterial activity as seen for compound **7**. The compounds consisting of the D-isomer of the most potent compounds **3** and **4** were also synthesized. The D-isomeric compounds **8** and **9** were found to display similar antibacterial activity when compared to their corresponding L-isomeric analogues, as can be seen from the data presented in Table 1. Compound **10**, which lacked an aliphatic group, did not show any antibacterial activity even up to 250 μg/mL, indicating the importance of the aliphatic lipophilic group for potent antibacterial activity.

Hemolytic Activity. To investigate the preliminary toxicity of these compounds, their toxicity was evaluated against human erythrocytes (hRBCs). The extent of lysis of RBCs was expressed as their HC_{50} values (the concentration corresponding to 50% cell lysis). Results suggested that none of these compounds caused significant hemolysis. To compare the effectiveness of the compounds as selective antibacterial agents, hemolytic assay for the common antiseptics (BAC-12 and DDAB-10) and other cationic amphiphiles such as DTAB and CTAB were also performed (Table 1). Results showed that these existing antibacterial molecules are highly toxic, resulting in RBC lysis at very low concentrations (HC_{50} in the range of 27−218 μg/mL), whereas our compounds being antibacterial at very low concentration remain nontoxic even at higher concentration (HC_{50} in the range of 108−1000 μg/mL). A decreasing trend of the HC_{50} value was observed with increasing length of lipophilic chain. Compounds **3** and **4**, the most potent compounds of the L-isomeric series, were found to have HC_{50} values of 588 and 264 μg/mL, respectively (Table 1) and the D-isomeric analogues **8** and **9** showed even less toxicity, with HC_{50} values of 730 and 283 μg/mL, respectively. Compound **8** emerged as the most effective compound, being >120-fold selective (HC_{50}/MIC) toward both classes of bacteria (*S. aureus* and *E. coli*) as shown in Supporting Information Figure S38.

Cytotoxicity. To further investigate this class of compounds as selective antibacterial agents, toxicity against the mammalian cell line (HeLa) was determined using the MTT assay with the most effective compound, **8**. As can be seen from Supporting Information Figure S39, this compound did not show any toxicity at its MIC, and the IC_{50} value (50% cells viability) for this compound was found to be >70 μg/mL (Supporting Information Figure S39), which is many fold higher compared to the concentration required for bactericidal activity.

Bactericidal Kinetics. The time-kill kinetics of compound **8** was performed to determine the rate at which this class of compounds kills bacteria. This investigation was carried out against both classes of bacteria, *S. aureus* (Gram-positive representative) and *E. coli* (Gram-negative representative). A rapid-bactericidal activity was observed against *S. aureus*,

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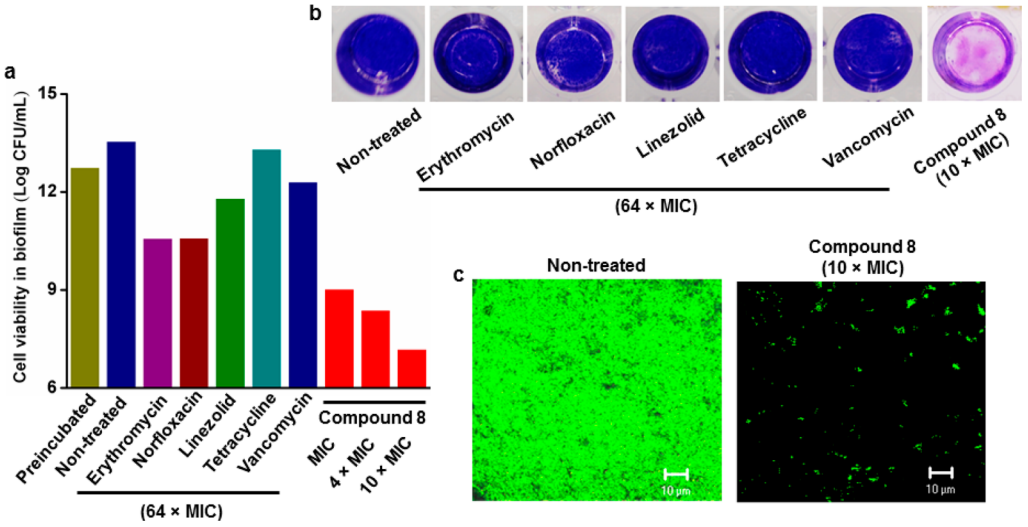

