Journal of Medicinal Chemistry

Article

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Membrane Active Phenylalanine Conjugated Lipophilic Norspermidine Derivatives with Selective Antibacterial Activity

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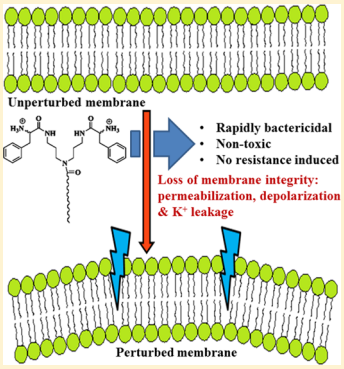
Supporting Information

ABSTRACT: Natural and synthetic membrane active antibacterial agents offer hope as potential solutions to the problem of bacterial resistance as the membrane-active nature imparts low propensity for the development of resistance. In this report norspermidine based antibacterial molecules were developed that displayed excellent antibacterial activity against various wild-type bacteria (Gram-positive and Gram-negative) and drug-resistant bacteria (methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecium*, and β-lactam-resistant *Klebsiella pneumoniae*). In a novel structure−activity relationship study it has been shown how incorporation of an aromatic amino acid drastically improves selective antibacterial activity. Additionally, the effect of stereochemistry on activity, toxicity, and plasma stability has also been studied. These rapidly bactericidal, membrane active antibacterial compounds do not trigger development of resistance in bacteria and hence bear immense potential as therapeutic agents to tackle multidrug resistant bacterial infections.



INTRODUCTION

The global threat of infectious diseases has been aggravated by the emergence of bacterial resistance to antibiotics.[1−3] The steady decline of antibiotics entering the clinical pipeline has created an urgent need for development of novel antibacterial agents.[4] Since most of the known antibiotics target cellular processes in bacteria, they are rendered ineffective, either by mutations or by enzymes and even by efflux pumps.[5] Membrane active molecules are considered to be better because they have been known to possess low propensity for triggering the development of bacterial resistance.[6−9] Antimicrobial peptides (AMPs) and lipopeptides are nature's own membrane active agents. Some of them are already being used in the clinic,[10] and some are undergoing clinical trials.[10,11] However, low in vivo potency, high cost of manufacture, and low selectivity limit the large-scale use of natural amphipathic peptides as clinical antibacterial agents. Moreover, some of these naturally occurring amphipathic peptides (lipopeptides) show antimicrobial activity toward narrow spectra of microorganisms. For example, daptomycin, a naturally occurring lipopeptide and active only against Gram-positive bacteria, acts through a Ca^{2+} ion dependent pathway that helps to interact with bacterial membrane component phospatidylglycerol (PG).[10,12,13] The polymyxins (polymyxin B and colistin) are a class of cationic lipopeptides that bind specifically to the lipopolysaccharide (LPS), the major component of outer cell membrane of Gram-negative bacteria, and are thus active only against this class of bacteria.[10,14−16]

Inspired from natural AMPs, several synthetic membrane active agents such as α-peptides,[17,18] β-peptides,[19,20] oligoacyl lysines,[21] oligoureas,[22] α-AApeptides,[23] arylamide foldamers,[6,24] antimicrobial polymers,[25−31] and cationic amphiphiles[32−34] also showed promising ability to be developed as future antibacterial agents. Likewise, natural lipopeptides have also inspired the design of several lipophilic membrane active agents.[9,35−42] In an exemplary work, Shai et al. made a series of compounds wherein long alkyl chains were appended to a tetrapeptide sequence of naturally occurring amino acids.[39] These compounds were found to have potent antibacterial activity. Cai et al. likewise have reported lipidated peptidomimetics[40] and lipo-γ-AApeptides[9] that exhibited potent antibacterial efficacy over a broad spectrum of bacterial strains. Recently, Gopi et al. have reported vinylogous hybrid lipopeptides that displayed self-assembled nanostructures dependent antimicrobial activitiy.[41] In another example, David et al. have developed a series of simple lipopolyamines in which peptides were replaced by a spermine moiety.[42] Effective as they may be in their structural designs, synthetic complexity associated with such molecules has left immense scope for development of much simpler membrane active antibacterial agents.

Herein, we report simple membrane active molecules with two positive charges and a lipophilic tail to enable stronger interaction with the bacterial cell envelope. In our design we chose to functionalize a norspermidine derivative to have at least two positive charges and a pendent aliphatic group. Norspermidine is an easily available and inexpensive compound that allows facile functionalization. Phenylalanine was conjugated to the primary amines of norspermidine, while variable aliphatic chain was appended to the secondary amine to create

Received: June 23, 2014

   dx.doi.org/10.1021/jm5013566 | J. Med. Chem. XXXX, XXX, XXX−XXX

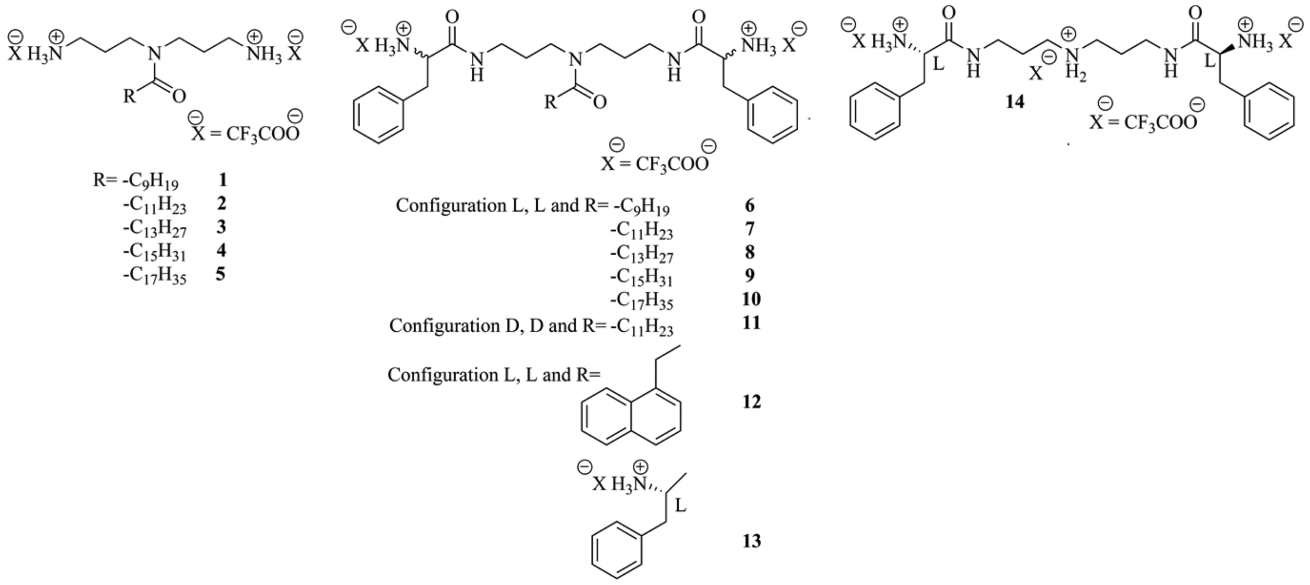
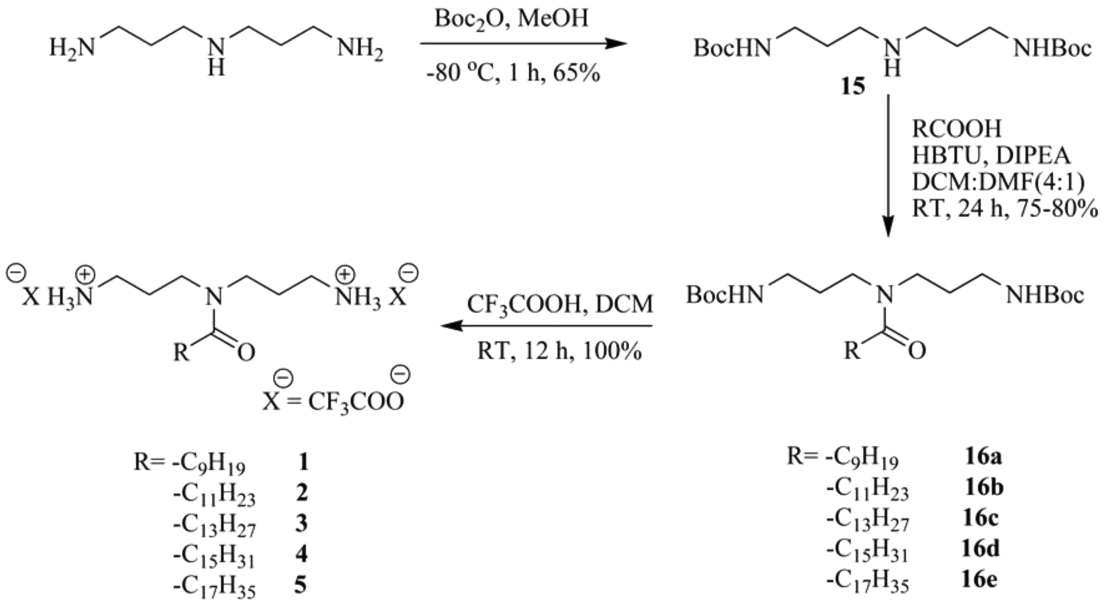


Figure 1. Structures of the compounds synthesized in this study.

Scheme 1. Preparation of Aliphatic Norspermidine Analogues 1−5



a library of compounds. Antibacterial activity against a panel of bacteria and toxicity against mammalian cells (both human erythrocytes and RAW macrophages) were evaluated for the library of compounds. A structure−activity relationship was delineated by varying the length of the long chain and varying the stereoisomerism and type of hydrophobicity. Notably, compounds with different stereoisomerism (L and D) were synthesized to understand the role of strereoisomerism on plasma stability, which will in turn contribute to retaining their antibacterial activity in plasma. To understand the role of phenylalanine conjugation and appended long chains, control compounds without the phenylalanine conjugation as well as those without long chains were also synthesized (Figure 1) and membrane active mechanism of action, plasma stability studies, activity in the presence of serum, and ability of these compounds to withstand bacterial resistance were evaluated.

RESULTS

Synthesis. Initially, the compounds containing an aliphatic chain appended to the secondary amine of norspermidine were synthesized. Synthesis of these compounds was achieved in three steps. First, the primary amine groups of norspermidine were selectively Boc protected. This was achieved by carrying out the reaction at −80 °C using di-*tert*-butyl dicarbonate (Boc_2O) for 1 h. Then aliphatic chains were introduced at the secondary amine of Boc-protected norspermidine through amide coupling reaction using *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) as coupling agent. Finally, deprotection of Boc groups with trifluoroacetic acid (TFA) yielded the aliphatic norspermidine derivatives (**1**−**5**) (Scheme 1).

The scheme and strategy used to conjugate phenylalanine to these aliphatic norspermidine derivatives are furnished in Scheme 2. The first step involved selective coupling of the

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Scheme 2. Preparation of Phenylalanine Conjugated Norspermidine Analogues 6−14

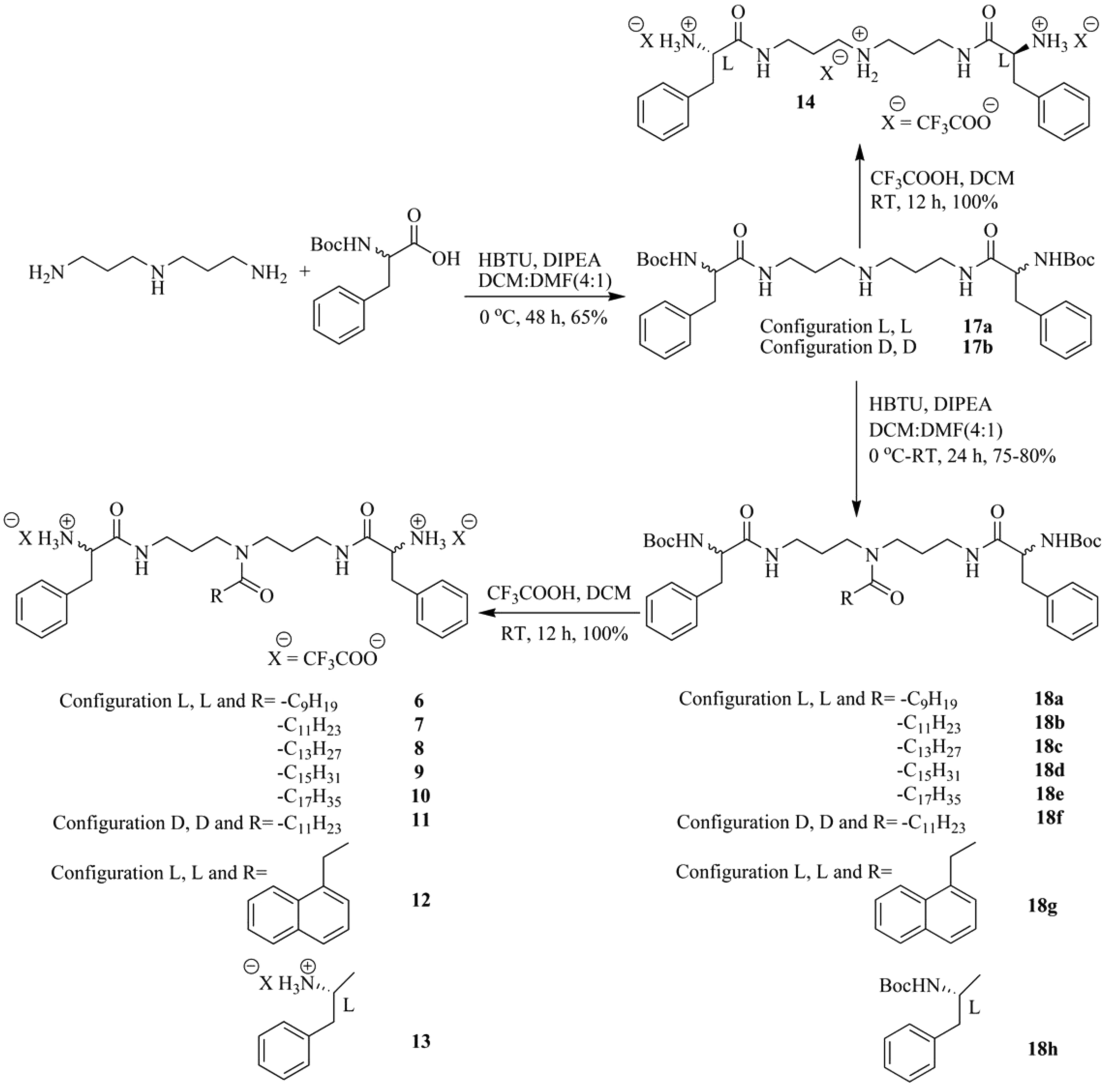

Table 1. Antibacterial and Hemolytic Activity of the Compounds

	MIC (μg/mL)						
	drug sensitive strains			drug resistant strains			
compd	*S. aureus*	*E. faecium*	*E. coli*	MRSA[a]	VRE[b]	*K. pneumoniae*[c]	HC_{50} (μg/mL)
1	240	>250	231	137	250	>250	>500
2	45	62	108	53	57	163	107
3	18	27	28	15	23	47	72
4	12	2.8	14	11	2.4	65	52
5	21	21	26	19	11	106	42
6	15	29	19	20	14	57	187
7	3.6	4	10	3.3	2.3	8	171
8	6	4.5	14	4.1	2.4	16	124
9	9	7.6	227	4.6	2.7	62	74
10	11	10	248	5.2	2.9	87	65
11	3.5	2.8	10	4.2	2.4	6.5	209
12	>250	>250	>250	ND[d]	>250	>250	ND
13	>250	>250	>250	ND	>250	>250	ND
14	>250	>250	>250	ND	>250	>250	ND
colistin	25	>250	0.5	68	>250	1.5	ND
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 stands for "not determined".

