Electronic Supplementary Information

Selective and Broad Spectrum Amphiphilic Small Molecules to Combat Bacterial Resistance and Eradicate Biofilm

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1. EXPERIMENTAL PROCEDURES

1.1. Materials and Instrumentation. *N,N*-Dimethyloctylamine, *N,N*-dimethyldecylamine, *N,N*-dimethyldodecylamine, *N,N*-dimethylhexadecylamine, bromoacetyl bromide, 1,2-diaminoethane, 1,3-diaminopropane, 1,4-diaminobutane and 1,6-diaminohexane were purchased from Sigma-Aldrich and used as received. Analytical grade anhydrous potassium carbonate (K_2CO_3), anhydrous sodium sulphate (Na_2SO_4), phosphorous pentaoxide (P_2O_5), calcium hydride (CaH_2), dichloromethane (DCM), chloroform, ethanol, acetonitrile, diethylether, acetone and molecular sieves (4 Å), were purchased from SD Fine, India. DCM and chloroform were dried over P_2O_5 and ethanol was dried over CaH_2 and stored over activated molecular sieves. Nuclear magnetic resonance (NMR) spectra in suitable deuterated solvents were recorded using Bruker AMX-400 (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR) spectrometer. The chemical shift (δ) values are expressed in parts per million (ppm) downfield from the peak for internal standard TMS for ^{1}H NMR and ^{13}C NMR. High resolution mass spectra were recorded on a 6538-UHD Accurate mass Q-TOF LC-MS high resolution mass-spectrometer (HRMS). Fourier transform infrared (FT-IR) spectra were recorded on an attenuated total reflectance (ATR) FT-IR spectrometer using diamond crystal as ATR crystal. Elemental analyses were performed using a Thermo Finnigan FLASH EA 1112 CHNS analyzer. For optical density (O. D.) and fluorescence measurements, Tecan Infinite Pro series M200 Microplate Reader was used. *Staphylococcus aureus* (MTCC 737) and *Escherichia coli* (MTCC 443) were obtained from MTCC (Chandigarh, India). *Pseudomonas aeruginosa* (ATCC 424), methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 33591), vancomycin-resistant *Enterococcus faecium* (VRE) (ATCC 51559) and beta-lactam-resistant *Klebsiella pneumoniae* (ATCC 700603) were purchased from ATCC (Rockville, MD, USA). Bacterial growth media and agar were supplied by HIMEDIA, India. Studies on human subjects were performed according to the protocols approved by Institutional Bio-Safety Committee (IBSC) of Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR).

1.2. Synthesis of small molecular compounds.

1.2.1. General procedure for the synthesis of 1a-1d: α-ω-Diaminoalkanes (0.1 M) was dissolved in chloroform (250 mL) and an aqueous solution of K_2CO_3 (0.3 M, 150 mL) was added to it. The binary solvent mixture was then cooled to 5 °C in a cold incubator connected to a chiller. Bromoacetyl bromide (0.3 M) was dissolved in dry chloroform (100 mL) and was added to the mixture drop wise for about 30 min. Then the reaction mixture was stirred at

room temperature for 24 h. After the reaction, the insoluble solid was directly filtered through a sintered glass funnel and washed repeatedly with water. Finally, the precipitate was dried in vacuum oven at 60 °C for 24 h to obtain white colored products. Also, the organic layer from the filtrate was separated using a separating funnel and the aqueous layer was subjected to repeated wash with chloroform (2 × 50 mL) to extract any remaining amount of product. All the organic solutions were then mixed and washed with water repeatedly (3 × 75 mL). The final organic layer was passed over anhydrous Na_2SO_4. Finally, the solvent was evaporated to obtain white coloured compounds as another portion of the products. The products were characterized by FT-IR, ^{1}H NMR and ^{13}C NMR spectroscopy. The products obtained in both the ways (as precipitate and from organic solution) were same and combined weight gave quantitative (>99%) yield of **1a-1d**.

1a. FT-IR (ATR): 3252 cm^{-1} (amide N-H str.), 2928 cm^{-1} (-CH_2-assym. str.), 2849 (-CH_2-sym. str.), 1678 cm^{-1} (Amide I, C=O str.), 1562 cm^{-1} (Amide II, N-H ben.), 1465 cm^{-1} (-CH_2-scissor). ^{1}H NMR: (400 MHz, $CDCl_3$): δ 0.879 (t, terminal –*CH₃*, 3H), 1.300 (m,-(*CH₂*)$_9$-, 18H), 1.549 (m, -*CH₂*(CH_2)$_9$-, 8H), 3.270 (t, -CONH*CH₂*-, 2H), 3.888 (s, -CO*CH₂*Br, 2H), 6.479 (br s, amide –*NH*CO, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.198, 22.778, 26.914, 29.314, 29.413, 29.578, 29.656, 29.718, 31.975, 40.403, 165.599; Yield 100%.

1b. FT-IR (ATR): 3251 cm^{-1} (amide N-H str.), 2928 cm^{-1} (-CH_2-assym. str.), 2855 (-CH_2-sym. str.), 1678 cm^{-1} (Amide I, C=O str.), 1560 cm^{-1} (Amide II, N-H ben.), 1470 cm^{-1} (-CH_2-scissor). ^{1}HNMR: (400 MHz, $CDCl_3$): δ 0.878 (t, terminal –*CH₃*, 3H), 1.310 (m,-(*CH₂*)$_9$-, 18H), 1.543 (m, -*CH₂*(CH_2)$_9$-, 8H), 3.278 (t, -CONH*CH₂*-, 2H), 3.881 (s, -CO*CH₂*Br, 2H), 6.475 (br s, amide –*NH*CO, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.195, 22.768, 26.904, 29.324, 29.423, 29.588, 29.646, 29.708, 31.995, 40.403, 165.589; Yield 100%.

1c. FT-IR (ATR): 3250 cm^{-1} (amide N-H str.), 2928 cm^{-1} (-CH_2-assym. str.), 2851 (-CH_2-sym. str.), 1681 cm^{-1} (Amide I, C=O str.), 1557 cm^{-1} (Amide II, N-H ben.), 1472 cm^{-1} (-CH_2-scissor). ^{1}H NMR: (400 MHz, $CDCl_3$): δ 0.872 (t, terminal –*CH₃*, 3H), 1.290 (m,-(*CH₂*)$_9$-, 18H), 1.551 (m, -*CH₂*(CH_2)$_9$-, 8H), 3.318 (t, -CONH*CH₂*-, 2H), 3.870 (s, -CO*CH₂*Br, 2H), 6.465 (br s, amide –*NH*CO, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.186, 22.778, 26.884, 29.335, 29.433, 29.578, 29.666, 29.718, 31.895, 40.393, 165.549; Yield 100%.

1d. FT-IR (ATR): 3254 cm^{-1} (amide N-H str.), 2929 cm^{-1} (-CH_2-assym. str.), 2849 (-CH_2-sym. str.), 1681 cm^{-1} (Amide I, C=O str.), 1563 cm^{-1} (Amide II, N-H ben.), 1472 cm^{-1} (-CH_2-scissor). ^{1}H NMR: (400 MHz, $CDCl_3$): δ 0.879 (t, terminal –*CH₃*, 3H), 1.285 (m,-(*CH₂*)$_9$-, 18H), 1.526 (m, -*CH₂*(CH_2)$_9$-, 8H), 3.198 (t, -CONH*CH₂*-, 2H), 3.905 (s, -CO*CH₂*Br, 2H),

6.475 (br s, amide –*NH*CO, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.201, 22.760, 26.884, 29.329, 29.429, 29.498, 29.716, 29.678, 31.995, 40.389, 165.599; Yield 100%.

1.2.2. General procedure for the synthesis of amphiphilic small molecules (2a-2d, 3a-3d, 4a-4d and **5a-5d):** *N,N*-Dimethylalkylamines (15 mM) were added to the organic solutions (EtOH for **1a**, $CHCl_3$ for **1b** and MeCN for **1c** and **1d**, 50 mL) of intermediates **1a-1d** (5 mM) separately in screw top pressure tubes and the reaction mixtures were stirred at 85 °C for about 24 h. After the reaction, the mixtures were allowed to cool down to room temperature and transferred to round bottom flask. Then, the organic solvents were removed and volume of the reaction mixtures was reduced to 1/10th to its original volume. Finally, the products were precipitated in excess of dry diethyl ether/acetone (150 mL). The precipitates were filtered and washed repeatedly with diethyl ether/acetone. The white precipitates were vacuum dried to give more than 99% yield of **2a-2d, 3a-3d, 4a-4d** and **5a-5d**. All the final products were characterized by FT-IR, ^{1}H NMR, ^{13}C NMR, HRMS and elemental analysis.

2a. FTIR (Solid): 3215 cm^{-1} (amide N–H str.), 2922 cm^{-1} (–CH_2– assym. str.), 2861 cm^{-1} (–CH_2– sym. str.), 1685 cm^{-1} (amide I, C=O str.), 1560 cm^{-1} (amide II, N–H ben.), 1468 cm^{-1} (–CH_2– scissor); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.868 (t, terminal –CH_3, 6H), 1.288 (m, –CH_3*(*CH_2*)*$_5$$CH_2$–, 20H), 1.789 (m, $CH_3(CH_2)_5$*CH_2*CH_2–, 4H), 3.392-3.515 (m, –CH_2N^+*(*CH_3*)*$_2$$CH_2$– and $CH_3(CH_2)_5CH_2$*CH_2*–, 16H), 3.619 (m, –NH*CH_2**CH_2*NH–, 4H), 4.663 (s, –$N^+(CH_3)_2$*CH_2*CO–, 4H), 8.882 (br s, –CO*NH*CH_2–, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.217, 22.782, 26.350, 29.424, 29.591, 29.678, 31.978, 38.514, 52.265, 63.624, 66.502, 163.623; HRMS (ESI): m/z calculated for $C_{26}H_{56}N_4O_2Br_2$ $[M–Br]^+$ and $[M–2Br]^{2+}$: 535.3575, 537.3554 and 228.2196 Found: 535.3619, 537.3605 and 228.2268; Elemental analysis: C 50.62, H 9.16, N 9.08 (calculated); C 50.58, H 9.14, N 9.01 (found).

