Supplementary Information

Selectively Targeting Bacteria by Tuning the Molecular Design of Membrane-Active Peptidomimetic Amphiphiles

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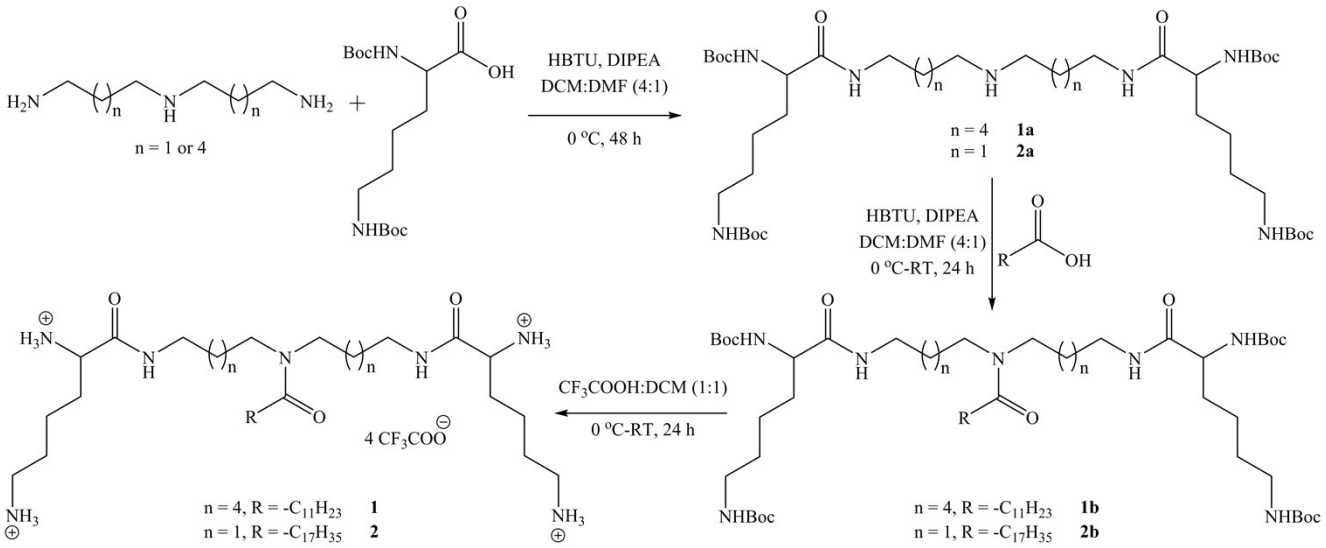
Details of Synthesis and Characterization

Scheme S1: Synthesis of compound **1** and **2**



Protocol for synthesizing 1a and 2a: At first, 2 equivalents of Boc-Lys (Boc)-OH was dissolved in dry DCM at 0 °C. Then, 6 equivalents of DIPEA was added to it followed by 2 equivalents of HBTU. Then, DMF (1/4 volume of DEM) was added to the reaction mixture and was stirred for 10-15 min. Next, 1 equivalent of bis(hexamethylene)triamine or bis(trimethylene)triamine was added drop wise after dissolving it in DCM and allowed to stir for 48 h. In the end, the reaction solvent was romoved by using rotary evaporator and crude residue was dissolved in ethyl acetate. Work-up was then performed with 1(N) HCl and saturated Na_2CO_3 solution, respectively and the organic layer was collected through anhydrous Na_2SO_4. Finally, the compounds was purified by performing column chromatography on 60-120 mesh silica gels (using various ratios of $CHCl_3$ and MeOH as eluent).

***N*^{1}-(Boc-Lys-Boc)-*N*^{6}-[{6-(Boc-Lys-Boc)amido}hexyl]hexane-1,6-diamine (1a).** Yield-60%, ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 8.230 (bs, NH(-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-*NH*-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 6.934 (bs, NH(-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-NH-CO-CH(*NH*Boc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 5.554 (bs, NH(-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-*NH*Boc)$_2$, 2H), 4.826 (bs, *NH*(-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 1H), 4.071 (bs, NH(-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-NH-CO-*CH*(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 3.236-3.005 (m, NH(-*CH*$_2$-CH_2-CH_2-CH_2-CH_2-*CH*$_2$-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-*CH*$_2$-NHBoc)$_2$, 12H), 2.165-2.144 (m, NH(-CH_2-CH_2-CH_2-CH_2-*CH*$_2$-CH_2-NH-CO-CH(NHBoc)-CH_2-

CH_2-CH_2-CH_2-NHBoc)$_2$, 4H), 1.805-1.498 (m, NH(-CH2-*C*$\underline{H_2}$-CH_2-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-*C*$\underline{H_2}$-*C*$\underline{H_2}$-*C*$\underline{H_2}$-CH_2-NHBoc)$_2$, 16H), 1.421 (bs, NH(-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C*(C*$\underline{H_3}$*)*$_3$)-CH_2-CH_2-CH_2-CH_2-NH-COO-C*(C*$\underline{H_3}$*)*$_3$)$_2$, 36H), 1.377-1.345 (m, NH(-CH_2-CH_2-*C*$\underline{H_2}$-*C*$\underline{H_2}$-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 8H); HRMS (m/z): 872.64698 [(M+H)$^+$] (Observed), 872.64362 (Calculated).

***N*^{1}-(Boc-Lys-Boc)-*N*^{3}-[{3-(Boc-Lys-Boc)amido}propyl]proane-1,3-diamine (2a).** Yield-66%, ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 8.870 (bs, NH(-CH_2-CH_2-CH_2-*N*$\underline{H}$-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 7.474 (bs, NH(-CH_2-CH_2-CH_2-NH-CO-CH(*N*$\underline{H}$Boc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 5.520 (bs, NH(-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-*N*$\underline{H}$Boc)$_2$, 2H), 4.785 (bs, *N*$\underline{H}$(-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 1H), 4.033 (bs, NH(-CH_2-CH_2-CH_2-NH-CO-*C*$\underline{H}$(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 3.513-3.008 (m, NH(-*C*$\underline{H_2}$-CH_2-*C*$\underline{H_2}$-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-*C*$\underline{H_2}$-NHBoc)$_2$, 12H), 1.772-1.4486 (m, NH(-CH_2-*C*$\underline{H_2}$-CH_2-NH-CO-CH(NHBoc)-*C*$\underline{H_2}$-*C*$\underline{H_2}$-*C*$\underline{H_2}$-CH_2-NHBoc)$_2$, 16H), 1.424 (s, NH(-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C*(C*$\underline{H_3}$*)*$_3$)-CH_2-CH_2-CH_2-CH_2-NH-COO-C*(C*$\underline{H_3}$*)*$_3$)$_2$, 36H); HRMS (m/z): 788.54937 [(M+H)$^+$] (Observed), 788.54972 [(M+H)$^+$] (Calculated).

Protocol for synthesizing 1b and 2b: At first, 1.5 equivalents of dodecanoic or octadecanoic acid was dissolved in dry DCM at 0 ºC. 4 equivalents of DIPEA and 1.5 equivalents of HBTU were then added to the reaction mixture followed by DMF (1/4 volume of DCM) and allowed to stirr for 10-15 min. After that, 1 equivalent of **1a** or **2a** was added to the reactiion mixture after dissolving it in DCM and reaction was allowed to stirr for 24 h. At the end, the reaction solvent was removed by using rotary evaporator and crude residue was diluted in ethyl acetate. This was then washed at first with 1N HCl followed by saturated Na_2CO_3 solution and the organic was collected through anhydrous Na_2SO_4. Finally, column was accomplished on 60-120 mesh silica gel (using vatious ratios of chloroform and methanol as eluent) to obtain pure compound.

***N,N*-bis-[{6-(Boc-Lys-Boc)amido}hexyl]dodecanamide (1b):** Yield-75%; ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 6.574-6.287 (d, R-CO-N(-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-*N*$\underline{H}$-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 5.370-5.225 (d, R-CO-N(-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-NH-CO-CH(*N*$\underline{H}$Boc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 4.712 (bs, R-CO-N(-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-*N*$\underline{H}$Boc)$_2$, 2H), 4.018 (bs, R-CO-N(-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-NH-CO-*C*$\underline{H}$(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 3.295-3.083 (m, R-CO-N(-*C*$\underline{H_2}$-CH_2-CH_2-CH_2-CH_2-*C*$\underline{H_2}$-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-

C$\underline{H_2}$-NHBoc)$_2$, 12H), 2.276-2.239 (t, *J* = 7.4 Hz, CH_3-$(CH_2)_8$-CH_2-*C*$\underline{H_2}$ of R group, 2H), 1.852-1.481 (m, CH_3-$(CH_2)_8$-*C*$\underline{H_2}$-CH_2-CO-N(-CH_2-*C*$\underline{H_2}$-CH_2-CH_2-*C*$\underline{H_2}$-CH_2-NH-CO-CH(NHBoc)-*C*$\underline{H_2}$-CH_2-*C*$\underline{H_2}$-CH_2-NHBoc)$_2$, 18H), 1.424 (s, R-CO-N(-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C*(C*$\underline{H_3}$*)*$_3$)-CH_2-CH_2-CH_2-CH_2-NH-COO-C*(C*$\underline{H_3}$*)*$_3$)$_2$, 36H), 1.372-1.243 (m, CH_3-*(C*$\underline{H_2}$*)*$_8$-CH_2-CH_2-CO-N(-CH_2-CH_2-*C*$\underline{H_2}$-*C*$\underline{H_2}$-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-*C*$\underline{H_2}$-CH_2-CH_2-NHBoc)$_2$, 24H), 0.881-0.847 (t, *J* = 6.8 Hz, *C*$\underline{H_3}$-$(CH_2)_8$-CH_2-CH_2- of R group, 3H); HRMS (m/z): 1054.81120 [$(M+H)^+$] (Observed), 1054.48908 (Calculated).