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primary amine groups of norspermidine with *N*-Boc-L/D-phenylalanine by carrying out amide coupling reaction at 0 °C using HBTU as coupling agent for 48 h. Then an aliphatic chain was introduced at the secondary amine through another amide coupling reaction with various aliphatic acids. In the third step the deprotection of Boc groups with trifluoroacetic acid (TFA) yielded the final compounds (**6**−**11**) (Scheme 2). Syntheses of **12** and **13** were achieved by using α-naphthylacetic acid and *N*-Boc-L-phenylalanine, respectively, instead of aliphatic carboxylic acids in the second amide coupling reaction. The control compound **14**, which is devoid of the long chain, was made by directly adding TFA to the first step product. The final compounds (**6**−**14**) were found to have more than 95% purity as determined by HPLC. Finally, all the compounds were characterized by IR, ^{1}H NMR, ^{13}C NMR, and HR-MS.

Antibacterial Activity. The antibacterial efficacy of both the aliphatic norspermidine derivatives (**1**−**5**) and phenylalanine conjugated norspermidine derivatives (**6**−**14**) was determined against various drug-sensitive and drug-resistant bacteria such as *S. aureus*, *E. faecium*, MRSA, VRE (Gram-positive), *E. coli*, and *K. pneumoniae* (Gram-negative). The activity was reported as their minimum inhibitory concentration (MIC) values (Table 1).

In order to estimate the required parameters for potent antibacterial activity, compounds **1**−**5** were tested for their antibacterial activity. Most of the compounds showed moderate antibacterial activity against all the bacteria tested. Among these derivatives, compound **1** (decanoyl analogue) was found to possess no significant activity against any of the tested bacteria. The dodecanoyl analogue, compound **2**, displayed much improved antibacterial activity with MIC values of 45 and 108 μg/mL against *S. aureus* and *E. coli*, respectively; however, the activity cannot be considered significant clinically. Further increase in the length of the aliphatic chain yielded compound **3** (tetradecanoyl analogue) that displayed significant activity with MIC values of 18 and 28 μg/mL against S. *aureus* and *E. coli*, respectively. The antibacterial efficacy was enhanced even further as the length of the aliphatic chain was increased to hexadecanoyl (compound **4**), which displayed excellent MIC values of 12 and 14 μg/mL against *S. aureus* and *E. coli*, respectively. However, further increase in the length of the aliphatic chain to octadecanoyl, as in compound **5**, resulted in decrease in antibacterial activity. Compound **5** displayed MIC values of 21 and 26 μg/mL against *S. aureus* and *E. coli*, respectively. Thus, compound **4** with hexadecanoyl aliphatic chain was found to be the most potent antibacterial agent among the first set of norspermidine series of compounds, the highlight being its activity against *E. faecium* (MIC = 2.8 μg/mL). Compounds **3** and **5** also exhibited considerable activity against *E. faecium* with MIC values of 27 and 21 μg/mL.

Against drug-resistant species such as MRSA and VRE these compounds showed moderate activity. Against MRSA compound **4** displayed an MIC of 11 μg/mL while the rest were a little less active. The activity of compound **4** against VRE was excellent, as it exhibited a MIC value of 2.4 μg/mL, but only moderate activities were observed for compounds **3** and **5**, with MIC values of 23 and 11 μg/mL, respectively. The activity of these compounds against *K. pneumoniae* was not significant.

The effect of phenylalanine conjugation was reflected on the antibacterial activity of the conjugated compounds. An enhanced antibacterial efficacy was observed compared to the nonconjugated aliphatic norspermidine derivatives (compounds **1**−**5**). Compound that consists of the shortest aliphatic chain (decanoyl chain) was found to be the least effective against all tested Gram-positive bacteria in this series. Compound **6** with decanoyl aliphatic group exhibited MIC values of 15 and 19 μg/mL against *S. aureus* and *E. coli*, respectively. The dodecanoyl analogue **7** displayed excellent antibacterial activity with MIC values of 3.6 and 10 μg/mL against *S. aureus* and *E. coli*, respectively. However, further increase in long chain to the tetradecanoyl chain as in compound **8** resulted in a decrease in antibacterial activity. Compound **8** displayed MIC values of 6 and 14 μg/mL against *S. aureus* and *E. coli*, respectively. Further increase in long chain yielded compound **9** (hexadecanoyl analogue), which displayed even decreased activity (MIC values of 9 and 227 μg/mL against *S. aureus* and *E. coli*, respectively). The highest long chain analogue (octadecanoyl) compound **10** followed the same trend and was found to be least active against Gram-negative bacteria. Thus, compound **7** with dodecanoyl aliphatic chain was found to be the most potent antibacterial agent among all the phenylalanine conjugated derivatives.

In fact compound **7** showed excellent activity against *E. faecium* (MIC = 4 μg/mL). Compounds **8**, **9**, and **10** also exhibited considerable activity against *E. faecium* with MIC values of 4.5, 7.6, and 10 μg/mL, respectively. The fact that introduction of phenylalanine moiety was indeed an improvement to the existing design was reflected in the activity of compound **7**, which showed MIC of 3.3 μg/mL against MRSA, while the unconjugated analogue compound **2** had MIC of 53 μg/mL. Among the others, compounds **8**, **9**, and **10** displayed excellent activity with MIC values of 4.1, 4.6, and 5.2 μg/mL, respectively, against MRSA. Compound **7** also showed superior activity against VRE (MIC = 2.3 μg/mL), highlighting the effectiveness of the design. This compound was more than 250 times more potent than vancomycin, which did not show activity even at a concentration of 1000 μg/mL (Supporting Information Figure S47). Compounds **8**, **9**, and **10**, with the MIC values of 2.4, 2.7, and 2.9 μg/mL, respectively, also exhibited potent activity against VRE. The most potent analogue **7** also showed good activity against the β-lactam-resistant *K. pneumoniae* with an MIC value of 8 μg/mL, whereas the other compounds in the series showed moderate activity against this bacterium.

Compound **11** (dodecanoyl long chain) consisting of "D" isomer of phenylalanine displayed similar antibacterial efficacy compared to the most potent compound **7**. But compound **12** consisting of a naphthalene moiety in the place of long chain did not show any antibacterial activity even at 250 μg/mL against all the tested bacteria. Similarly, compound **13** having a phenylalanine group instead of the aliphatic chain also remained inactive even at a concentration of 250 μg/mL. Finally, compound **14** lacking the aliphatic chain showed complete loss of activity, emphasizing the importance of the aliphatic chain in these derivatives.

Critical Micellar Concentration (cmc). In order to investigate the effect of aggregation toward antibacterial activities, the cmc values of compounds **6**−**11** were determined (the conditions used were same as that of MIC experiment). Chain-length dependent variation of cmc values was observed. Compound **6**, the decanoyl analogue, was found to have the highest cmc value (Supporting Information Figure S48) of 156 μg/mL, while the cmc of dodecyl analogue, compound **7**, sharply fell to 40 μg/mL. The octadecanoyl analogue (compound **10**) had the lowest value (20 μg/mL) in the

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series. The corresponding D-isomer of compound **7**, compound **11**, was found to have the same cmc value (40 μg/mL).

Bactericidal Kinetics. In order to understand the antibacterial nature of the compounds, time–kill kinetics experiments of compounds **7** and **11** against *S. aureus* were performed. The compounds showed rapid-bactericidal activity. From the initial assay both the tested compounds revealed more than 3 log (CFU/mL) reduction in the number of viable bacteria within an hour at a concentration of 6 × MIC (Figure 2 and Supporting Information Figure S49). In order to ascertain the exact time required to exhibit bactericidal activity, the experiment was done for compound **11** in a smaller time gap ("minute" scale) with the same concentration of 6 × MIC of compound. Compound **11** showed more than 3 log (CFU/mL) reduction within 15 min only (inset of Figure 2).

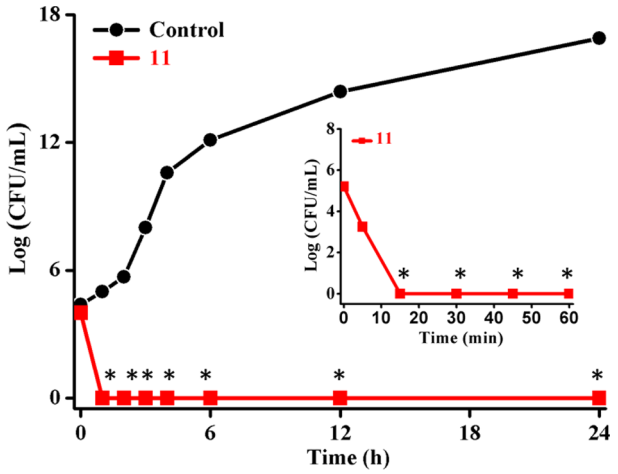

Figure 2. Time–kill kinetics of compound **11** (6 × MIC) against *S. aureus* (asterisks correspond to <50 CFU/mL).

Hemolytic Activity. Toxicity of these compounds was evaluated against human erythrocytes (RBCs). The ability of the compounds to lyse RBCs was expressed as their HC_{50} values (HC_{50} is defined as the concentration at which 50% of the red blood cells are lysed). In general, the HC_{50} values of both series displayed a decreasing trend with increasing aliphatic chain length. The most potent compound in the aliphatic norspermidine derivatives (**1**–**5**), compound **4**, was found to have an HC_{50} value of 52 μg/mL. However, the HC_{50} values of phenylalanine conjugated norspermidine derivatives ranged from 209 to 65 μg/mL. The compound with the shortest long chain in the series, compound **6**, had an HC_{50} value of 187 μg/mL. Compound **7**, which displayed the most potent antibacterial efficacy, was found to have an HC_{50} value of 171 μg/mL (Table 1). The corresponding "D" isomer, compound **11**, showed even less toxicity with an HC_{50} value of 209 μg/mL. This study suggests that the compounds are selectively active toward bacterial cell over human erythrocytes. In the field of membrane active agents, selectivity of the compounds is adequately represented by HC_{50}/MIC.[23−25,31] Selectivity ratios (HC_{50}/MIC_{VRE}) described here were calculated with respect to activity against VRE (Supporting Information Figure S50), and compounds **7** and **11** were found to be the most selective compounds.

Cytotoxicity. Toxicity of the compounds against a mammalian cell line (RAW 264.7 TIB-71) was also determined using the MTT assay. The compound selected for the study was the most potent compound **11**. No toxicity was observed even at a concentration of 16 μg/mL, which was 7-fold more compared to its MIC value against VRE (Figure 3). The EC_{50} value (50% cells viability) for compound **11** was found to be 26 μg/mL, which was determined from the sigmoidal plot of cell viability against concentration. This result further advocates the potential of compound **11** for use as an antimicrobial therapeutic.

Antibacterial Efficacy in Human Plasma. Protease degradation is responsible for instability of antibacterial peptides (AMPs and lipopeptides), which subsequently results in decreased antibacterial activity in the plasma condition.[43] In order to determine the stability of the phenylalanine conjugated norspermidine derivatives, antibacterial activity in the presence of 50% plasma was determined. The model compounds selected for the study were the most potent compounds **7** and **11**. In the case of compound **7**, in the presence of 50% human plasma, ~2-fold and ~4-fold decrease in antibacterial activity was observed after 2 and 4 h of incubation, respectively. Interestingly, "D" analogue compound **11** did not reveal any loss in activity even after 24 h incubation (Supporting Information Figure S51). This indicates that the corresponding "D" analogue (compound **11**) was stable in plasma and did not lose antibacterial efficacy.