2b: FTIR (Solid): 3222 cm^{-1} (amide N–H str.), 2930 cm^{-1} (–CH_2– assym. str.), 2865 cm^{-1} (–CH_2– sym. str.), 1685 cm^{-1} (amide I, C=O str.), 1565 cm^{-1} (amide II, N–H ben.), 1471 cm^{-1} (–CH_2– scissor); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.887 (t, terminal –CH_3, 6H), 1.298 (m, –CH_3*(*CH_2*)*$_5$$CH_2$–, 20H), 1.789 (m, $CH_3(CH_2)_5$*CH_2*CH_2–, 4H), 1.882 (m, –NHCH_2*CH_2*CH_2NH–, 2H), 3.269-3.466 (m, –CH_2N^+*(*CH_3*)*$_2$$CH_2$– and $CH_3(CH_2)_5CH_2$*CH_2*–, 16H), 3.678 (m, –NH*CH_2*CH_2*CH_2*NH–, 4H), 4.687 (s, –$N^+(CH_3)_2$*CH_2*CO–, 4H), 8.860 (br s, –CO*NH*CH_2–, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.201, 22.675, 22.981, 26.267, 27.779, 29.201, 29.439, 29.475, 31.920, 52.106, 63.543, 65.913, 163.207; HRMS (ESI): m/z calculated for $C_{27}H_{58}N_4O_2Br_2$ $[M–Br]^+$ and $[M–2Br]^{2+}$: 549.3137, 551.3116 and 235.2274. Found:

549.3729, 551.3712 and 235.2274; Elemental analysis: C 51.41, H 9.27, N 8.88 (calculated); C 51.37, H 9.18, N 8.76 (found).

2c: FTIR (Solid): 3215 cm^{-1} (amide N–H str.), 2915 cm^{-1} (–CH_2– assym. str.), 2859 cm^{-1} (–CH_2– sym. str.), 1675 cm^{-1} (amide I, C=O str.), 1560 cm^{-1} (amide II, N–H ben.), 1476 cm^{-1} (–CH_2– scissor); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.889 (t, terminal –*CH_3*, 6H), 1.290 (m, –CH_3*$(CH_2)_5$*CH_2–, 20H), 1.639 (m, $CH_3(CH_2)_5$*CH_2*CH_2–, 4H), 1.781 (m, –$NHCH_2$*$(CH_2)_2$*CH_2NH–, 4H), 3.302 (m, –NH*CH_2*$(CH_2)_2$*CH_2*NH–, 4H), 3.423 (s, –CH_2N^+*$(CH_3)_2$*CH_2–, 12H), 3.624 (m, $CH_3(CH_2)_5CH_2$*CH_2*–, 4H), 4.641 (s, –$N^+(CH_3)_2$*CH_2*CO–, 4H), 8.6798 (br s, –CO*NH*CH_2–, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.241, 22.829, 26.270, 26.344, 29.245, 29.448, 29.569, 32.123, 38.890, 52.201, 63.609, 66.403, 163.179; HRMS (ESI): m/z calculated for $C_{28}H_{60}N_4O_2Br_2$ $[M–Br]^+$ and $[M–2Br]^{2+}$: 563.3961, 565.3886 and 242.2353. Found: 563.3961, 565.3946 and 242.2427; Elemental analysis: C 52.15, H 9.38, N 8.69 (calculated); C 52.11, H 9.29, N 8.62 (found).

2d: FTIR (Solid): 3216 cm^{-1} (amide N–H str.), 2921 cm^{-1} (–CH_2– assym. str.), 2862 cm^{-1} (–CH_2– sym. str.), 1675 cm^{-1} (amide I, C=O str.), 1561 cm^{-1} (amide II, N–H ben.), 1465 cm^{-1} (–CH_2– scissor); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.849 (t, terminal –*CH_3*, 6H), 1.278 (m, –CH_3*$(CH_2)_5$*CH_2–, 20H), 1.418 (m, –$NHCH_2CH_2$*$(CH_2)_2$*CH_2CH_2NH–, 4H), 1.544 (m, $CH_3(CH_2)_5$*CH_2*CH_2–, 4H), 1.769 (m, –$NHCH_2$*CH_2*$(CH_2)_2$*CH_2*CH_2NH–, 4H), 3.267 (m, –NH*CH_2*$(CH_2)_4$*CH_2*NH–, 4H), 3.487 (s, –CH_2N^+*$(CH_3)_2$*CH_2–, 12H), 3.667 (m, $CH_3(CH_2)_5CH_2$-*CH_2*–, 4H), 4.667 (m, –$N^+(CH_3)_2$*CH_2*CO–, 4H), 8.691 (br s, –CO*NH*CH_2–, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.211, 22.817, 23.226, 26.423, 28.201, 29.317, 29.518, 29.614, 29.696, 32.160, 39.479, 52.209, 63.392, 66.116, 163.219; HRMS (ESI): m/z calculated for $C_{30}H_{64}N_4O_2Br_2$ $[M–Br]^+$ and $[M–2Br]^{2+}$: 591.4203, 593.4182 and 256.2510. Found: 591.4242, 593.4228 and 256.2595; Elemental analysis: C 53.55, H 9.59, N 8.33 (calculated); C 53.48, H 9.49, N 8.29 (found).

3a: FTIR (Solid): 3215 cm^{-1} (amide N–H str.), 2925 cm^{-1} (–CH_2– assym. str.), 2858 cm^{-1} (–CH_2– sym. str.), 1685 cm^{-1} (amide I, C=O str.), 1560 cm^{-1} (amide II, N–H ben.), 1468 cm^{-1} (–CH_2– scissor); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.856 (t, terminal –*CH_3*, 6H), 1.288 (m, –CH_3*$(CH_2)_7$*CH_2–, 28H), 1.765 (m, $CH_3(CH_2)_7$*CH_2*CH_2–, 4H), 3.402-3.465 (m, –CH_2N^+*$(CH_3)_2$*CH_2– and $CH_3(CH_2)_7CH_2$*CH_2*–, 16H), 3.620 (m, –NH*CH_2*CH_2*NH–, 4H), 4.654 (s, –$N^+(CH_3)_2$*CH_2*CO–, 4H), 8.889 (br s, –CO*NH*CH_2–, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.220, 22.778, 23.105, 26.297, 29.329, 29.514, 29.554, 29.787, 31.987, 38.511, 52.248, 63.614, 66.410, 163.613; HRMS (ESI): m/z calculated for $C_{30}H_{64}N_4O_2Br_2$ $[M–Br]^+$ and [M–

2Br]$^{2+}$: 591.4203, 593.4182 and 256.2510. Found: 591.4244, 593.4218 and 256.2504; Elemental analysis: C 53.55, H 9.59, N 8.33 (calculated); C 53.44, H 9.45, N 8.24 (found).

3b: FTIR (Solid): 3215 cm^{-1} (amide N–H str.), 2930 cm^{-1} (–CH_2– assym. str.), 2865 cm^{-1} (–CH_2– sym. str.), 1680 cm^{-1} (amide I, C=O str.), 1565 cm^{-1} (amide II, N–H ben.), 1470 cm^{-1} (–CH_2– scissor); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.887 (t, terminal –*CH_3*, 6H), 1.285 (m, –CH_3*$(CH_2)_7$*CH_2–, 28H), 1.787 (m, $CH_3(CH_2)_7$*CH_2*CH_2–, 4H), 1.877 (m, –$NHCH_2$*CH_2*CH_2NH–, 2H), 3.264-3.455 (m, –CH_2N^+*$(CH_3)_2$*CH_2– and $CH_3(CH_2)_7CH_2$*CH_2*–, 16H), 3.648 (m, –NH*CH_2*CH_2*CH_2*NH–, 4H), 4.678 (s, –$N^+(CH_3)_2$*CH_2*CO–, 4H), 8.859 (br s, –CO*NH*CH_2–, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.147, 22.710, 22.957, 26.277, 27.790, 29.165, 29.434, 29.472, 31.915, 52.104, 63.347, 65.884, 163.128; HRMS (ESI): m/z calculated for $C_{31}H_{66}N_4O_2Br_2$ [M–Br]$^+$ and [M–2Br]$^{2+}$: 605.4359, 607.4338 and 263.2588. Found: 605.4347, 607.4333 and 263.2598; Elemental analysis: C 54.20, H 9.69, N 8.16 (calculated); C 54.14, H 9.51, N 8.10 (found).

3c: FTIR (Solid): 3215 cm^{-1} (amide N–H str.), 2925 cm^{-1} (–CH_2– assym. str.), 2862 cm^{-1} (–CH_2– sym. str.), 1675 cm^{-1} (amide I, C=O str.), 1563 cm^{-1} (amide II, N–H ben.), 1478 cm^{-1} (–CH_2– scissor); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.874 (t, terminal –*CH_3*, 6H), 1.284 (m, –CH_3*$(CH_2)_7$*CH_2–, 28H), 1.644 (m, $CH_3(CH_2)_7$*CH_2*CH_2–, 4H), 1.788 (m, –$NHCH_2$*$(CH_2)_2$*CH_2NH–, 4H), 3.311 (m, –NH*CH_2*$(CH_2)_2$*CH_2*NH–, 4H), 3.420 (s, –CH_2N^+*$(CH_3)_2$*CH_2–, 12H), 3.630 (m, $CH_3(CH_2)_7CH_2$*CH_2*–, 4H), 4.639 (s, –$N^+(CH_3)_2$*CH_2*CO–, 4H), 8.670 (br s, –CO*NH*CH_2–, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.230, 22.804, 23.145, 26.270, 26.344, 29.239, 29.512, 29.550, 29.711, 32.113, 38.965, 52.194, 63.549, 66.370, 163.179; HRMS (ESI): m/z calculated for $C_{32}H_{68}N_4O_2Br_2$ [M–Br]$^+$ and [M–2Br]$^{2+}$: 619.4516, 621.4495 and 270.2666. Found: 619.4498, 621.4482 and 270.2674; Elemental analysis: C 54.83, H 9.79, N 7.99 (calculated); C 54.79, H 9.68, N 7.82 (found).

3d: FTIR (Solid): 3212 cm^{-1} (amide N–H str.), 2922 cm^{-1} (–CH_2– assym. str.), 2860 cm^{-1} (–CH_2– sym. str.), 1675 cm^{-1} (amide I, C=O str.), 1565 cm^{-1} (amide II, N–H ben.), 1468 cm^{-1} (–CH_2– scissor); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.866 (t, terminal –*CH_3*, 6H), 1.291 (m, –CH_3*$(CH_2)_7$*CH_2–, 28H), 1.410 (m, –$NHCH_2CH_2$*$(CH_2)_2$*CH_2CH_2NH–, 4H), 1.574 (m, $CH_3(CH_2)_7$*CH_2*CH_2–, 4H), 1.809 (m, –$NHCH_2$*CH_2*$(CH_2)_2$*CH_2*CH_2NH–, 4H), 3.287 (m, –NH*CH_2*$(CH_2)_4$*CH_2*NH–, 4H), 3.432 (s, –CH_2N^+*$(CH_3)_2$*CH_2–, 12H), 3.649 (m, $CH_3(CH_2)_7CH_2$*CH_2*–, 4H), 4.691 (m, –$N^+(CH_3)_2$*CH_2*CO–, 4H), 8.670 (br s, –CO*NH*CH_2–, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.206, 22.783, 23.126, 26.343, 26.720, 28.192, 29.225, 29.440, 29.554, 29.706, 32.120, 39.532, 52.219, 63.402, 66.116, 163.209; HRMS (ESI): m/z calculated for $C_{34}H_{72}N_4O_2Br_2$ [M–Br]$^+$ and [M–2Br]$^{2+}$: 647.4516, 649.4495 and 284.3192.

