

Supporting information

L-lysine based lipidated biphenyls as agents with anti-biofilm and anti-inflammatory properties that also inhibit intracellular bacteria

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I. Materials and Instrumentation.

All the solvents were of reagent grade and were distilled and dried prior to use wherever required. Chloroform and methanol were supplied by Merck-India. Dimethylformamide, Dichloromethane, Diethyl ether and other solvents were supplied either by SDFCL (India) or Spectrochem (India). L-Lysine, Di-tert-butyl carbonate, Diisopropylethylamine, HBTU, Butylamine, Hexylamine, Octylamine, Trifluoroacetic acid were purchased from Spectrochem (India). 4,4'-Bis(chloromethyl)-1,1'-biphenyl, Decylamine and Dodecylamine were purchased from Sigma-Aldrich. All the chemicals were used as supplied. Analytical thin layer chromatography (TLC) was performed on E. Merck TLC plates pre-coated with silica gel 60 F254 (250 μm thickness). Visualization was accomplished using UV light and Iodine. Column chromatography was performed on silica gel (60-120 \AA pore size). HPLC analysis was performed on a Shimadzu-LC 8A Liquid Chromatograph instrument (C18 column, 10 mm diameter, 250 mm length) with UV detector monitoring at 254 nm. Nuclear magnetic resonance spectra were recorded on Bruker (AV-400) 400 MHz spectrometer in deuterated solvents. Infrared (IR) spectra of the solid compounds were recorded on Bruker IFS66 V/s spectrometer using KBr pellets. IR spectra of the compounds soluble in low-boiling solvents were recorded with the same instrument using NaCl crystal. High-resolution Mass Spectrometry was recorded on Agilent 6538 Q-TOF LC-MS system and Shimadzu LC-MS 2020 spectrometer. Optical density was measured by TecanInfinitePro series M200 Microplate Reader. Bacterial strains, *Staphylococcus aureus* (MTCC 737) was obtained from MTCC (Chandigarh, India). Methicillin resistant *S. aureus* (ATCC 33591), was obtained from ATCC. MRSA 3545. MRSA 3889 and MRSA 3890 were clinical isolates obtained from NIMHANS, Bangalore.

Animals: 6-8-week old Balb/c male mice weighing 20 to 25 g were used for all studies. The animal experiments were approved by the Institutional Animal Ethics Committee (IAEC) and carried out as per the guidelines of Committee for the purpose of Supervision and Experiments on Animals (CPCSEA), Ministry of Environment and Forests, New Delhi.

II. Synthetic procedure and characterization of compounds

General procedure for synthesizing secondary amines of biphenyls (1a to 5a) :

In a typical reaction, 4,4'-Bis(chloromethyl)-1,1'-biphenyl (4 mmols) and alkylamines (8.8 mmols) were dissolved in 20 mL DMF. To that K_2CO_3 (8.8 mmols) was added and stirred for 48 h at $60^\circ C$ in a pressure tube. Completion of the reaction was confirmed by TLC analysis. After completion of the reaction, DMF was removed under reduced pressure and then the mixture was diluted by addition of ethyl acetate (30 mL). The organic layer was subsequently washed four times water, brine and dried over anhydrous Na_2SO_4 . The organic layer was evaporated under reduced pressure to obtain the products in 65-80% yield and characterized by 1H NMR and HRMS. The details of characterization are provided below.

(1a): Yield-78%. 1H NMR ($CDCl_3$, 400 MHz) δ/ppm : 1H NMR ($CDCl_3$) δ/ppm : 7.56-7.54 (t, ArH , 4H), 7.45-7.38 (m, ArH , 4H), 3.82 and 3.61(s, $Ar(-CH_2-NH-R)_2$, 4H), 2.65 (m, $Ar(-CH_2-NH-CH_2-C_3H_7)_2$, 4H), 2.5 (m, $Ar(-CH_2-NH-R)_2$, 2H), 1.67-1.1 ((-NH₂-CH₂-(CH₂)₂-CH₃)₂, 8H), 0.82 (m, terminal CH₃ groups, 6H). HR-MS (m/z): [M+H]⁺ obsd. = 325.2631 (calc. = 325.2644).

(2a): Yield-80%. 1H NMR ($CDCl_3$, 400 MHz) δ/ppm : 1H NMR ($CDCl_3$) δ/ppm : 7.56-7.54 (t, ArH , 4H), 7.45-7.38 (m, ArH , 4H), 3.82 and 3.61(s, $Ar(-CH_2-NH-R)_2$, 4H), 2.65 (m, $Ar(-CH_2-NH-CH_2-C_5H_{11})_2$, 4H), 2.5 (m, $Ar(-CH_2-NH-R)_2$, 2H), 1.67-1.1 ((-NH₂-CH₂-(CH₂)₄-CH₃)₂, 16H), 0.82 (m, terminal CH₃ groups, 6H). HR-MS (m/z): [M+H]⁺ obsd. = 381.3264 (calc. = 381.3270).

(3a): Yield-78%. 1H NMR ($CDCl_3$, 400 MHz) δ/ppm : 1H NMR ($CDCl_3$) δ/ppm : 7.56-7.54 (t, ArH , 4H), 7.45-7.38 (m, ArH , 4H), 3.82 and 3.61(s, $Ar(-CH_2-NH-R)_2$, 4H), 2.65 (m, $Ar(-CH_2-NH-CH_2-C_7H_{15})_2$, 4H), 2.5 (t, $Ar(-CH_2-NH-R)_2$, 2H), 1.67-1.1 ((-NH₂-CH₂-(CH₂)₆-CH₃)₂, 24H), 0.82 (m, terminal CH₃ groups, 6H). HR-MS (m/z): [M+H]⁺ obsd. = 437.3883 (calc. = 437.3896).

(4a) Yield-65%. $^1\text{H NMR}$ (CDCl_3 , 400 MHz) δ/ppm : $^1\text{H NMR}$ (CDCl_3) δ/ppm : 7.56-7.54 (t, ArH , 4H), 7.45-7.38 (m, ArH , 4H), 3.82 and 3.61(s, $\text{Ar}(-\text{CH}_2-\text{NH}-\text{R})_2$, 4H), 2.65 (m, $\text{Ar}(-\text{CH}_2-\text{NH}-\text{CH}_2-\text{C}_9\text{H}_{19})_2$, 4H), 2.5 (t, $\text{Ar}(-\text{CH}_2-\text{NH}-\text{R})_2$, 2H), 1.67-1.1 (($-\text{NH}_2-\text{CH}_2-(\text{CH}_2)_8-\text{CH}_3$)₂, 32H), 0.82 (m, terminal CH_3 groups, 6H). HR-MS (m/z): 493.4511 $[\text{M}+\text{H}]^+$ obsd. = (calc. = 493.4522)

(5a): Yield-65%. $^1\text{H NMR}$ (CDCl_3 , 400 MHz) δ/ppm : $^1\text{H NMR}$ (CDCl_3) δ/ppm : 7.56-7.54 (t, ArH , 4H), 7.45-7.38 (m, ArH , 4H), 3.82 and 3.61(s, $\text{Ar}(-\text{CH}_2-\text{NH}-\text{R})_2$, 4H), 2.65 (m, $\text{Ar}(-\text{CH}_2-\text{NH}-\text{CH}_2-\text{C}_{11}\text{H}_{25})_2$, 4H), 2.5 (t, $\text{Ar}(-\text{CH}_2-\text{NH}-\text{R})_2$, 2H), 1.67-1.1 (($-\text{NH}_2-\text{CH}_2-(\text{CH}_2)_{10}-\text{CH}_3$)₂, 40H), 0.82 (m, terminal CH_3 groups, 6H). HR-MS (m/z): $[\text{M}+\text{H}]^+$ obsd. = 549.5115 (calc. = 549.5148).

General procedure for amide coupling (1b to 5b):

The protocol for amide coupling was performed using a previously published protocol with some modifications. In a typical amide coupling reaction, to a stirred solution of Boc-Lys(Boc)-OH (4.4 mmols) in 20 mL DMF and 10 mL of CHCl_3 , *N,N*-Diisopropylethylamine (DIPEA, 5.49 mmols) was added at 0°C . To the solution HBTU (4.4 mmols) was added to the mixture and stirred for five minutes before the addition of secondary amines of biphenyl (1a-5a, 1.83 mmols). The mixture was stirred at 0°C for 30 min and subsequently at RT for 24 h typically. At the end, CHCl_3 was evaporated under reduced pressure and the resulting solution was diluted to 2 times its original volume by addition of ethyl acetate. This mixture was subsequently washed with 0.5 M KHSO_4 , H_2O (thrice), and brine. After passage through anhydrous Na_2SO_4 , the organic layer was evaporated under reduced pressure and the residue was purified using column chromatography (5% MeOH/ CHCl_3 mixture) to obtain the product in 50%-68% yield. The purified compound was subsequently characterized using $^1\text{H NMR}$ and mass spectrometry.