Antibacterial Activity in Human Serum. Activity in the presence of 50% serum was also determined in order to establish the efficacy of the compounds further. Here too, the

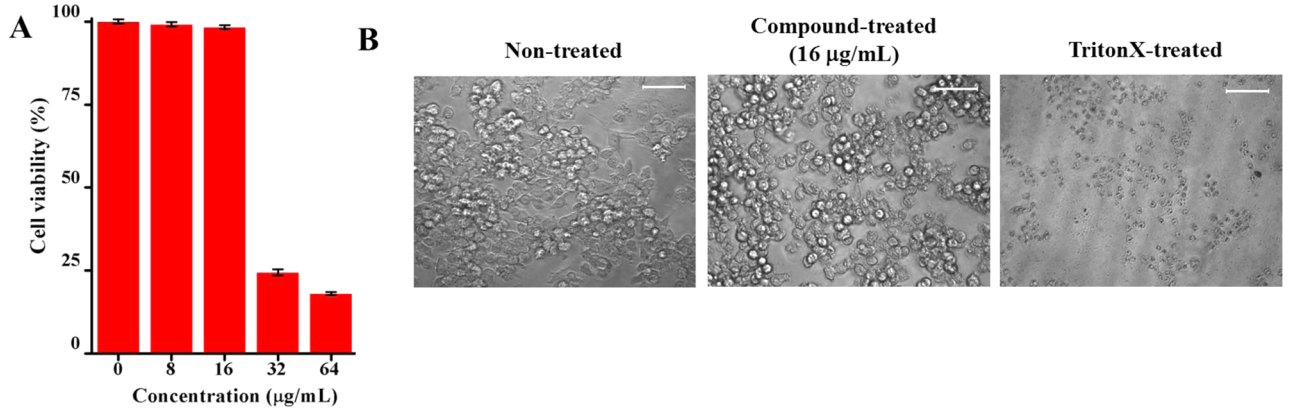

Figure 3. Cytotoxicity against RAW cell line: (A) cell viability after treatment of different concentrations of compound **11**; (B) microscopic images of cells at different conditions (scale bar = 100 μm).

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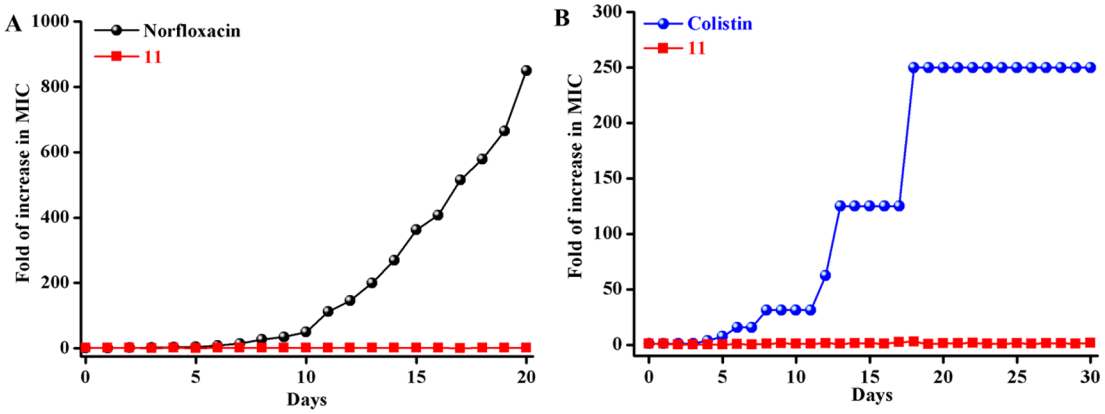

Figure 4. Propensity to induce bacterial resistance: comparison of fold of increase in MIC of (A) norfloxacin and compound **11** against *S. aureus* and (B) colistin and compound **11** against *E. coli*.

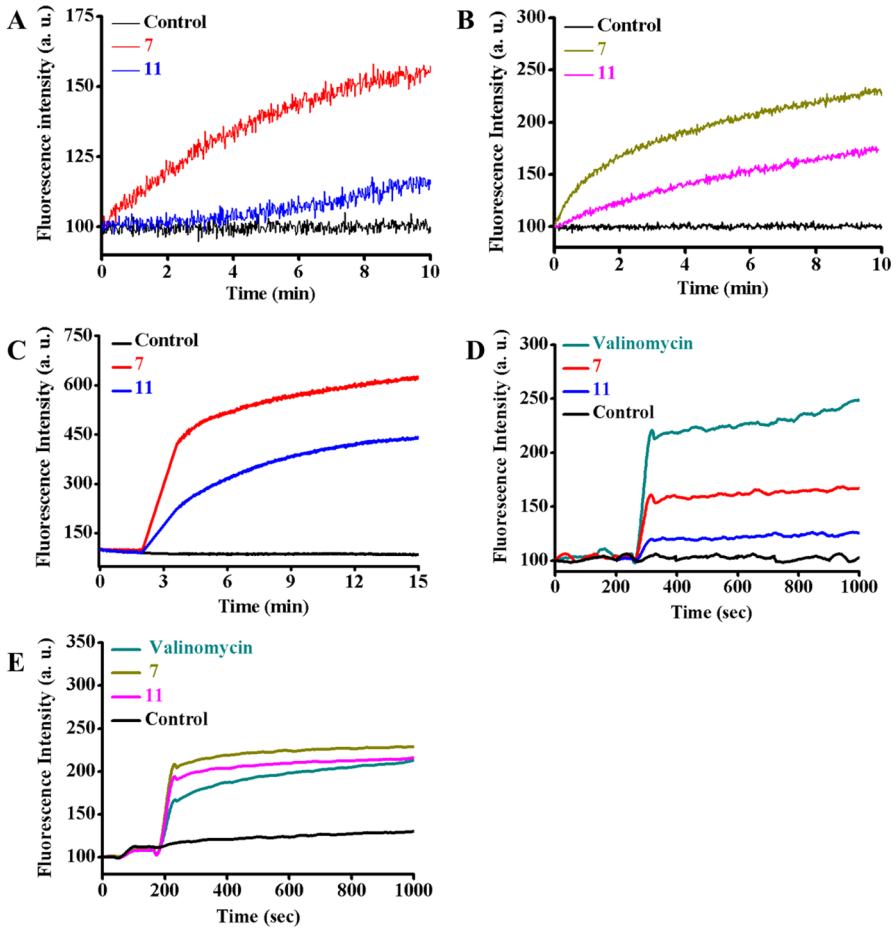

Figure 5. Mechanism of antibacterial action of compounds **7** and **11** at concentrations of 12 × MIC: (A) membrane permeabilization of *S. aureus*; (B) membrane permeabilization of *E. coli*; (C) membrane depolarization of *S. aureus*; (D) K^+ leakage of *S. aureus*; (E) K^+ leakage of *E. coli*.

experiment was performed with the most potent compound **11**. The MIC value of the compound was found to increase by 2-fold and remained the same even up to 4 h of incubation (Supporting Information Figure S52). This indicates that the compound is stable and retains sufficient antibacterial activity in serum condition.

Propensity To Induce Bacterial Resistance. The emergence of antibiotic-resistant bacteria is a major problem to global health.[1−3] Hence, to investigate the potential of these compounds as an antibacterial agent with sufficient longevity, the ability of *S. aureus* (Gram-positive representative) and *E. coli* (Gram-negative representative) bacteria to develop resistance against these compounds was investigated. For this study, compound **11** was chosen as the model compound. Norfloxacin was used as a positive control for *S. aureus*, whereas colistin was used in the case of *E. coli*. Results showed no change in the MIC for compound **11** against *S. aureus* and *E. coli* even after 20 and 30 passages, respectively (Figure 4A and Figure 4B), whereas around 800-fold increase in the MIC was observed in the case of norfloxacin and 250-fold in the case of

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colistin. This study suggested that bacteria find it difficult to develop resistance against this type of compound.

Mechanism of Action. *Bacterial Membrane Permeabilization.* The ability of compounds **7** and **11** to permeabilize the bacterial cell membrane was studied using propidium iodide (PI). This dye can pass through the membrane of compromised bacterial cells and fluoresces upon binding to the DNA.[8,30,44] As can be seen from Figure 5A and Figure 5B, the test compounds were efficient in permeabilizing the membranes of both Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria.

Cytoplasmic Membrane Depolarization. In order to ascertain if the compounds act by depolarizing the bacterial cytoplasmic membrane, fluorescence spectroscopic studies were carried out using the membrane-potential sensitive dye $DiSC_3$. This dye can distribute itself between the cell interior and the medium depending on the membrane potential gradient.[8,30,44] Loss of membrane potential leads to an increase in fluorescence. Figure 5C shows that both compounds **7** and **11** were able to depolarize the membranes of *S. aureus*.

K^+ Ion Leakage. Whether or not these compounds cause leakage of K^+ ions was confirmed by fluorescence spectroscopic studies with PBFI-AM dye. This dye shows an increase in fluorescence after binding to K^+ ions which leak out from the cells.[8] As can be observed in Figure 5D and Figure 5E, both the compounds **7** and **11** caused significant leakage of K^+ ions.

DISCUSSION

Natural and synthetic membrane active agents, such as lipopeptides, are a promising class of antibacterial agents, and some of them are already being used in the clinics for treatment against bacterial infection.[10] However, because of several inherent problems, the clinically approved lipopeptides have limited applications. Design of non-natural membrane active agents may open up a promising new avenue toward the development of better antibiotics. Herein, we have designed simple membrane active antibacterial agents based on a norspermidine backbone involving only three synthetic steps. In the synthetic design positive charges were contributed by the primary amine functionalities of the norspermidine in the case of aliphatic norspermidine derivatives (**1**−**5**) and from the amine group of phenylalanine residues for phenylalanine conjugated norspermidine derivatives (**6**−**11**). In both the cases an aliphatic chain has been assembled into the system by a tertiary amide linkage with the secondary amine of the norspermidine backbone. In order to assess the importance of the aliphatic chains, compounds devoid of pendent long chains were also prepared. Compounds with different stereoisomerism (D-amino acids) were also synthesized to understand the role of stereoisomerism in antibacterial activity, stability, and toxicity. Compounds with fused aromatic rings instead of aliphatic long chains were also prepared to study the importance of flexible hydrophobicity over rigid hydrophobicity.

Although the aliphatic norspermidine derivatives (compounds **1**−**5**) were designed as control compounds to show the efficacy of the phenylalanine conjugated compounds, they showed moderate antibacterial activity. In the structure−activity relationship (SAR) studies of these compounds, the trend of chain length dependent activity was found to be parabolic in nature (Supporting Information Figure S53A). Compound **4** was the most active compound in the series with high activity against VRE. Moreover, this series of compounds exhibited a narrow spectrum of antibacterial activity and considerable amount of toxicity toward human erythrocytes. What can be deduced from this series of compounds is that even two positive charges with a single aliphatic group are good enough for antibacterial activity. There could be several reasons as to why these compounds did not exhibit high, broad-spectrum antibacterial activity. We surmised that the aliphatic norspermidine derivatives might not be hydrophobic enough to impart significant activity. Further, there were no functional moieties that could be a source of hydrogen bonding. In contrast, AMPs and lipopeptides bear amide bonds, which can provide additional hydrogen bonding interaction in order to exhibit high antibacterial activity.

On surveying the literature, we observed that aromatic moieties were a common feature in several effective antibacterial peptidomimetic designs. Svendsen et al. reported several potent cationic antibacterial tripeptides most of which involved aromatic moieties in their designs.[43] All of the antibacterial small molecules reported from the groups of DeGrado and Tew are based on aryl scaffolds.[7,24,45] Recently we have reported from our group several potent antibacterial compounds based on fused aromatic rings.[44] Barron et al. have also used aromatic moieties in the design of antibacterial peptoids.[46] Thus, the introduction of phenylalanine moiety into the design was expected to bring about significant changes in the physical properties of the compounds. The two amide linkages resulting from the coupling of the amino acids also provides the possibility of H-bonding interactions. As mentioned earlier, several groups around the world have used aromatic moieties in order to bring about higher hydrophobicity.[7,24,43−46] We too have observed in this study that introduction of such aromatic moieties indeed plays an important role toward improvement of antibacterial activity. This set of compounds was selective broad spectrum antibacterial agents, and like in the earlier case, these too showed a parabolic pattern of chain length dependent activity (Supporting Information Figure S53B). Whether the improved antibacterial activity and selectivity are specifically because of the introduction of the aromatic moiety or are a culmination of all the properties put together is not very clear. However, we strongly believe that all the properties put together play an important part toward achieving such potent antibacterial activity.

A well-known fact in the literature is that optimization of amphiphilicity is the most important factor directing selective antibacterial activity.[21,24,44] Thus, it was important to study the length of alkyl chain that provided optimum hydrophobicity for selective and potent antibacterial activity. The importance of aliphatic chain is also established by the fact that neither compound **12** (naphthalene moiety in place of aliphatic group) nor compound **13** (phenylalanine moiety in place of aliphatic group) nor compound **14** (lacking aliphatic group) showed any significant antibacterial activity. The long flexible chain allows better interaction with the bacterial cell membrane. Even the naphthalene moiety with 10 carbon atoms (compound **12**) was not able to show any significant activity. This is probably because structural flexibility is an essential condition for potent antibacterial activity.

Such small molecules, with only two amino acid residues, are not expected to change much with respect to structure in solution. However, if the molecules rely on specific interactions for antibacterial action other than membrane damaging abilities, then such changes are expected to affect antibacterial activity significantly. Indeed, it was observed that the replacement of L-

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isomer with D-isomer brings about no significant change in antibacterial activity.

We further investigated if these compounds had propensity to aggregate and whether their aggregation properties played any role toward their antibacterial efficacy. The compounds indeed aggregated in media, and the cmc values decreased with increasing long chain. This trend differed from the parabolic trend observed in case of MIC. Moreover, these cmc values were found to be much higher compared to the concentration at which these compounds inhibited bacterial growth. Thus, it was concluded that aggregation properties of these molecules do not contribute to their superior antibacterial efficacy.

Coming to activity against various classes of bacteria, we observe that in all the cases the activity against Gram-positive bacteria is around 1.5- to 2-fold better than the corresponding activity against Gram-negative bacteria. This might be due to the difference in the cell membrane of Gram-positive and Gram-negative bacteria. Another interesting observation is that the compounds showed good antibacterial activity against drug-resistant species especially Gram-positive species MRSA and VRE. Despite the advent of new molecules, MRSA and VRE infections is a major cause of concern for human health today. Extended spectrum β-lactamase producing Gram-negative pathogens, particularly *K. pneumonia*, cause severe infections, which are increasingly becoming difficult to treat.[3] Any compound with significant activity against such β-lactam resistant *Klebsiella* sp. is welcome in the field. Although the compounds described herein have not achieved excellent activity against β-lactam resistant *K. pneumoniae,* these can serve as lead molecules with possibility of further optimization. Overall, such superior activity against drug-resistant bacteria is definitely the highlight of this series of compounds.