Found: 647.4813, 649.4800 and 284.2868; Elemental analysis: C 56.01, H 9.97, N 7.69 (calculated); C 55.91, H 9.89, N 7.57 (found).

4a: FTIR (Solid): 3215 cm^{-1} (amide N–H str.), 2922 cm^{-1} (–CH_2– assym. str.), 2861 cm^{-1} (–CH_2– sym. str.), 1685 cm^{-1} (amide I, C=O str.), 1560 cm^{-1} (amide II, N–H ben.), 1470 cm^{-1} (–CH_2– scissor); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.859 (t, terminal –CH_3, 6H), 1.279 (m, –$CH_3(CH_2)_9CH_2$–, 36H), 1.764 (m, $CH_3(CH_2)_9CH_2CH_2$–, 4H), 3.412-3.445 (m, –$CH_2N^+(CH_3)_2CH_2$– and $CH_3(CH_2)_9CH_2CH_2$–, 16H), 3.619 (m, –NHCH_2CH_2NH–, 4H), 4.653 (s, –$N^+(CH_3)_2CH_2$CO–, 4H), 8.878 (br s, –CO$NH$$CH_2$–, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.214, 22.780, 23.005, 26.347, 29.229, 29.419, 29.503, 29.544, 29.687, 31.999, 38.509, 52.255, 63.603, 66.401, 163.603; HRMS (ESI): m/z calculated for $C_{34}H_{72}N_4O_2Br_2$ $[M–Br]^+$ and $[M–2Br]^{2+}$: 647.4516, 649.4495 and 284.3192. Found: 647.4601, 649.4594 and 284.2854; Elemental analysis: C 56.01, H 9.97, N 7.69 (calculated); C 55.92, H 9.85, N 7.54 (found).

4b: FTIR (Solid): 3215 cm^{-1} (amide N–H str.), 2930 cm^{-1} (–CH_2– assym. str.), 2865 cm^{-1} (–CH_2– sym. str.), 1685 cm^{-1} (amide I, C=O str.), 1565 cm^{-1} (amide II, N–H ben.), 1470 cm^{-1} (–CH_2– scissor); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.881 (t, terminal –CH_3, 6H), 1.305 (m, –$CH_3(CH_2)_9CH_2$–, 36H), 1.790 (m, $CH_3(CH_2)_9CH_2CH_2$–, 4H), 1.880 (m, –NH$CH_2CH_2CH_2$NH–, 2H), 3.270-3.453 (m, –$CH_2N^+(CH_3)_2CH_2$– and $CH_3(CH_2)_9CH_2CH_2$–, 16H), 3.649 (m, –NH$CH_2CH_2CH_2$NH–, 4H), 4.677 (s, –$N^+(CH_3)_2CH_2$CO–, 4H), 8.857 (br s, –CO$NH$$CH_2$–, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.131, 22.690, 22.950, 26.270, 27.793, 29.161, 29.334, 29.432, 29.471, 31.911, 52.006, 63.343, 65.893, 163.127; HRMS (ESI): m/z calculated for $C_{34}H_{70}N_4O_2Br_2$ $[M–Br]^+$ and $[M–2Br]^{2+}$: 661.4986, 663.4964 and 291.2901. Found: 661.4959, 661.4945 and 291.2890; Elemental analysis: C 56.57, H 10.05, N 7.57 (calculated); C 56.49, H 9.92, N 7.49 (found).

4c: FTIR (Solid): 3212 cm^{-1} (amide N–H str.), 2915 cm^{-1} (–CH_2– assym. str.), 2858 cm^{-1} (–CH_2– sym. str.), 1675 cm^{-1} (amide I, C=O str.), 1560 cm^{-1} (amide II, N–H ben.), 1475 cm^{-1} (–CH_2– scissor); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.869 (t, terminal –CH_3, 6H), 1.288 (m, –$CH_3(CH_2)_9CH_2$–, 36H), 1.646 (m, $CH_3(CH_2)_9CH_2CH_2$–, 4H), 1.776 (m, –NH$CH_2(CH_2)_2CH_2$NH–, 4H), 3.302 (m, –NH$CH_2(CH_2)_2CH_2$NH–, 4H), 3.419 (s, –$CH_2N^+(CH_3)_2CH_2$–, 12H), 3.624 (m, $CH_3(CH_2)_9CH_2CH_2$–, 4H), 4.641 (s, –$N^+(CH_3)_2CH_2$CO–, 4H), 8.699 (br s, –CO$NH$$CH_2$–, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.237, 22.804, 23.045, 26.270, 26.341, 29.235, 29.440, 29.503, 29.555, 29.705, 32.023, 38.980, 52.197, 63.449, 66.363, 163.171; HRMS (ESI): m/z calculated for $C_{36}H_{76}N_4O_2Br_2$ $[M–Br]^+$ and [M–

2Br]$^{2+}$: 675.5142, 677.5121 and 298.2979. Found: 675.5119, 677.5104 and 298.2942; Elemental analysis: C 57.11, H 10.13, N 7.40 (calculated); C 57.04, H 9.98, N 7.33 (found).

4d: FTIR (Solid): 3212 cm^{-1} (amide N–H str.), 2920 cm^{-1} (–CH_2– assym. str.), 2860 cm^{-1} (–CH_2– sym. str.), 1675 cm^{-1} (amide I, C=O str.), 1560 cm^{-1} (amide II, N–H ben.), 1465 cm^{-1} (–CH_2– scissor); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.869 (t, terminal –CH_3, 6H), 1.288 (m, –CH_3*$(CH_2)_9$*CH_2–, 36H), 1.414 (m, –$NHCH_2CH_2$*$(CH_2)_2$*CH_2CH_2NH–, 4H), 1.586 (m, $CH_3(CH_2)_9$*CH_2*CH_2–, 4H), 1.779 (m, –$NHCH_2$*CH_2*$(CH_2)_2$*CH_2*CH_2NH–, 4H), 3.284 (m, –NH*CH_2*$(CH_2)_4$*CH_2*NH–, 4H), 3.429 (s, –CH_2N^+*$(CH_3)_2$*CH_2–, 12H), 3.653 (m, $CH_3(CH_2)_9CH_2$-*CH_2*–, 4H) 4.701 (m, –$N^+(CH_3)_2$*CH_2*CO–, 4H), 8.688 (br s, –CO*NH*CH_2–, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.236, 22.803, 23.046, 26.343, 26.720, 28.191, 29.227, 29.439, 29.498, 29.554, 29.706, 32.020, 39.529, 52.119, 63.412, 66.106, 163.119; HRMS (ESI): m/z calculated for $C_{38}H_{80}N_4O_2Br_2$ [M–Br]$^+$ and [M–2Br]$^{2+}$: 703.5456, 705.5434 and 312.3136. Found: 703.5440, 705.5427 and 312.3124; Elemental analysis: C 58.13, H 10.27, N 7.14 (calculated); C 58.04, H 10.21, N 7.06 (found).

5a: FTIR (Solid): 3219 cm^{-1} (amide N–H str.), 2928 cm^{-1} (–CH_2– assym. str.), 2861 cm^{-1} (–CH_2– sym. str.), 1680 cm^{-1} (amide I, C=O str.), 1555 cm^{-1} (amide II, N–H ben.), 1470 cm^{-1} (–CH_2– scissor); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.872 (t, terminal –CH_3, 6H), 1.311 (m, –CH_3*$(CH_2)_{13}$*CH_2–, 52H), 1.780 (m, $CH_3(CH_2)_{13}$*CH_2*CH_2–, 4H), 3.410-3.456 (m, –CH_2N^+*$(CH_3)_2$*CH_2– and $CH_3(CH_2)_{13}CH_2$*CH_2*–, 16H), 3.611 (m, –NH*CH_2CH_2*NH–, 4H), 4.671 (s, –$N^+(CH_3)_2$*CH_2*CO–, 4H), 8.943 (br s, –CO*NH*CH_2–, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.256, 22.830, 23.026, 26.371, 29.248, 29.503, 29.530, 29.583, 29.727, 29.801, 29.839, 32.066, 38.452, 52.311, 63.523, 66.112, 163.453; HRMS (ESI): m/z calculated for $C_{42}H_{88}N_4O_2Br_2$ [M–Br]$^+$ and [M–2Br]$^{2+}$: 759.6082, 761.6061 and 340.3449 Found: 759.6044, 761.6035 and 340.3584; Elemental analysis: C 59.96, H 10.55, N 6.66 (calculated); C 59.87, H 10.48, N 6.58 (found).

5b: FTIR (Solid): 3215 cm^{-1} (amide N–H str.), 2929 cm^{-1} (–CH_2– assym. str.), 2865 cm^{-1} (–CH_2– sym. str.), 1683 cm^{-1} (amide I, C=O str.), 1565 cm^{-1} (amide II, N–H ben.), 1475 cm^{-1} (–CH_2– scissor); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.871 (t, terminal –CH_3, 6H), 1.325 (m, –CH_3*$(CH_2)_{13}$*CH_2–, 52H), 1.785 (m, $CH_3(CH_2)_{13}$*CH_2*CH_2–, 4H), 1.883 (m, –$NHCH_2$*CH_2*CH_2NH–, 2H), 3.383-3.425 (m, –CH_2N^+*$(CH_3)_2$*CH_2– and $CH_3(CH_2)_{13}CH_2$*CH_2*–, 16H), 3.604 (m, –NH*CH_2CH_2*CH_2*NH–, 4H), 4.814 (s, –$N^+(CH_3)_2$*CH_2*CO–, 4H), 8.876 (br s, –CO*NH*CH_2–, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.250, 22.824, 22.989, 26.354, 27.646, 29.213, 29.497, 29.564, 29.717, 29.802, 29.828, 32.059, 52.088, 63.518, 66.144, 163.252; HRMS (ESI): m/z calculated for $C_{43}H_{90}N_4O_2Br_2$ [M–Br]$^+$ and [M–2Br]$^{2+}$: 773.6238,

775.6218 and 347.3527. Found: 773.6192, 775.6183 and 347.3605; Elemental analysis: C 60.39, H 10.61, N 6.55 (calculated); C 60.31, H 10.53, N 6.46 (found).