Figure 3. Biofilm disruption of *S. aureus*: (a) quantification of cell viability in biofilms; (b) visualization by crystal violet staining; (c) confocal microscopy images of biofilms.

compared to slower bactericidal kinetics against *E. coli*. From the initial experiment (in "hour scale") against *S. aureus*, a >3 $\log_{10}$ (CFU/mL) reduction in bacterial cell viability was observed within the first hour of compound exposure at a concentration of 6 × MIC (Supporting Information Figure S40). The next experiment in a smaller time gap (in "minute scale") suggested that the exact time required to kill this bacterium was about 30 min (inset of Supporting Information Figure S40). In the case of *E. coli*, a >3 $\log_{10}$ (CFU/mL) reduction in cell viability was observed within 4 h of compound (6 × MIC) exposure as shown in Supporting Information Figure S41. These results suggested that lipophilic lysine−norspermidine conjugates act much more quickly on Gram-positive compared to Gram-negative bacteria. Probably, the complex cell envelope structure of Gram-negative bacteria does not allow fast interaction of the compound, unlike with Gram-positive bacteria.

Antibacterial Activity against Stationary Phase of Bacteria. As mentioned earlier, because conventional antibiotics primarily target the cellular processes of cells, their activity depends upon the metabolic state of the bacteria.[31] Therefore, most of the known antibiotics are ineffective against the stationary phase of bacteria, when all of the biological functions become slow and the cell division process almost stops.[49] Hence, the kinetics of killing for this class of compounds was determined against the stationary phase cultures of *S. aureus*. As shown in Figure 2a, compound **8** displayed a >3 log CFU/mL reduction in cell viability within 1 h of exposure at a concentration even as low as 50 μg/mL (around 8 × MIC of compound concentration). These results suggested that these lipophilic lysine−norspermidine conjugates showcase a significant potency even against nondividing stationary phase of bacteria.

Antibacterial Efficacy in Human Plasma. Antibacterial peptides (AMPs and lipopeptides) suffer from the serious limitation of protease degradation,[50] which is responsible for instability of the peptides and results in decreased antibacterial activity under in vivo conditions. To overcome this limitation, we have synthesized compounds containing unnatural amino acids (D-lysine). The stability of compound **8** (containing D-lysine) in the presence of serum proteases was investigated by determining the antibacterial activity in the presence of 50% plasma. Results suggested that compound **8** retained antibacterial activity even after 24 h of incubation with 50% plasma as shown in Supporting Information Figure S42. Hence, this class of compounds showed promise in retaining antibacterial activity in the presence of serum protease.

Mechanism of Action. The ability of this class of compounds to kill both metabolically active and inactive cells suggested that a membrane disruption might be a possible mechanism of action of these compounds. To establish the mechanism of action of this compound class, the most effective compound, **8**, was selected for further investigation. Experiments with the membrane-potential sensitive dye $DiSC_3(5)$ showed that the test compound rapidly depolarized the membrane of *S. aureus* (Figure 2b) at concentrations 12 times its MIC. Similarly, another dye-based (PBFI-AM dye) experiment suggested that the compound induced a significant amount of leakage of K^+ from bacterial cells (Figure 2c). Taken together, these results confirm the compounds disrupt bacterial membranes.