(1b): Yield-60 %. $^1\text{H NMR}$ (CDCl_3 , 400 MHz) δ/ppm : 7.56-7.50 (t, ArH , 4H), 7.27-7.24 (m, ArH , 4H), 5.5-5.3 (m, NH of Boc-Lys(Boc), 4H), 4.9-4.3 ($\text{Ar}(-\text{CH}_2-\text{NH}-\text{R})_2$ and $\alpha\text{-H}$ of Boc-Lys(Boc), 6H) 3.4-3.0 ($\epsilon\text{-H}$ of Boc-Lys(Boc) and $\text{Ar}(-\text{CH}_2-\text{N}(\text{Boc}_2\text{Lys})-\text{CH}_2-\text{R})_2$, 8H), 1.9-1.1 (-

CO-[CH- $\underline{CH_2}$ - $\underline{CH_2}$ - $\underline{CH_2}$ -CH₂-NH-COO-C($\underline{CH_3}$)₃]-NH-COO-C($\underline{CH_3}$)₃ of Lys(boc)₂ and[-($\underline{CH_2}$)₂-] ₂ of R groups, 56H) and 0.88 (m, terminal CH₃ groups, 6H). FT-IR (cm⁻¹): 3330 (carbamate N-H str.), 2984-2863 (sp³ C-H str.), 1710 (C=O str. of carbamate), 1680 (C=O str. of tertiary amide). HR-MS (m/z): [M+H]⁺ obsd. = 981.6589 (calc. =981.6640).

(2b): Yield-61 %. ¹H NMR (CDCl₃, 400 MHz) δ/ppm: 7.56-7.50 (t, ArH, 4H), 7.27-7.24 (m, ArH, 4H), 5.5-5.3 (m, NH of Boc-Lys(Boc), 4H), 4.9-4.3 (Ar(- $\underline{CH_2}$ -NH-R)₂ and α-H of Boc-Lys(Boc), 6H) 3.4-3.0 (ε-H of Boc-Lys(Boc) and Ar-(CH₂-N(Boc₂Lys) $\underline{CH_2}$ -R)₂, 8H), 1.9-1.1 (-CO-[CH- $\underline{CH_2}$ - $\underline{CH_2}$ - $\underline{CH_2}$ -CH₂-NH-COO-C($\underline{CH_3}$)₃]-NH-COO-C($\underline{CH_3}$)₃ of Lys(boc)₂ and[-($\underline{CH_2}$)₄-] ₂ of R groups, 64 H) and 0.88 (m, terminal CH₃ groups, 6H). FT-IR (cm⁻¹): 3330 (carbamate N-H str.), 2984-2863 (sp³ C-H str.), 1710 (C=O str. of carbamate), 1680 (C=O str. of tertiary amide). HR-MS (m/z): [M+H]⁺ obsd. = 1059.7058 (calc. = 1059.7086).

(3b): Yield-61 %. ¹H NMR (CDCl₃, 400 MHz) δ/ppm: 7.56-7.50 (t, ArH, 4H), 7.27-7.24 (m, ArH, 4H), 5.5-5.3 (m, NH of Boc-Lys(Boc), 4H), 4.9-4.3 (Ar(- $\underline{CH_2}$ -NH-R)₂ and α-H of Boc-Lys(Boc), 6H) 3.4-3.0 (ε-H of Boc-Lys(Boc) and Ar-(CH₂-N(Boc₂Lys) $\underline{CH_2}$ -R)₂, 8H), 1.9-1.1 (-CO-[CH- $\underline{CH_2}$ - $\underline{CH_2}$ - $\underline{CH_2}$ -CH₂-NH-COO-C($\underline{CH_3}$)₃]-NH-COO-C($\underline{CH_3}$)₃ of Lys(boc)₂ and[-($\underline{CH_2}$)₆-] ₂ of R groups, 72H) and 0.88 (m, terminal CH₃ groups, 6H). FT-IR (cm⁻¹): 3330 (carbamate N-H str.), 2984-2863 (sp³ C-H str.), 1710 (C=O str. of carbamate), 1680 (C=O str. of tertiary amide). HR-MS (m/z): [M+H]⁺ obsd. = 1093.7881 (calc. = 1093.7892).

(4b): Yield-50 %.¹H NMR (CDCl₃, 400 MHz) δ/ppm: 7.56-7.50 (t, ArH, 4H), 7.27-7.24 (m, ArH, 4H), 5.5-5.3 (m, NH of Boc-Lys(Boc), 4H), 4.9-4.3 (Ar(- $\underline{CH_2}$ -NH-R)₂ and α-H of Boc-Lys(Boc), 6H) 3.4-3.0 (ε-H of Boc-Lys (Boc) and Ar-(CH₂-N(Boc₂Lys) $\underline{CH_2}$ -R)₂, 8H), 1.9-1.1 (-CO-[CH- $\underline{CH_2}$ - $\underline{CH_2}$ - $\underline{CH_2}$ -CH₂-NH-COO-C($\underline{CH_3}$)₃]-NH-COO-C($\underline{CH_3}$)₃ of Lys(boc)₂ and[-($\underline{CH_2}$)₈-] ₂ of R groups, 80H) and 0.88 (m, terminal CH₃ groups, 6H). FT-IR (cm⁻¹): 3330 (carbamate N-H str.), 2984-2863 (sp³ C-H str.), 1710 (C=O str. of carbamate), 1680 (C=O str. of tertiary amide). HR-MS (m/z): [M+H]⁺ obsd. = 1149.8481 (calc. = 1149.8518).

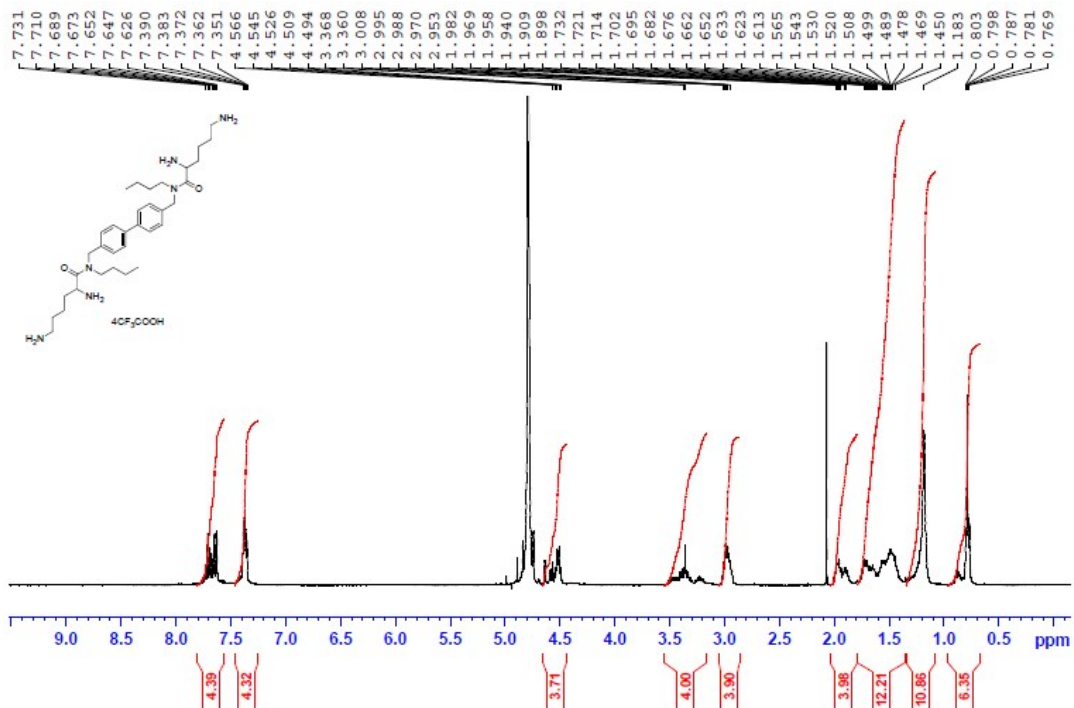
(5b): Yield-68%.¹H NMR (CDCl₃, 400 MHz) δ/ppm: ¹H NMR (CDCl₃) δ/ppm: 7.56-7.50 (t, ArH, 4H), 7.27-7.24 (m, ArH, 4H), 5.5-5.3 (m, NH of Boc-Lys(Boc), 4H), 4.9-4.3 (Ar(- $\underline{CH_2}$ -

NH-R)₂ and α -H of Boc-Lys(Boc), 6H) 3.4-3.0 (ϵ -H of Boc-Lys (Boc) and Ar-(CH₂-N(Boc₂Lys)CH₂-R)₂, 8H), 1.9-1.1 (-CO-[CH-CH₂-CH₂-CH₂-CH₂-NH-COO-C(CH₃)₃]-NH-COO-C(CH₃)₃ of Lys(boc)₂ and-[-(CH₂)₁₀-]₂ of R groups, 88H) and 0.88 (m, terminal CH₃ groups, 6H). FT-IR (cm⁻¹): 3330 (carbamate N-H str.), 2984-2863 (sp³ C-H str.), 1710 (C=O str. of carbamate), 1680 (C=O str. of tertiary amide). HR-MS (m/z): [M+Na]⁺ obsd. = 1227.8914 (calc. = 1227.8964).