5c: FTIR (Solid): 3215 cm^{-1} (amide N–H str.), 2925 cm^{-1} (–CH_2– assym. str.), 2861 cm^{-1} (–CH_2– sym. str.), 1675 cm^{-1} (amide I, C=O str.), 1565 cm^{-1} (amide II, N–H ben.), 1475 cm^{-1} (–CH_2– scissor); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.868 (t, terminal –*CH_3*, 6H), 1.289 (m, –CH_3*$(CH_2)_{13}$*CH_2–, 52H), 1.645 (m, $CH_3(CH_2)_{13}$*CH_2*CH_2–, 4H), 1.787 (m, –$NHCH_2$*$(CH_2)_2$*CH_2NH–, 4H), 3.299 (m, –$NHCH_2(CH_2)_2$*CH_2*NH–, 4H), 3.418 (s, –CH_2N^+*$(CH_3)_2$*CH_2–, 12H), 3.624 (m, $CH_3(CH_2)_9CH_2$*CH_2*–, 4H), 4.648 (s, –$N^+(CH_3)_2$*CH_2*CO–, 4H), 8.711 (br s, –CO*NH*CH_2–, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.243, 22.816, 23.048, 26.278, 26.350, 29.247, 29.487, 29.516, 29.574, 29.720, 29.784, 29.807, 29.826, 32.053, 38.974, 52.1990, 63.453, 66.379, 163.179; HRMS (ESI): m/z calculated for $C_{44}H_{92}N_4O_2Br_2$ $[M–Br]^+$ and $[M–2Br]^{2+}$: 787.6395, 789.6374 and 354.3606. Found: 787.6352, 789.6343 and 354.3759; Elemental analysis: C 60.79, H 10.68, N 6.45 (calculated); C 60.71, H 10.59, N 6.41 (found).

5d: FTIR (Solid): 3215 cm^{-1} (amide N–H str.), 2921 cm^{-1} (–CH_2– assym. str.), 2865 cm^{-1} (–CH_2– sym. str.), 1674 cm^{-1} (amide I, C=O str.), 1560 cm^{-1} (amide II, N–H ben.), 1465 cm^{-1} (–CH_2– scissor); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.876 (t, terminal –*CH_3*, 6H), 1.337 (m, –CH_3*$(CH_2)_{13}$*CH_2–, 52H), 1.421 (m, –$NHCH_2CH_2$*$(CH_2)_2$*CH_2CH_2NH–, 4H), 1.587 (m, $CH_3(CH_2)_{13}$*CH_2*CH_2–, 4H), 1.779 (m, –$NHCH_2$*CH_2*$(CH_2)_2$*CH_2*CH_2NH–, 4H), 3.299 (m, –NH*CH_2*$(CH_2)_4$*CH_2*NH–, 4H), 3.429 (s, –CH_2N^+*$(CH_3)_2$*CH_2–, 12H), 3.644 (m, $CH_3(CH_2)_{13}CH_2$*CH_2*–, 4H), 4.681 (m, –$N^+(CH_3)_2$*CH_2*CO–, 4H), 8.682 (br s, –CO*NH*CH_2–, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.241, 22.815, 23.050, 26.350, 26.737, 28.180, 29.237, 29.486, 29.511, 29.571, 29.717, 29.783, 29.805, 29.824, 32.052, 39.538, 52.122, 63.420, 66.111, 163.136; HRMS (ESI): m/z calculated for $C_{46}H_{96}N_4O_2Br_2$ $[M–Br]^+$ and $[M–2Br]^{2+}$: 815.6708, 817.6687 and 368.3763. Found: 815.6661, 817.6652 and 368.3823; Elemental analysis: C 61.57, H 10.79, N 6.25 (calculated); C 61.47, H 10.71, N 6.20 (found).

1.3. Microorganisms and culture conditions: The antibacterial activity of all the amphiphilic small molecules was evaluated against both Gram-positive bacteria (*S. aureus*, MRSA and VRE) and Gram-negative bacteria (*E. coli*, *P. aeruginosa*, *K. pneumoniae*). *S. aureus*, MRSA and *K. pneumoniae* were cultured in nutrient broth (1.0 g of beef extract, 2.0 g of yeast extract, 5.0 g of peptone, and 5.0 g of NaCl in 1000 mL of distilled water) while *E. coli* was grown in Luria-Bertani broth (10.0 g of tryptone, 5.0 g of yeast extract, and 10.0 g of NaCl in 1000 mL of distilled water). Brain-heart infusion broth (5.0 g beef heart infusion

form, 12.5 g of calf brains infusion form, 2.5 g Na_2HPO_4, 2.0 g D-glucose, 10 g of peptone and 5.0 g NaCl in 100 mL of sterile distilled water) was used for VRE as growth medium. For solid media, 2.0-2.5% agar was used along with the above mentioned growth medium. The freeze dried stock samples of bacteria in 30% glycerol were stored at −80 ºC. About 3 µL of these stock solutions were added to 3 mL of the respective growth medium and the cultures were grown at 37 ºC for 6 h prior to the antibacterial experiments.

1.4. Antibacterial assay (minimum inhibitory concentration): Antibacterial efficacy of all the small molecular compounds (**2a-2d, 3a-3d, 4a-4d** and **5a-5d**) were assayed in a micro-dilution broth method as described in CLSI guideline.[1] The 6 h grown culture as described in the microorganism and culture condition section gives about 10^9 CFU/mL of bacteria (determined by spread plating method). The bacterial cultures were then diluted to give approximately 10^5 CFU/mL in different media (nutrient media for *S. aureus*, MRSA, *P. aeruginosa* and *K. pneumonia*, Luria-Bertani for *E. coli* and brain-heart infusion media for VRE) which were then used for determining antibacterial efficacy. All the final compounds except **5c** and **5d** were water soluble at room temperature. Compounds **5c** and **5d** were solubilised in water by heating. Stock solutions were prepared by serial dilution of all the compounds using sterilized Milli-Q water. These dilutions (50 µL) were added to the wells of 96 well plate followed by the addition of about 150 µL of bacterial suspension (~10^5 CFU/mL). The plates were then incubated at 37 °C for 24 h. After the incubation, the optical density (OD) of the bacterial suspension was recorded using TECAN (Infinite series, M200 pro) Plate Reader at 600 nm. Each concentration was added in triplicate and the whole experiment was repeated at least twice. Finally, the antibacterial efficacy was determined by taking the average of triplicate OD values for each concentration and plotting it against concentration. The data was then subjected to sigmoidal fitting and from the curve the antibacterial activity was determined as the point where the OD value was similar to that of control having no bacteria. The antibacterial activity was thus expressed as minimum inhibitory concentration (MIC). A glycopeptides antibiotic vancomycin and a lipopeptide antibiotic colistin were used to compare the antibacterial efficacy in this study. For determining the minimum bactericidal concentration (MBC), the bacterial suspension that appeared to have less/little turbidity in the MIC experiment was plated (3 µL) and the agar plates were incubated for 24 h at 37 °C. Concentration at which no bacterial growth (no bacterial colony) was observed was taken as the MBC of the respective small molecules.

1.5. Antibacterial assay in presence of human plasma (Plasma stability): Bacteria (*S. aureus*) was grown in a similar way as mentioned in the microorganism and culture condition and finally diluted in the respective media to give ~10^5 CFU/mL. Fresh heparinised human blood donated by a healthy donor was centrifuged at 3500 rpm for 5 min. The plasma, separated from the blood cells after centrifugation, was carefully collected. The test molecule **2d** was dissolved in sterile Milli-Q water at a concentration of 2000 μg/mL. This was further diluted 2-fold into the plasma so that the final concentration of compound **2d** was 1000 μg/mL in 50% plasma. Three such test samples were preincubated at 37 °C in 50% plasma for 0 h, 3 h and 6 h respectively. Then 50 μL of the above solutions was added to wells of a 96-well plate and 150 μL of the bacterial suspension (~10^5 CFU/mL) was added to wells containing the test samples in 50% plasma. The well plate was then incubated for 24 h at 37 °C and antibacterial efficacy of the test compound was determined as described above for antibacterial assay in the previous section.

1.6. Antibacterial assay in complex mammalian fluids: Blood was donated by healthy human donors (sodium heparin was used as anticoagulant). Plasma was obtained by centrifugation of the blood at 3500 rpm for 5 min as mentioned above. Serum was isolated by using SST™ II Advance serum tube (BD vacutainer) (Ref 367956) containing human blood and then centrifuging the blood at 3500 rpm for 5 min. Methicillin-resistant *S. aureus* (MRSA) was grown in way as mentioned in the microorganism and culture conditions. Finally, MRSA was diluted in minimum essential medium (MEM) and mixed with the mammalian media (serum, plasma, blood) individually in a way to give ~10^5 CFU/mL of MRSA in 50% serum, 50% plasma, and 50% blood (having 50% MEM medium). The test molecule **2d** was dissolved in sterile water at a concentration of 4000 μg/mL. This was further diluted 2-fold and then 50 μL of the solutions was added to the wells of a 96-well plate. Then, 150 μL of the bacterial suspension (10^5 CFU/mL) in 50% serum, 50% plasma and 50% blood was added separately to the wells containing the dilutions. The plate was then incubated for 24 h at 37 °C and bactericidal efficacy of the test compound was determined by plating the bacterial suspension (20 μL) directly from the wells onto nutrient agar plate. The agar plates were incubated at 37 °C for 24 h and colonies were observed to determine the MBC.

1.7. Bactericidal time-kill assay: The bactericidal kinetics, i.e. the rate at which the compounds kill bacteria was evaluated by performing time kill kinetics.[2] Briefly, bacteria (*S. aureus* and *E. coli*) were grown in suitable growth medium at 37 ºC for 6 h as mentioned in

microorganism and culture conditions. The compound **2d** was added to the bacterial suspension (150 μL of *S. aureus* of approximately 1.8×10^5 CFU/mL and 50 μL of 7.8 and 45.6 μg/mL of **2d**) and (150 μL of *E. coli* of approximately 1.5×10^5 CFU/mL and 50 μL of 15.6 and 93.6 μg/mL of **2d**) in 96-well plate. The plate was then incubated at 37 °C. At different time intervals (0, 30, 60, 90, 120, 240 and 360 min), 10 μL of aliquots from the bacterial suspension was withdrawn and was subjected to 10-fold serial dilution in 0.9 % saline. 20 μL of the dilution was plated on solid agar plates and incubated at 37 ºC for 24 h. A similar experiment was performed by suspending both *S. aureus* and *E. coli* bacteria in HEPES: glucose buffer (1:1) with **2d** at 24 μg/mL. The bacterial colonies were counted and results are represented in logarithmic scale, i.e. $\log_{10}$ (CFU/mL). Water (50 μL) was used as control in all the experiments.