The rapid bactericidal properties of the compounds suggest that the possible mechanism of action of the compounds might be through disruption of bacterial membrane integrity. It was also observed that the phenylalanine conjugated aliphatic norspermidine derivatives kill bacteria in less than 15 min of exposure. These compounds also showed selective toxicity toward bacterial cell over human erythrocytes (RBCs). The antibiotics colistin and vancomycin were not toxic until 100 μg/mL, whereas the most potent compound **11** was found to be nontoxic against RAW cell line until a concentration of 16 μg/mL (7-fold more than its MIC value against VRE). Thus, the compounds warrant further optimization in their design to reach the selected efficacy reflected in the activity of marketed antibiotics. Further, the D-isomeric compound (**11**) did not lose antibacterial efficacy in 50% plasma or in 50% serum, which proves that this type of compound is resistant toward proteases degradation. The membrane lytic properties of the compounds were confirmed by the membrane destabilization experiments carried out with the compounds. Although concentration of compounds (12 × MIC) required to show the membrane permeabilization of bacteria was higher than their corresponding MIC, even a small perturbation is enough to bring about significant damage. What was interesting in this study was the difference in fluorescence intensity brought about by the two compounds **7** and **11**. Similar observation was also made in their abilities to depolarize the cell membrane. This opens up the possibility of compound **11** having alternative modes of action, since in all the cases the membrane damaging property of compound **7** was more, but there was no significant difference in their antibacterial activities. We are currently investigating the reason for such kinds of observation. The fact that bacteria find it difficult to grow resistance against this type of compound enhances the potential use of these compounds as possible clinical candidates. Although several toxicity studies, both in vitro and in vivo, as well as their in vivo activities, need to be performed in order to ascertain the actual potential of the compounds as future antibiotics, it is beyond doubt that they bear much promise.

CONCLUSIONS

A series of phenylalanine conjugated aliphatic norspermidine derivatives have been developed as membrane active antibacterial agents through a simple synthetic strategy. The compounds displayed improved antibacterial efficacy compared to nonconjugated aliphatic norspermidine derivatives. The most potent compound **7** (dodecanoyl analogue) and its "D" isomer analogue compound **11** not only displayed >250-fold more antibacterial activity against VRE compared to vancomycin (last resort antibiotic for Gram-positive bacterial infection) but also were nontoxic at that concentration. Unlike the clinically approved lipopeptide colistin which is active only against Gram-negative bacteria, these compounds were also active toward Gram-positive bacteria. These derivatives primarily damage bacterial cell membrane and kill the bacteria very quickly (within 15 min). Additionally, bacteria could not develop resistance against them. Hence, these compounds have immense potential to be developed as therapeutic agents in order to tackle multidrug resistant bacterial infections.

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 *N*-Boc-D-phenylalanine were purchased from Sigma-Aldrich. All the fatty acids (decanoic, dodecanoic, tetradecanoic, hexadecanoic, and octadecanoic acids) were obtained from Alfa-Aesar. *N*,*N*,*N*′,*N*′-Tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), *N*,*N*-diisopropylethylamine (DIPEA), *N*-Boc-L-phenylalanine, α-naphthylacetic acid, and di-*tert*-butyl dicarbonate (Boc_2O) were purchased from Spectrochem (India). All 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 UV light and iodine. Column chromatography was performed on silica gel (60−120 mesh) using different ratios of chloroform and methanol solvent system as eluent. HPLC analysis of the final compounds was accomplished by using a Shimadzu LC-8A liquid chromatograph instrument (C_{18} column, 10 mm diameter, 250 mm length) with the UV detector monitoring at 254 nm. 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 NaCl crystal. For optical density (OD) measurements, Tecan Infinite Pro series M200 microplate reader was used. Bacterial strains *Staphylococcus aureus* (MTCC 737) and *Escherichia coli* (MTCC 443) were purchased from MTCC (Chandigarh, India). Methicillin-resistant *Staphylococcus aureus* (ATCC 33591), *Enterococcus faecium* (ATCC 19634), vancomycin-resistant *Enterococcus faecium* (ATCC 51559), and β-lactam-resistant *Klebsiella 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), while *S. aureus*, MRSA, and *K. pneumonia* 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

dx.doi.org/10.1021/jm5013566 | J. Med. Chem. XXXX, XXX, XXX−XXX

infusion broth (BHI) was used as growth medium. And for solid media, 2.5% agar was used along with the above-mentioned growth medium.

Synthesis and Characterization. N^1-Boc-N^3-(3-(Boc-amino)-propyl)propane-1,3-diamine (15). An amount of 10 g (1 equiv) of norspermidine was dissolved in MeOH (50 mL), and the solution was kept at −80 °C. Then 24.94 g (1.5 equiv) of di-*tert*-butyl dicarbonate (Boc_2O) was dissolved in MeOH (50 mL) and added to the reaction mixture dropwise. The reaction was continued for 1 h at −80 °C. Then the reaction mixture was allowed to come to room temperature. MeOH was removed under reduced pressure and purification was done through column chromatography on silica gel (60−120 mesh) using methanol and chloroform (7:93) as eluent to afford the product with 65% yield. FT-IR (NaCl): 3344 cm^{-1} (−NH− str), 2974 cm^{-1} (−CH_2− asym str), 2871 cm^{-1} (−CH_2− sym str), 1696 cm^{-1} (C═O str). ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 5.182 (s, NH(−CH_2−CH_2−CH_2−$N\underline{H}$Boc)$_2$, 2H), 3.190−3.175 (m, NH(−$C\underline{H}_2$−CH_2−CH_2−NHBoc)$_2$, 4H), 2.649−2.616 (m, NH(−CH_2−CH_2−$C\underline{H}_2$−NHBoc)$_2$, 4H), 1.860 (s, $N\underline{H}$(−CH_2−CH_2−CH_2−NHBoc)$_2$, 1H), 1.666−1.602 (m, NH(−CH_2−$C\underline{H}_2$−CH_2−NH−Boc)$_2$, 4H), 1.417 (s, NH(−CH_2−CH_2−CH_2−NH−COO−C($C\underline{H}_3$)$_3$)$_2$, 18H).

General Procedure for Synthesizing *N,N*-Bis(3-(Boc-amino)-propyl)alkanamide (16a−e). An amount of about 15 mmol (1.5 equiv) of saturated aliphatic acid (decanoic, dodecanoic, tetradecanoic, hexadecanoic, or octadecanoic) was dissolved in dry DCM (12 mL) at 0 °C. In the reaction mixture 4 equiv of *N,N*-diisopropylethylamine (DIPEA) was added followed by 1.5 equiv of *N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU). Then DMF (3 mL) was added to the reaction mixture. After 10 min, 1 equiv of **15** in dry DCM (1 mL) was added dropwise. The reaction mixture was brought to room temperature and allowed to stir for 24 h. Solvent was evaporated, and residue was diluted in ethyl acetate (50 mL). Then workup was carried out first with 1 N HCl (50 mL, 3 times) followed by saturated Na_2CO_3 solution (50 mL, 3 times). The crude product was extracted in ethyl acetate layer. Finally purification was accomplished through column chromatography on silica gel (60−120 mesh) using different ratios of methanol and chloroform as eluent to afford **16a−e** with 75−80% yield.

*N,N-Bis(3-(Boc-amino)propyl)decanamide (**16a**).* Yield 78%. FT-IR (NaCl): 3340 cm^{-1} (−NH− str), 2929 cm^{-1} (−CH_2− asym str), 2860 cm^{-1} (−CH_2− sym str), 1705 (C═O str). ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 5.388−4.707 (d, R−CO−N(−CH_2−CH_2−CH_2−$N\underline{H}$Boc)$_2$, 2H), 3.386−3.016 (m, R−CO−N(−$C\underline{H}_2$−CH_2−$C\underline{H}_2$−NHBoc)$_2$, 8H), 2.293−2.248 (t, CH_3−(CH_2)$_6$−CH_2−$C\underline{H}_2$− of R group, 2H), 1.760−1.611 (m, CH_3−(CH_2)$_6$−$C\underline{H}_2$−CH_2−CO−N-(−CH_2−$C\underline{H}_2$−CH_2−NHBoc)$_2$, 6H), 1.408 (s, R−CO−N(−CH_2−CH_2−CH_2−NH−COO−C($C\underline{H}_3$)$_3$)$_2$, 18H), 1.241 (m, CH_3−($C\underline{H}_2$)$_6$−CH_2−CH_2− of R group, 12H), 0.871−0.837 (t, $C\underline{H}_3$−(CH_2)$_6$−CH_2−CH_2− of R group, 3H).

*N,N-Bis(3-(Boc-amino)propyl)dodecanamide (**16b**).* Yield 80%. FT-IR (NaCl): 3288 cm^{-1} (−NH− str), 2928 cm^{-1} (−CH_2− asym str), 2859 cm^{-1} (−CH_2− sym str), 1699 cm^{-1} (C═O str). ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 5.379−4.629 (d, R−CO−N(−CH_2−CH_2−CH_2−$N\underline{H}$Boc)$_2$, 2H), 3.400−3.029 (m, R−CO−N(−$C\underline{H}_2$−CH_2−$C\underline{H}_2$−NHBoc)$_2$, 8H), 2.296−2.258 (t, CH_3−(CH_2)$_8$−CH_2−$C\underline{H}_2$− of R group, 2H), 1.764−1.594 (m, CH_3−(CH_2)$_8$−$C\underline{H}_2$−CH_2−CO−N(−CH_2−$C\underline{H}_2$−CH_2−NHBoc)$_2$, 6H), 1.422 (s, R−CO−N-(−CH_2−CH_2−CH_2−NH−COO−C($C\underline{H}_3$)$_3$)$_2$, 18H), 1.249 (m, CH_3−($C\underline{H}_2$)$_8$−CH_2−CH_2− of R group, 16H), 0.887−0.853 (t, $C\underline{H}_3$−(CH_2)$_8$−CH_2−CH_2− of R group, 3H).

*N,N-Bis(3-(Boc-amino)propyl)tetradecanamide (**16c**).* Yield 75%. FT-IR (NaCl): 3337 cm^{-1} (−NH− str), 2926 cm^{-1} (−CH_2− asym str), 2852 cm^{-1} (−CH_2− sym str), 1693 cm^{-1} (C═O str). ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 5.377−4.643 (d, R−CO−N(−CH_2−CH_2−CH_2−$N\underline{H}$Boc)$_2$, 2H), 3.400−3.030 (m, R−CO−N(−$C\underline{H}_2$−CH_2−$C\underline{H}_2$−NHBoc)$_2$, 8H), 2.299−2.261 (t, CH_3−(CH_2)$_{10}$−CH_2−$C\underline{H}_2$− of R group, 2H), 1.774−1.592 (m, CH_3−(CH_2)$_{10}$−$C\underline{H}_2$−CH_2−CO−N(−CH_2−$C\underline{H}_2$−CH_2−NHBoc)$_2$, 6H), 1.422 (s, R−CO−N-(−CH_2−CH_2−CH_2−NH−COO−C($C\underline{H}_3$)$_3$)$_2$, 18H), 1.246 (m, CH_3−($C\underline{H}_2$)$_{10}$−CH_2−CH_2− of R group, 20H), 0.887−0.853 (t, $C\underline{H}_3$−(CH_2)$_{10}$−CH_2−CH_2− of R group, 3H).

*N,N-Bis(3-(Boc-amino)propyl)hexadecanamide (**16d**).* Yield 76%. FT-IR (NaCl): 3337 cm^{-1} (−NH− str), 2922 cm^{-1} (−CH_2− asym str), 2854 cm^{-1} (−CH_2− sym str), 1699 cm^{-1} (C═O str). ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 5.377−4.643 (d, R−CO−N(−CH_2−CH_2−CH_2−$N\underline{H}$Boc)$_2$, 2H), 3.400−3.030 (m, R−CO−N(−$C\underline{H}_2$−CH_2−$C\underline{H}_2$−NH−Boc)$_2$, 8H), 2.299−2.261 (t, CH_3−(CH_2)$_{12}$−CH_2−$C\underline{H}_2$− of R group, 2H), 1.774−1.592 (m, CH_3−(CH_2)$_{12}$−$C\underline{H}_2$−CH_2−CO−N(−CH_2−$C\underline{H}_2$−CH_2−NH−Boc)$_2$, 6H), 1.422 (s, R−CO−N(−CH_2−CH_2−CH_2−NH−COO−C($C\underline{H}_3$)$_3$)$_2$, 18H), 1.246 (m, CH_3−($C\underline{H}_2$)$_{12}$−CH_2−CH_2− of R group, 24H), 0.887−0.853 (t, $C\underline{H}_3$−(CH_2)$_{12}$−CH_2−CH_2− of R group, 3H).

*N,N-Bis(3-(Boc-amino)propyl)octadecanamide (**16e**).* Yield 78%. FT-IR (NaCl): 3340 cm^{-1} (−NH− str), 2929 cm^{-1} (−CH_2− asym str), 2854 cm^{-1} (−CH_2− sym str), 1708 cm^{-1} (C═O str). ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 5.376−4.628 (d, R−CO−N(−CH_2−CH_2−CH_2−$N\underline{H}$Boc)$_2$, 2H), 3.400−3.029 (m, R−CO−N(−$C\underline{H}_2$−CH_2−$C\underline{H}_2$−NHBoc)$_2$, 8H), 2.299−2.260 (t, CH_3−(CH_2)$_{14}$−CH_2−$C\underline{H}_2$− of R group, 2H), 1.773−1.592 (m, CH_3−(CH_2)$_{14}$−$C\underline{H}_2$−CH_2−CO−N(−CH_2−$C\underline{H}_2$−CH_2−NHBoc)$_2$, 6H), 1.421 (s, R−CO−N-(−CH_2−CH_2−CH_2−NH−COO−C($C\underline{H}_3$)$_3$)$_2$, 18H), 1.243 (m, CH_3−($C\underline{H}_2$)$_{14}$−CH_2−CH_2− of R group, 28H), 0.889−0.852 (t, $C\underline{H}_3$−(CH_2)$_{14}$−CH_2−CH_2− of R group, 3H).