***N,N*-bis-[{3-(Boc-Lys-Boc)amido}propyl]octadecanamide (2b):** Yield-75%; ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 7.324-7.036 (d, R-CO-N(-CH_2-CH_2-CH_2-*N*$\underline{H}$-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 5.321 (bs, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(*N*$\underline{H}$Boc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 4.776 (bs, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-*N*$\underline{H}$Boc)$_2$, 2H), 4.073 (bs, R-CO-N(-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-NH-CO-*C*$\underline{H}$(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 3.527-3.078 (m, R-CO-N(-*C*$\underline{H_2}$-CH_2-*C*$\underline{H_2}$-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-*C*$\underline{H_2}$-NHBoc)$_2$, 12H), 2.303-2.265 (t, *J* = 7.6 Hz, CH_3-$(CH_2)_{14}$-CH_2-*C*$\underline{H_2}$ of R group, 2H), 1.822-1.475 (m, CH_3-$(CH_2)_{14}$-*C*$\underline{H_2}$-CH_2-CO-N(-CH_2-*C*$\underline{H_2}$-CH_2-NH-CO-CH(NHBoc)-*C*$\underline{H_2}$-CH_2-*C*$\underline{H_2}$-CH_2-NHBoc)$_2$, 14H), 1.422 (s, R-CO-N(-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C*(C*$\underline{H_3}$*)*$_3$)-CH_2-CH_2-CH_2-CH_2-NH-COO-C*(C*$\underline{H_3}$*)*$_3$)$_2$, 36H), 1.374-1.238 (m, CH_3-*(C*$\underline{H_2}$*)*$_{14}$-CH_2-CH_2-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-*C*$\underline{H_2}$-CH_2-CH_2-NHBoc)$_2$, 32H), 0.882-0.848 (t, *J* = 6.8 Hz, *C*$\underline{H_3}$-$(CH_2)_{14}$-CH_2-CH_2- of R group, 3H); HRMS (m/z): 1054.81120 [$(M+H)^+$] (Observed), 1054.48908 (Calculated).

Protocol for synthesizing 1 and 2: At first, **1b** and **2b** were dissolved in 2 mL of DCM, then 2 mL of TFA was added to it and kept for stirring. At the end of 2h, the reaction solvent and unused TFA were removed to afford pure **1**and **2** with 100% yield.

***N,N*-bis-[{6-(Lys)amido}hexyl]dodecanamide Tetrakis(trifluoroacetate) (1):** ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 8.491-8.439 (m, R-CO-N(-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-*N*$\underline{H}$-CO-CH(NH_3^+)-CH_2-CH_2-CH_2-CH_2-NH_3^+)$_2$, 2H), 8.183 and 7.850 (bs, R-CO-N(-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-NH-CO-CH*(N*$\underline{H_3}^+$*)*-CH_2-CH_2-CH_2-CH_2-*N*$\underline{H_3}^+$)$_2$, 12H), 3.703 (bs, R-CO-N(-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-NH-CO-*C*$\underline{H}$(NH_3^+)-CH_2-CH_2-CH_2-CH_2-NH_3^+)$_2$, 2H), 3.186-3.074 (m, R-CO-N(-*C*$\underline{H_2}$-CH_2-CH_2-CH_2-CH_2*C*$\underline{H_2}$-NH-CO-CH(NH_3^+)-CH_2-CH_2-CH_2-CH_2-NH_3^+)$_2$, 8H), 2.742 (bs, R-CO-N(-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-NH-CO-CH(NH_3^+)-CH_2-CH_2-CH_2-*C*$\underline{H_2}$-NH_3^+)$_2$, 4H), 2.232-2.195 (t, *J* = 7.2 Hz, CH_3-$(CH_2)_8$-CH_2-*C*$\underline{H_2}$- of R group, 2H), 1.717-1.396 (m, CH_3-$(CH_2)_8$-*C*$\underline{H_2}$-CH_2-CO-N(-CH_2-*C*$\underline{H_2}$-CH_2-CH_2-*C*$\underline{H_2}$-CH_2-NH-CO-CH(NH_3^+)-*C*$\underline{H_2}$-CH_2-

CH$_2$-CH$_2$-NH$_3^+$)$_2$, 18H), 1.330-1.236 (m, CH$_3$-*(CH*$_2$*)*$_8$-CH$_2$-CH$_2$-CO-N(-CH$_2$-CH$_2$-*CH*$_2$-*CH*$_2$-CH$_2$-CH$_2$-NH-CO-CH(NH$_3^+$)-CH$_2$-*CH*$_2$-CH$_2$-CH$_2$-NH$_3^+$, 28H)$_2$, 0.867-0.834 (t, *J* = 6.6 Hz, *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, 168.27, 158.79, 158.46, 118.26, 115.30, 52.23, 47.20, 44.94, 38.83, 38.62, 32.19, 31.38, 30.60, 29.07, 29.02, 28.95, 28.78, 27.34, 26.58, 26.27, 26.23, 26.18, 26.05, 25.16, 22.18, 21.34, 14.03. HRMS (m/z): 654.59804 [(M+H)$^+$] (Observed), 654.60096 [(M+H)$^+$] (Calculated).

***N,N*-bis-[{3-(Lys)amido}propyl]octadecanamide Tetrakis(trifluoroacetate) (2):** 8.668-8.534 (m, R-CO-N(-CH$_2$-CH$_2$-CH$_2$-*NH*-CO-CH(NH$_3^+$)-CH$_2$-CH$_2$-CH$_2$-CH$_2$-NH$_3^+$)$_2$, 2H), 8.108 (bs, R-CO-N(-CH$_2$-CH$_2$-CH$_2$-NH-CO-CH*(NH*$_3^+$*)*-CH$_2$-CH$_2$-CH$_2$-CH$_2$-*NH*$_3^+$)$_2$, 12H), 3.770 (bs, R-CO-N(-CH$_2$-CH$_2$-CH$_2$-CH$_2$-CH$_2$-CH$_2$-NH-CO-*CH*(NH$_3^+$)-CH$_2$-CH$_2$-CH$_2$-CH$_2$-NH$_3^+$)$_2$, 2H), 3.283-2.803 (m, R-CO-N(-*CH*$_2$-CH$_2$-*CH*$_2$-NH-CO-CH(NH$_3^+$)-CH$_2$-CH$_2$-CH$_2$-*CH*$_2$-NH$_3^+$)$_2$, 12H), 2.267-2.231 (t, *J* = 7.2 Hz, CH$_3$-(CH$_2$)$_{14}$-CH$_2$-*CH*$_2$- of R group, 2H), 1.750-1.461 (m, CH$_3$-(CH$_2$)$_{14}$-*CH*$_2$-CH$_2$-CO-N(-CH$_2$-*CH*$_2$-CH$_2$-NH-CO-CH(NH$_3^+$)-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH$_2$-NH$_3^+$)$_2$, 18H), 1.232 (bs, CH$_3$-*(CH*$_2$*)*$_{14}$-CH$_2$-CH$_2$-CO-N(-CH$_2$- CH$_2$-CH$_2$-NH-CO-CH(NH$_3^+$)-CH$_2$-CH$_2$-CH$_2$-CH$_2$-NH$_3^+$, 28H)$_2$, 0.865-0.832 (t, *J* = 6.6 Hz, *CH*$_3$-(CH$_2$)$_{14}$-CH$_2$-CH$_2$- of R group, 3H); ^{13}C-NMR (100 MHz, DMSO-d$_6$) δ/ppm: 171.05, 168.02, 158.57, 158.26, 118.64, 115.67, 51.72, 44.95, 42.92, 38.33, 38.23, 36.60, 36.44, 32.04, 31.26, 29.03, 28.80, 28.67, 28.17, 27.38, 25.01, 22.59, 22.52, 22.06, 13.91. HRMS (m/z): 654.60147 [(M+H)$^+$] (Observed), 654.60096 [(M+H)$^+$] (Calculated).