1.8. Biofilm disruption assay.

1.8.1. Determination of viable count and imaging.

Method 1. Bacteria (*S. aureus* and *E. coli*) (6 h grown, midlog phase) were diluted to ~10^5 CFU/mL into suitable media (nutrient media supplemented with 1% glucose and 1% NaCl for *S. aureus* and M9 media supplemented with 0.02% casamino acid and 0.5% glycerol for *E. coli* respectively). The 96-well plates containing 100 μL of these suspensions were incubated under stationary conditions (for about 24 h for *S. aureus* and 72 h for *E. coli*).[3] After incubation, the medium was decanted and the wells were washed with 1X PBS once. Compound **2d** (100 μL at 4, 8, 16, 32 and 64 μg/mL) was then added to the wells containing preformed bacterial biofilms and allowed to incubate for 24 h. A control was made where 100 μL of the above medium was added. After 24 h, medium was discarded and the planktonic cells were removed by washing with 1X PBS. Then 100 μL of trypsin-EDTA solution was added to the treated biofilm to make the suspension of bacterial cells present within the biofilm. Cell suspension was then assessed by plating the 10-fold serial dilutions of the suspension on suitable agar plates. After 24 h of incubation, bacterial colonies were counted and cell viability was expressed as $\log_{10}$ (CFU/well) along with the control. For visualizing the disruption of biofilm by the small molecules, 100 μL of 0.1% of crystal violet (CV) was added into the wells and incubated for 10 min. Then the crystal violet solution was discarded and the plates were washed once with 1X PBS. Finally, imaging of the stained wells was taken using a digital camera.

Method 2. Cover slips (18 mm diameter) were first sterilised by dipping them in ethanol followed by drying in flame. The sterilised cover slips were then placed in wells of a 6-well

plate. Midlog phase (6 h grown) culture were then diluted to approximately 10^5 CFU/mL (2 mL) in nutrient media supplemented with 1% glucose and 1% NaCl for *S. aureus* and M9 medium supplemented with 0.02% casamino acid and 0.5% glycerol for *E. coli* and were added to the wells containing cover slips. The plate was then incubated under stationary conditions to allow the formation of bacterial biofilm. After 24 h for *S. aureus* and 72 h for *E. coli*, medium was removed and planktonic bacteria were carefully washed once with 1X PBS (pH = 7.4). Biofilm containing cover slips were then placed into the wells of another 6-well plate. Compound **2d** (2 mL at 4, 8, 16, 32 and 64 μg/mL) was then added to the wells containing cover slip with preformed bacterial biofilms onto it and allowed to incubate for 24 h. A control was made where 2 mL of the above medium was added. After 24 h, medium was discarded and the planktonic cells were removed by washing with 1X PBS. Then 2 mL of trypsin-EDTA solution (diluted in saline at 1:4 ratio) was added to the treated biofilm to make the suspension of bacterial cells present within the biofilm. Cell suspension was then assessed by plating the 10-fold serial dilutions of the suspension on suitable agar plates. After 24 h of incubation of the agar plate, bacterial colonies were counted and cell viability was expressed as $\log_{10}$(CFU/mL) along with the control.

1.8.1. Confocal laser scanning microscopy (CLSM) imaging of biofilms. Cover slips were first sterilised by dipping them in ethanol followed by drying in flame. The sterilised cover slips were then placed in wells of a 12-well plate. Midlog phase (6 h grown) culture of *S. aureus* and *E. coli* were then diluted to approximately 10^5 CFU/mL in nutrient medium supplemented with 1% glucose and 1% NaCl for *S. aureus* and M9 medium supplemented with 0.02% casamino acid and 0.5% glycerol for *E. coli* respectively) for *E. coli* and were added to the wells containing cover slips (2 mL). The plate was then incubated under stationary conditions at 37 ºC to allow the formation of bacterial biofilm. After 24 h, medium was removed and planktonic bacteria were carefully washed once with 1X PBS (pH = 7.4). Biofilm containing cover slips were then placed into the wells of another 12-well plate and 2 mL of test compound **2d** dissolved in the above media (at 64 μg/mL) was added to the wells containing cover slips with preformed bacterial biofilm and allowed to incubate for 24 h. In case of control, 2 mL of media was used instead of the compound. After 24 h, medium was discarded and cover slips were washed once with 1X PBS. Cover slips containing biofilms (both treated and non-treated) were stained with SYTO-9 (5 μM, 10 μL) and imaged using a confocal laser-scanning microscopy (Zeiss 510 Meta Confocal Microscope).

1.9. Mechanism of action.

1.9.1. Cytoplasmic membrane depolarization assay: The 6 h grown bacteria (midlog phase) were harvested (3500 rpm, 5 min), washed in 5 mM glucose and 5 mM HEPES buffer (1:1) (pH = 7.2) and resuspended in 5 mM HEPES buffer, 5 mM glucose and 100 mM KCl solution at 1:1:1 ratio. Bacterial suspension (~10^8 CFU/mL, 150 μL) was added to the wells of a 96-well plate (Black plate, clear bottom with lid). Then 3, 3′-dipropylthiadicarbocyanine iodide ($diSC_35$) (8 μM, 50 μL) was added to the wells containing bacterial suspension and pre-incubated for about 30 min for *S. aureus* and 40 min for *E. coli* (additional 50 μL of 200 μM of EDTA was also added in case of *E. coli*). After the incubation, fluorescence was measured for the next 8 min at every 2 min interval at an excitation wavelength of 622 nm (slit width: 10 nm) and emission wavelength of 670 nm (slit width: 5 nm). Bacterial suspensions were then transferred to another well-plate containing 10 μL of 840 μg/mL of small molecules and fluorescence intensity was monitored immediately for another 12 min at every 2 min interval. A control experiment was performed by treating the preincubated bacterial suspension and dye solution only with sterile Milli-Q water (50 μL).

1.9.2. K^+ ion leakage assay: Bacteria were grown similarly and were harvested (3500 rpm, 5 min), washed and resuspended in 10 mM HEPES buffer and 0.5% glucose in 1:1 ratio. Then 150 μL of the bacterial suspension (~10^8 CFU/mL) was transferred into the wells of a 96-well plate (Black plate, clear bottom with lid). Then PBFI-AM dye (4 μM, 50 μL) was added to the wells containing bacterial suspension and preincubated for about 30 min for *S. aureus* and 40 min for *E. coli*. After the incubation, fluorescence intensity was measured for next 8 min at every 2 min interval at an excitation wavelength of 346 nm (slit width: 10 nm) and emission wavelength of 505 nm (slit width: 5 nm). Then bacterial suspensions were transferred to another black well-plate containing 10 μL of 840 μg/mL of small molecules and fluorescence intensity was monitored immediately for another 12 min at every 2 min interval. A control experiment was similarly performed by treating the preincubated bacterial suspension and dye solution only with Milli-Q water (50 μL). None of the amphiphilic small molecules showed K^+ ion leakage (data not shown).

1.9.3. Outer membrane permeabilization assay: The outer membrane permeabilization activity of the small molecules was determined by the *N*-phenylnapthylamine (NPN) assay. Midlog phase bacteria (*E. coli*) were harvested similarly as mentioned in earlier experiments, washed, and resuspended in 5 mM glucose/5 mM HEPES buffer (1:1) pH 7.2. Bacterial suspension (~10^8 CFU/mL, 150 μL) was transferred into the wells of a black 96-

well plate. Then NPN dye (10 μM, 50 μL) was added to the wells containing bacterial suspension and preincubated for about 30 min for *S. aureus* and 40 min for *E. coli*. After the incubation, fluorescence was monitored for next 8 min at every 2 min interval at an excitation wavelength of 350 nm (slit width: 10 nm) and emission wavelength of 420 nm (slit width: 5 nm). Then, the bacterial suspensions were transferred to another black well-plate containing 10 μL of 840 μg/mL of small molecules and fluorescence intensity was monitored immediately for another 12 min at every 2 min interval. A control experiment was similarly performed by treating the preincubated bacterial suspension and dye solution only with Milli-Q water (50 μL).

1.9.4. Inner membrane permeabilization assay: Bacteria (*S. aureus* and *E. coli*) were grown similarly and harvested, washed, and resuspended in 5 mM glucose and 5 mM HEPES buffer (1:1) (pH = 7.2). Bacterial suspension (~10^8 CFU/mL, 150 μL) was added to wells of a black 96-well plate. Then propidium iodide (PI) (10 μM, 50 μL) was added to the wells containing bacterial suspension and pre-incubated for about 30 min for *S. aureus* and 40 min for *E. coli*. After the incubation, fluorescence was measured for next 8 min at every 2 min interval at an excitation wavelength of 535 nm (slit width: 10 nm) and emission wavelength of 617 nm (slit width: 5 nm). Then, the bacterial suspensions were transferred to another black well-plate containing 10 μL of 840 μg/mL of small molecules and fluorescence intensity was monitored immediately for another 12 min at every 2 min interval. A control experiment was similarly performed by treating the preincubated bacterial and dye solution only with Milli-Q water (50 μL).

1.10. Propensity of bacterial resistance development: In order to evaluate the propensity of developing bacterial resistance towards the small molecules, one of the potent compounds **2d** was used in the study.[4] First MIC of **2d** was determined against both Gram-positive and Gram-negative *S. aureus* and *E. coli* in a way as described in antibacterial assay previously and subsequently the compound was challenged repeatedly at the sub-MIC level. Two control antibiotics norfloxacin and colistin were chosen for *S. aureus* and for *E. coli*, respectively. In case of norfloxacin and colistin also, the initial MIC values were determined against respective bacteria. After the initial MIC experiment, serial passaging was initiated by transferring bacterial suspension grown at the sub-MIC of the compound/antibiotics (at MIC/2) and was subjected to another MIC assay. After 24 h incubation period, cells grown at the sub-MIC of the test compound/antibiotics were once again transferred and assayed for MIC experiment. The process was repeated for 20 passages for both *S. aureus* and *E. coli*

respectively. The fold increase in MIC for test compound to the control antibiotics was plotted against the number of passages/days to determine the propensity of bacterial resistance development.

1.11. Cytotoxicity assays.

1.11.1. Hemolytic activity: Red blood cells (RBCs) were isolated from freshly drawn, heparinised human blood and resuspended in 1X PBS (5 vol%). RBC suspension (150 μL) was then added to solutions of serially diluted small molecules in a 96-well plate (50 μL). Two controls were prepared, one without the compounds and the other with 50 μL of 0.1 vol% solution of Triton X-100. The plate was then incubated for 1 h at 37 °C. After the incubation, the plate was centrifuged at 3500 rpm for 5 minutes. Supernatant (100 μL) from each well was then transferred to a fresh 96-well plate and absorbance at 540 nm was measured. Percentage of hemolysis was determined as $(A–A_o)/(A_{total}–A_o)\times100$, where A is the absorbance of the test well, A_o is the absorbance of the negative control (the wells having no compound), and A_{total} the absorbance of completely lysed cells (wells with Triton X-100), all at 540 nm.[5]

1.11.2. LDH assay: Cytotoxicity assay kit (CytoTox 96 Non-Radioactive, Promega) was used for determining the cytotoxicity of the small molecules. In brief, human embryo kidney (HEK 293) cells, maintained in complete DMEM media (Gibco) supplemented with 10% FBS (Gibco) and penicillin-streptomycin solution (Gibco), were seeded in 96 well plates at a concentration of 10^4 cells/well. Cells were then allowed to adhere to the plate overnight. Triton-X (0.5 vol%) and only media were used as positive and negative controls respectively. The seeded cells were then treated with the most potent compound (**2d**). After 24 hrs of incubation, the plate was centrifuged at 1100 rpm for 5 min. The supernatants from respective wells were transferred and the assay was performed according to the manufacturer's protocol. Supernatant (100 μL) was transferred to a fresh 96-well plate and absorbance at 490 nm was measured using a Tecan InfinitePro series M200 Micro plate Reader. Percentage of cell death was determined as $(A –A_0)/ (A_{total}–A_0) \times 100$, where A is the absorbance of the test well, A_0 is the absorbance of the negative controls, and A_{total} is the absorbance of triton-X treated wells, all at 490 nm. Percentage of LDH release was plotted as a function of concentration of the small molecule and the half-maximal inhibitory concentration (IC_{50}) was defined as the compound concentration that caused 50% LDH release relative to the positive control.