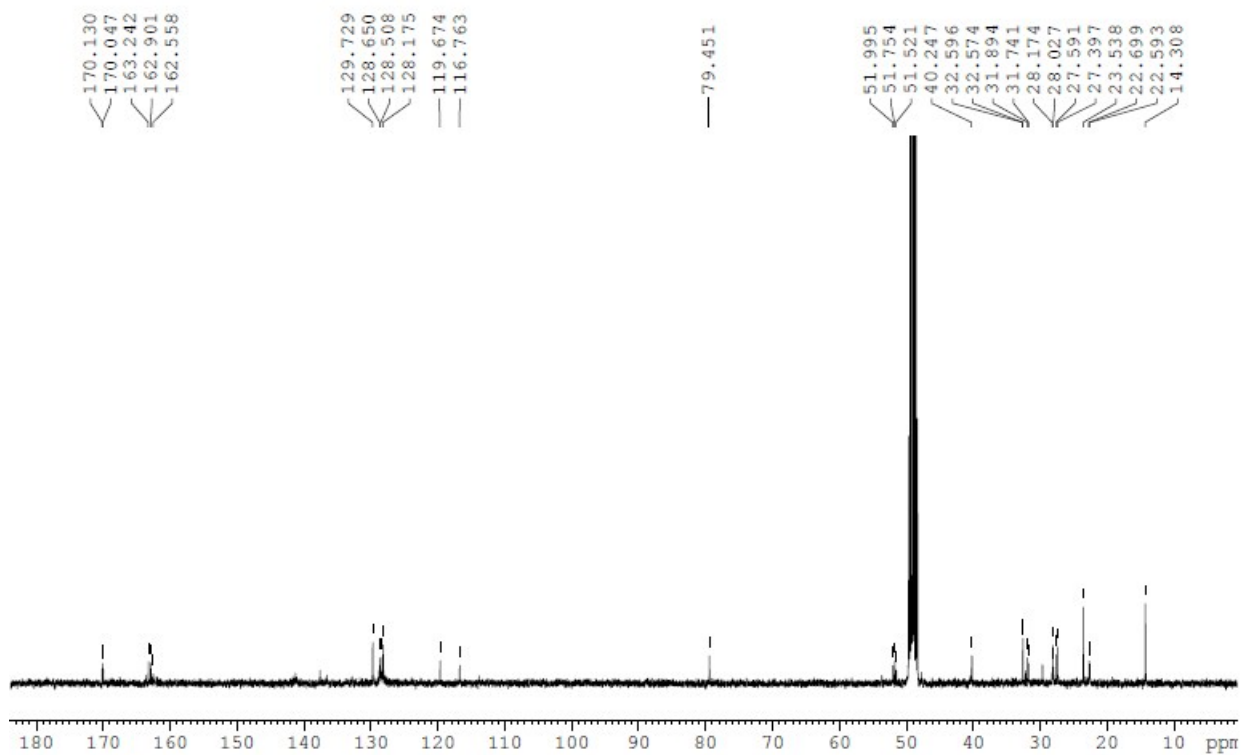
General procedure for deprotection of Boc groups (1 to 5):

Typically, the compounds (1b–5b, 0.76 mmol) were dissolved in DCM (2 mL) and subsequently CF₃COOH (2 mL) was added and stirred at RT. The reactions were monitored by TLC until complete removal of starting material was observed. All the volatile components were removed and the product was purified by reverse phase HPLC using 0.1% trifluoroacetic acid (TFA) in water/acetonitrile (0–100%) as mobile phase to more than 95% purity. A C18 column (10 mm diameter, 250 mm length) and a UV detector (at 256 nm wavelength) were used. After drying the compounds in a freeze dryer, the compounds were characterized by ¹H NMR, ¹³C NMR, IR and mass spectrometry. The ¹³C NMR have not been assigned and the spectra are furnished directly.

(1): ¹H NMR (D₂O, 400 MHz) δ /ppm: 7.7-7.2 (t, ArH, 8H), 4.6-4.3 (Ar(-CH₂-NH-R)₂ and α -H of Lys, 4H) 3.6-3.3 (Ar-(CH₂-N(Lys)CH₂-R)₂, 2H), 3-2.8 (ϵ of CH₂ of Lys) 2.1-1 (-CO-[CH-CH₂-CH₂-CH₂-CH₂-NH₂ of Lys and-[-(CH₂)₂-]₂ of R groups, 20H) and 0.88 (m, terminal CH₃ groups, 6H). 4Hs have merged with the residual solvent peak of CD₃OD. FT-IR (cm⁻¹): 2963-2853 (sp³ C-H str.), 1679 (C=O str. of tertiary amide). HR-MS (m/z): [M+H]⁺ obsd. = 581.4513 (calc. = 581.4543).

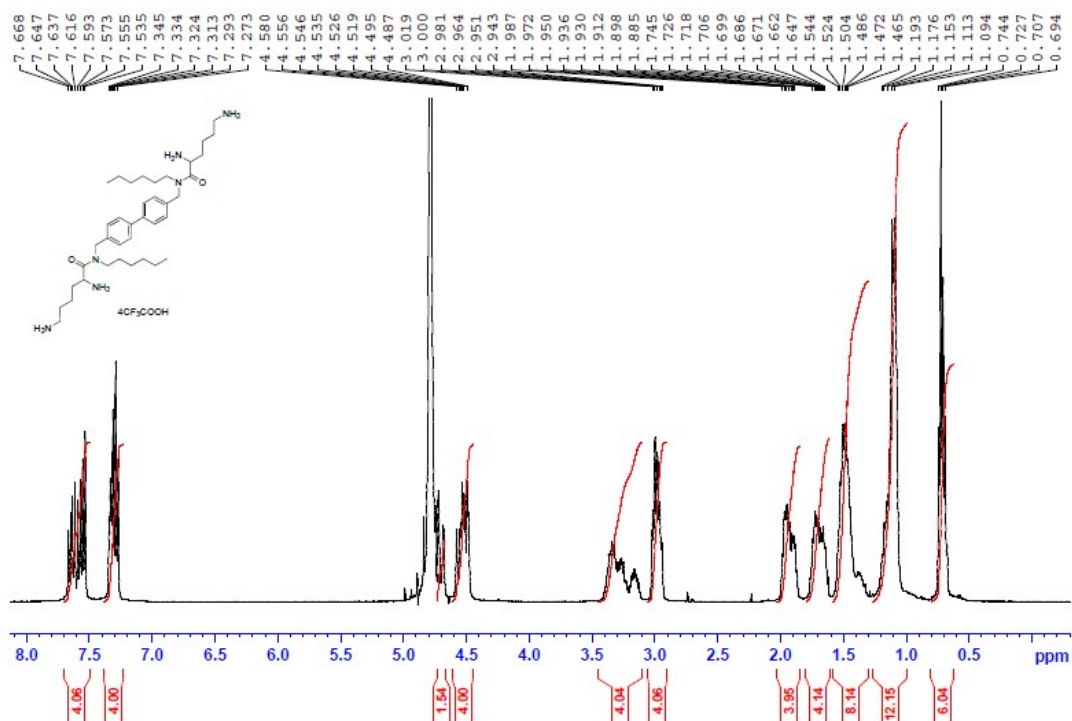


¹H NMR spectra of 1. The NMR was taken in D₂O and the solvent peak was calibrated at δ value of 4.79 ppm.

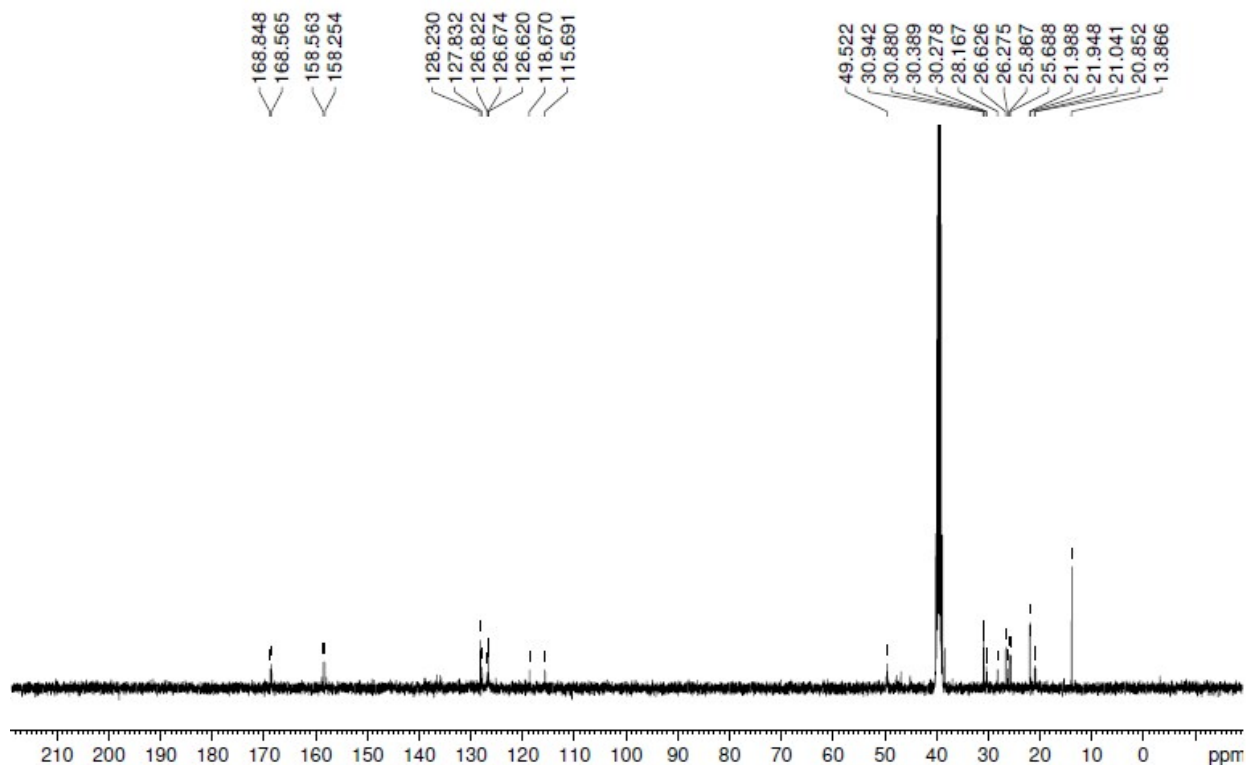


¹³C NMR spectra of 1. The NMR was taken in CD₃OD and the solvent peak was calibrated at δ value of 49 ppm.