Experimental Section

Antibacterial Assay: The experiment was performed by following established protocol.[1] Briefly, 100 µL of the compounds were serially diluted by 2-fold in 96-well plate in autoclaved Millipore water. After that, 6 h grown mid-log phase bacterial culture (~10^8 CFU/mL) were diluted to ~10^5 CFU/mL in nutrient broth. 150 µL of these bacterial suspensions were then added to wells containing compound solutions. After that, the plate was incubated for 24 h at 37 °C under shaking condition. At the end, the optical density (OD) of the plates were measured at 600 nm using TECAN Plate Reader (Infinite series, M200 pro). The MIC values were then determined by observing the OD values of the wells.

Hemolytic Assay: The assay was performed by following reported experimental protocol.[1] Like the antibacterial assay, 100 μL of compounds were serially diluted in by 2-fold in 96-well plate. Then, freshly collected hRBCs were centrifuged down (at 3500 rpm for 5 minutes) from the heparinized blood and suspended to 5 vol% in 1X PBS (pH = 7.4) and 150 μL of these suspensions were added to wells containing 50 μL of compound solutions. Both, positive as well as negative controls were included in the study. In case of negative control same volume of miliore water was added instead of compound solution and for the positive control 50 μL of Triton X-100 (1 vol% solution in water) was used. After addition of hRBCs, the plate was incubated at 37 ºC for 1 h. At the end, it was centrifuged (at 3500 rpm for 5 minutes) and 100 μL of the supernatant was transferred to another 96-well plate. The OD of the supernatant containing plate was then measured at 540 nm and the percentage of hemolysis was determined. To determine the percentage of hemolysis, the following formula was used: $(A_t - A_{nt})/(A_{TXt} - A_{nt})\times 100$ where, A_t is the absorbance of the compound treated well, A_{nt} the absorbance of the negative controls, and A_{TXt} the absorbance of the Triton X-100 containing well. Experiment was performed in triplicate and the HC_{50} was determined by sigmoidal fitting of the average values against compound concentartion.

Toxicity study against HEK and RAW cells:

LDH Assay.[1b] By following our reported experimental protocol, further toxicity of the compounds was investigated through LDH assay by using cytotoxicity assay kit (CytoTox 96 Non-Radioactive, Promega). 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 various concentrations of the compounds. Two control experiments were also performed; one containing no compound (non-treated cells, negative control) and the other one treated with 0.1 vol % Triton-X solution (positive control). However, the treated plate was incubated for 24 h at 37 °C under 5% CO_2

atmosphere. At the end, 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. The supernatant (100 μL) was transferred to a fresh 96-well plate and absorbance at 490 nm was measured using a Tecan Infinite Pro series M200 Micro plate Reader. Percentage of cell death was determined as $(A_t \times A_0)/ (A_{TX} \times A_0) \times 100$, where A_t is the absorbance of the compound treated well, A_0 is the absorbance of the negative controls, and A_{TX} is the absorbance of Triton-X treated wells, all at 490 nm. Percentage of LDH release, i.e. cell death was plotted as a function of concentration of the compound and the EC_{50} (defined as the compound concentration that caused 50% LDH release relative to the positive control) was determined. The experiment was performed in triplicate and the average data was plotted to obtain EC_{50} values.

Live-dead assay.[1d] Like the LDH assay, the cells were seeded into 96-well plate in complete DMEM media (supplemented with 10% fetal bovine serum and 5% penicillin-streptomycin) and grown to 70-80% confluency. After that, the cells were treated with 40 μg/mL of compound **1** and **2**. Two control experiments were also performed; while for the negative control the cells were left untreated, the cells were treated with 0.1 vol % Triton X-100 solution in positive control experiment. The treated plate was then incubated for 24 h at 37 ºC under 5% CO_2 atmosphere. At the end, the media was discarded and cells were washed once with 1X PBS and stained with calcein AM (2 μM, Fluka) and propidium iodide (PI, 4.5 μM) (Sigma-Aldrich) for 15 min. After that, the dye containing solution was discarded and the cells were washed with 1X PBS to remove the excess dyes. The images of the cells were then captured with a 40X objective in a Leica DM2500 fluorescence microscope. A band-pass filter for calcein AM at 500-550 nm and a long-pass filter for PI at 590-800 nm were used while imaging. For bright field images another experiment was performed by following the same protocol as mentioned above, where the images of the cells were directly captured after 24 h of compound treatment instead of staining with dyes.

Antibacterial efficacy in co-culture with RAW cells

RAW 264.7 macrophage in complete DMEM media (supplemented with 10% fetal bovine serum and penicillin-streptomycin) were added to the wells of 24-well plate (2×10^5 cells/well) and allowed to attach for 12 hrs at 37 °C under 5% CO_2 atmosphere. At the end, the medium was discarded and the cells were treated with 500 µL of 1.4×10^5 CFU/mL MRSA, which was immediately prepared in antibiotic-free DMEM supplemented with 10% FBS containing 40 µg/mL of compound **1**. Various control experiments were performed. To understand the anti-MRSA efficacy of the compound in co-culture, same experiment was performed in absence of mammalian cells. Similarly, to understand the effect of compound on mammalian cells, control experiment was performed where the cells were treated with compound in absence of MRSA. Two negative controls were also included, in one case the MRSA cells were left untreated with compound in absence of mammalian cells and in other case RAW cells were left untreated with both compound as well as MRSA where same volume of antibiotic-free DMEM (supplemented with 10% FBS) were added. The plate was then incubated for 3 h at 37 °C under 5% CO_2 atmosphere. To determine the bacterial cell viability, the soups were collected into eppendorf tube and the wells were washed twice with 500 µL of 1X PBS and added to corresponding samples. The samples were then serially diluted in 1X PBS (by 10-fold) and spot plated on to nutrient agar. The plates were then incubated for 24 h at 37 °C and bacterial colony were counted and cell viability was represented in terms of precentage by considering 100% cell viability for negative control. The mammalian cells viability was also determined by performing MTT assay and results were represented in terms of percentage by considering 100% cell viability for negative control.[1a] The mammalian cell viability was also investigated through live-dead assay by following the protocol as described in the previous experiment.

Membrane depolarization assay

The assay was performed by following a reported experimental protocol with little modification.[1b] Briefly, 1 mL of mid-log phase (6 h grown) MRSA culture was centrifuged at 9000 rpm for 2 min washed with 1:1 of 5 mM glucose and HEPES buffer. It was resuspended into 4 mL of cocktail media containing 5 mM HEPES buffer, 5 mM glucose and 100 mM KCl solution in 1:1:1 ratio. To this bacterial suspension then 2 μM working concentration of of 3, 3′-Dipropylthiadicarbocyanine iodide ($DiSC_3$) was added and allowed to incubate for 20 min. At the end, 190 μL of dye containing bacterial suspensions were placed into wells of black and clear bottom 96-well plates. The fluorescence was then monitored for 4 min at excitation wavelength of 622 nm and emission wavelength of 670 nm. At the end, 10 μL of compound **1** (at various working concentrations) was added to dye containing bacterial suspension and the fluorescence was monitored for another 20 min. The results were normalized with respect to the negative control, where 10 μL of milipore water was added instead of compound solution.

Membrane hydration studies using Laurdan dye

Dye embedded liposomes were first prepared by following the reported protocol.[1d] To compare the effects of compounds on bacterial and mammalian membranes, liposomes were prepared with lipids mimicking the anionic properties of the bacterial membrane (DPPG and DPPE lipids with 88:12 ratio) or the zwitterionic character of the outer layer of mammalian membranes (DPPC lipid). The freshly prepared liposome suspension was placed into the fluorescence cuvette (2 mL) and fluorescence emission of this untreated sample was measured upon excitation at 350 nm. The fluorescence emission of liposome sample treated with compounds was measured for both bacterial as well as mammalian model membranes. All the measurements were performed at 37 °C by using the Peltier system attached to a PerkinElmer LS-55 luminescence spectrometer.

Determination of critical aggregation concentration (CAC)

The CAC values of the compounds were determined by following previously reported protocol.[1a] Briefly, the compounds were dissolved in nutient broth (condition for MIC experiment) at a concentration of 500 μg/mL. 2 mL of these solutions were placed into the cuvette and the scattering intensity was measured upon 2-fold successive dilutions in nutient broth. Intensities of the scattered light were measured at an angle of 90°, fixing both the excitation and the emission at 400 nm. The intensities of scattered signal were then plotted against the concentration. The CAC was determined from the inflection point which is defined as the abscissa where the intensity rises steeply and decreases after reaching a local maximum.