1.11.3. Fluorescence microscopy: As mentioned above for the LDH assay, cells were seeded into the wells of a 96-well plate and then treated with compounds (**2d** and **3a**) at various

concentrations (8, 16, 32, 64 and 128 μg/mL). For positive control 0.1% triton-X was used. All the treated and untreated cells (as negative control) were washed once with 1X PBS and stained with 2 μM calcein AM (Fluka) and 4.5 μM propidium iodide (PI) for (Sigma-Aldrich) (50 μL of 1:1 calcein AM:PI) 15 min at 37 °C under 5% CO_2-95% air atmosphere. Finally, the cells were washed and images were captured with a 10X objective in Leica DM2500 fluorescence microscope using a band-pass filter for calcein AM at 500-550 nm and a long-pass filter for PI at 590-800 nm.

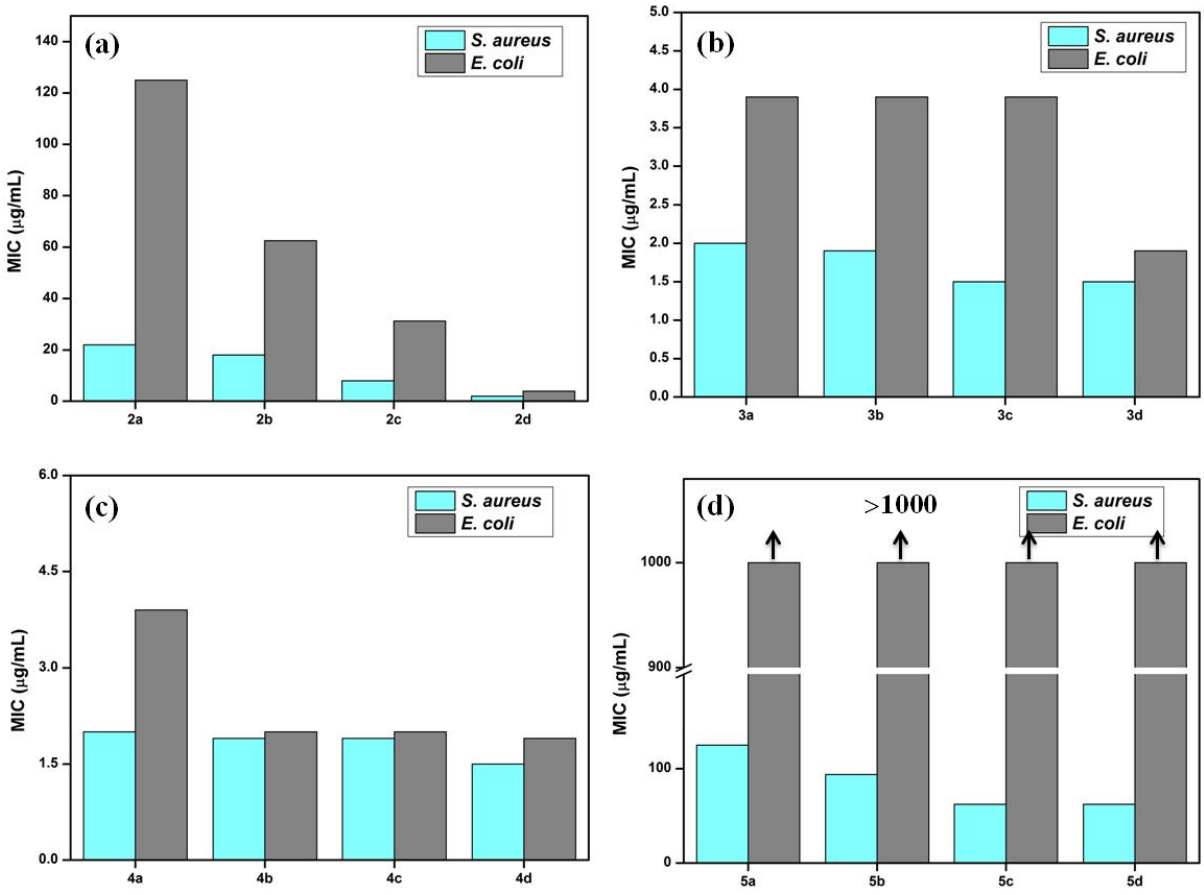

Fig. S1. Antibacterial activity of the small molecules. MIC of compounds **2a**, **2b**, **2c** and **2d** (a); **3a**, **3b**, **3c** and **3d** (b); **4a**, **4b**, **4c** and **4d** (c); **5a**, **5b**, **5c** and **5d** (d) in growth medium aganist *S. aureus* and *E. coli* respectively.

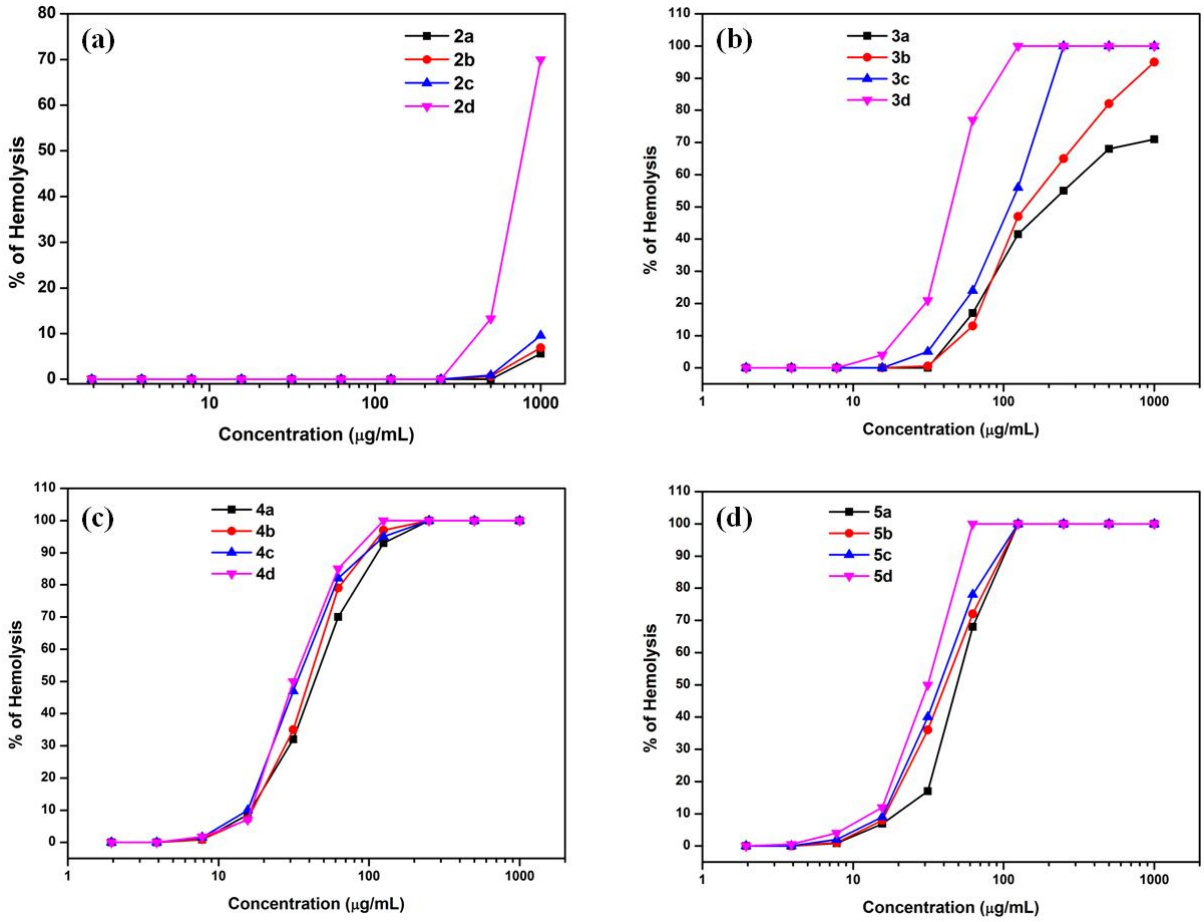

Fig. S2. Hemolytic activity of small molecules. % of Hemolysis of compounds **2a**, **2b**, **2c** and **2d** (a); **3a**, **3b**, **3c** and **3d** (b); **4a**, **4b**, **4c** and **4d** (c); **5a**, **5b**, **5c** and **5d** (d) at different concentrations.

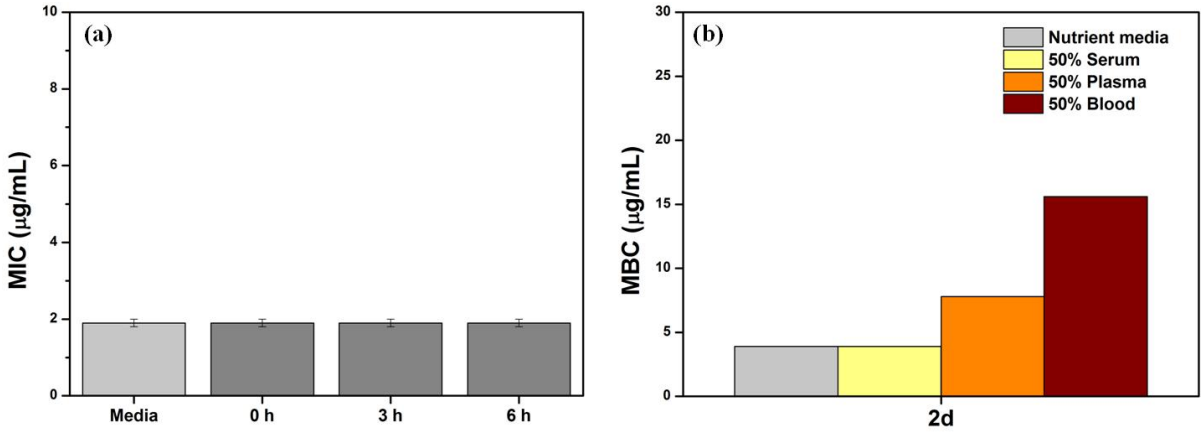

Fig S3. Plasma stability and antibacterial activity in complex mammalian fluids of small molecule: (a) antibacterial efficacy of compound **2d** against *S. aureus* after preincubating in 50% plasma for different period of time (0 h, 3 h and 6 h; (b) minimum bactericidal concentrations (MBCs) of **2d** in 50% serum, 50% plasma and 50% blood against MRSA.