General Procedure for Synthesizing 17a and 17b. An amount of 5 g (2 equiv, 18.85 mmol) of *N*-Boc-L/D-phenylalanine was dissolved in dry DCM (30 mL) at 0 °C. To the reaction mixture DIPEA (9.8 mL, 6 equiv, 56.55 mmol) was added followed by 7.2 g of HBTU (2 equiv, 18.85 mmol). Then DMF (8 mL) was added to solubilize the reaction mixture. After 10 min, 1.2 g of norspermidine (1 equiv, 9.43 mmol) was added dropwise. The reaction mixture was allowed to stir for 48 h at 0 °C. At the end of 48 h, the solvent was evaporated under reduced pressure and residue was diluted with ethyl acetate (100 mL). It was then washed with 1 N HCl (100 mL, 3 times) followed by saturated Na_2CO_3 solution (100 mL, 3 times). The crude product was collected in ethyl acetate layer and was passed through anhydrous sodium sulfate. Finally purification was done through column chromatography on silica gel (60−120 mesh) using different ratios of methanol and chloroform as eluent to obtain the product with 65% yield.

*N^1-(Boc-^{L}Phe)-N^3-[3-(Boc-^{L}Phe)amidopropyl]propane-1,3-diamine (**17a**).* Yield 65%. FT-IR (NaCl): 3311 cm^{-1} (−NH− str), 3033 cm^{-1} (aromatic C−H str), 2929 cm^{-1} (−CH_2− asym str), 2854 cm^{-1} (−CH_2− sym str), 1700 and 1684 cm^{-1} (C═O str), 1654 cm^{-1}, 1635 cm^{-1} (aromatic C═C str). ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 7.300−7.204 (m, NH(−CH_2−CH_2−CH_2−NH−CO−CH(NHBoc)−CH_2−Ph$\underline{H}$)$_2$, 10H), 7.194 (s, NH(−CH_2−CH_2−CH_2−$N\underline{H}$−CO−CH(NHBoc)−CH_2−Ph)$_2$, 2H), 5.368 (s, NH(−CH_2−CH_2−CH_2−NH−CO−CH($N\underline{H}$Boc)−CH_2−Ph)$_2$, 2H), 4.314−4.298 (t, NH-(−CH_2−CH_2−CH_2−NH−CO−$C\underline{H}$(NHBoc)−CH_2−Ph)$_2$, 2H), 3.370−2.985 (m, NH(−$C\underline{H}_2$−CH_2−$C\underline{H}_2$−NH−CO−CH(NHBoc)−$C\underline{H}_2$−Ph)$_2$, 12H), 2.570 (s, $N\underline{H}$(−CH_2−CH_2−CH_2−NH−CO−CH-(NHBoc)−CH_2−Ph)$_2$, 1H), 1.687−1.676 (m, NH(−CH_2−$C\underline{H}_2$−CH_2−NH−CO−CH(NHBoc)−CH_2−Ph)$_2$, 4H), 1.386 (s, NH-(−CH_2−CH_2−CH_2−NH−CO−CH(NH−COO−C($C\underline{H}_3$)$_3$)−CH_2−Ph)$_2$, 18H). HRMS (m/z): 626.3891 [(M + H)$^+$] (observed), 626.3918 [(M + H)$^+$] (calculated).

*N^1-(Boc-^{D}Phe)-N^3-[3-(Boc-^{D}Phe)amidopropyl]propane-1,3-diamine (**17b**).* Yield 65%. FT-IR (NaCl): 3315 cm^{-1} (−NH− str), 3027 cm^{-1} (aromatic C−H str), 2932 cm^{-1} (−CH_2− asym str), 2856 cm^{-1} (−CH_2− sym str), 1704 and 1682 cm^{-1} (C═O str), 1652 cm^{-1}, 1637 cm^{-1} (aromatic C═C str). ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 7.303−7.201 (m, NH(−CH_2−CH_2−CH_2−NH−CO−CH(NHBoc)−CH_2−Ph$\underline{H}$)$_2$, 10H), 7.197 (s, NH(−CH_2−CH_2−CH_2−$N\underline{H}$−CO−CH(NHBoc)−CH_2−Ph)$_2$, 2H), 5.365 (s, NH(−CH_2−CH_2−CH_2−NH−CO−CH($N\underline{H}$Boc)−CH_2−Ph)$_2$, 2H), 4.319−4.294 (t, NH-(−CH_2−CH_2−CH_2−NH−CO−$C\underline{H}$(NHBoc)−CH_2−Ph)$_2$, 2H), 3.367−2.983 (m, NH(−$C\underline{H}_2$−CH_2−$C\underline{H}_2$−NH−CO−CH(NHBoc)−$C\underline{H}_2$−Ph)$_2$, 12H), 2.569 (s, $N\underline{H}$(−CH_2−CH_2−CH_2−NH−CO−CH-(NHBoc)−CH_2−Ph)$_2$, 1H), 1.684−1.673 (m, NH(−CH_2−$C\underline{H}_2$−

dx.doi.org/10.1021/jm5013566 | J. Med. Chem. XXXX, XXX, XXX−XXX

CH_2−NH−CO−CH(NHBoc)−CH_2−Ph)$_2$, 4H), 1.383 (s, NH-(−CH_2−CH_2−CH_2−NH−CO−CH(NH−COO−C(CH_3)$_3$)−CH_2−Ph)$_2$, 18H). HRMS (*m*/*z*): 626.3898 [(M + H)$^+$] (observed), 626.3918 [(M + H)$^+$] (calculated).

General Procedure for Synthesizing 18a−h. About 2.4 mmol (1.5 equiv) of saturated aliphatic acid (decanoic, dodecanoic, tetradecanoic, hexadecanoic, or octadecanoic), α-naphthylacetic acid, *N*-Boc-L-phenylalanine were dissolved in dry DCM (12 mL) at 0 °C. To the reaction mixture 4 equiv of DIPEA was then added followed by 1.5 equiv of HBTU. Then DMF (3 mL) was added to the reaction mixture. After 10 min, 1 equiv of **17a** or **17b** in dry DCM (2 mL) was added dropwise. The reaction mixture was brought to room temperature and allowed to stir for 24 h. At the end of 24 h, solvent was evaporated and residue was diluted in ethyl acetate (50 mL). The reaction mixture was washed at first with 1 N HCl (50 mL, 3 times) followed by saturated Na_2CO_3 solution (50 mL, 3 times). The crude product was extracted in ethyl acetate layer and dried over anhydrous sodium sulfate, and finally purification was accomplished through column chromatography on silica gel (60−120 mesh) using different percentage of methanol and chloroform as eluent to afford **18a−h** with 75−80% yield.

*N,N-Bis[3-(Boc-^{L}Phe)amidopropyl]decanamide (**18a**).* Yield 76%. FT-IR (NaCl): 3304 cm^{-1} (−NH− str), 3029 cm^{-1} (aromatic C−H str), 2927 cm^{-1} (−CH_2− asym str), 2855 cm^{-1} (−CH_2− sym str), 1699 and 1684 cm^{-1} (C═O str), 1652 cm^{-1}, 1635 cm^{-1} (aromatic C═C str). ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 7.246−7.097 (m, R−CO−N(−CH_2−CH_2−CH_2−*N*H−CO−CH(NHBoc)−CH_2−Ph*H*)$_2$, 12H), 5.350−5.229 (m, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH(*N*HBoc)−CH_2−Ph)$_2$, 2H), 4.355−4.315 (t, R−CO−N-(−CH_2−CH_2−CH_2−NH−CO−*C*H(NHBoc)−CH_2−Ph)$_2$, 2H), 3.384−2.871 (m, R−CO−N(−*C*H_2−CH_2−*C*H_2−NH−CO−CH-(NHBoc)−*C*H_2−Ph)$_2$, 12H), 2.215−2.195 (t, CH_3−(CH_2)$_6$−CH_2−*C*H_2− of R group, 2H), 1.597−1.463 (m, CH_3−(CH_2)$_6$−*C*H_2−CH_2−CO−N(−CH_2−*C*H_2−CH_2−NH−CO−CH(NHBoc)−CH_2−Ph)$_2$, 6H), 1.353 (s, R−CO−NH(−CH_2−CH_2−CH_2−NH−CO−CH-(NH−COO−C(*C*H_3)$_3$)−CH_2−Ph)$_2$, 18H), 1.255 (m, CH_3−(*C*H_2)$_6$−CH_2−CH_2− of R group, 12H), 0.889−0.855 (t, *C*H_3−(CH_2)$_6$−CH_2−CH_2− of R group, 3H). HRMS (*m*/*z*): 780.5268 [(M + H)$^+$] (observed), 780.5275 [(M + H)$^+$] (calculated).

*N,N-Bis[3-(Boc-^{L}Phe)amidopropyl]dodecanamide (**18b**).* Yield 78%. FT-IR (NaCl): 3300 cm^{-1} (−NH− str), 3030 cm^{-1} (aromatic C−H str), 2928 cm^{-1} (−CH_2− asym str), 2854 cm^{-1} (−CH_2− sym str), 1699 and 1683 cm^{-1} (C═O str), 1652 cm^{-1}, 1635 cm^{-1} (aromatic C═C str). ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 7.215−7.096 (m, R−CO−N(−CH_2−CH_2−CH_2−*N*H−CO−CH(NHBoc)−CH_2−Ph*H*)$_2$, 12H), 5.417−5.277 (m, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH(*N*HBoc)−CH_2−Ph)$_2$, 2H), 4.360−4.328 (t, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−*C*H(NHBoc)−CH_2−Ph)$_2$, 2H), 3.356−2.869 (m, R−CO−N(−*C*H_2−CH_2−*C*H_2−NH−CO−CH(NHBoc)−*C*H_2−Ph)$_2$, 12H), 2.209−2.189 (t, CH_3−(CH_2)$_8$−CH_2−*C*H_2− of R group, 2H), 1.714−1.463 (m, CH_3−(CH_2)$_8$−*C*H_2−CH_2−CO−N(−CH_2−*C*H_2−CH_2−NH−CO−CH(NHBoc)−CH_2−Ph)$_2$, 6H), 1.351 (s, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH(NH−COO−C(*C*H_3)$_3$)−CH_2−Ph)$_2$, 18H), 1.244 (m, CH_3−(*C*H_2)$_8$−CH_2−CH_2− of R group, 16H), 0.885−0.851 (t, *C*H_3−(CH_2)$_8$−CH_2−CH_2− of R group, 3H). HRMS (*m*/*z*): 808.5593 [(M + H)$^+$] (observed), 808.5588 [(M + H)$^+$] (calculated).

*N,N-Bis[3-(Boc-^{L}Phe)amidopropyl]tetradecanamide (**18c**).* Yield 75%. FT-IR (NaCl): 3301 cm^{-1} (−NH− str), 3028 cm^{-1} (aromatic C−H str), 2926 cm^{-1} (−CH_2− asym str), 2855 cm^{-1} (−CH_2− sym str), 1699 and 1684 cm^{-1} (C═O str), 1654 cm^{-1}, 1637 cm^{-1} (aromatic C═C str). ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 7.215−7.100 (m, R−CO−N(−CH_2−CH_2−CH_2−*N*H−CO−CH(NHBoc)−CH_2−Ph*H*)$_2$, 12H), 5.495−5.345 (m, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH(*N*HBoc)−CH_2−Ph)$_2$, 2H), 4.352−4.333 (t, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−*C*H(NHBoc)−CH_2−Ph)$_2$, 2H), 3.451−2.861 (m, R−CO−N(−*C*H_2−CH_2−*C*H_2−NH−CO−CH(NHBoc)−*C*H_2−Ph)$_2$, 12H), 2.202−2.182 (t, CH_3−(CH_2)$_{10}$−CH_2−*C*H_2− of R group, 2H), 1.644−1.547 (m, CH_3−(CH_2)$_{10}$−*C*H_2−CH_2−CO−N(−CH_2−*C*H_2−CH_2−NH−CO−CH(NHBoc)−CH_2−Ph)$_2$, 6H), 1.345 (s, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH-(NH−COO−C(*C*H_3)$_3$)−CH_2−Ph)$_2$, 18H), 1.235 (m, CH_3−(*C*H_2)$_{10}$−CH_2−CH_2− of R group, 20H), 0.879−0.845 (t, *C*H_3−(CH_2)$_{10}$−CH_2−CH_2− of R group, 3H). HRMS (*m*/*z*): 836.5908 [(M + H)$^+$] (observed), 836.5901 [(M + H)$^+$] (calculated).