MD simulations of compounds in water: A single copy of the compound was placed at the center of a 4.8 × 4.8 × 4.8 nm box, and hydrated with approximately 3600 pre-equilibrated SPC water molecules. Electroneutrality was ensured by substituting 4 Cl- atoms to randomly selected water molecules. MD simulations were carried out with the GROMACS 4.6 software package.[2a] Each system was energy-minimized and then equilibrated during a 100 ps MD, where the positions of the peptidomimetic atoms were restrained. Production simulations were performed for 40 ns, at a temperature of 300 K, with the same parameters and settings used in the membrane simulations. The first ns of the trajectories was not considered in the analyses. The conformation-independent hydrophobicity was calculated with the ALOGPS 2.1 server.[2b] The molecular lipole was calculated with VEGA ZZ.[2c] The lipole is defined as:[2d]

$$L = \sum_{i=1}^{A} r_i \cdot l_i$$

where r_i is the distance of the ith atom from the center of mass and l_i is the corresponding lipophilicity, calculated with the Broto parameters.[2e] The most representative structures of each simulation were defined by cluster analysis conducted with GROMACS (g_cluster), using a 0.22 nm root mean square deviation cutoff. The structural figures were produced by using VMD.[2f]

MD simulations of compounds in presence of membrane lipids: A common approach to study the insertion of membrane-active molecules into lipid bilayers is to start the simulation with a preformed membrane. However, several studies have demonstrated that in this case the time-range achievable with present day computational resources (usually hundreds of ns to μs) is not long enough to reach the system configuration corresponding to the global free energy minimum.[3a] Often, the molecule interacts with the membrane but remains trapped at the water/bilayer interface, due to the high viscosity of the lipid environment. For this reason, often the simulations are started from configuration with the active-molecules already embedded in the membrane. This approach is strongly biased from the often arbitrary choice of the starting configuration. To overcome these problems, we have adopted a third approach, demonstrating this effectiveness in terms of time required and reliability. We termed this method "minimum bias", since it minimizes the effect of the initial configuration on the final results. In this method, the simulation is started from a random mixture of the membrane-active molecule, lipids and water, and the bilayer forms spontaneously, usually in 50–100 ns. During this self-assembly process, the system is much more fluid than a fully formed bilayer, especially in the first stages of the simulation. This ensures that the bioactive molecule can sample different environments in a relatively short time, and, as a consequence, it is able to find its minimum free energy configuration. We recently demonstrated that the simulative results obtained with this approach are consistent with the depth of membrane insertion and the orientation determined experimentally by fluorescence, ATR-FTIR and solid-state NMR spectroscopies.[3a-c]

By following this established "minimum-bias" method, the molecular dynamics simulations were performed to characterize the interaction of compounds with lipid membranes, and to elucidate the molecular determinants of their different activities/selectivities. Membranes of DPPG:DPPE (88:12) and DPPC lipids were used to parallel the conditions of the experimental studies on lipid vesicles and to mimic eukaryotic and bacterial membranes, respectively. Briefly,

a single copy of the compound was placed at the center of a 9 × 9 × 9 nm box. 128 lipid molecules and 7500 water molecules were randomly added into the box. The simulations with charged lipids were carried out with a DDPG/DPPE ratio equal of 88:12; 113 Na^+ atoms were added as counterions of the negative charges on the lipids and 4 Cl^- atoms as counterions of the +4 charge on the compounds. In simulations with neutral DPPC lipids, 117 Cl^- and 113 Na^+ were included, to ensure electrostatic conditions similar to those used with charged lipids. In all cases, Na^+ and Cl^- ions were introduced in replacement of water molecules. MD simulations were carried out with the GROMACS 4.5 software package.[2a] The parameters for the DPPC molecule were taken from reported literature,[3d] similarly to our previous studies of peptide-membrane systems.[3a-3c] The PG and PE moiety were modeled as in reported literature.[3e,3f] The parameters for compounds were obtained by starting from those provided by the Prodrg server.[3g] The charges were modified according to those of analogous groups in the gromos 43a1 force field.[3h] The hydrophobic chains in the compounds were modeled with the Berger parameters used for lipids. The simple point charge (SPC) model was used for water.[3i] Each system was energy-minimized and then equilibrated using a 100 ps MD, where the positions of the peptidomimetic atoms were restrained. Production simulations were performed for 200 ns, at a temperature of 333 K, controlled by using a Berendsen thermostat.[3j] Pressure coupling was applied anisotropically, using the Berendsen scheme, with a time constant of 1.0 ps and a reference pressure of 1 bar. Bond lengths were constrained with the LINCS algorithm.[3k] Short-range electrostatic interactions were cut-off at 1.4 nm and long range electrostatic interactions were calculated using the particle mesh Ewald (PME) algorithm.[3l] Simulations were run with a 2 fs time step. The analyses were conducted on the last 20 ns of the simulations. To evaluate the Solvent Accessible Surface Area (SAS), the g_sas tool in GROMACS was used with the default settings. The density profiles along the bilayer normal were determined by means of the g_density tool in GROMACS. The center of the bilayer was determined by symmetrizing the

density profile of the lipid aliphatic chains. The structural figures were produced by using VMD.[2f]

Supplementary Figures

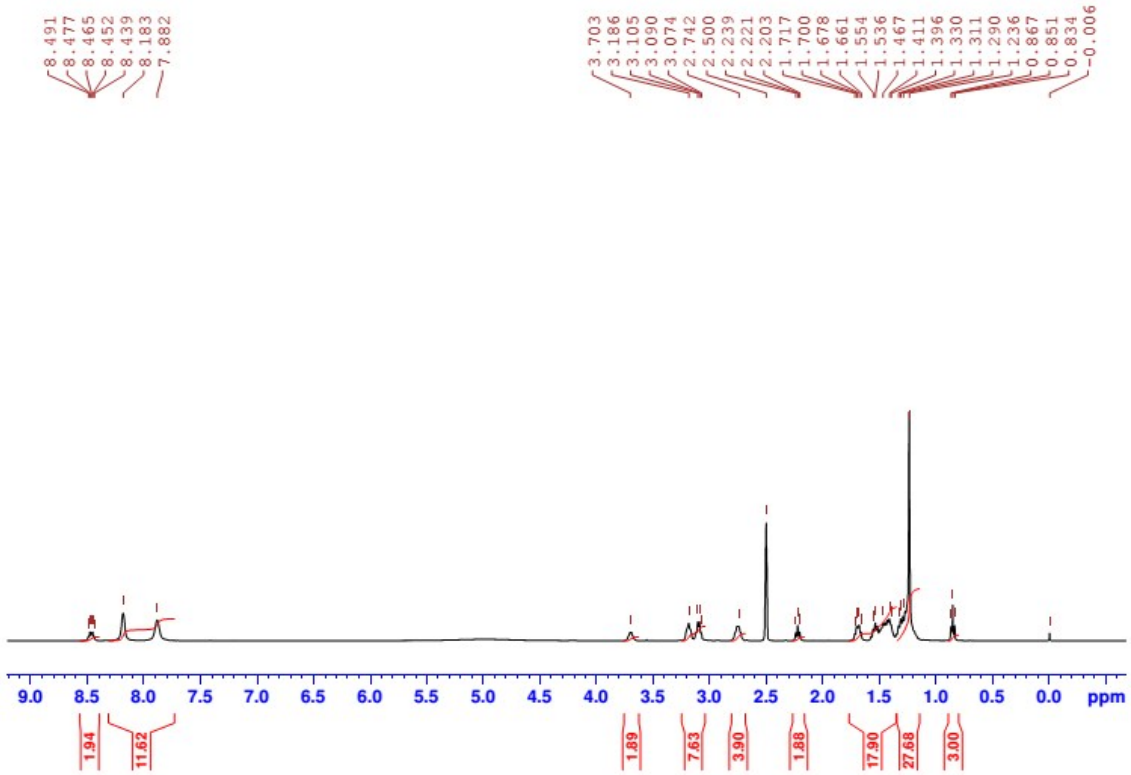


Figure S1: ^{1}H NMR of compound **1**. The NMR was taken in DMSO-d_6 and the solvent peak was calibrated at δ value of 2.500 ppm.