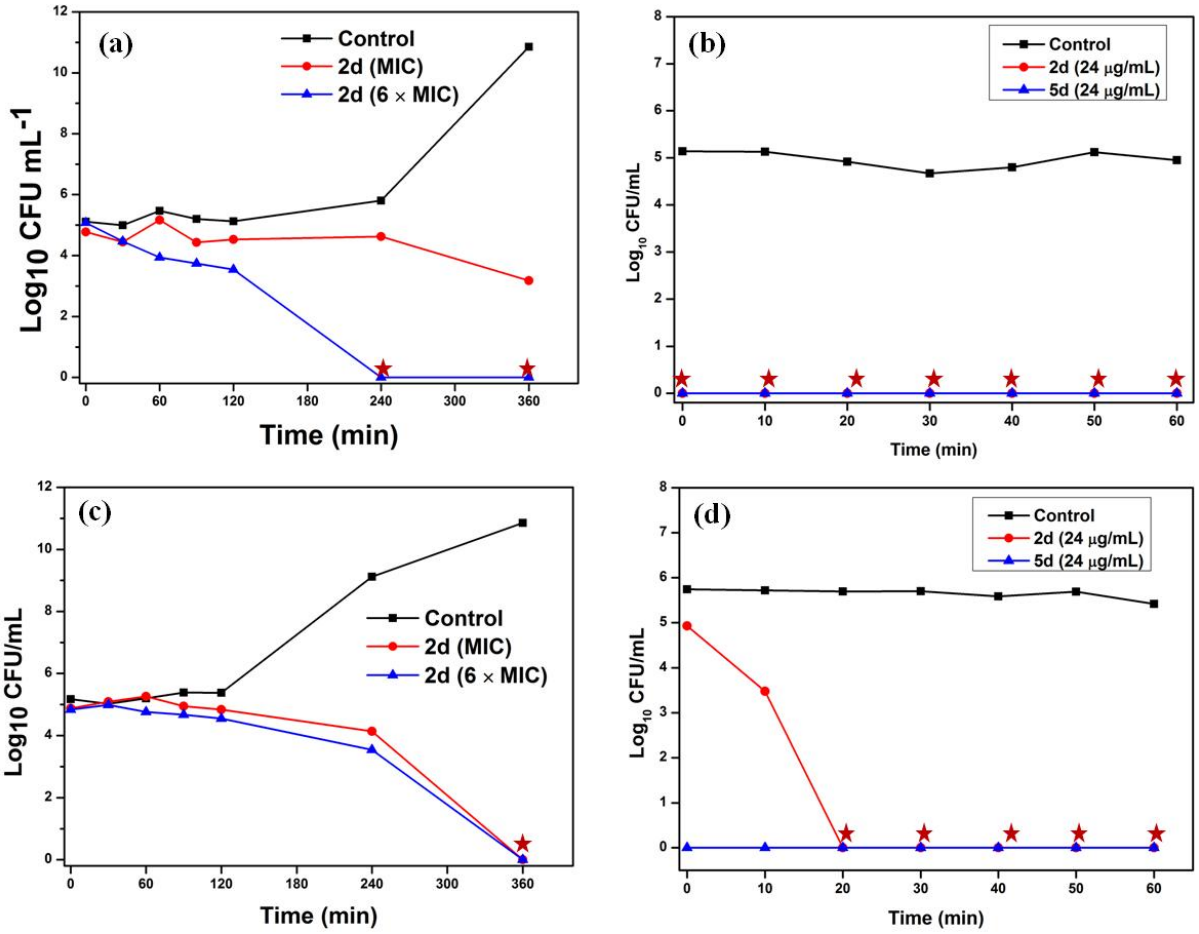

Fig S4. Time-kill kinetics of compound **2d** against *S. aureus* (a) at MIC and 6 × MIC in growth medium (nutrient medium) and (b) in buffer (HEPES: glucose = 1:1) at 24 μg/mL. Time-kill kinetics of compounds **2d** against *E. coli* (c) at MIC and 6 × MIC in growth medium (LB) and (d) in buffer (HEPES: glucose = 1:1) at 24 μg/mL respectively. Star represents < 50 CFU/mL.

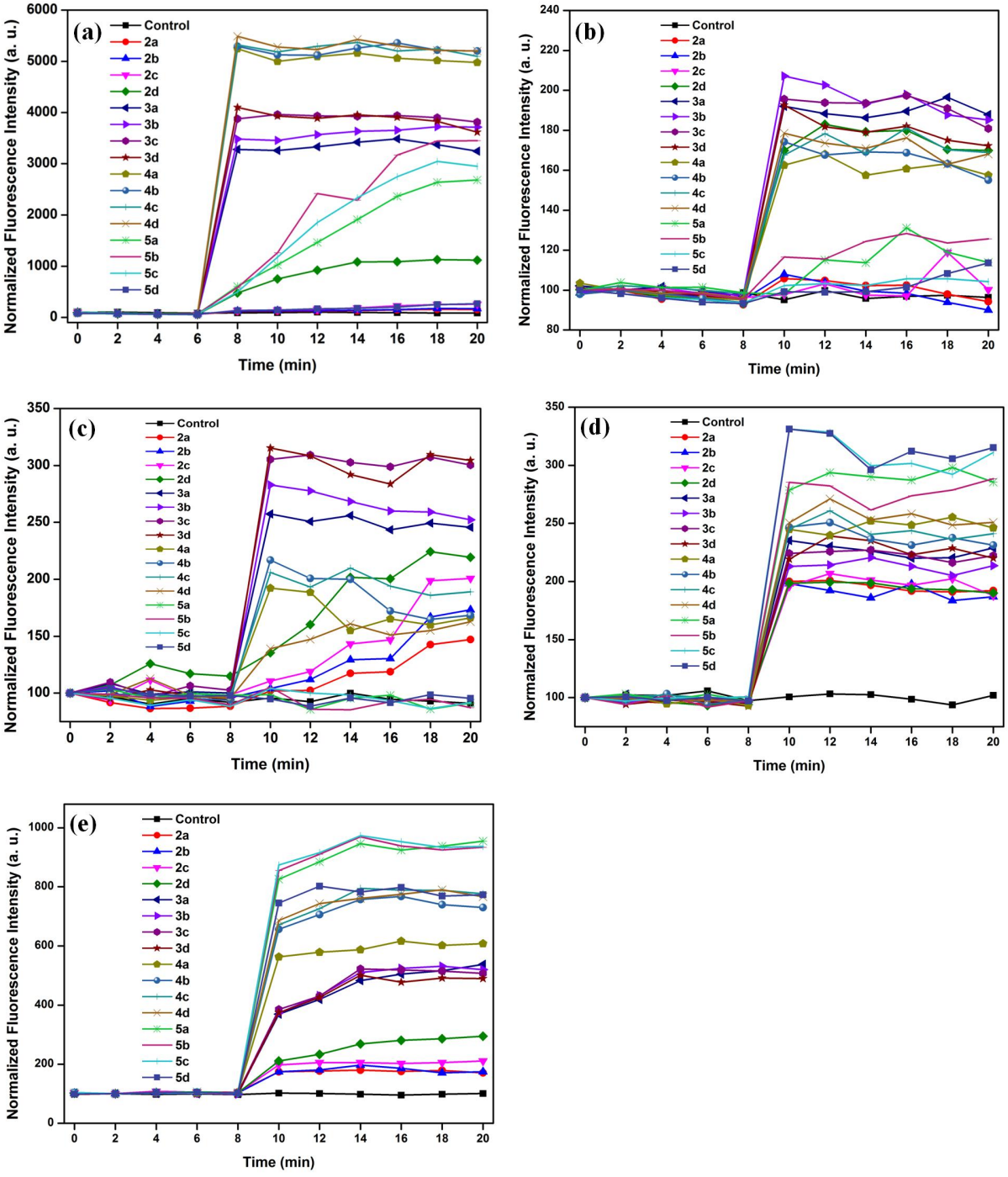

Fig. S5. Mechanism of antibacterial action of the cationic small molecules: (a) and (b) membrane depolarization of *S. aureus* and *E. coli* respectively; (c) and (d) cytoplasmic membrane permeabilization of *S. aureus* and *E. coli* respectively; (e) outer membrane permeabilization of *E. coli* by the amphiphilic small molecules.

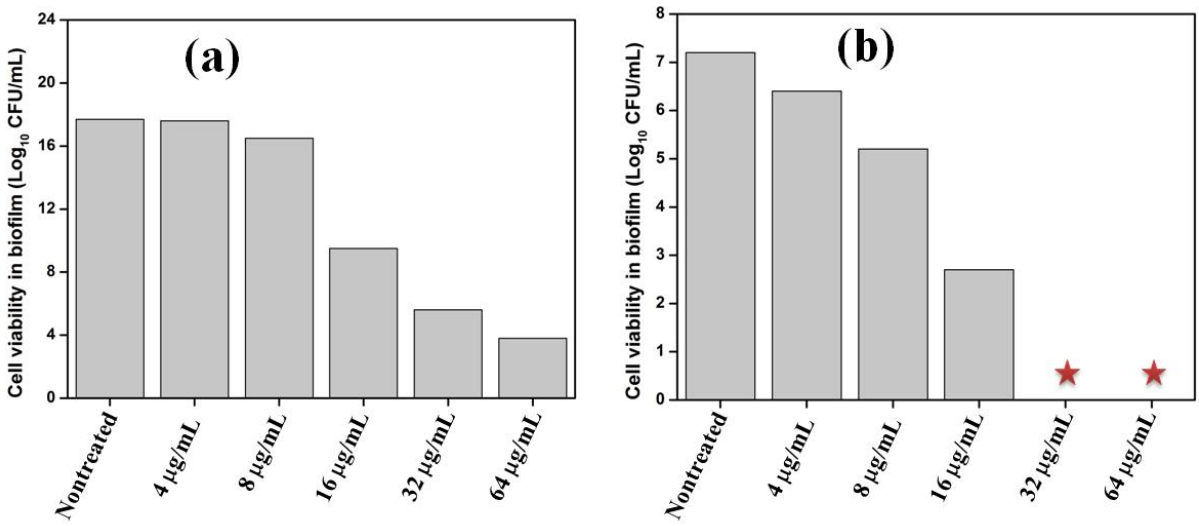

Fig. S6. Antibiofim activity of small molecule **2d**. (a) Cell viability in non-treated and treated biofilms of *S. aureus* grown on cover slip for 24 h and after treating the biofilms at different concentration of **2d**; (b) Cell viability in non-treated and treated biofilms of *E. coli* grown on cover slip for 72 h and after treating the biofilms at different concentrations of **2d**. Star represents <50 CFU/mL.