(2): ¹H NMR (CD₃OD, 400 MHz) δ/ppm: 7.7-7.2 (t, ArH, 8H), 4.6-4.3 (Ar(-CH₂-NH-R)₂ and α-H of Lys, 4H) 3.6-3.3 (Ar-(CH₂-N(Lys)CH₂-R)₂, 2H), 3-2.8 (ε of CH₂ of Lys) 2.1-1 (-CO-[CH-CH₂-CH₂-CH₂-CH₂-NH₂ of Lys and -(CH₂)₄]₂ of R groups, 28H) and 0.88 (m, terminal CH₃ groups, 6H). 4Hs have merged with the residual solvent peak of CD₃OD. FT-IR (cm⁻¹): 2963-2853 (sp³ C-H str.), 1679 (C=O str. of tertiary amide). HR-MS (m/z): [M+H]⁺ obsd. = 637.5145 (calc. = 637.5169).

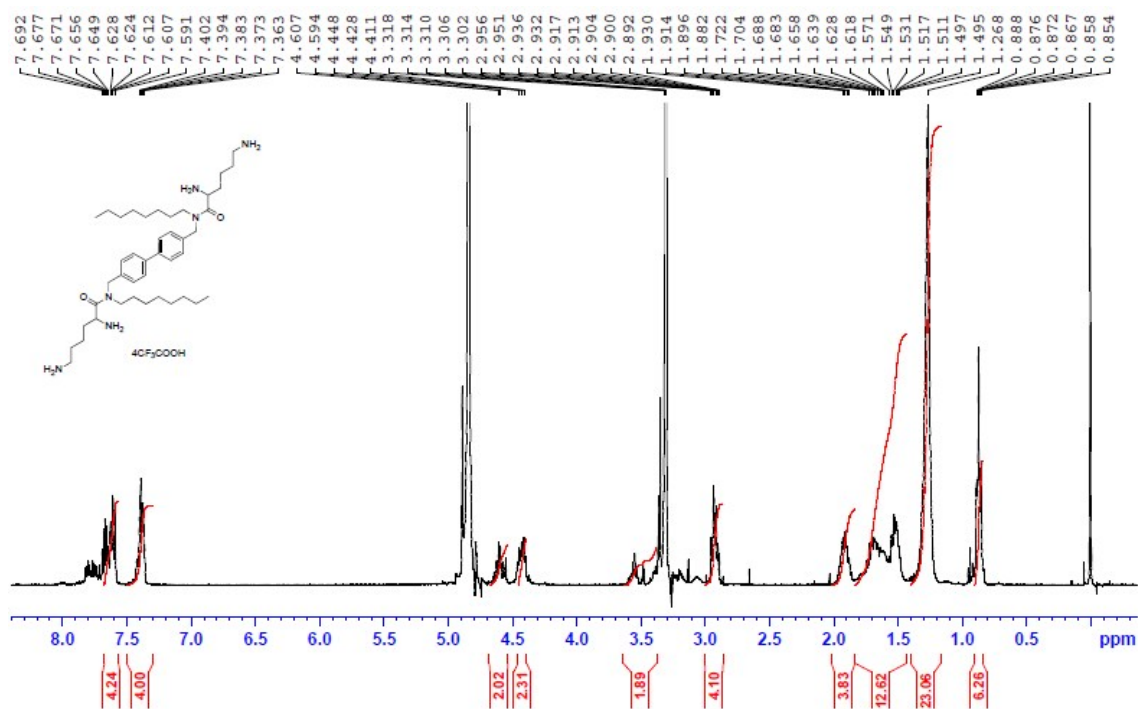


¹H NMR spectra of 2. The NMR was taken in D₂O and the solvent peak was calibrated at δ value of 4.79 ppm.

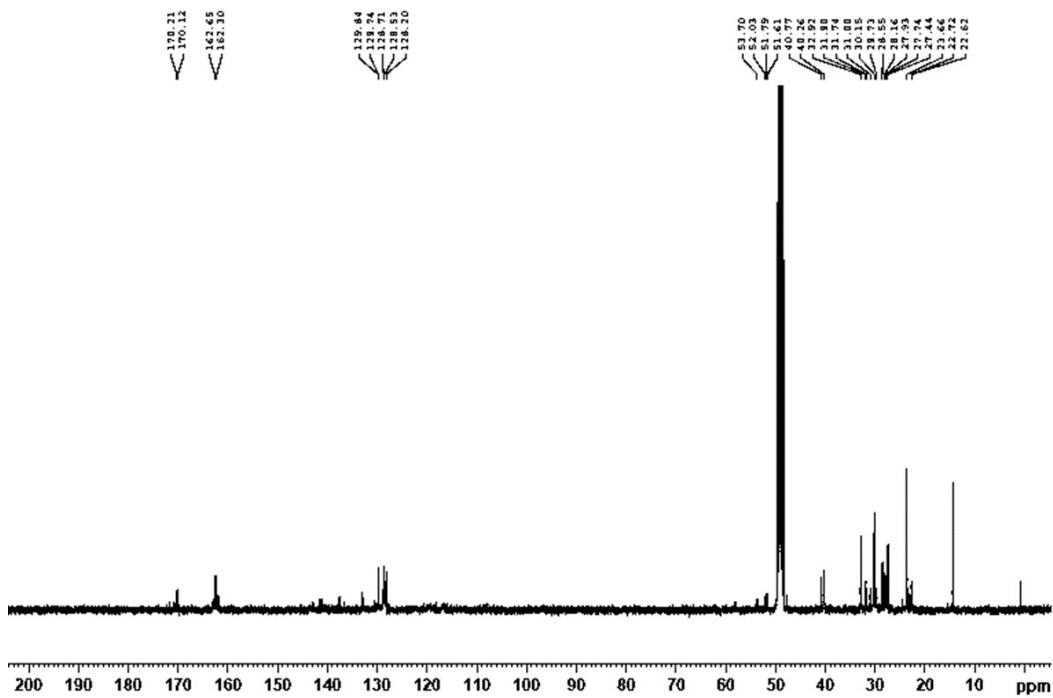


^{13}C NMR spectra of 2. The NMR was taken in DMSO-d_6 and the solvent peak was calibrated at δ value of 39.5 ppm.

(3): ^1H NMR (CD_3OD , 400 MHz) δ/ppm : 7.7-7.2 (t, ArH , 8H), 4.6-4.3 ($\text{Ar}(-\text{CH}_2-\text{NH-R})_2$ and α -H of Lys, 4H) 3.6-3.3 ($\text{Ar}(-\text{CH}_2-\text{N}(\text{Lys})\text{CH}_2-\text{R})_2$, 2H), 3-2.8 (ϵ of CH_2 of Lys) 2.1-1 (-CO-[$\text{CH}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}_2$ of Lys and $-(\text{CH}_2)_6-$] of R groups, 36H) and 0.88 (m, terminal CH_3 groups, 6H). 4Hs have merged with the residual solvent peak of CD_3OD . FT-IR (cm^{-1}): 2963-2853 (sp^3 C-H str.), 1679 (C=O str. of tertiary amide). HR-MS (m/z): $[\text{M}+\text{H}]^+$ obsd. = 693.5787 (calc. = 693.5795).

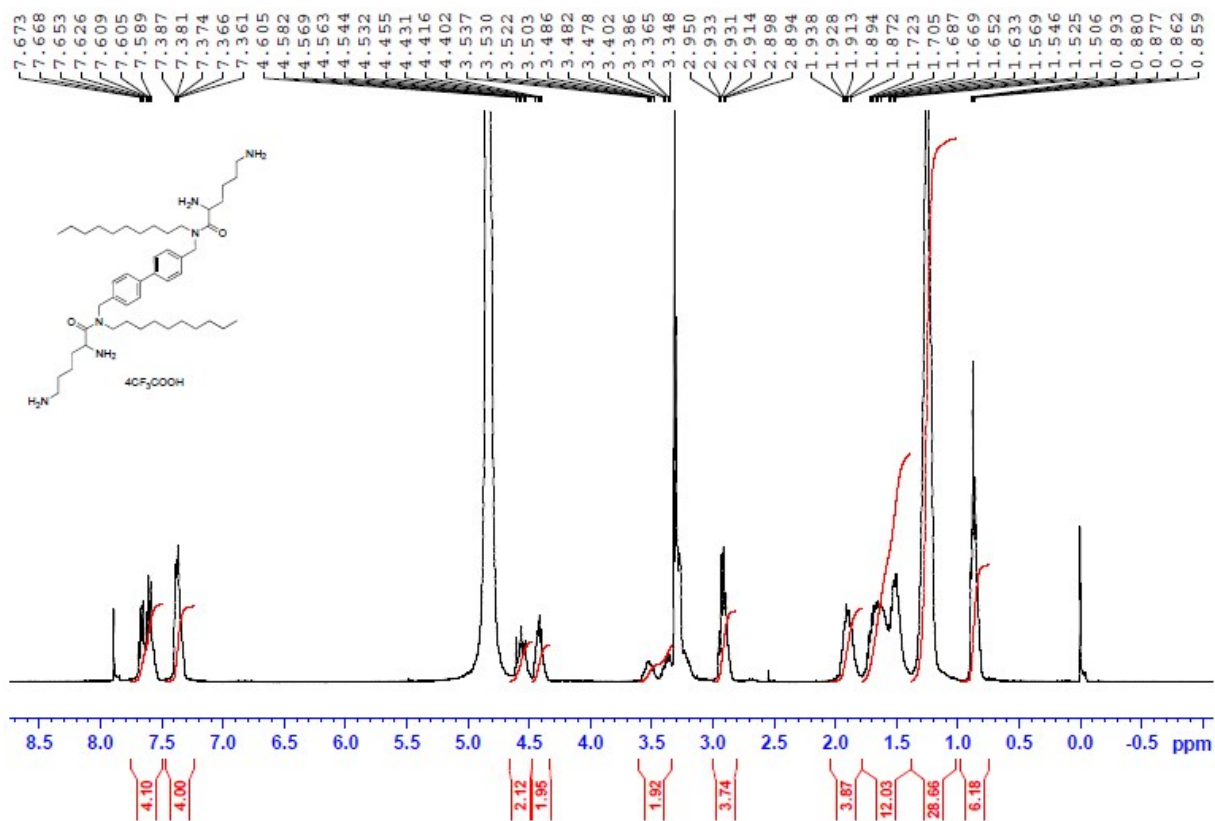


¹H NMR spectra of **3**. The NMR was taken in CD₃OD and the solvent peak was calibrated at δ value of 3.31 ppm.