Propensity To Induce Bacterial Resistance. To investigate the longevity of this class of compounds as future antibacterial agents, resistance development studies against *S. aureus* were performed. Results indicated no change in the MIC for the test compound **8** against this bacterium even after 20 passages, as shown in Figure 2d. The control antibiotic norfloxacin, however, showed about 50-, 300-, and 900-fold increases in the MIC after 10, 15, and 20 passages, respectively. This result demonstrates that it is difficult for bacteria to develop resistance against this class of compounds. This is probably because of their membrane-targeted mode of action, and this study indicates the potential of these compounds as long-lasting antibacterial agents.

Disruption of Bacterial Biofilms. Having established the long-lasting antibacterial potency of the lipophilic lysine−norspermidine conjugates against planktonic and stationary phase bacterial cells, we next investigated the efficacy of this class of compounds as agents that disrupt bacterial biofilms. Compound **8**, the model compound for the study, was compared with antibiotics such as erythromycin, norfloxacin, linezolid, tetracycline, and vancomycin. Results suggested that

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ACS Infect. Dis. 2015, 1, 469−478

compared to the nontreated control, compound **8** displayed significant reduction (>4 log CFU/mL) of cell viability in the preformed biofilms of pathogenic *S. aureus* bacteria at its MIC, whereas none of the antibiotics showed significant reduction even at very high concentrations (64 × MIC) as shown in Figure 3a. We further observed a dependence of concentration on the anti-biofilm activity of compound **8**, which displayed around 5 and 6 $\log_{10}$ (CFU/mL) reductions in cell viability at concentrations of 4 × MIC and 10 × MIC, respectively. Visualization of the extent of biofilm disruption was achieved by crystal violet staining and confocal imaging. Crystal violet staining clearly illustrated that compound **8** (at a concentration of 10 × MIC) displayed an enormous reduction in preformed biofilm mass compared to the nontreated control or the antibiotics as shown in Figure 3b. At the same concentration, confocal microscopy also revealed significant biofilm disruption as shown in Figure 3c. Although the actual mechanism of biofilm disruption is under investigation, we strongly believe that greater electrostatic and hydrogen bonding interactions with the extracellular matrix components of the biofilm play a major role. Furthermore, the potency of this class of compounds as anti-biofilm agents was investigated by performing the minimum biofilm eradication concentration (MBEC) assay. The MBEC values for these compounds varied in the concentration range of 116−1000 μM (Supporting Information Figure S43). To understand the importance of these compounds as anti-biofilm agents, we have directly compared MBEC values to those of common antiseptics (BAC-12 and DDAB-10) and other cationic amphiphiles such as DTAB and CTAB (Supporting Information Table 1). Results suggested that this class of compounds was as good as anti-biofilm agents compared to these existing antiseptics and cationic amphiphiles. Therefore, this class of compounds has great potential to be developed as selective therapeutic agents to tackle biofilm-associated infections.

■ CONCLUSIONS

This paper illustrates the development of lipophilic lysine−norspermidine conjugates from inexpensive starting materials (norspermidine, amino acids, and fatty acids) involving only three simple synthetic steps. These compounds primarily acted on bacteria by destabilization of the bacterial cell membrane. This membrane active nature of the compounds resulted in rapid killing of planktonic cells as well as cells in stationary phase. Furthermore, bacteria could not develop resistance against this class of compounds. Most importantly, the compounds eradicated preformed biofilms of *S. aureus* and killed the bacteria embedded in the biofilms. Hence, this class of compounds, capable of showing simultaneous antibacterial and biofilm-disrupting activities, bears the promise of being developed as next-generation antibacterial therapeutics.