*N,N-Bis[3-(Boc-^{L}Phe)amidopropyl]hexadecanamide (**18d**).* Yield 80%. FT-IR (NaCl): 3299 cm^{-1} (−NH− str), 3031 cm^{-1} (aromatic C−H str), 2927 cm^{-1} (−CH_2− asym str), 2854 cm^{-1} (−CH_2− sym str), 1699 and 1684 cm^{-1} (C═O str), 1656 cm^{-1}, 1635 cm^{-1} (aromatic C═C str). ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 7.211−7.097 (m, R−CO−N(−CH_2−CH_2−CH_2−*N*H−CO−CH(NHBoc)−CH_2−Ph*H*)$_2$, 12H), 5.515−5.357 (m, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH(*N*HBoc)−CH_2−Ph)$_2$, 2H), 4.356−4.337 (t, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−*C*H(NHBoc)−CH_2−Ph)$_2$, 2H), 3.425−2.861 (m, R−CO−N(−*C*H_2−CH_2−*C*H_2−NH−CO−CH(NHBoc)−*C*H_2−Ph)$_2$, 12H), 2.200−2.181 (t, CH_3−(CH_2)$_{12}$−CH_2−*C*H_2− of R group, 2H), 1.690−1.435 (m, CH_3−(CH_2)$_{12}$−*C*H_2−CH_2−CO−N(−CH_2−*C*H_2−CH_2−NH−CO−CH(NHBoc)−CH_2−Ph)$_2$, 6H), 1.343 (s, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH-(NH−COO−C(*C*H_3)$_3$)−CH_2−Ph)$_2$, 18H), 1.233 (m, CH_3−(*C*H_2)$_{12}$−CH_2−CH_2− of R group, 24H), 0.877−0.843 (t, *C*H_3−(CH_2)$_{12}$−CH_2−CH_2− of R group, 3H). HRMS (*m*/*z*): 864.6227 [(M + H)$^+$] (observed), 864.6214 [(M + H)$^+$] (calculated).

*N,N-Bis[3-(Boc-^{L}Phe)amidopropyl]octadecanamide (**18e**).* Yield 77%. FT-IR (NaCl): 3302 cm^{-1} (−NH− str), 3028 cm^{-1} (aromatic C−H str), 2927 cm^{-1} (−CH_2− asym str), 2855 cm^{-1} (−CH_2− sym str), 1699 and 1684 cm^{-1} (C═O str), 1652 cm^{-1}, 1635 cm^{-1} (aromatic C═C str). ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 7.245−7.100 (m, R−CO−N(−CH_2−CH_2−CH_2−*N*H−CO−CH(NHBoc)−CH_2−Ph*H*)$_2$, 12H), 5.338−5.216 (m, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH(*N*HBoc)−CH_2−Ph)$_2$, 2H), 4.338−4.319 (t, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−*C*H(NHBoc)−CH_2−Ph)$_2$, 2H), 3.484−2.802 (m, R−CO−N(−*C*H_2−CH_2−*C*H_2−NH−CO−CH(NHBoc)−*C*H_2−Ph)$_2$, 12H), 2.214−2.194 (t, CH_3−(CH_2)$_{14}$−CH_2−*C*H_2− of R group, 2H), 1.631−1.3578 (m, CH_3−(CH_2)$_{14}$−*C*H_2−CH_2−CO−N(−CH_2−*C*H_2−CH_2−NH−CO−CH(NHBoc)−CH_2−Ph)$_2$, 6H), 1.354 (s, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH(NH−COO−C(*C*H_3)$_3$)−CH_2−Ph)$_2$, 18H), 1.250 (m, CH_3−(*C*H_2)$_{14}$−CH_2−CH_2−, 28H), 0.894−0.860 (t, *C*H_3−(CH_2)$_{14}$−CH_2−CH_2− of R group, 3H). HRMS (*m*/*z*): 892.6535 [(M + H)$^+$] (observed), 892.6527 [(M + H)$^+$] (calculated).

*N,N-Bis[3-(Boc-^{D}Phe)amidopropyl]dodecanamide (**18f**).* Yield 75%. FT-IR (NaCl): 3300 cm^{-1} (−NH− str), 3030 cm^{-1} (aromatic C−H str), 2928 cm^{-1} (−CH_2− asym str), 2854 cm^{-1} (−CH_2− sym str), 1699 and 1683 cm^{-1} (C═O str), 1652 cm^{-1}, 1635 cm^{-1} (aromatic C═C str). ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 7.260−7.094 (m, R−CO−N(−CH_2−CH_2−CH_2−*N*H−CO−CH(NHBoc)−CH_2−Ph*H*)$_2$, 12H), 5.555−5.442 (m, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH(*N*HBoc)−CH_2−Ph)$_2$, 2H), 4.353−4.334 (t, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−*C*H(NHBoc)−CH_2−Ph)$_2$, 2H), 3.452−2.892 (m, R−CO−N(−*C*H_2−CH_2−*C*H_2−NH−CO−CH(NHBoc)−*C*H_2−Ph)$_2$, 12H), 2.205−2.185 (t, CH_3−(CH_2)$_8$−CH_2−*C*H_2− of R group, 2H), 1.647−1.434 (m, CH_3−(CH_2)$_8$−*C*H_2−CH_2−CO−N(−CH_2−*C*H_2−CH_2−NH−CO−CH(NHBoc)−CH_2−Ph)$_2$, 6H), 1.343 (s, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH(NH−COO−C(*C*H_3)$_3$)−CH_2−Ph)$_2$, 18H), 1.243 (m, CH_3−(*C*H_2)$_8$−CH_2−CH_2− of R group, 16H), 0.878−0.844 (t, *C*H_3−(CH_2)$_8$−CH_2−CH_2− of R group, 3H). HRMS (*m*/*z*): 808.5593 [(M + H)$^+$] (observed), 808.5588 [(M + H)$^+$] (calculated).

*N,N-Bis[3-(Boc-^{L}Phe)amidopropyl]α-naphthylacetamide (**18g**).* Yield 75%. FT-IR (NaCl): 3301 cm^{-1} (−NH− str), 3033 cm^{-1} (aromatic C−H str), 2933 cm^{-1} (−CH_2− asym str), 2856 cm^{-1} (−CH_2− sym str), 1700 and 1684 cm^{-1} (C═O str), 1654 cm^{-1}, 1635 cm^{-1} (aromatic C═C str). ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 7.775−7.082 (m, Naph*H*−CH_2−CO−N(−CH_2−CH_2−CH_2−*N*H−CO−CH(NHBoc)−CH_2−Ph*H*)$_2$, 19H), 5.365−5.217 (m, Naph−CH_2−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH(*N*HBoc)−CH_2−Ph)$_2$, 2H), 4.334−4.298 (t, Naph−CH_2−CO−N(−CH_2−CH_2−CH_2−NH−CO−*C*H(NHBoc)−CH_2−Ph)$_2$, 2H), 4.105−4.070 (m, Naph−*C*H_2−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH(NHBoc)−CH_2−

dx.doi.org/10.1021/jm5013566 | J. Med. Chem. XXXX, XXX, XXX−XXX

Ph)$_2$, 2H), 3.217−2.832 (m, Naph−CH$_2$−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH−CO−CH(NHBoc)−CH$_2$−Ph)$_2$, 12H), 1.512−1.446 (m, Naph−CH$_2$−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH−CO−CH(NHBoc)−CH$_2$−Ph)$_2$, 4H), 1.346 (s, Naph−CH$_2$−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH−CO−CH(NH−COO−C(CH$_3$)$_3$)−CH$_2$−Ph)$_2$, 18H). HRMS (*m*/*z*): 794.4512 [(M + H)$^+$] (observed), 794.4493 [(M + H)$^+$] (calculated).

*N^1,N^3-Bis(Boc-^{L}Phe)-N^1-[3-(Boc-^{L}Phe)amidopropyl]propane-1,3-diamine (**18h**).* Yield 78%. FT-IR (NaCl): 3311 cm^{-1} (−NH− str), 3029 cm^{-1} (aromatic C−H str), 2929 cm^{-1} (−CH$_2$− asym str), 2854 cm^{-1} (−CH$_2$− sym str), 1699 and 1686 cm^{-1} (C═O str), 1655 cm^{-1}, 1637 cm^{-1} (aromatic C═C str). ^{1}H NMR (400 MHz, CDCl$_3$) δ/ppm: 7.236−7.093 (m, PhH−CH$_2$−CH(NHBoc)−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH−CO−CH(NHBoc)−CH$_2$−PhH)$_2$, 17H), 5.346−5.217 (m, Ph−CH$_2$−CH(NHBoc)−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH−CO−CH(NHBoc)−CH$_2$−Ph)$_2$, 3H), 4.347−4.307 (t, Ph−CH$_2$−CH(NHBoc)−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH−CO−CH(NHBoc)−CH$_2$−Ph)$_2$, 3H), 3.387−2.869 (m, Ph−CH$_2$−CH(NHBoc)−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH−CO−CH(NHBoc)−CH$_2$−Ph)$_2$, 14H), 1.537−1.455 (m, Ph−CH$_2$−CH(NHBoc)−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH−CO−CH(NHBoc)−CH$_2$−Ph)$_2$, 4H), 1.343 (s, Ph−CH$_2$−CH(NH−COO−C(CH$_3$)$_3$)−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH−CO−CH(NH−COO−C(CH$_3$)$_3$)−CH$_2$−Ph)$_2$, 27H). HRMS (*m*/*z*): 873.5239 [(M + H)$^+$] (observed), 873.5126 [(M + H)$^+$] (calculated).

General Procedure for Synthesizing 1−14. About 7.5 mmol (1 equiv) of **16a−e**, 1.6 mmol of **17a** (1 equiv), and 1.2 mmol of **18a−h** (1 equiv) were dissolved in DCM (3 mL). To the intensely stirred solution 4 equiv (excess amount) of trifluoroacetic acid (TFA) was added, and the mixture was stirred at room temperature for 12 h. Then solvent and unused TFA were removed to afford pure compounds **1−14** with 100% yield.

*N,N-Bis(3-aminopropyl)decanamide Bis(trifluoroacetate) (**1**).* FT-IR (NaCl): 3367 cm^{-1}(−NH− str), 2929 cm^{-1} (−CH$_2$− asym str), 2866 cm^{-1} (−CH$_2$− sym str), 1671 cm^{-1} (C═O str). ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 7.971−7.828 (d, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH$_3^+$)$_2$, 6H), 3.327−3.268 (m, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH$_3^+$)$_2$, 4H), 2.848−2.709 (m, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH$_3^+$)$_2$, 4H), 2.297−2.260 (t, CH$_3$−(CH$_2$)$_6$−CH$_2$−CH$_2$− of R group, 2H), 1.804−1.711 (m, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH$_3^+$)$_2$, 4H), 1.484 (m, CH$_3$−(CH$_2$)$_6$−CH$_2$−CH$_2$− of R group, 2H), 1.242 (bs, CH$_3$−(CH$_2$)$_6$−CH$_2$−CH$_2$− of R group, 12H), 0.868−0.835 (t, CH$_3$−(CH$_2$)$_6$−CH$_2$−CH$_2$− of R group, 3H). HRMS (*m*/*z*): 286.2873 [(M + H)$^+$] (observed), 286.2858 [(M + H)$^+$] (calculated).

*N,N-Bis(3-aminopropyl)dodecanamide Bis(trifluoroacetate) (**2**).* FT-IR (NaCl): 3375 cm^{-1} (−NH− str), 2928 cm^{-1} (−CH$_2$− asym str), 2861 cm^{-1} (−CH$_2$− sym str), 1681 cm^{-1} (C═O str). ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 7.931−7.797 (d, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH$_3^+$)$_2$, 6H), 3.326−3.268 (m, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH$_3^+$)$_2$, 4H), 2.832−2.688 (m, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH$_3^+$)$_2$, 4H), 2.299−2.262 (t, CH$_3$−(CH$_2$)$_8$−CH$_2$−CH$_2$− of R group, 2H), 1.821−1.693 (m, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH$_3^+$)$_2$, 4H), 1.502−1.470 (m, CH$_3$−(CH$_2$)$_8$−CH$_2$−CH$_2$−of R group, 2H), 1.242 (m, CH$_3$−(CH$_2$)$_8$−CH$_2$−CH$_2$− of R group, 16H), 0.871−0.837 (t, CH$_3$−(CH$_2$)$_8$−CH$_2$−CH$_2$− of R group, 3H). HRMS (*m*/*z*): 314.3172 [(M + H)$^+$] (observed), 314.3171 [(M + H)$^+$] (calculated).

*N,N-Bis(3-aminopropyl)tetradecanamide Bis(trifluoroacetate) (**3**).* FT-IR (NaCl): 3394 cm^{-1} (−NH− str), 2922 cm^{-1} (−CH$_2$− asym str), 2869 cm^{-1} (−CH$_2$− sym str), 1680 cm^{-1} (C═O str). ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 7.966−7.825 (d, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH$_3^+$)$_2$, 6H), 3.328−3.269 (m, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH$_3^+$)$_2$, 4H), 2.851−2.710 (m, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH$_3^+$)$_2$, 4H), 2.297−2.260 (t, CH$_3$−(CH$_2$)$_{10}$−CH$_2$−CH$_2$− of R group, 2H), 1.787−1.713 (m, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH$_3^+$)$_2$, 4H), 1.483 (m, CH$_3$−(CH$_2$)$_{10}$−CH$_2$−CH$_2$− of R group, 2H), 1.236 (m, CH$_3$−(CH$_2$)$_{10}$−CH$_2$−CH$_2$− of R group, 20H), 0.867−0.833 (t, CH$_3$−(CH$_2$)$_{10}$−CH$_2$−CH$_2$− of R group, 3H). HRMS (*m*/*z*): 342.3482 [(M + H)$^+$] (observed), 342.3484 [(M + H)$^+$] (calculated).

*N,N-Bis(3-aminopropyl)hexadecanamide Bis(trifluoroacetate) (**4**).* FT-IR (NaCl): 3403 cm^{-1} (−NH− str), 2924 cm^{-1} (−CH$_2$− asym str), 2855 cm^{-1} (−CH$_2$− sym str), 1680 cm^{-1} (C═O str). ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 7.890−7.765 (d, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH$_3^+$)$_2$, 6H), 3.328−3.272 (m, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH$_3^+$)$_2$, 4H), 2.851−2.713 (m, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH$_3^+$)$_2$, 4H), 2.304−2.267 (t, CH$_3$−(CH$_2$)$_{12}$−CH$_2$−CH$_2$− of R group, 2H), 1.801−1.695 (m, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH$_3^+$)$_2$, 4H), 1.491 (m, CH$_3$−(CH$_2$)$_{12}$−CH$_2$−CH$_2$− of R group, 2H), 1.241 (m, CH$_3$−(CH$_2$)$_{12}$−CH$_2$−CH$_2$− of R group, 24H), 0.875−0.840 (t, CH$_3$−(CH$_2$)$_{12}$−CH$_2$−CH$_2$− of R group, 3H). HRMS (*m*/*z*): 370.3787 [(M + H)$^+$] (observed), 370.3797 [(M + H)$^+$] (calculated).