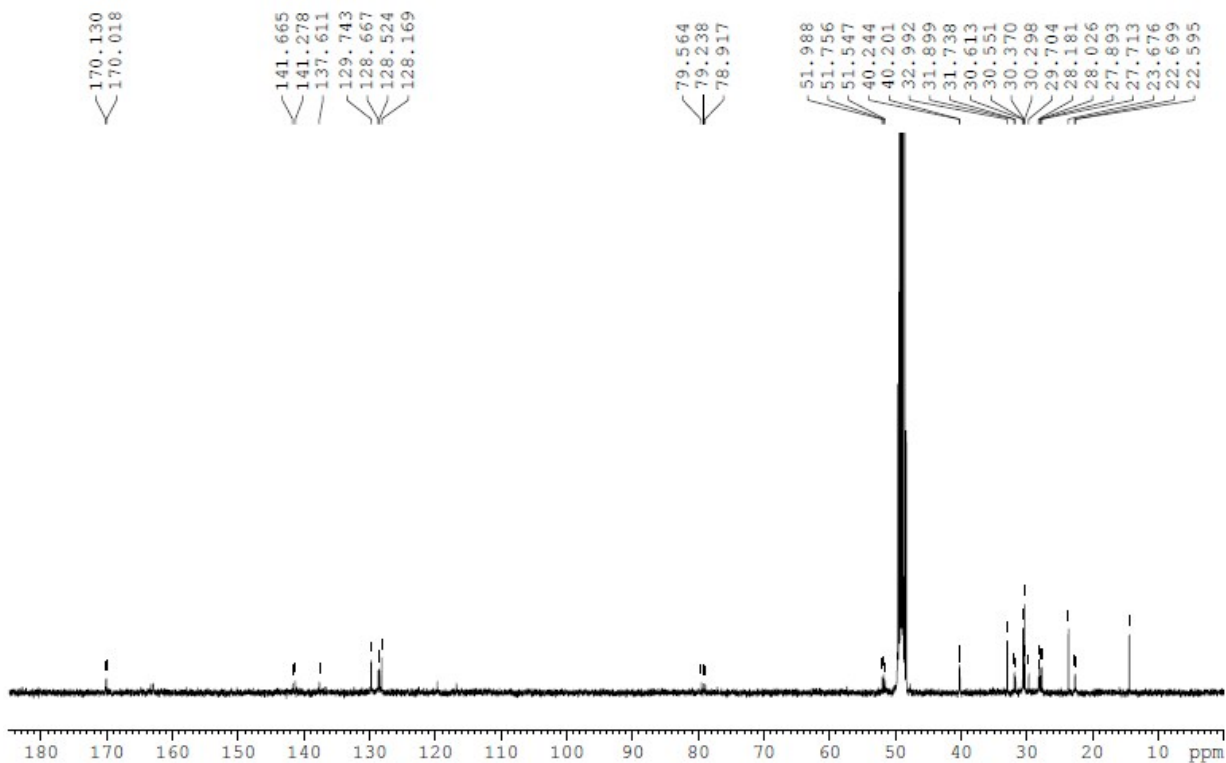


¹³C NMR spectra of **3**. The NMR was taken in CD₃OD and the solvent peak was calibrated at δ value of 49.0 ppm.

(4): ^1H NMR (CD_3OD , 400 MHz) δ/ppm : 7.7-7.2 (t, ArH, 8H), 4.6-4.3 (Ar(-CH₂-NH-R)₂ and α -H of Lys, 4H) 3.6-3.3 (Ar-(CH₂-N(Lys)CH₂-R)₂, 2H), 3-2.8 (ϵ of CH₂ of Lys) 2.1-1 (-CO-[CH-CH₂-CH₂-CH₂-CH₂-NH₂ of Lys and -(CH₂)₈]₂ of R groups, 44H) and 0.88 (m, terminal CH₃ groups, 6H). 4Hs have merged with the residual solvent peak of CD₃OD. FT-IR (cm⁻¹): 2963-2853 (sp³ C-H str.), 1679 (C=O str. of tertiary amide). HR-MS (m/z): [M+H]⁺ obsd. = 749.6419 (calc. = 749.6421).

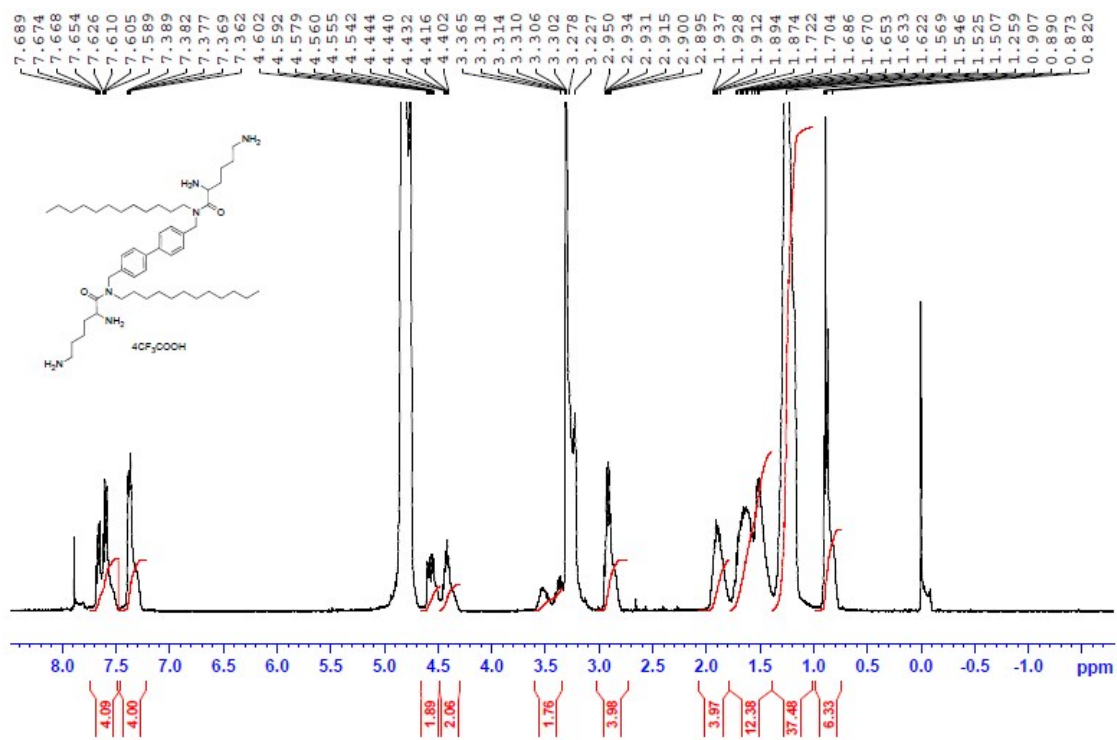


^1H NMR spectra of 4. The NMR was taken in CD_3OD and the solvent peak was calibrated at δ value of 3.31 ppm.