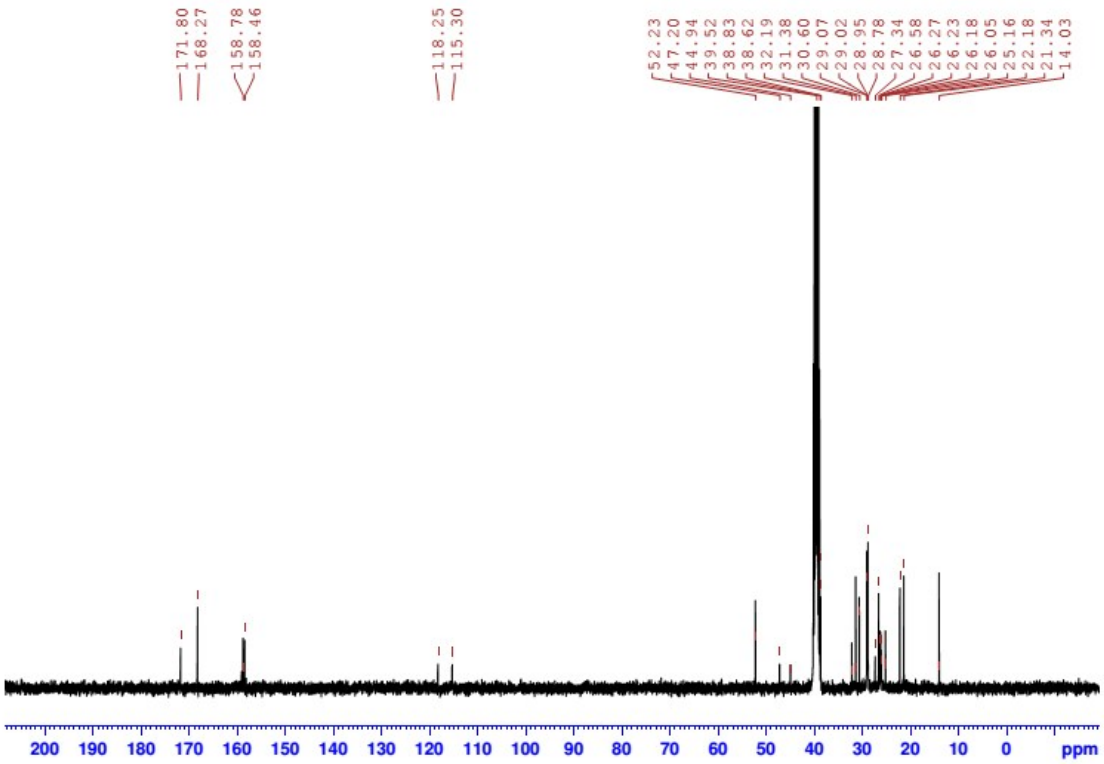


Figure S2: ^{13}C NMR of compound **1**. The NMR was taken in DMSO-d_6 and the solvent peak was calibrated at δ value of 39.52 ppm.

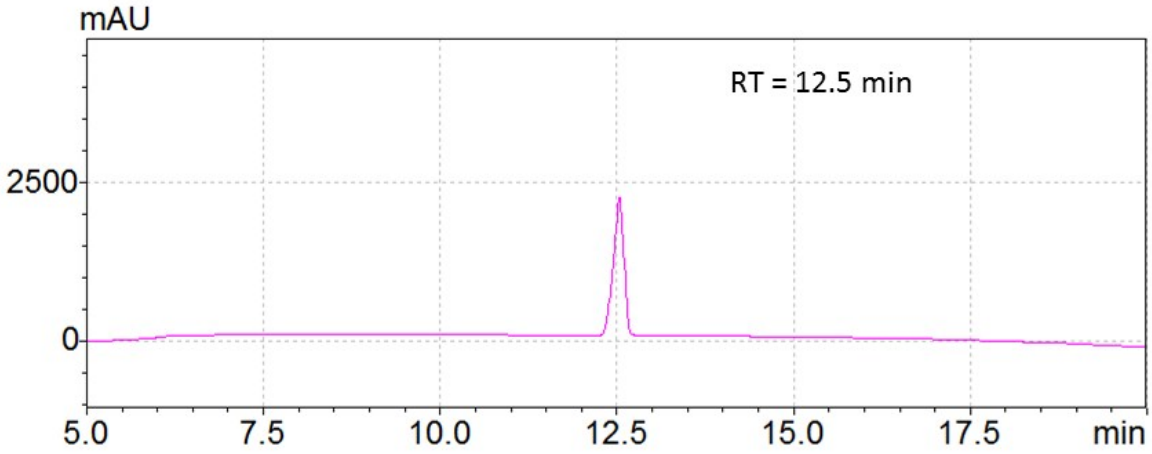


Figure S3: HPLC trace of compound **1**.

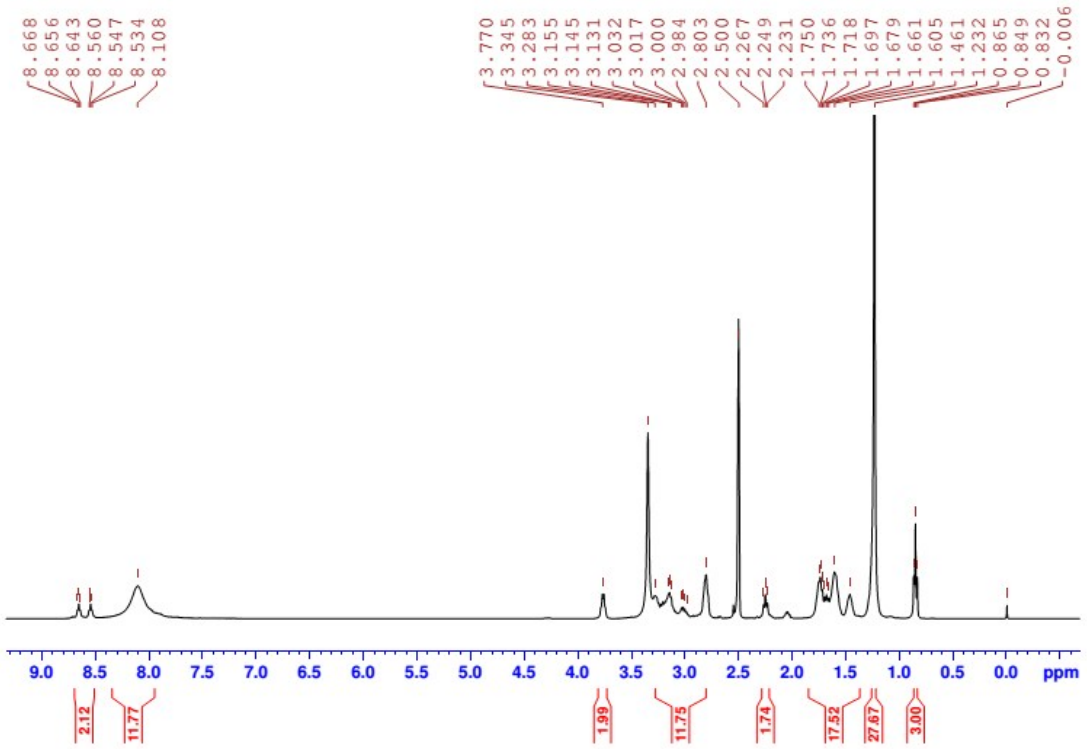


Figure S4: ^{1}H NMR of compound **2**. The NMR was taken in DMSO-d_6 and the solvent peak was calibrated at δ value of 2.500 ppm.

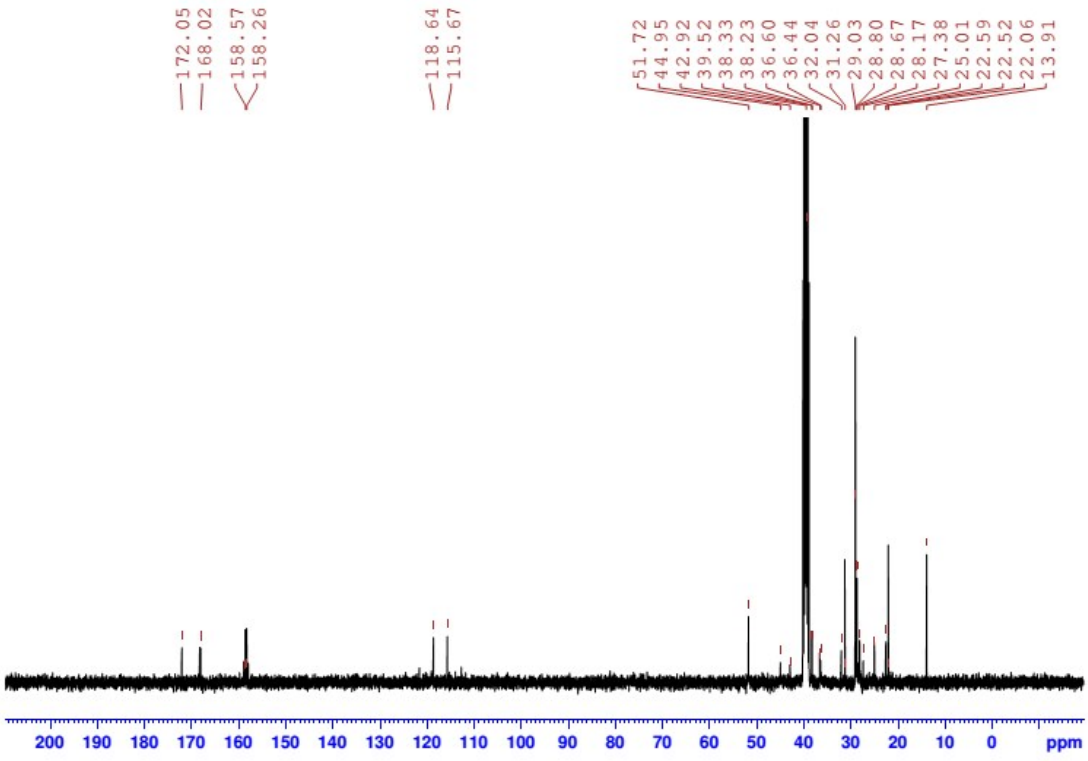


Figure S5: ^{13}C NMR of compound **2**. The NMR was taken in DMSO-d_6 and the solvent peak was calibrated at δ value of 39.52 ppm.