*N,N-Bis(3-aminopropyl)octadecanamide Bis(trifluoroacetate) (**5**).* FT-IR (NaCl): 3382 cm^{-1} (−NH− str), 2928 cm^{-1} (−CH$_2$− asym str), 2855 cm^{-1} (−CH$_2$− sym str), 1679 cm^{-1} (C═O str). ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 7.885−7.760 (d, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH$_3^+$)$_2$, 6H), 3.324−3.268 (m, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH$_3^+$)$_2$, 4H), 2.847−2.669 (m, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH$_3^+$)$_2$, 4H), 2.299−2.262 (t, CH$_3$−(CH$_2$)$_{14}$−CH$_2$−CH$_2$− of R group, 2H), 1.796−1.707 (m, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH$_3^+$)$_2$, 4H), 1.487 (m, CH$_3$−(CH$_2$)$_{14}$−CH$_2$−CH$_2$− of R group, 2H), 1.237 (m, CH$_3$−(CH$_2$)$_{14}$−CH$_2$−CH$_2$− of R group, 28H), 0.870−0.836 (t, CH$_3$−(CH$_2$)$_{14}$−CH$_2$−CH$_2$− of R group, 3H). HRMS (*m*/*z*): 398.4111 [(M + H) $^+$] (observed), 398.4110 [(M + H) $^+$] (calculated).

*N,N-Bis[3-(^{L}Phe)amidopropyl]decanamide Bis(trifluoroacetate) (**6**).* FT-IR (NaCl): 3270 cm^{-1} (−NH− str), 3034 cm^{-1} (aromatic C−H str), 2930 cm^{-1} (−CH$_2$− asym str), 2859 cm^{-1} (−CH$_2$− sym str), 1673 cm^{-1} (C═O str), 1618 cm^{-1}, 1577 cm^{-1} (aromatic C═C str). ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.473−8.231 (m, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH−CO−CH(NH$_3^+$)−CH0$_2$-Ph)$_2$, 8H), 7.339−7.219 (m, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH−CO−CH(NH$_3^+$)−CH$_2$−PhH)$_2$, 10H), 3.939 (t, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH−CO−CH(NH$_3^+$)−CH$_2$−Ph)$_2$, 2H), 3.171−2.925 (m, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH−CO−CH(NH$_3^+$)−CH$_2$−Ph)$_2$, 12H), 2.200−2.166 (t, CH$_3$−(CH$_2$)$_6$−CH$_2$−CH$_2$− of R group, 2H), 1.527−1.446 (m, CH$_3$−(CH$_2$)$_6$−CH$_2$−CH$_2$−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH−CO−CH(NH$_3^+$)−CH$_2$−Ph)$_2$, 6H), 1.221 (m, CH$_3$−(CH$_2$)$_6$−CH$_2$−CH$_2$− of R group, 12H), 0.864−0.830 (t, CH$_3$−(CH$_2$)$_6$−CH$_2$−CH$_2$− of R group, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ/ppm: 171.81, 167.81, 167.59, 158.54, 158.22, 135.04, 129.44, 128.47, 127.09, 53.64, 45.00, 42.79, 37.15, 37.09, 36.57, 36.29, 32.07, 31.30, 29.04, 28.96, 28.80, 28.69, 28.36, 27.28, 25.09, 22.09, 13.94. HRMS (*m*/*z*): 580.4225 [(M + H)$^+$] (observed), 580.4227 [(M + H)$^+$] (calculated).

*N,N-Bis[3-(^{L}Phe)amidopropyl]dodecanamide Bis(trifluoroacetate) (**7**).* FT-IR (NaCl): 3275 cm^{-1} (−NH− str), 3034 cm^{-1} (aromatic C−H str), 2927 cm^{-1} (−CH$_2$− asym str), 2855 cm^{-1} (−CH$_2$− sym str), 1680 cm^{-1} (C═O str), 1620 cm^{-1}, 1576 cm^{-1} (aromatic C═C str). ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.479−8.235 (m, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH−CO−CH(NH$_3^+$)−CH$_2$−Ph)$_2$, 8H), 7.341−7.218 (m, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH−CO−CH(NH$_3^+$)−CH$_2$−PhH)$_2$, 10H), 3.940 (t, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH−CO−CH(NH$_3^+$)−CH$_2$−Ph)$_2$, 2H), 3.171−2.924 (m, R−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH−CO−CH(NH$_3^+$)−CH$_2$−Ph)$_2$, 12H), 2.199−2.165 (t, CH$_3$−(CH$_2$)$_8$−CH$_2$−CH$_2$− of R group, 2H), 1.526−1.444 (m, CH$_3$−(CH$_2$)$_8$−CH$_2$−CH$_2$−CO−N(−CH$_2$−CH$_2$−CH$_2$−NH−CO−CH(NH$_3^+$)−CH$_2$−Ph)$_2$, 6H), 1.221 (m, CH$_3$−(CH$_2$)$_8$−CH$_2$−CH$_2$− of R group, 16H), 0.865−0.831 (t, CH$_3$−(CH$_2$)$_8$−CH$_2$−CH$_2$− of R group, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ/ppm: 171.80, 167.79, 167.58, 158.38, 158.07, 135.01, 129.42, 128.47, 127.09, 53.63, 44.98, 42.76, 37.14, 37.09, 36.56, 36.28, 32.06, 31.29, 29.03, 29.01, 28.80, 28.70, 28.35, 27.28, 25.08, 22.09, 13.94. HRMS (*m*/*z*): 608.4510 [(M + H)$^+$] (observed), 608.4540 [(M + H)$^+$] (calculated).

*N,N-Bis[3-(^{L}Phe)amidopropyl]tetradecanamide Bis(trifluoroacetate) (**8**).* FT-IR (NaCl): 3275 cm^{-1} (−NH− str), 3033 cm^{-1} (aromatic C−H str), 2927 cm^{-1} (−CH$_2$− asym str), 2856 cm^{-1} (−CH$_2$− sym str), 1679 cm^{-1} (C═O str), 1620 cm^{-1}, 1576 cm^{-1}

dx.doi.org/10.1021/jm5013566 | J. Med. Chem. XXXX, XXX, XXX−XXX

(aromatic C=C str). ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.506−8.257 (m, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−$CH(NH_3^+)$−CH_2−Ph)$_2$, 8H), 7.333−7.218 (m, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−$CH(NH_3^+)$−CH_2−PhH)$_2$, 10H), 3.944 (t, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−$CH(NH_3^+)$−CH_2−Ph)$_2$, 2H), 3.184−2.905 (m, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH-(NH_3^+)−CH_2−Ph)$_2$, 12H), 2.197−2.163 (t, CH_3−$(CH_2)_{10}$−CH_2−CH_2− of R group, 2H), 1.525−1.443 (m, CH_3−$(CH_2)_{10}$−CH_2−CH_2−CO−N(−CH_2−CH_2−CH_2−NH−CO−$CH(NH_3^+)$−CH_2−Ph)$_2$, 6H), 1.222 (m, CH_3−$(CH_2)_{10}$−CH_2−CH_2−, of R group, 20H), 0.865−0.831 (t, CH_3−$(CH_2)_{10}$−CH_2−CH_2− of R group, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ/ppm: 171.80, 167.80, 167.59, 135.03, 129.43, 128.46, 127.07, 53.63, 45.00, 42.79, 37.14, 37.08, 36.56, 36.29, 32.07, 31.30, 29.05, 29.02, 28.81, 28.71, 28.35, 27.27, 25.09, 22.10, 13.93. HRMS (m/z): 636.4843 [(M + H)$^+$] (observed), 636.4853 [(M + H)$^+$] (calculated).

N,N-Bis[3-(^{L}*Phe)amidopropyl]hexadecanamide Bis-(trifluoroacetate) (**9**).* FT-IR (NaCl): 3277 cm^{-1} (−NH− str), 3034 cm^{-1} (aromatic C−H str), 2928 cm^{-1} (−CH_2− asym str), 2857 cm^{-1} (−CH_2− sym str), 1676 cm^{-1} (C=O str), 1618 cm^{-1}, 1577 cm^{-1} (aromatic C=C str). ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.500−8.254 (m, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−$CH(NH_3^+)$−CH_2−Ph)$_2$, 8H), 7.334−7.218 (m, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH(NH_3^+)−CH_2−PhH)$_2$, 10H), 3.944 (t, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−$CH(NH_3^+)$−CH_2−Ph)$_2$, 2H), 3.202−2.891 (m, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH-(NH_3^+)−CH_2−Ph)$_2$, 12H), 2.197−2.163 (t, CH_3−$(CH_2)_{12}$−CH_2−CH_2− of R group, 3H), 1.525−1.444 (m, CH_3−$(CH_2)_{12}$−CH_2−CH_2−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH(NH_3^+)−CH_2−Ph)$_2$, 6H), 1.226 (m, CH_3−$(CH_2)_{12}$−CH_2−CH_2− of R group, 24H), 0.866−0.832 (t, CH_3−$(CH_2)_{12}$−CH_2−CH_2− of R group, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ/ppm: 171.78, 167.76, 167.55, 158.44, 134.99, 129.39, 128.43, 127.06, 53.61, 44.97, 37.12, 36.55, 36.28, 35.75, 32.05, 31.26, 30.75, 29.01, 28.97, 28.78, 28.66, 28.34, 27.26, 25.05, 22.05, 13.89. HRMS (m/z): 664.5156 [(M + H)$^+$] (observed), 664.5166 [(M + H)$^+$] (calculated).

N,N-Bis[3-(^{L}*Phe)amidopropyl]octadecanamide Bis-(trifluoroacetate) (**10**).* FT-IR (NaCl): 3275 cm^{-1} (−NH− str), 3034 cm^{-1} (aromatic C−H str), 2926 cm^{-1} (−CH_2− asym str), 2854 cm^{-1} (−CH_2− sym str), 1679 cm^{-1} (C=O str), 1619 cm^{-1}, 1576 cm^{-1} (aromatic C=C str). ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.500−8.253 (m, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−$CH(NH_3^+)$−CH_2−Ph)$_2$, 8H), 7.334−7.217 (m, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH(NH_3^+)−CH_2−PhH)$_2$, 10H), 3.943 (t, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−$CH(NH_3^+)$−CH_2−Ph)$_2$, 2H), 3.201−2.891 (m, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH-(NH_3^+)−CH_2−Ph)$_2$, 12H), 2.196−2.163 (t, CH_3−$(CH_2)_{14}$−CH_2−CH_2- of R group, 2H), 1.524−1.443 (m, CH_3−$(CH_2)_{14}$−CH_2−CH_2−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH(NH_3^+)−CH_2−Ph)$_2$, 6H), 1.226 (s, CH_3−$(CH_2)_{14}$−CH_2−CH_2− of R group, 28H), 0.866−0.832 (t, CH_3−$(CH_2)_{14}$−CH_2−CH_2− of R group, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ/ppm: 171.80, 167.81, 167.59, 158.57, 158.25, 135.05, 129.44, 128.45, 127.07, 53.64, 45.01, 42.80, 37.15, 37.09, 36.57, 36.29, 32.08, 31.30, 29.06, 29.02, 28.82, 28.72, 28.35, 27.27, 25.09, 22.10, 13.93. HRMS (m/z): 692.5433 [(M + H)$^+$] (observed), 692.5479 [(M + H)$^+$] (calculated).

N,N-Bis[3-(^{D}*Phe)amidopropyl]dodecanamide Bis-(trifluoroacetate) (**11**).* FT-IR (NaCl): 3275 cm^{-1} (−NH− str), 3034 cm^{-1} (aromatic C−H str), 2927 cm^{-1} (−CH_2− asym str), 2855 cm^{-1} (−CH_2− sym str), 1680 cm^{-1} (C=O str), 1620 cm^{-1}, 1576 cm^{-1} (aromatic C=C str). ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.458−8.240 (m, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−$CH(NH_3^+)$−CH_2−Ph)$_2$, 8H), 7.346−7.223 (m, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH(NH_3^+)−CH_2−PhH)$_2$, 10H), 3.945 (t, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−$CH(NH_3^+)$−CH_2−Ph)$_2$, 2H), 3.208−2.878 (m, R−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH-(NH_3^+)−CH_2−Ph)$_2$, 12H), 2.204−2.169 (t, CH_3−$(CH_2)_8$−CH_2−CH_2− of R group, 2H), 1.531−1.449 (m, CH_3−$(CH_2)_8$−CH_2−CH_2−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH(NH_3^+)−CH_2−Ph)$_2$, 6H), 1.226 (m, CH_3−$(CH_2)_8$−CH_2−CH_2− of R group, 16H), 0.870−0.836 (t, CH_3−$(CH_2)_8$−CH_2−CH_2− of R group, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ/ppm: 171.78, 167.78, 167.56, 158.33, 158.03, 135.00, 129.41, 128.45, 127.08, 53.62, 44.97, 42.76, 37.13, 37.07, 36.55, 36.27, 32.05, 31.28, 29.01, 29.00, 28.78, 28.68, 28.34, 27.26, 25.06, 22.07, 13.92. HRMS (m/z): 608.4511 [(M + H)$^+$] (observed), 608.4540 [(M + H)$^+$] (calculated).