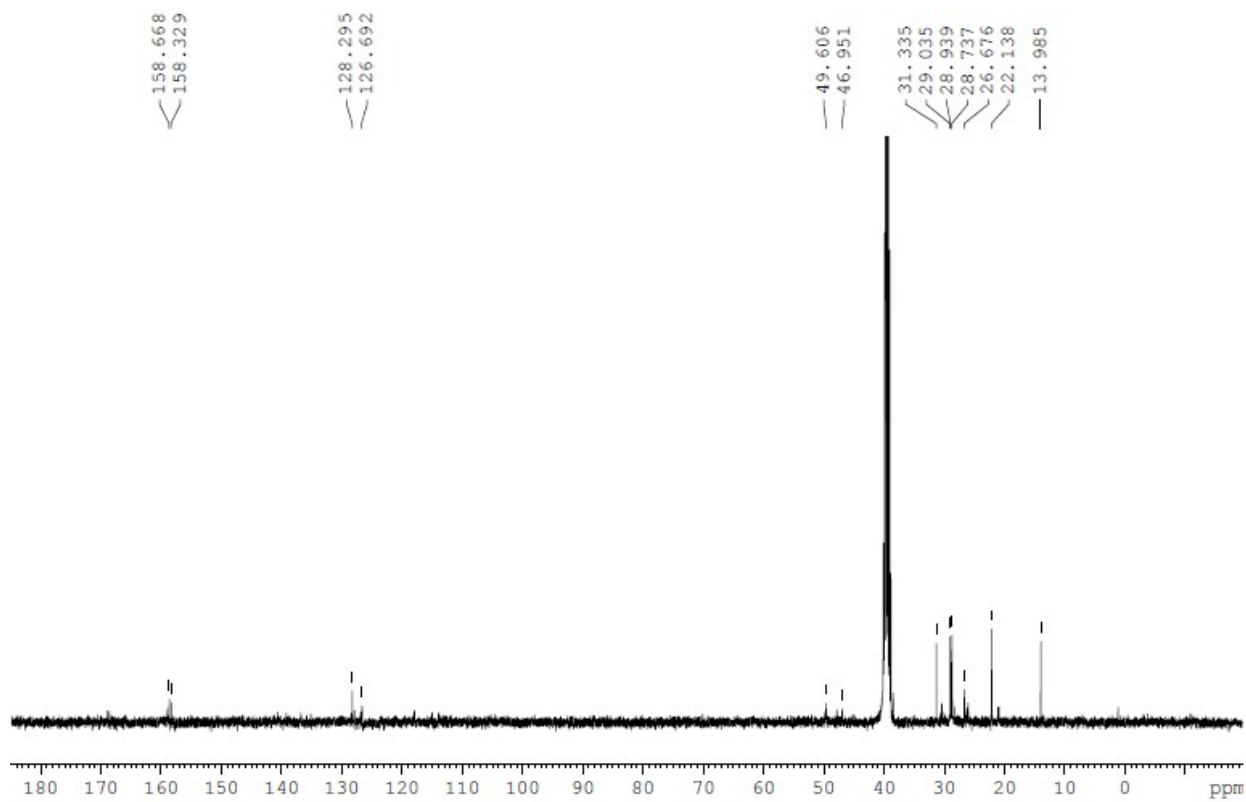


^{13}C NMR spectra of 4. The NMR was taken in CD_3OD and the solvent peak was calibrated at δ value of 49.0 ppm.

(5): ^1H NMR (CD_3OD , 400 MHz) δ /ppm: 7.7-7.2 (t, ArH , 8H), 4.6-4.3 ($\text{Ar}(-\text{CH}_2-\text{NH}-\text{R})_2$ and α -H of Lys, 4H) 3.6-3.3 ($\text{Ar}(-\text{CH}_2-\text{N}(\text{Lys})\text{CH}_2-\text{R})_2$, 2H), 3-2.8 (ϵ of CH_2 of Lys, 4H), 2.1-1 ($-\text{CO}-[\text{CH}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}_2$ of Lys and $-(\text{CH}_2)_{10}-$] of R groups, 52H) and 0.88 (m, terminal CH_3 groups, 6H). 4Hs have merged with the residual solvent peaks of CD_3OD . FT-IR (cm^{-1}): 2963-2853 (sp^3 C-H str.), 1679 (C=O str. of tertiary amide). HR-MS (m/z): $[\text{M}+\text{H}]^+$ obsd. = 805.7035 (calc. = 805.7047).



¹H NMR spectra of 5. The NMR was taken in CD₃OD and the solvent peak was calibrated at δ value of 3.31 ppm.



¹³C NMR spectra of 5. The NMR was taken in DMSO-d₆ and the solvent peak was calibrated at δ value of 39.5 ppm.

III. *In-vitro* biological assays

Bacterial strains: *S. aureus* (MTCC 737) were obtained from Microbial Type Culture Collection (Chandigarh, India), Methicillin-resistant *S. aureus* (MRSA) ATCC 33591 was obtained from the American Type Culture Collection (ATCC). Clinical samples MRSA R3889 and R3990 were obtained from the Department of Neuromicrobiology, National Institute of Mental Health and Neuro Sciences, Hosur Road, Bangalore 560029, India. Bacterial identification was performed by the Vitek 2 Compact 60 system, bioMerieux, France. Culture media and all the antibiotics were from HiMedia and Sigma-Aldrich (India) respectively.

***In-vitro* susceptibility studies:** The *in-vitro* susceptibility assay was performed as reported earlier.¹ Staphylococcal strains were grown in nutrient broth at 37°C and subcultured in fresh nutrient broth medium to the desired inoculum concentration and MICs were determined by using a slightly modified broth microdilution method according to CLSI guidelines. (CLSI. Performance Standards for Antimicrobial Susceptibility Testing, M100-S22. Vol. 32 No. 3) Briefly, 150 µL of media containing cells at 10⁵ CFU/mL were added to wells of a 96 well plate containing 50 µL of serially diluted compound. The plate was then incubated at 37 °C for 24 h and then the O. D. value was measured at 600 nm using TECAN (Infinite series, M200 pro) Plate Reader. Each concentration had triplicate values and the whole experiment was done at least twice and the MIC value was determined as the concentration at which the O. D. is similar to that of control having no bacteria. The results furnished are an average value of at least two independent experiments and each experiment was performed in duplicates/triplicates.

Kinetics of antibacterial activity: The experiment was performed exactly as reported earlier.¹ *S. aureus* cells were isolated as described above and diluted to approximately 10⁵ cells. 100 µL of the test compound (2) was then added to 300 µL of the bacterial solution with the working concentration of MIC, 2 × MIC, 5 × MIC. After specified time intervals (0min , 30 min, 60 min, 120 min, 180 min and 360 min), 20 µL aliquots were serially diluted 10 fold in 0.9 % saline,

plated on sterile nutrient agar plates and incubated at 37 °C overnight. The viable colonies were counted the next day and represented as log (CFU/mL). The experiment was performed twice and the results furnished are an average of the two experiments.

Cytoplasmic membrane depolarization assay: The cytoplasmic membrane depolarization studies were performed as described previously.¹ Briefly, to a solution of 10⁸ CFU/ml of mid-log phase *S. aureus* cells in 5 mM glucose, 5 mM HEPES buffer and 100 mM KCl solution (1:1:1 ratio) was added DiSC₃(5) dye (obtained from Sigma-Aldrich) to a final concentration of 2 μM. After 20 min of preincubation (for dye uptake, and resultant self-quenching) in a well of a black 96-well plate with transparent bottom, the fluorescence of the bacterial suspension was measured (excitation wavelength: 622 nm; emission wavelength: 670 nm). Stabilization was allowed for 6 min at room temperature before the addition of 2 μL of Compound 2 (final concentration of 10 μg mL⁻¹). After addition fluorescence intensity was measured every minute or two for 20 min. The resultant plot was obtained by joining the data points. The experiment was repeated twice and one representative plot has been furnished.

Cytoplasmic membrane permeabilization assay: The experiment was performed as reported earlier.¹ Briefly, to a solution of 10⁸ CFU/ml of mid-log phase *S. aureus* cells in 5 mM HEPES and 5 mM glucose (pH 7.2) was added propidium iodide (PI) dye to a final concentration of 10 μM. The suspension containing the dye (200 μL) was then added to the well of a 96-well plate (black plate, clear bottom with lid) and then after 4 minutes 2μL of Compound 2 was added to the solution to a final concentration of 10 μg mL⁻¹. Fluorescence intensity was measured at excitation wavelength of 535 nm (slit width: 10 nm) and emission wavelength of 617 nm (slit width: 5 nm). The uptake of PI was measured by the increase in fluorescence of PI for 10 min as a measure of inner membrane permeabilization. The experiment was repeated twice.

Intracellular potassium ion leakage assay: The experiment was performed as reported earlier with minor modifications.² Mid-log phase (grown for 6 h) *S. aureus* cells were harvested, washed twice with 10mM HEPES (pH 7.2) and 0.5% glucose and were resuspended in the same amount of 10 mM HEPES (pH 7.2) and 0.5% glucose (10⁸ CFU/mL). The bacterial suspension (0.2 mL) was placed in a 96 well plate. The fluorescence of the bacterial suspension was measured and

allowed to stabilize for 2 min at room temperature before the addition of PBF1-AM dye (2 μ M). Data was collected for an additional 2 min to establish a baseline signal before the addition of test compound (Compound 2). The fluorescence signals were collected for each sample over 10 min. The fluorescence of the dye was monitored at excitation wavelength of 346 nm and emission wavelength of 505 nm.

Intracellular accumulation of UDP-N-acetyl-muramyl-pentadepsipeptide: The experiment was performed as reported earlier.² Analysis of the cytoplasmic peptidoglycan nucleotide precursor pool was examined using *S. aureus* grown in 25 mL MHB. First, cells grown to an OD₆₀₀ of 0.6 were incubated with 130 μ g/mL of chloramphenicol for 15 min. Then, test compounds vancomycin (5 μ M), compound 2 (15 μ M) were added and incubated for another 60 min. Subsequently, bacterial cells were centrifuged and washed with sterile water to remove the antimicrobial agents before being subjected to boiling water treatment for about 30 min. Following the boiling water treatment the cell extract was centrifuged and the supernatant was lyophilized. The lyophilized powder obtained was subsequently dissolved in 2 mL of water and pH was adjusted to 2.0 with 20% phosphoric acid. The UDP-linked cell wall precursors in the solution were then analyzed by RP-HPLC monitoring the UV absorbance peak at 260 nm. The presence of the precursor was confirmed by HR-MS mass spectrometry.