■ EXPERIMENTAL SECTION

Materials and Bacterial Strains. Dichloromethane (DCM), *N*,*N*-dimethylformamide (DMF), and methanol were obtained from Spectrochem (India) and were dried before their use. Norspermidine and all of the quaternary ammonium salts such as benzyldimethyldodecylammonium chloride (BAC-12), didecyldimethylammonium bromide (DDAB-10), dodecyltrimethylammonium bromide (DTAB), and cetyltrimethylammonium bromide (CTAB) were purchased from Sigma-Aldrich. All of the saturated and unsaturated fatty acids, decanoic, dodecanoic, tetradecanoic, hexadecanoic, octadecanoic, oleic, and linoleic acids, were obtained from Alfa-Aesar. *N*,*N*,*N*′,*N*′-Tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), *N*,*N*-diisopropylethylamine (DIPEA), and di-*tert*-butyl dicarbonate (Boc_2O) were purchased from Spectrochem (India). All of these chemicals were used for reaction directly without any further purification. Analytical thin layer chromatography (TLC) was performed on E. Merck TLC plates precoated with silica gel 60 F_{254}, and visualization was carried out using iodine. Column chromatography was performed on silica gel (60−120 mesh) using different ratios of chloroform and methanol solvent system as eluent. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker (AV-400) 400 MHz spectrometer in deuterated solvents. Mass spectra were obtained using a 6538-UHD Accurate mass Q-TOF LC-MS instrument. Infrared (IR) spectra of the compounds (in chloroform or methanol) were recorded on a Bruker IFS66 V/s spectrometer using a NaCl crystal. For optical density (OD) measurement, a Tecan Infinite Pro series M200 microplate reader was used. Bacterial strains *S. aureus* (MTCC 737) and *E. coli* (MTCC 443) were purchased from MTCC (Chandigarh, India). MRSA (ATCC 33591), *E. faecium* (ATCC 19634), VRE (ATCC 51559), and β-lactam-resistant *K. pneumoniae* (ATCC 700603) were obtained from ATCC (Rockville, MD, USA). *E. coli* was cultured in Luria−Bertani broth (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl in 1000 mL of sterile distilled water), whereas *S. aureus*, MRSA, and *K. pneumoniae* were grown in yeast−dextrose broth (1 g of beef extract, 2 g of yeast extract, 5 g of peptone, and 5 g of NaCl in 1000 mL of sterile distilled water). For *E. faecium* and VRE, brain−heart infusion broth (BHI) was used as growth medium. For solid media, 2.5% agar was used along with the above-mentioned growth medium.

Antibacterial Assay. The antibacterial activity of the compounds is reported as their MIC, which is the lowest concentration of the antibacterial agent required to inhibit the growth of microorganism after overnight incubation. All synthesized compounds (**1**−**10**) were assayed in a microdilution broth format as described in the CLSI guideline.[51] The bacterial freeze-dried stock samples were stored at −80 °C. About 5 μL of these stocks was added to 3 mL of the respective broth, and the culture was grown for 6 h at 37 °C prior to the experiments. This 6 h grown culture gives about 10^9 CFU/mL in the cases of *S. aureus* and MRSA and 10^8 CFU/mL in the cases of *E. coli*, *E. faecium*, VRE, and *K. pneumoniae*, which were determined by spread plating method. This 6 h grown culture was diluted to give an approximate cell concentration of 10^5 CFU/mL, which was then used for determining the MIC. Compounds were serially diluted, in sterile Millipore water (in a 2-fold manner), and 50 μL of these serial dilutions was added to the wells of a 96-well plate followed by the addition of about 150 μL of bacterial solution. The plates were then incubated for 24 h at 37 °C. The OD value at 600 nm was recorded using a TECAN (Infinite series, M200 pro) plate reader. Each concentration had triplicate values, and the whole experiment was done at least twice; the MIC value was determined by taking the average of triplicate OD values for each concentration and plotting it against concentration. The data were then subjected to sigmoidal fitting. From the curve the MIC value was determined as the point where the OD was similar to that of control having no bacteria.