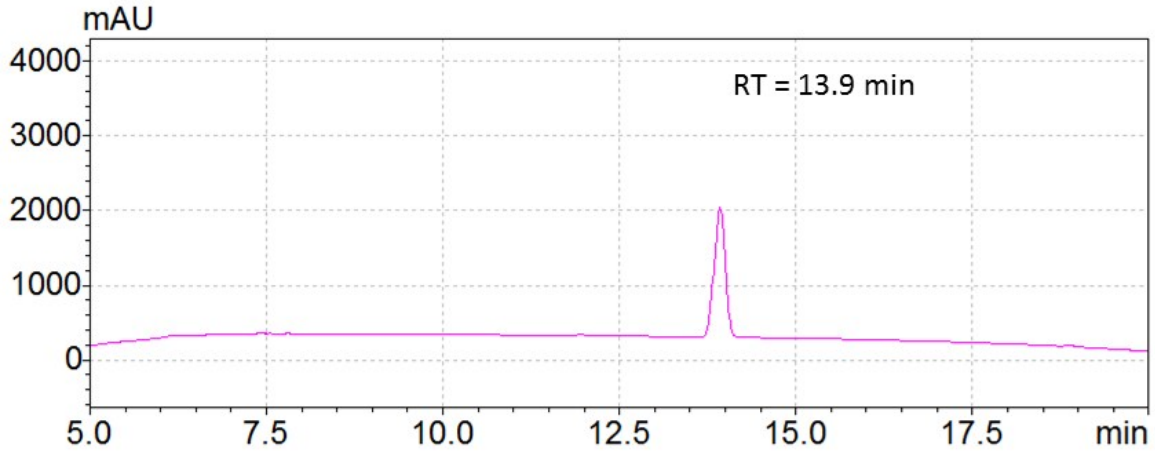


Figure S6: HPLC trace of compound **2**.

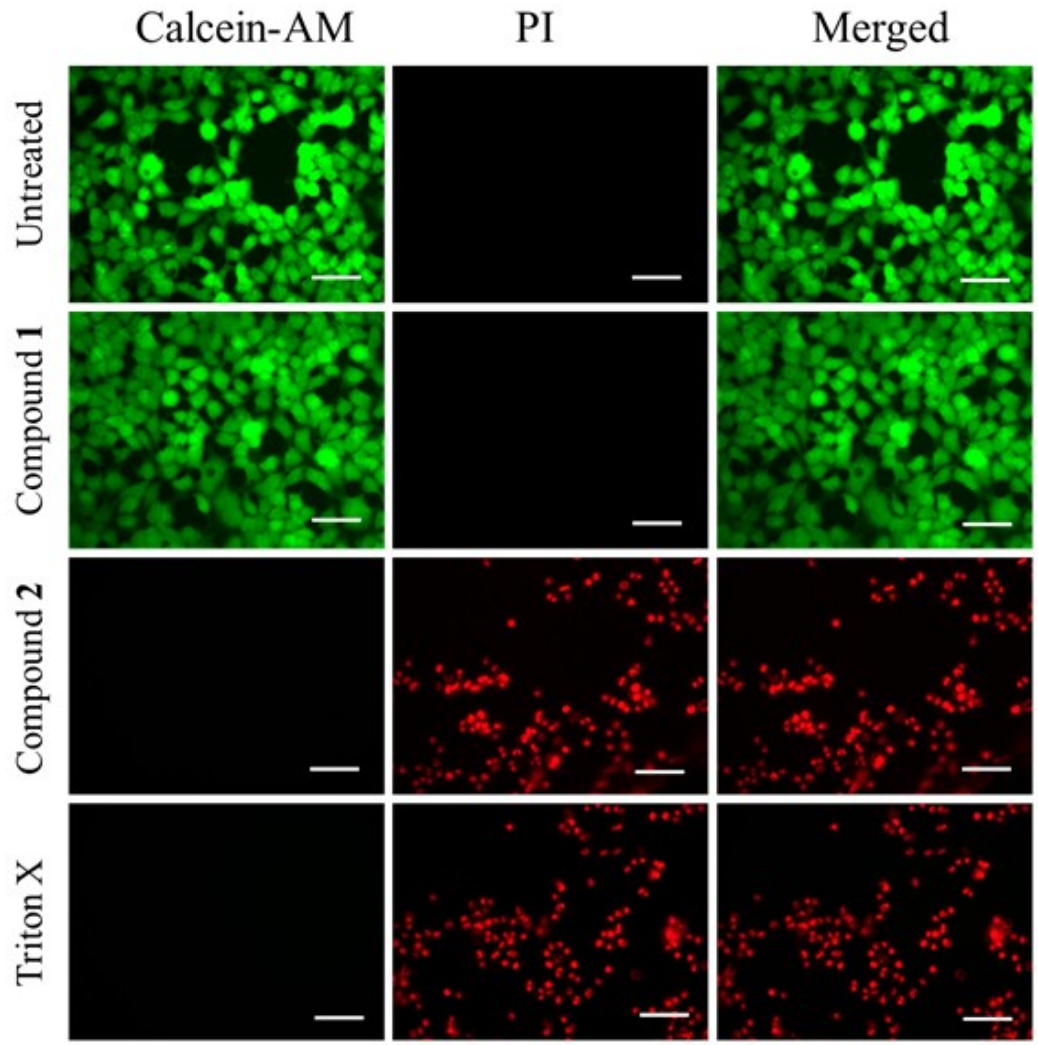


Figure S7: Fluorescence microscopy images of HEK cells treated with compound **1** and **2** (40 µg/mL each). Scale bar: 50 µm.

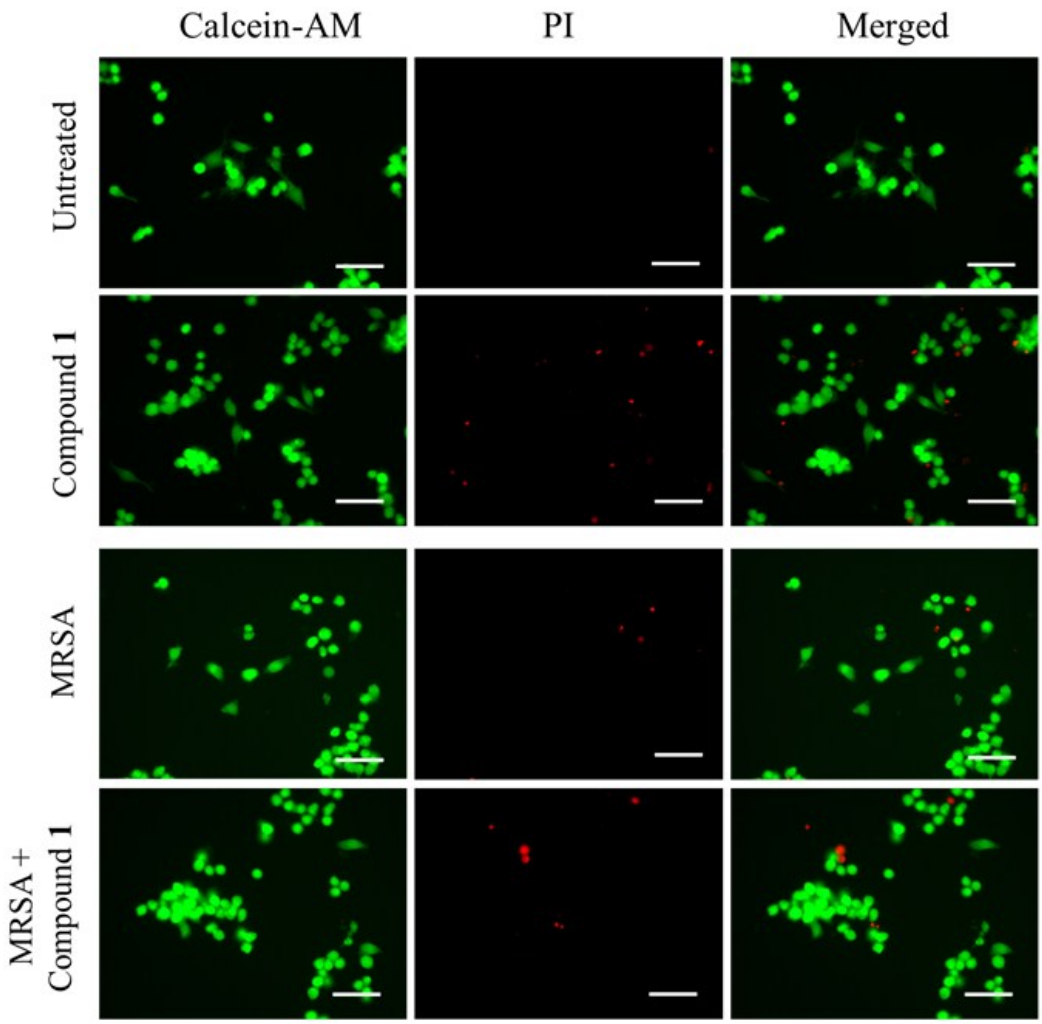


Figure S8: Fluorescence microscopy images of RAW cells at different conditions in co-culture studies. Scale bar: 50 μm.