Activity against Stationary-phase bacteria: This experiment was performed exactly as reported earlier.¹ Briefly, *S. aureus* was grown for 6 h in nutrient broth and contained $\sim 10^9$ cfu/mL (determined through dilution plate technique by spread plate method). This was then diluted to 1000 fold and incubated 37 °C for 16 h to obtain stationary-phase cultures. At the end of 16h, the cells were centrifuged down, washed twice with Minimum Essential Media (MEM) and resuspended in MEM media at concentration of 10^5 CFU/mL. 50 μ L of the test compound 2 was then added to 150 μ L of the stationary-phase bacteria (in wells of a 96 well plate) with the working concentrations of MIC, 2X MIC and 5X MIC respectively. In the negative control the well contained 200 μ L of MEM. The 96 well plate was then incubated at 37 °C with shaking at 150 rpm. At the end of 2h, 20 μ L of aliquots from that solution were serially diluted 10-fold in MEM (GIBCO) media. Then from the dilutions, 20 μ L was plated on nutrient agar plates and

incubated at 37 °C. After 24 h the bacterial colonies were counted and results represented in logarithmic scale, i.e. log (CFU/mL).

Isolation of Persister cells: Persister cells of *S. aureus* were isolated exactly as described earlier and the experiment was performed as reported earlier.¹ After growing the respective bacteria to their stationary phases using the protocol mentioned above, *S. aureus* cells were then treated with 100 µg/ml ampicillin for 3h. Then they were centrifuged down, and washed with MEM twice and resuspended again in the same media. These cells were then diluted to 10⁵ cells and treated with the compound (at concentrations of 5×MIC, 2×MIC and MIC) and also with another round of Ampicillin (20 µg/mL) for 2h or left untreated in wells of a 96 well plate as mentioned above. It was then incubated at 37°C with shaking at 150 rpm. At the end of 2h, 20 µL of aliquots from that solution were serially diluted 10-fold in corresponding media. Then from the dilutions, 20 µL was plated on nutrient agar plates and incubated at 37 °C. After 24 h the bacterial colonies were counted and results represented in logarithmic scale, i.e. log (CFU/mL).

Biofilm disruption assay: The experiment was performed as reported earlier.¹ Cover slips were first sterilized by drying in flame after soaking them in ethanol and then they were placed in well of a 6-well plate. To the wells containing the cover slips, 2 mL of midlog phase (6 h grown) culture of culture of *S. aureus* diluted to approximately 10⁵ CFU/mL in a nutrient broth supplemented with 1% glucose and 1% NaCl was added. Subsequently, the plate was incubated under stationary conditions at 37 °C for 24 h after which, medium was removed and planktonic bacteria were carefully washed out with 1X PBS (pH = 7.4). Biofilm containing cover slips were then placed into another 6-well plates and 2 mL of fresh media containing compound 2 (at 25 µg mL⁻¹ and 50 µg mL⁻¹) and allowed to incubate for 24 h. In case of control, 2 mL of complete medium was added instead of compound. At the end of 24 h, medium were then removed and planktonic cells were removed by washing with 1X PBS. The coverslips were carefully removed from the well and placed on glass slides. The biofilms were stained with 10 µL SYTO-9 (5 µM) and imaged using a Zeiss 510 Meta confocal laser-scanning microscope. The images were prepared using LSM 5 Image examiner.

***In-vitro* cytotoxicity against RAW macrophages.** Cytotoxicity of the compound 2 was assessed against RAW 264.7 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 μ L of serially diluted compound. Two controls were made; one containing no compound (non-treated cells) and the other one was treated with 10 vol % Triton-X 100 solution. The plate was incubated for 1 h at 37 °C under 5% CO₂ atmosphere. After 24 h, the medium was carefully removed and 100 μ L 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% CO₂ atmosphere. Then it was centrifuged at 1100 rpm for 5 min and the supernatant was removed. After that 100 μ L of DMSO was added to solubilize formazan crystals. The O. D. 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-treated})/(A_{non-treated}-A_{tritonX-treated}) \times 100. Each concentration had triplicate values and the average of triplicate O. D. values were plotted against concentration followed by fitted with sigmoidal plot. From the curve the values were determined corresponding to 50% cell viability. For bright-field microscopic images, a 40x objective was used and images were captured using a Leica DM2500 microscope.

Intracellular antibacterial activity: The intracellular activity of the test compounds (compound 2 and vancomycin) was determined by modification of a previously published protocol.³ Briefly, 500 μ L complete growth medium (1X DMEM, 10% FBS and 1% Penicillin-Streptomycin) containing \sim 10⁵ RAW 264.7 cells were seeded in 24-well tissue-culture plates at 37 °C with 5% CO₂ for 12 h to ensure cell adherence. Next, MRSA cells (4 \times 10⁵ CFU/mL) were suspended in complete growth medium during 1 h for opsonization. Then, the growth media of aforesaid 24-well tissue-culture plates containing RAW 264.7 cells was replaced with 500 μ L of opsonized bacterial suspension and incubated further for 1 h at 37 °C in humidified air containing 5% CO₂ to allow phagocytosis. After incubation, macrophages were washed with 1X PBS (pH \sim 7.2) twice and treated with gentamicin (50 μ g/mL) for 1 h in complete growth medium to remove any extracellular or non-phagocytic bacteria. Gentamicin was removed by washing the cells with PBS twice. Then compound 2 (15 μ g mL⁻¹) and vancomycin (60 μ M) in growth medium (500 μ L) were added and incubated for 6 h and 24 h at 37 °C with 5% CO₂. At the end of 6h or 24 h, the supernatant was withdrawn carefully and the adhered cells were lysed with 1 mL of ice cold

water for 1 h. Dilutions of lysates in saline were plated on nutrient agar plates. The plates were then incubated at 37 °C for 24 h, and viable bacterial colonies were counted on the next day. The results furnished are averaged values of two independent experiments.

***In-vitro* cytotoxicity against human PBMCs.** Fresh human blood was drawn and human peripheral blood mononuclear cells (PBMCs) were isolated using a standard Ficoll-Hypaque density centrifugation technique and the number of PBMCs (and viability) was determined by trypan blue exclusion. More than 95% of cells were viable. All the media components used were certified and contained low endotoxin levels. After isolation, human PBMCs were resuspended in RPMI 1640 growth medium (with L-glutamine and sodium bicarbonate, Gibco, Life Technologies) supplemented with 10% of heat inactivated and low endotoxin fetal bovine serum (FBS, Life Technologies) and 1% penicillin-streptomycin at 37 °C in a with 5% CO₂. Human PBMCs (2×10⁴) were seeded into 96 well plates and incubated overnight at 37 °C in a humidified-air atmosphere (5% CO₂/95% air). Microscopic images of the appearance of the cells (in presence and absence of compound **2**) were compared to determine the toxicity of the cells.

Stimulation of human PBMCs with LTA. Freshly isolated human PBMCs were seeded into 24- well plates (1×10⁶ cells) in 1 mL of RPMI 1640 complete medium. After 3 h of resting, the cells were stimulated with 10 µg mL⁻¹ of LTA (Sigma Aldrich) either in the presence or absence of compound **2** (3µg mL⁻¹ and 10 µg mL⁻¹). A control experiment was performed using HBSS (Life Technologies) as vehicle control. The cells were incubated for 18-24 hours and then cell culture supernatants were analyzed for tumor necrosis factor (TNF- α) using the human ELISA kits (BD Biosciences) following the manufacturer's instructions. The results furnished are averages of two independent experiments and each experiment was performed in duplicates.

Propensity to induce resistance development in bacteria: This assay was performed as reported previously.¹ Briefly, MIC values of the compounds (**2** and Norfloxacin) were determined against *S. aureus* (MTCC 737) as described above. After determination of MIC, bacterial cells growing at the highest concentration of the compound (usually half of MIC) were harvested and inoculated into fresh media. This inoculum was subjected to another MIC assay. After 24 h incubation period, cells growing in the highest concentration of the compound from

the previous passage were once again harvested and inoculated for another MIC experiment. The process was repeated for 20 passages. The MIC value of the compound was plotted against the number of passages, and the fold increase in MIC was determined. Each MIC experiment was performed in triplicates.

IV. *In-vivo* studies

Animal studies were performed according to the protocols approved by Institutional Animal Ethics Committee (IAEC). The mice were housed in individually ventilated cages (IVC) maintained with controlled environment. They were housed in pathogen free conventional caging systems, bedding material used were corncob. The husbandry conditions:-Light: dark cycle-12:12 hours, Animal Room Temp: $22 \pm 2^{\circ}\text{C}$, Relative humidity: 30–40%, Access to feed and water: ad libitum and Water: RO Water. Animals were randomly selected, marked to permit individual identification and kept in their cages for at least 5 days before the experiment to allow for acclimatization to the experimental conditions. Animal handling and experimentation protocols were followed according to OECD Guidelines for the Testing of Chemicals (OECD 425). All care was taken to cause no pain to the animals. Humane endpoints were used to avoid unnecessary distress and suffering in animals following an experimental intervention that would lead to death.

Acute dermal toxicity: The acute dermal toxicity was performed exactly as reported earlier.⁴ Briefly, the fur of 6 to 8 weeks-old male BALB/c mice (n=5, 18-22g) was removed from the back of the mice, first by clipping and then shaving, 24h prior to the start of the experiment. Care was taken to avoid abrasion of the skin. The area of the shaved portion was roughly 2 cm². Compound 2 was dissolved in saline at concentrations of 100 mg/mL to make working stocks. From this stock, 40 μL was added to the shaved area of the skin to give a concentration of 200 mg/kg per animal and spread around the shaved region gently. Following the application of the compound, the animals were observed carefully for first 6h and then observed carefully once every day for 14 days. Particular attention was paid to the changes in fur, eyes and mucous membranes, and observations of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma.