Hemolytic Assay.[27] Compounds were serially diluted in Millipore water, and 50 μL of these serial dilutions was added

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ACS Infect. Dis. 2015, 1, 469−478

to the wells of 96-well plates. Human erythrocytes were centrifuged down from the fresh heparinized blood and suspended to 5 vol % in 1× PBS (pH 7.4). After that, 150 μL of the erythrocyte suspension was added to the compound-containing plates. Two controls were made, one without compound as negative control and the other as a positive control by addition with 50 μL of 1 vol % solution of Triton X-100 instead of compound. After that, the plate was incubated at 37 °C for 1 h. Then it was centrifuged at 3500 rpm for 5 min, and 100 μL of the supernatant was then transferred to another 96-well plate to measure the absorbance at 540 nm. To determine the percentage of hemolysis the formula $(A_t - A_0)/(A_{TX} - A_0) \times 100$ has been used, where A_t is the absorbance of the compound-treated well, A_0 the absorbance of the negative control (without compound), and A_{TX} the absorbance of the Triton X-100 containing well. Each concentration had triplicate values, and the HC_{50} was determined by taking the average of triplicate OD values and plotting it against concentration fitted with a sigmoidal plot. From the curve the values were determined corresponding to 50% hemolysis.

Cytotoxicity Assay.[27] The cytotoxicity of compound **8** was assessed against the HeLa cell line. Briefly, the cells were grown in a 96-well plate in DMEM (supplemented with 10% fetal bovine serum and 5% penicillin−streptomycin) until they reached around 70−80% confluency. The cells were then treated with 50 μL of serially diluted compound. Two controls were made, one containing no compound (nontreated cells) and the other one with 10 vol % Triton-X 100 solution. The plate was incubated for 1 h at 37 °C under 5% CO_2 atmosphere. After 24 h, the medium was carefully removed, and 100 μL of MTT solution (5 mg/mL concentration) was then added to each well. The plate was incubated for 4 h at 37 °C under 5% CO_2 atmosphere. Then it was centrifuged at 1100 rpm for 5 min, and the supernatant was removed. After that, 100 μL of DMSO was added to solubilize formazan crystals. The OD of the plate was then recorded at 570 nm. The percentage of cell survival was calculated using the following equation: cell viability (%) = $(A_{treated} - A_{TritonX\text{-}treated})/(A_{nontreated} - A_{TritonX\text{-}treated}) \times 100$. Each concentration had triplicate values, and the average of triplicate OD values was plotted against concentration followed by fitting with a sigmoidal plot. From the curve the values were determined corresponding to 50% cell viability. For bright-field microscopic images, a 40× objective was used, and images were captured using a Leica DM2500 microscope.

Bactericidal Time-Kill Kinetics.[27] *Against Planktonic Bacteria.* The bactericidal activity of the compounds was evaluated by performing a time-kill kinetics assay. Briefly, *S. aureus* and *E. coli* were grown in respective broth at 37 °C for 6 h to their exponential phase. Test compound **8** was then added to the bacterial solution (approximately 10^5 CFU/mL) with the working concentration of 36 μg/mL (about 6 × MIC) and was incubated at 37 °C. For control, the same experiment was performed without compound. At different time intervals 20 μL aliquots from that solution were serially diluted 10-fold in 0.9% saline. Then from the dilutions, 20 μL was plated on yeast−dextrose agar plates and incubated at 37 °C for 24 h. The bacterial colonies were counted, and results are represented in logarithmic scale, that is, $\log_{10}$ (CFU/mL). The second experiment was performed using a similar protocol at shorter time gap of 0, 5, 15, 30, 45, and 60 min to find the exact time required to show bactericidal activity against exponential phase *S. aureus*.

Against Stationary Phase Bacteria. Briefly, *S. aureus* was grown in yeast−dextrose broth at 37 °C for 18 h to achieve stationary phase. Test compound **8** was then added to the stationary phase bacteria with the working concentrations of 50 and 100 μg/mL. It was then incubated at 37 °C with shaking at 150 rpm. At different time intervals (0, 1, and 3 h) 20 μL aliquots from that solution were serially diluted 10-fold in 0.9% saline. Then from the dilutions, 20 μL was plated on yeast−dextrose agar plates and incubated at 37 °C. After 24 h, the bacterial colonies were counted, and the results were represented in logarithmic scale, that is, $\log_{10}$ (CFU/mL). For control, the same experiment was performed without compound.