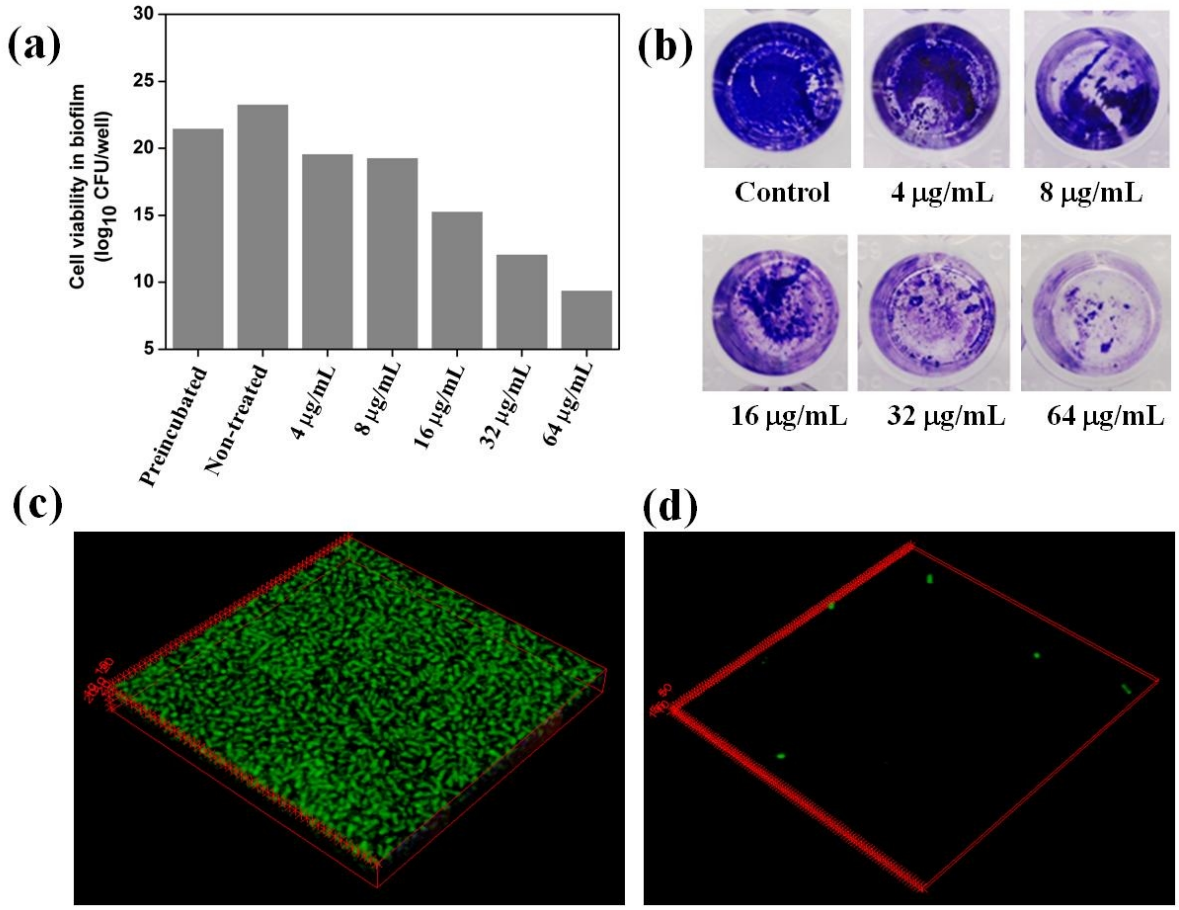

Fig. S7. Antibiofim activity of small molecule **2d**. (a) Cell viability in non-treated and treated biofilms of *E. coli* grown in 96-well plate for 72 h; (b) Images of the small molecule treated and non-treated biofilms of *E. coli* (grown in 96-well plate for 72 h) after staining with crystal violet; (c) and (d) CLSM images of non-treated and treated *E. coli* biofilm (grown on cover slips for 72 h) after treating the biofilm with **2d** and staining with SYTO 9.

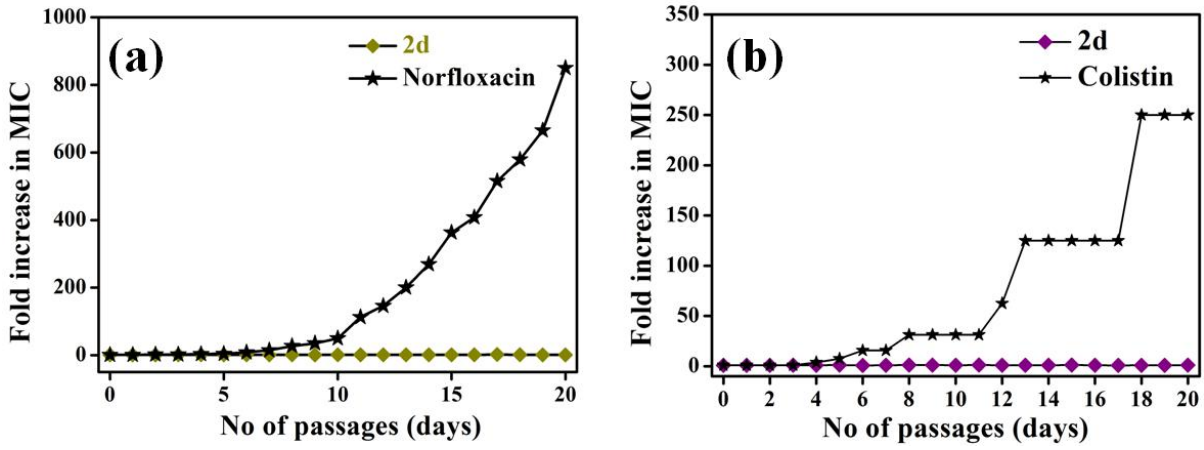

Fig. S8. Propensity of development of bacterial resistance against compound **2d**: (a) for *S. aureus* where antibiotic norfloxacin was used as control (b) for *E. coli* where lipopeptide colistin was used as control.

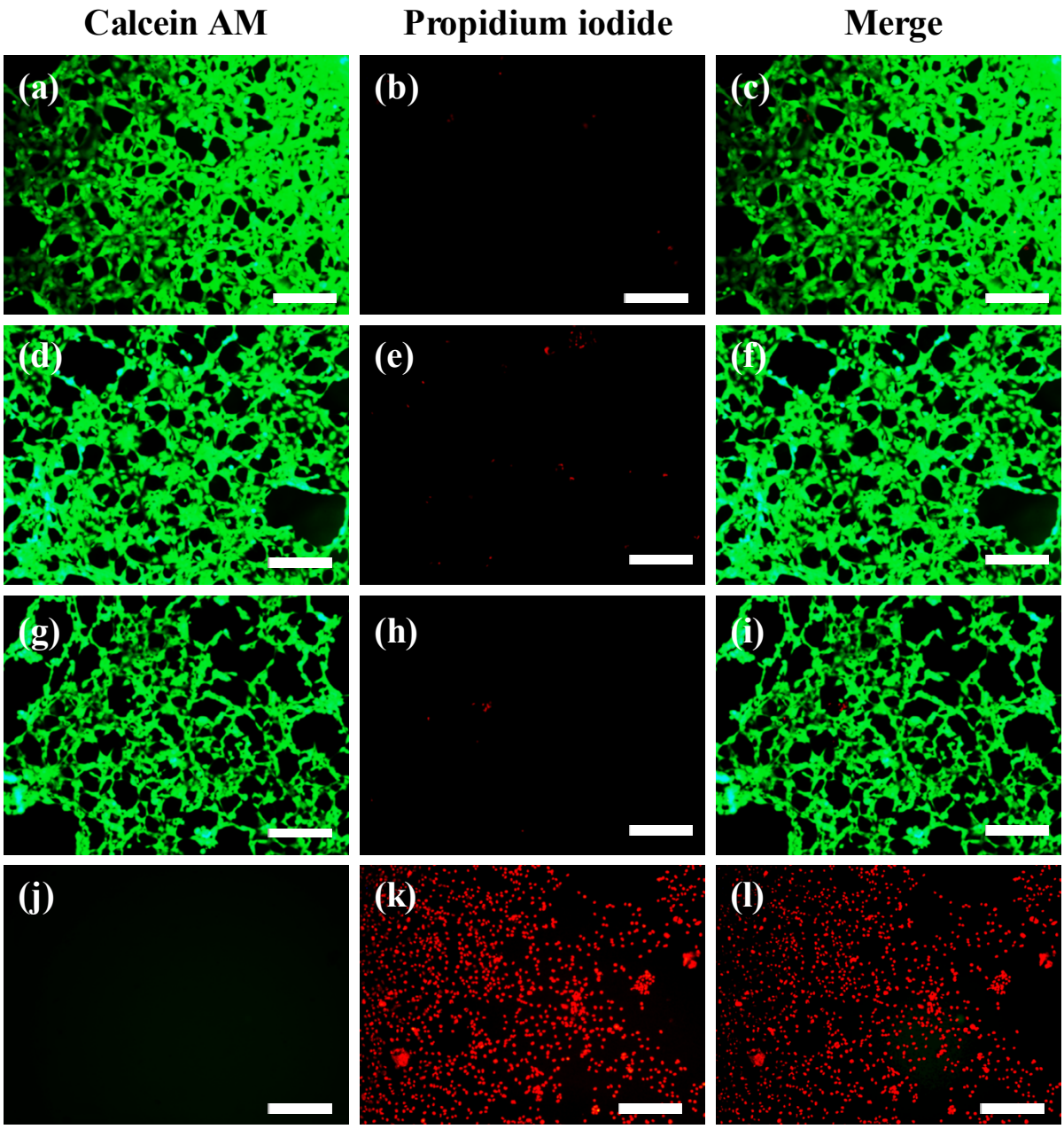

Fig. S9. Fluorescence microscopy images of HEK cells after treatment with small molecule **3a** for 24 h and staining with calcein AM and propidium iodide (PI): (a-c) non-treated cells (negative control); (d-f) cells treated with **3a** (64 μg/mL); (g-i) cells treated with **3a** (128 μg/mL); and (j-l) cells treated with 0.1% triton X (positive control). Scale bar is 20 μm.

Table S1. Minimum bactericidal concentration (MBC) of small molecules in buffer and LB medium

Compound	MBC (μg/mL)							
	Buffer (5 mM HEPES: 5 mM glucose = 1:1)				LB broth			
	1 h	2 h	4 h	6 h	1 h	2 h	4 h	6 h
2d	31.2	3.9	3.9	3.9	125	62.5	31.2	15.6
5d	7.8	3.9	3.9	3.9	>1000	>1000	>1000	>1000

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