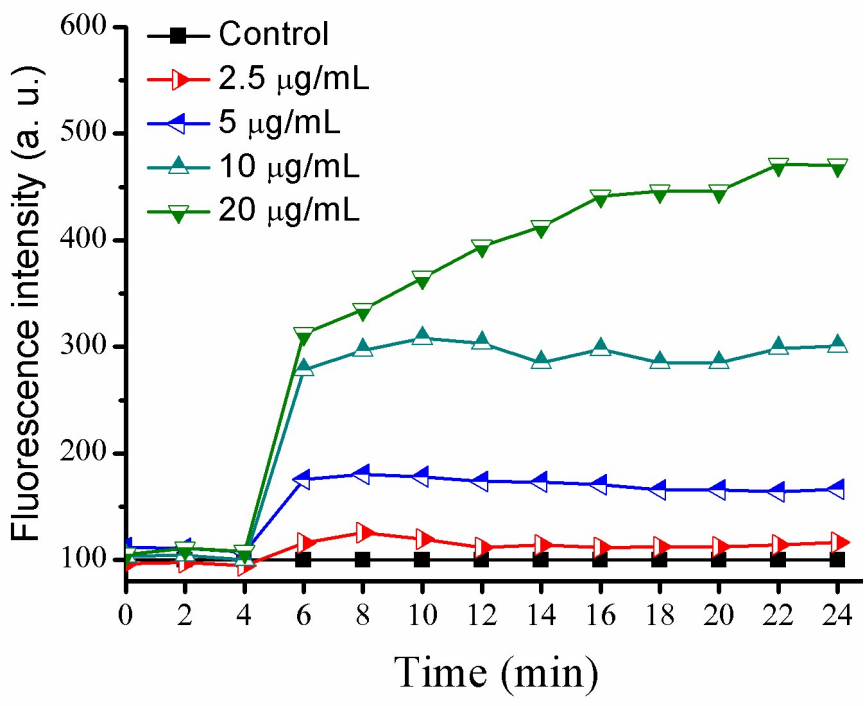


Figure S9: Membrane depolarization of MRSA by compound **1**.

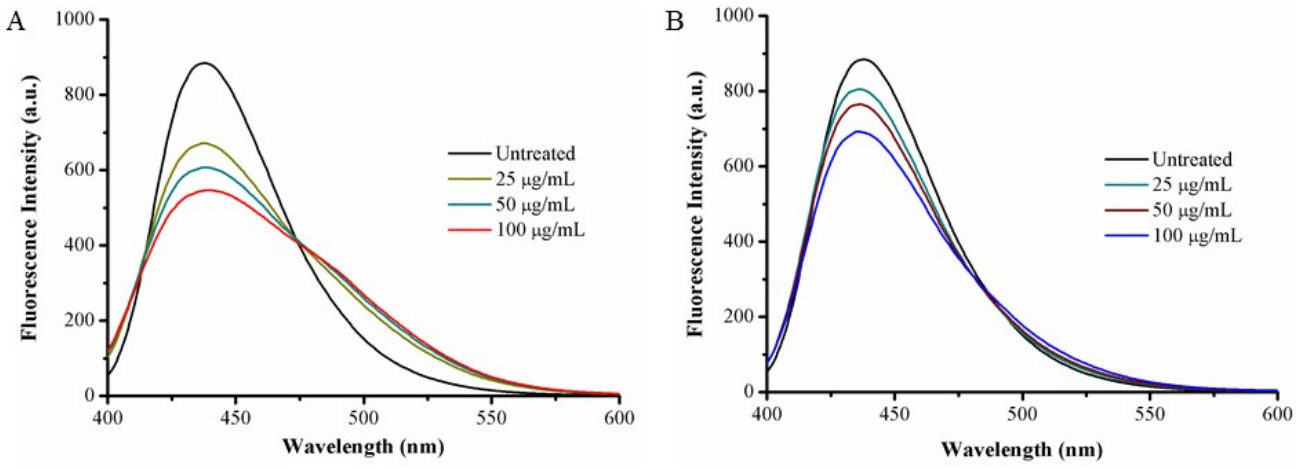


Figure S10: Laurdan fluorescence in DPPG:DPPE (88:12) liposomes. (A) For compound **1**. (B) For compound **2.**

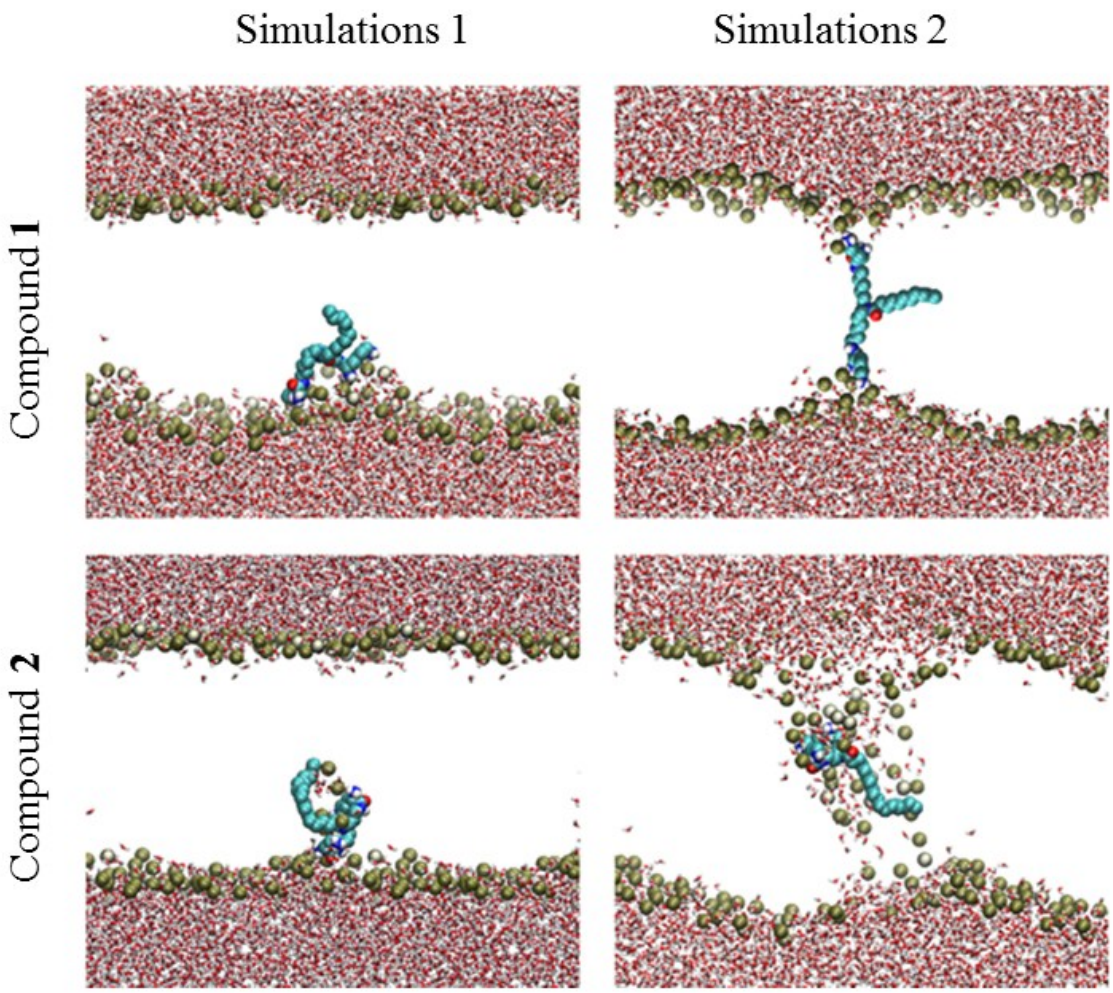


Figure S11: Final frames in the four simulations with DPPG:DPPE (88:12) lipids. Water molecules and compounds are shown in sticks and spheres representation, respectively. The phospholipids are not reported, for the sake of clarity, with the exception of phosphorus atoms (spheres). Color code: H: white; C: cyan; N: blue; O: red; P: light gold.