Antibacterial Assay in the Presence of Human Plasma.[27] To examine the susceptibility of the new compounds toward serum proteases, the antibacterial activities were tested in the presence of 50% of plasma. Briefly, 250 μL of compound **8** was added into 250 μL of fresh human plasma and incubated at 37 °C. At specified time intervals of 0, 2, 4, 6, 12, and 24 h, the aliquot was 2-fold diluted in 0.9% saline. Then antibacterial activities were determined against *S. aureus* by following the same protocol as described above for the antibacterial assay.

Membrane Active Mechanism of Action.[27] *Cytoplasmic Membrane Depolarization Assay.* The 6 h grown culture (mid log phase) of *S. aureus* was harvested (3500 rpm, 5 min), washed in 5 mM glucose and 5 mM HEPES buffer (pH 7.2) in a 1:1 ratio, and resuspended in 5 mM HEPES buffer, 5 mM glucose, and 100 mM KCl solution in a 1:1:1 ratio. Then 2 μM 3,3′-dipropylthiadicarbocyanine iodide ($DiSC_3$) was added to a cuvette containing 2 mL of bacterial suspension and pre-incubated for 20 min. The fluorescence was monitored at an excitation wavelength of 622 nm (slit width = 10 nm) and an emission wavelength of 670 nm (slit width = 5 nm). Then 10 μL of test compound **8** (12 × MIC) was added to the cuvette containing bacterial suspension and $DiSC_3$. As a measure of membrane depolarization, the increase in fluorescence was monitored for 15 min.

K^+ Leakage Assay. The 6 h grown cultures (mid log phase) of *S. aureus* and *E. coli* were harvested (3500 rpm, 5 min), washed, and resuspended in 10 mM HEPES buffer and 0.5% glucose in a 1:1 ratio. Then 2 mL of the bacterial suspension was placed in a cuvette, and fluorescence was measured at an excitation wavelength of 346 nm (slit width = 10 nm) and an emission wavelength of 505 nm (slit width = 5 nm) for 50 s at room temperature. PBFI-AM dye (1 μM) was then added followed by 10 μL of test compound **8** (12 × MIC), and the increase in fluorescence was monitored as a measure of K^+ leakage.

Resistance Studies.[27] For resistance study the antibiotic norfloxacin was chosen as the positive control. At first, the MIC values of compound **8** and norfloxacin were determined against *S. aureus* as described above for the antibacterial assay. For the next-day MIC experiment, the bacterial dilution was made by using the bacteria from sub-MIC (MIC/2) concentration of the compound **8** and norfloxacin, respectively. After a 24 h incubation period, again bacterial dilution was prepared like the previous day by using the bacterial suspension from sub-MIC concentration and assayed for the next MIC experiment. The process was repeated for 20 passages. The fold of MIC increase for test compound **8** and control antibiotic was plotted against the number of days.

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ACS Infect. Dis. 2015, 1, 469−478