MRSA skin infection: The experiment was performed exactly following previously published protocol.⁵ 6 to 8 weeks-old male BALB/c mice (18-22g) were anesthetized by intraperitoneal injection of xylazine-ketamine cocktail. The fur on the back of the mice were then shaved using a sterile razor. The fur was shaved in a manner to induce a wound, that is, reddening and glistening of the exposed skin was observed without bleeding. To this visibly damaged area (~2 cm²) of the skin, a bacterial infection was initiated by placing on the skin a 20 µL droplet containing 10⁷ cells of MRSA. This was allowed to dry for 20 minutes and it was ensured that the droplet stayed within the shaved area. In the first experiment one group of mice (n=5) were treated after 4h with 40 µL of compound 2 (concentration of 20 mg/mL) at the site of infection (on the shaved area of the skin where bacteria was added). The droplet was gently spread on the entire surface of the wound to avoid any drop from rolling down the sides. Another group of mice (n=4) were dosed with Fusidic acid, a comparator drug, at exactly the same concentration. The dosage for both the compounds were continued for seven days. One group of mice (n=4) were left untreated and served as a control. 18h after the last dose (to prevent carryover effects) the mice were sacrificed using isoflurane and the infected skin (on the back of the mice) was severed aseptically. The severed part was weighed and placed into about 10 mL of sterile saline and homogenized. The dilutions of the homogenate were plated onto agar plates, which were incubated overnight at about 37°C. The bacterial titer was expressed as log₁₀ CFU/g of weight of the tissue collected and expressed as mean ± S.E.M (standard error of mean). P value was calculated using unpaired Student's t test (2 tailed 2 samples assuming equal variances) between the control group and the treatment group and a value of P < 0.05 was considered significant.

V. Figures

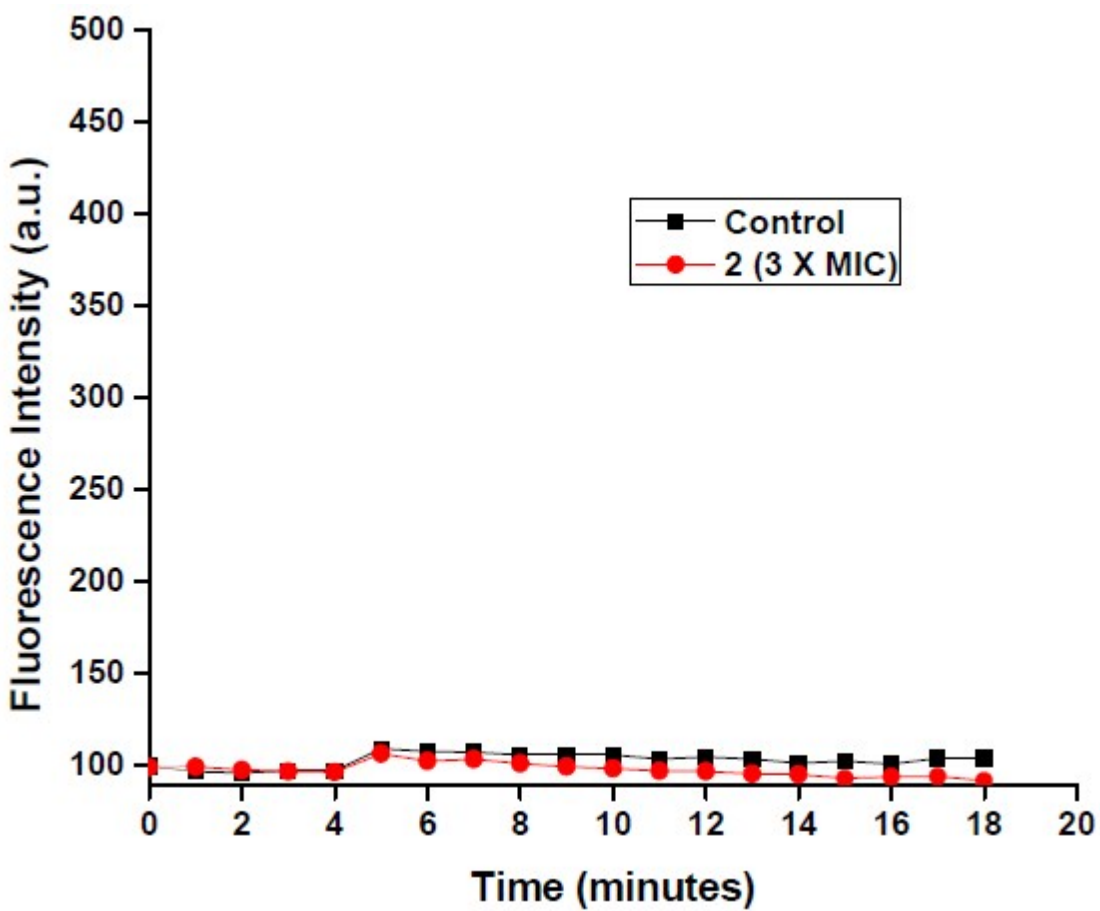


Figure S1. Compound 2 was not able to permeabilize *S. aureus* membrane as indicated by no change in fluorescence intensity of dye Propidium iodide (PI).

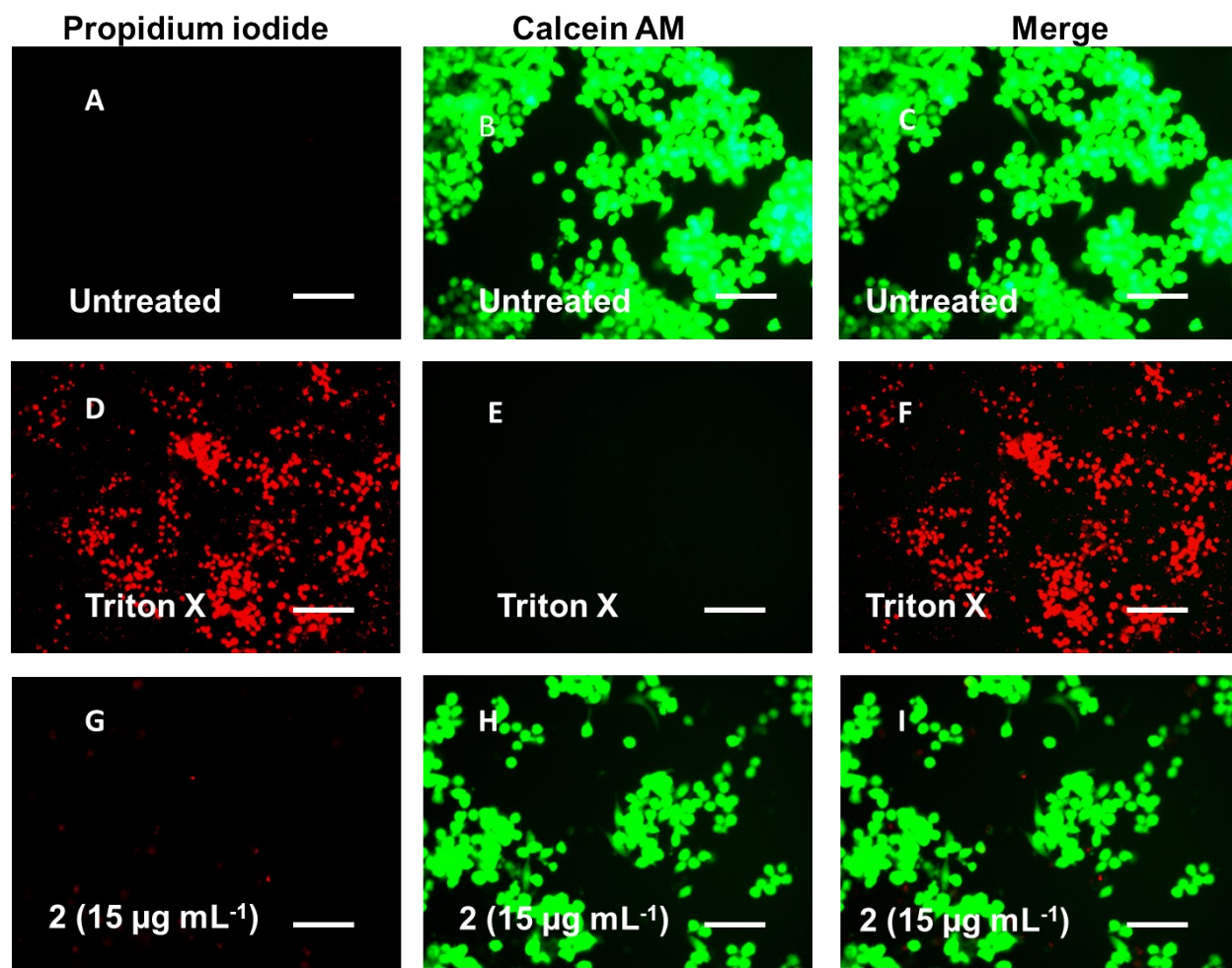


Figure S2. Toxicity studies. Fluorescence microscopy images of RAW cells after treatment with or without Triton X and compound 2 for 24h. Staining was done with propidium iodide (PI) and calcein AM. (A–C) Untreated cells (negative control); (D–F) cells treated with 0.1% Triton X (positive control); and (G–I) cells treated with compound 2 at 15 µg mL⁻¹.

VI. References

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