N,N-Bis[3-(^{L}*Phe)amidopropyl]α-naphthylacetamide Bis-(trifluoroacetate) (**12**).* FT-IR (NaCl): 3273 cm^{-1} (−NH− str), 3036 cm^{-1} (aromatic C−H str), 2924 cm^{-1} (−CH_2− asym str), 2857 cm^{-1} (−CH_2− sym str), 1678 cm^{-1} (C=O str), 1618 cm^{-1}, 1580 cm^{-1} (aromatic C=C str). ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.535−8.316 (m, Naph−CH_2−CO−N(−CH_2−CH_2−CH_2−NH−CO−$CH(NH_3^+)$−CH_2−Ph)$_2$, 8H), 7.931−7.81 and 7.524−7.206 (m, Naph$H$−CH_2−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH-(NH_3^+)−CH_2−PhH)$_2$, 17H), 4.061−4.018 (t, Naph−CH_2−CO−N(−CH_2−CH_2−CH_2−NH−CO−$CH(NH_3^+)$−CH_2−Ph)$_2$, 2H), 3.963−3.947 (m, Naph−CH_2−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH(NH_3^+)−CH_2−Ph)$_2$, 2H), 3.271−2.954 (m, Naph−CH_2−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH(NH_3^+)−CH_2−Ph)$_2$, 12H), 1.648−1.487 (m, Naph−CH_2−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH(NH_3^+)−CH_2−Ph)$_2$, 4H). ^{13}C NMR (100 MHz, DMSO-d_6) δ/ppm: 170.10, 167.88, 167.66, 158.75, 158.44, 135.09, 133.40, 132.93, 132.17, 129.48, 128.50, 127.12, 125.98, 125.66, 125.49, 124.16, 118.62, 115.65, 53.68, 45.60, 43.25, 36.69, 36.45, 28.26, 27.35. HRMS (m/z): 594.3410 [(M + H)$^+$] (observed), 594.3444 [(M + H)$^+$] (calculated).

N^1*,N*^{3}*-Bis(*^{L}*Phe)-N*^{1}*-[3-(*^{L}*Phe)amidopropyl]propane-1,3-diamine Tris(trifluoroacetate) (**13**).* FT-IR (NaCl): 3275 cm^{-1} (−NH− str), 3036 cm^{-1} (aromatic C−H str), 2923 cm^{-1} (−CH_2− asym str), 2856 cm^{-1} (−CH_2− sym str), 1680 cm^{-1} (C=O str), 1617 cm^{-1}, 1579 cm^{-1} (aromatic C=C str). ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.444−8.300 (m, Ph−CH_2−$CH(NH_3^+)$−CO−N(−CH_2−CH_2−CH_2−NH−CO−$CH(NH_3^+)$−CH_2−Ph)$_2$, 11H), 7.323−7.201 (m, PhH−CH_2−$CH(NH_3^+)$−CO−N(−CH_2−CH_2−CH_2−NH−CO−$CH(NH_3^+)$−CH_2−PhH)$_2$, 15H), 4.428−4.394 (t, Ph−CH_2−$CH(NH_3^+)$−CO−N(−CH_2−CH_2−CH_2−NH−CO−$CH(NH_3^+)$−CH_2−Ph)$_2$, 1H), 3.988−3.936 (t, Ph−CH_2−$CH(NH_3^+)$−CO−N(−CH_2−CH_2−CH_2−NH−CO−$CH(NH_3^+)$−CH_2−Ph)$_2$, 2H), 3.072−2.645 (m, Ph−CH_2−$CH(NH_3^+)$−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH(NH_3^+)−CH_2−Ph)$_2$, 14H), 1.438−1.290 (m, Ph−CH_2−$CH(NH_3^+)$−CO−N(−CH_2−CH_2−CH_2−NH−CO−CH-(NH_3^+)−CH_2−Ph)$_2$, 4H). ^{13}C NMR (100 MHz, DMSO-d_6) δ/ppm: 167.86, 167.83, 167.74, 158.50, 158.21, 135.04, 134.98, 134.36, 129.66, 129.47, 129.44, 128.61, 128.55, 128.52, 127.44, 127.18, 53.63, 53.59, 50.15, 44.77, 43.61, 37.36, 37.16, 36.67, 36.23, 27.80, 26.76. HRMS (m/z): 573.3569 [(M + H)$^+$] (observed), 573.3553 [(M + H)$^+$] (calculated).

N^1*-(*^{L}*Phe)-N*^{3}*-[3-(*^{L}*Phe)amidopropyl]propane-1,3-diamine Tris-(trifluoroacetate) (**14**).* FT-IR (NaCl): 3273 cm^{-1} (−NH− str), 3036 cm^{-1} (aromatic C−H str), 2924 cm^{-1} (−CH_2− asym str), 2857 cm^{-1} (−CH_2− sym str), 1678 cm^{-1} (C=O str), 1617 cm^{-1}, 1579 cm^{-1} (aromatic C=C str). ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 8.502−8.242 (m, NH_2^+(−CH_2−CH_2−CH_2−NH−CO−$CH(NH_3^+)$−CH_2−Ph)$_2$, 10H), 7.360−7.214 (m, NH_2^+(−CH_2−CH_2−CH_2−NH−CO−$CH(NH_3^+)$−CH_2−PhH)$_2$, 10H), 3.927−3.912 (t, NH_2^+(−CH_2−CH_2−CH_2−NH−CO−$CH(NH_3^+)$−CH_2−Ph)$_2$, 2H), 3.171−2.719 (m, NH_2^+(−CH_2−CH_2−CH_2−NH−CO−CH(NH_3^+)−CH_2−Ph)$_2$, 12H), 1.644−1.614 (m, NH_2^+(−CH_2−CH_2−CH_2−NH−CO−CH(NH_3^+)−CH_2−Ph)$_2$, 4H). ^{13}C NMR (100 MHz, DMSO-d_6) δ/ppm: 171.07, 161.54, 161.21, 135.98, 130.43, 129.94, 128.58, 119.21, 116.27, 113.34, 55.59, 45.85, 37.12, 26.38, 23.85. HRMS (m/z): 426.2842 [(M + H)$^+$] (observed), 426.2869 [(M + H)$^+$] (calculated).

Antibacterial Assay. The antibacterial activity of the compounds is reported as their minimum inhibitory concentrations (MICs), which is the lowest concentration of the antibacterial agent required to inhibit the growth of microorganism after overnight incubation. A glycopeptide antibiotic, vancomycin, and a lipopeptide, colistin, were also used in this study to compare the result. All synthesized compounds (**1**−**14**) were assayed in a microdilution broth format as described in

dx.doi.org/10.1021/jm5013566 | J. Med. Chem. XXXX, XXX, XXX−XXX

CLSI guideline.[47] The bacterial freeze-dried stock samples were stored at −80 °C. An amount of 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 with prior to the experiments. This 6 h grown culture gives about 10^9 CFU/mL in the case of *S. aureus*, MRSA, and 10^8 CFU/mL in the cases of *E. coli*, *E. faecium*, VRE, and *K. pneumoniae*, which were determined by the 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 MIC. Compounds were serially diluted in sterile Millipore water (as 2-fold manner), and an amount of 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 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.

Determination of Critical Micellar Concentration (cmc).[33] The cmc values of the phenylalanine conjugated aliphatic norspermidine derivatives were determined by static light scattering (SLS) measurements on a PerkinElmer LS-55 luminescence spectrometer. Briefly, the compounds **6**−**11** were dissolved in yeast−dextrose broth (condition for MIC experiment) at room temperature at a concentration of 500 μg/mL. These solutions (2 mL) were then used to measure the scattering intensity upon successive dilutions. Intensities of the scattered light were measured at an angle of 90°, fixing both the excitation and the emission at 400 nm with slit width of 2.5 nm. The intensities of scattered signal were then plotted against the concentration. The cmc was determined from the inflection point which is defined as the abscissa where the intensity rises steeply and decreases after reaching a local maximum.

Time−Kill Kinetics Assay.[8,30,44] The bactericidal activity of the compounds was evaluated by performing time kill kinetics assay. This gives the information about the rate at which the compounds are acting on bacteria. Briefly, *S. aureus* was grown in yeast−dextrose broth at 37 °C for 6 h. Compounds **7** and **11** were added to the bacterial solution (*S. aureus* of approximately 1.8×10^5 CFU/mL) with the working concentration of 25 μM (about 6 × MIC). This was incubated at 37 °C. At different time intervals (0, 1, 2, 3, 4, 6, 12, and 24 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 for 24 h. The bacterial colonies were counted, and results are represented in logarithmic scale, i.e., log (CFU/mL). The second experiment has been performed using a similar protocol at a shorter time gap of 0, 5, 15, 30, 45, and 60 min to find out the exact time required to show bactericidal activity.

Hemolytic Assay.[30,33,44] Compounds were serially diluted in Millipore water, and an amount of 50 μL of these serial dilutions was added to the wells of 96-well plates. Human erythrocytes were centrifuged down from the fresh heparinised blood and suspended to 5 vol % in PBS (pH 7.4). In the compound containing 96-well plates, 150 μL of the erythrocyte suspension was added. 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 transferred to another 96-well plate to measure the absorbance at 540 nm (A_{540}). To determine the percentage of hemolysis, the formula $[(A - A_0)/(A_{total} - A_0)] \times 100$ was used, where A is the absorbance of the compound containing well, A_0 the absorbance of the negative controls (without compound), and A_{total} 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 sigmoidal plot. From the curve the values were determined corresponding to 50% hemolysis.

Cytotoxicity Assay.[8] Cytotoxicity of the compound **11** was assessed against RAW macrophage cell line. Briefly, the cells were grown in a 96-well plate in DMEM media (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 treated with 10 vol % Triton-X 100 solution. The plate was incubated for 1 h at 37 °C under 5% CO_2 atmosphere. After 1 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. 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 were plotted against concentration followed by fitting with 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.

Antibacterial Assay in the Presence of Human Plasma.[30] To examine the susceptibility of the new derivatives toward plasma proteases, the antibacterial activities were tested in the presence of 50% of plasma. Briefly, an amount of 250 μL of compounds **7** and **11** was added to 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. Now antibacterial activities were tested against *S. aureus* by following the same protocol as described above for antibacterial assay.

Antibacterial Assay in the Presence of Human Serum. Activity of compound **11** was tested in the presence of 50% of serum in order to gauge the availability of the compound in physiological conditions. In short, compound **11** (250 μL) was added to 250 μL of human serum and incubated at 37 °C. At specified time intervals of 0, 2, and 4 h this solution was serially diluted 2-fold in 0.9% saline. Then antibacterial activities of this solution were tested (in triplicate) against *S. aureus* by following the same protocol as described above for antibacterial assay.

Resistance Studies.[7] For resistance study the control antibiotics norfloxacin and colistin were chosen for *S. aureus* and for *E. coli*, respectively. At first, the MIC value of compound **11** was determined against *S. aureus* and *E. coli* as described above in antibacterial assay. In the cases of norfloxacin and colistin the initial MIC values were also determined against respective bacteria. For the next day MIC experiment, the bacterial dilution was made by using the bacteria from sub-MIC concentration of the compounds (at MIC/2). After a 24 h incubation period, again bacterial dilution was prepared by using the bacterial suspension from sub-MIC concentration of the compound (at MIC/2) and assayed for the next MIC experiment. The process was repeated for 20 and 30 passages in the cases of *S. aureus* and *E. coli*, respectively. The fold of MIC increased for test compound, and control antibiotics were plotted against the number of days.

Inner Membrane Permeabilization Assay.[8,30,44] The 6 h grown culture (mid log phase) of *S. aureus* and *E. coli* were harvested (3500 rpm, 5 min), washed, and resuspended in 5 mM glucose and 5 mM HEPES buffer (pH 7.2) in 1:1 ratio. Then an amount of 10 μL of test compounds **7** and **11** (12 × MIC) was added to a cuvette containing 2 mL of bacterial suspension and 10 μM propidium iodide (PI). Fluorescence was monitored at excitation wavelength of 535 nm (slit width of 10 nm) and emission wavelength of 617 nm (slit width of 5 nm). As a measure of inner membrane permeabilization, the uptake of PI was monitored by the increase in fluorescence for 10 min.

Cytoplasmic Membrane Depolarization Assay.[8,30,44] 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 1:1 ratio, and resuspended in 5 mM HEPES buffer, 5 mM glucose, and

dx.doi.org/10.1021/jm5013566 | J. Med. Chem. XXXX, XXX, XXX−XXX

100 mM KCl solution in 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 preincubated for 20 min. The fluorescence was monitored at excitation wavelength of 622 nm (slit width of 10 nm) and emission wavelength of 670 nm (slit width of 5 nm). Then an amount of 10 μL of test compounds **7** and **11** (12 × MIC) was added to the cuvette containing bacterial suspension and $DiSC_3$ after 2 min of fluorescence measurement. As a measure of membrane depolarization, fluorescence was monitored for another 13 min.

K^+ Leakage Assay.[8] The 6 h grown cultures (mid log phase) of *S. aureus* and *E. coli* and were harvested (3500 rpm, 5 min), washed, and resuspended in 10 mM HEPES buffer and 0.5% glucose in 1:1 ratio. Then 2 mL of the bacterial suspension was placed in a cuvette. Then fluorescence was measured at excitation wavelength of 346 nm (slit width of 10 nm) and emission wavelength of 505 nm (slit width of 5 nm) for 50 s at room temperature. Then PBFI-AM dye (1 μM) was added, and fluorescence was monitored for another 150 s. Finally an amount of 10 μL of test compounds **7** and **11** (12 × MIC) was added to the cuvette containing bacterial suspension and PBFI-AM dye and fluorescence was monitored for another 800 s. As a measure of K^+ leakage, the increase in fluorescence signals was measured.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

HPLC data, NMR data, mass spectra of the final compounds, and other experimental figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

Jawaharlal Nehru Centre for Advanced Scientific Research has filed a patent application based on the work described in the manuscript.

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Prof. C. N. R. Rao (Jawaharlal Nehru Centre for Advanced Scientific Research) for his constant support and encouragement. J.H. acknowledges Department of Science and Technology (DST), Government of India, for Ramanujan Fellowship [Grant SR/S2/RJN-43/2009].

■ ABBREVIATIONS USED

HBTU, *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)-uronium hexafluorophosphate; $(Boc)_2O$, di-*tert*-butyl dicarbonate; 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 unit; $DiSC_3(5)$, 3,3′-dipropylthiadicarbocyanine iodide; PI, propidium iodide; PBFI-AM, potassium binding benzofuran isophthalate

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