Biofilm Disruption Assay.[42,46,48] *Quantification of Cell Viability in Biofilms and Crystal Violet Staining.* A mid log phase (6 h grown) culture of *S. aureus* was diluted to a concentration of approximately 10^5 CFU/mL in a nutrient broth supplemented with 1% glucose and 1% NaCl to make the bacterial stock solution. Then 100 μL of this stock was added into 96-well plates. The plates were then incubated under stationary conditions for 24 h at 37 °C. The medium was then removed, and planktonic bacteria were washed out with 1× PBS (pH 7.4) to obtain the biofilms. Then, 100 μL of the test compound **8** (MIC, 4 × MIC and 10 × MIC) and control antibiotics such as erythromycin, norfloxacin, linezolid, tetracycline, and vancomycin (64 × MIC) was added to the biofilm and allowed to incubate for 24 h. One control was made where 100 μL of complete medium was added instead of compound or antibiotics. At the end of 24 h, medium was discarded and planktonic cells were removed by washing with 1× PBS. Then 100 μL of trypsin−EDTA solution was added to the disrupted biofilms to dissolve it. Cell suspension was then assessed by plating serial 10-fold dilutions of biofilm on nutrient agar plates. After 24 h of incubation, the plates were counted and cell viability was expressed as $\log_{10}$ (CFU/mL) and compared with the nontreated control. For visualization of the biofilm disruption, 100 μL of 0.1% of crystal violet (CV) was added into the wells and incubated for 10 min at 37 °C. CV solution was then discarded, and the plates were washed once with 1× PBS (pH 7.4) solution. Finally, stained images were captured using a normal digital camera.

Confocal Imaging of Biofilms. Coverslips were first sterilized by soaking them in ethanol followed by drying in flame, and then placing in well of a 6-well plate. Midlog phase (6 h grown) culture of *S. aureus* was then diluted to approximately 10^5 CFU/mL in a nutrient broth supplemented with 1% glucose and 1% NaCl, and 2 mL of this stock was added to coverslips containing wells. The plate was then incubated under stationary conditions at 37 °C. After 24 h, medium was removed and planktonic bacteria were carefully washed out with 1× PBS (pH 7.4). Biofilms containing coverslips were then placed into another 6-well plate, and 2 mL of test compound **8** (10 × MIC) was added to it and allowed to incubate for 24 h. In the case of control, 2 mL of complete medium was added instead of compound. At the end of 24 h, medium was then removed and planktonic cells were removed by washing with 1× PBS. Coverslips containing biofilm were carefully removed from the well, stained with SYTO-9 (3 μM), and imaged using a confocal laser-scanning microscope.

MBEC Assay.[46] To find out the minimum concentration required to completely eradicate the preformed biofilms, we have also performed the MBEC assay. Briefly, midlog phase culture of *S. aureus* was diluted to a concentration of ∼10^5 CFU/mL in nutrient broth supplemented with 1% glucose and 1% NaCl. Then, 100 μL of the suspension was added into the wells of 96-well plates. The plates were then allowed to incubate under stationary conditions for 24 h. At the end of 24 h, the medium and planktonic bacteria were removed and the plate was carefully washed with 1× PBS. After that, 100 μL of the test compounds of various concentrations (diluted in the medium) was added to the preformed biofilms and allowed to incubate for 24 h. Control experiments were performed in which 100 μL of media was added instead of compound. At the end of 24 h, the media along with disrupted biofilms were removed, and the plate was washed carefully with 1× PBS. Two hundred microliters of fresh media was then added to the disrupted biofilms and allowed to regrow for 24 h. The OD value (at 600 nm) of the regrown biofilms was then recorded by using a TECAN (Infinite series, M200 pro) plate reader. The experiment was performed in triplicate, and the MBEC value was considered as the lowest concentration at which the OD (average of the triplicate values) was found to be <0.1.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.5b00056.

Details of characterization of the compounds, spectral data (HPLC, NMR, and HR-MS spectra), and supplementary figures (PDF)

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Notes

The authors declare the following competing financial interest(s): JNCASR has filed a patent application based on the work described in the manuscript.

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■ ABBREVIATIONS

HBTU, *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)-uronium hexafluorophosphate; $(Boc)_2O$, di-*tert*-butyldicarbonate; DIPEA, *N*,*N*-diisopropylethylamine; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; MRSA, methicillin-resistant *S. aureus*; VRE, vancomycin-resistant *E. faecium*; MIC, minimum inhibitory concentration; HC_{50}, 50% hemolytic concentration; CFU, colony-forming units; $DiSC_3(5)$, 3,3′-dipropylthiadicarbocyanine iodide; PBFI-AM, potassium-binding benzofuran isophthalate

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