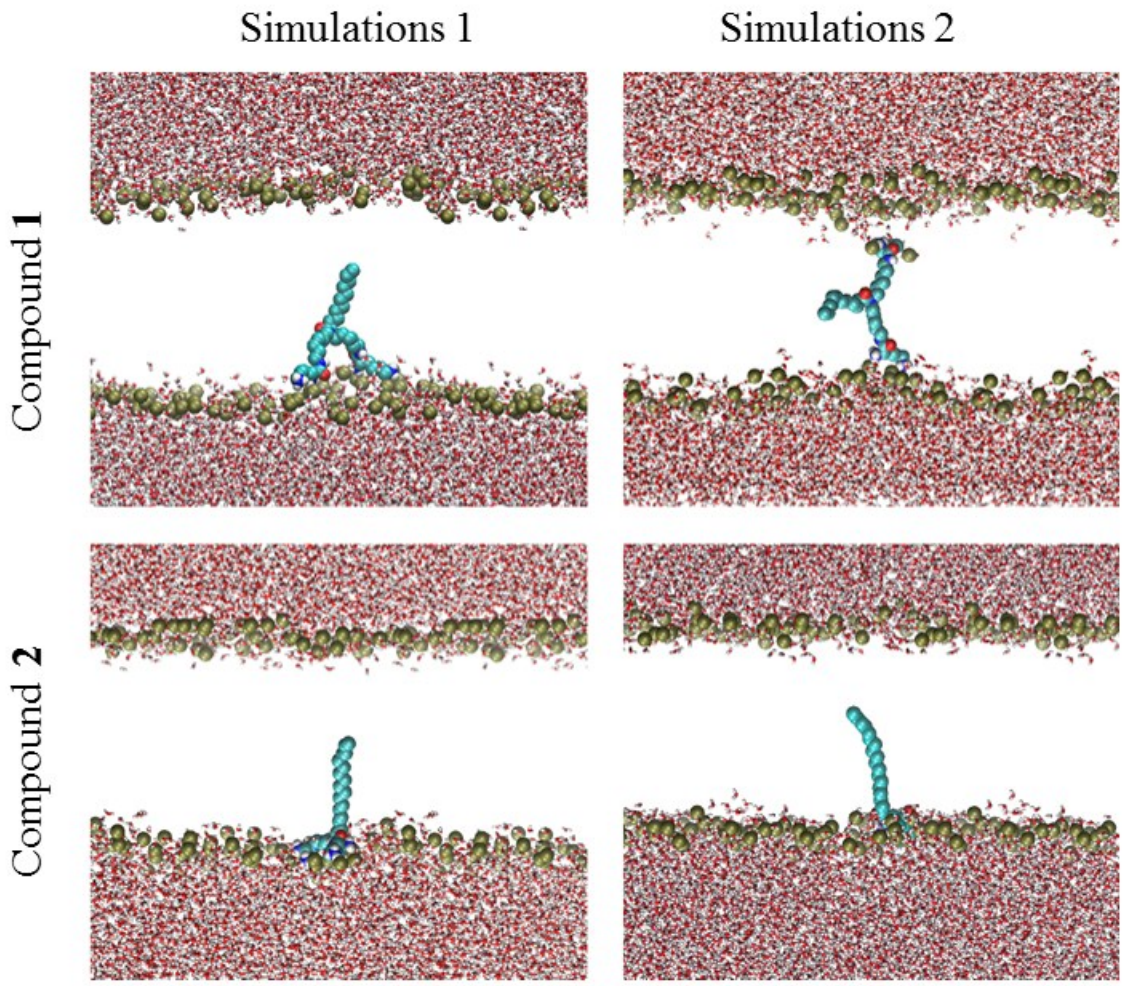


Figure S12: Final frames in the four simulations with DPPC lipids. Water molecules and compounds are shown in sticks and spheres representation, respectively. The phospholipids are not reported, for the sake of clarity, with the exception of phosphorus atoms (spheres). Color code: H: white; C: cyan; N: blue; O: red; P: gold.

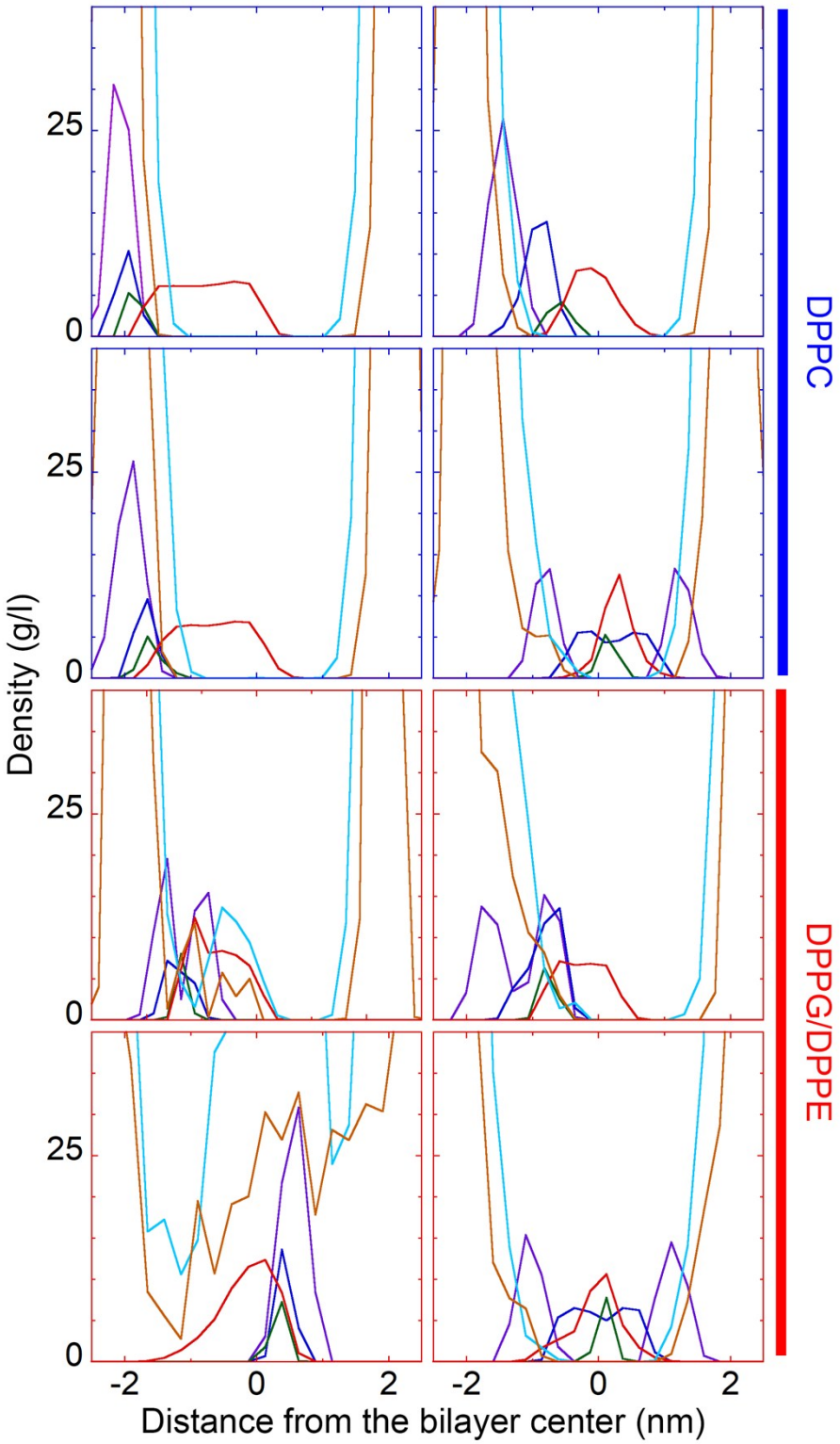


Figure S13: density profiles of the different molecules and moieties in the final section of the MD trajectories (last 20 ns). Cyan: water; orange: phosphorus atoms; purple: lysine moieties; blue: aliphatic linkers; green: secondary amide group; red: aliphatic tail. Left: compound **2**, right: compound **1**. Upper panels DPCC lipids, Lower panels DPPG/DPPE lipids.

Supplementary Table

Table S1: Antibacterial activity against MRSA clinical isolates and toxicity against mammalian cells (HEK and RAW cells).

Antibacterial agents	MIC (µg/mL)			EC_{50} (µg/mL)	
	MRSA R3545	MRSA R3889	MRSA R3890	RAW cells	HEK cells
1	3.1	3.1	3.1	84	95
2	3.1	3.1	6.3	28	35
DTAB	6.3	6.3	25	25	31
BAC-12	0.8	1.6	3.1	22	18

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