Bio-inspired Resin Acid-Derived Materials as Anti-Bacterial Resistance Agents with Unexpected Activities

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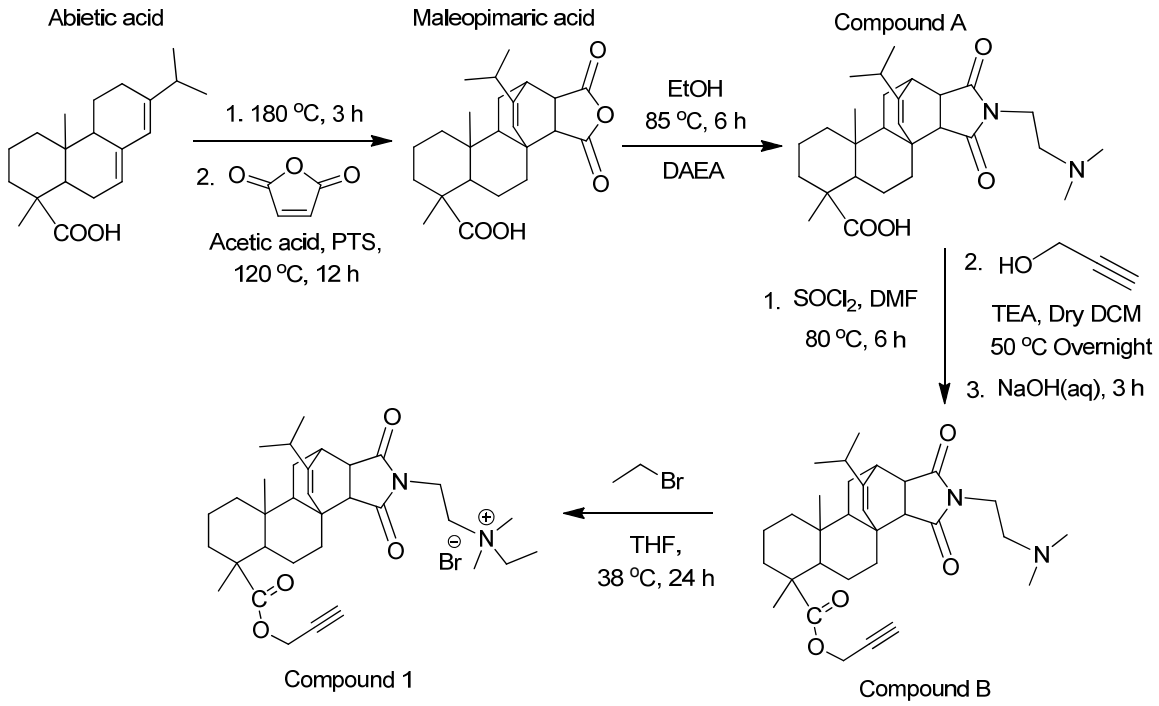
Supporting Information

Materials. Maleic anhydride, furan, ethyl acetate, ethanol, toluene, N,N-dimethylaminoethylamine (DAEA), abietic acid (85%), propargyl alcohol, triethylamine, p-toluene sulfonic acid (PTS), bromoethane, 2-chlorocyclohexanone, m-chloroperoxybenzoic acid (mCPBA), Sn(II)2-ethylhexanoate(Sn(Oct)2), sodium azide, copper iodide and 1,8-diazabicyclo[5.4.0] undec-7-ene (DBU) were purchased from Sigma Aldrich. Acetic acid, dichloromethane (DCM), ethyl acetate, ethanol, hexane, methanol, toluene, tetrahydrofuran (THF), N,N-dimethylformamide (DMF) were obtained as ACS grade solvents. Triethylamine was distilled after drying over K_2CO_3. Propargyl alcohol was distilled from CaH_2 under reduced pressure. Triton™ X-100, Calcein (97%), Sephadex® G-25 Medium and Peptidoglycan extract from *Staphylococcus aureus* were obtained from Sigma Aldrich and used as received. The phospholipids 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)(sodium salt) (DOPG) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were obtained as chloroform solutions from Avanti Polar Lipids, Inc. and used as received.

Characterization. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Varian Mercury spectrometer with tetramethylsilane (TMS) as an internal reference. Fourier transform infrared (FTIR) analysis was performed using a Perkin-Elmer spectrum with Attenuated Total Reflectance (ATR) mode. Gel permeation chromatography (GPC) was performed in DMF at a flow rate of 0.8 mL/min at 50 ºC on a Varian system equipped with a ProStar 210 pump and a Varian 356-LC RI detector and three 5 μm phenogel columns (Phenomenex Co.) with narrow dispersed polystyrene as standards. Mass spectrometry was conducted on a Waters Micromass Q-Tof mass spectrometer, and the ionization source was positive ion electrospray. Transmission electron microscopy (TEM) images were obtained on a Hitachi 8000 transmission electron microscope at an operating voltage of 150 kV. The TEM samples were prepared by placing a drop of sample on carbon-coated copper grids, dried and stained (with RuO_4) before observation. Fluorescence kinetic measurements were performed on a Varian Cary Eclipse fluorescence spectrophotometer with temperature controlled cells. A Zeiss

LSM 410 confocal laser scan microscope (CLSM) was used for fluorescent imaging. Zetasizer Nano ZSP, Malvern Instruments Corporate was used for the determination of liposome size and surface charge. Wallac 1420 VICTOR^{2TM} Multilabel Counter (PerkinElmer, Shelton, CT) was used for absorbance measurements.

Synthesis of Resin acid-Derived Antimicrobial Compound 1 and polymer 2. Compound **1** and polymer **2** were prepared using a modified synthetic strategy from our previous report.[1] The synthetic strategy illustrated in scheme S1 allowed us to prepare the compound **1** at large scale without any chromatography purification.



Scheme S1. Synthesis of resin acid-derived compound **1**.

Synthesis of maleopimaric acid: Maleopimaric acid was prepared according to our previous report.[1] Abietic acid (100.0 g, 0.28 mol) was heated to 180 °C under a nitrogen atmosphere and maintained for 3h. After adjusting the reaction temperature to 120 °C, maleic anhydride (27.5 g, 0.28 mol) and PTS (0.5 g, 0.0028 mol) dissolved in acetic acid (300.0 mL) were added. The reaction was refluxed at 120 °C for 12 h. The resultant yellow crystals were recrystallized from acetic acid thrice. Then washed with 5 % $NaHCO_3$ the final product was obtained as white crystals. (83 g, yield 73%) ^{1}H NMR (Figure S1) (300 MHz, $CDCl_3$) δ (ppm): 5.54 (s, 1H, C*H*=C); 3.10 (d, 1H, C*H*C=O); 2.73 (d, 1H, C*H*C=O); 2.50 (d, 1H, C*H*C=CH); 2.27 (m, 1H, CC*H*$(CH_3)_2$); 2.10 (m, 2H, CCH_2CH_2); 1.16 (s, 3H, CCH_3); 0.98 (m, 6H, CCH(CH_3)$_2$); 0.59 (s, 3H, CCH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 185.4(*C*OOH); 172.7-170.9 (O=*C*O*C*=O); 148.1

(*C*=CH); 125.1 (C=*C*H); 49.1 (C=O*C*H*C*HC=O); 46.8(*C*C=O). ES-MS: m/z 400 (theoretical m/z: 399+H^+)

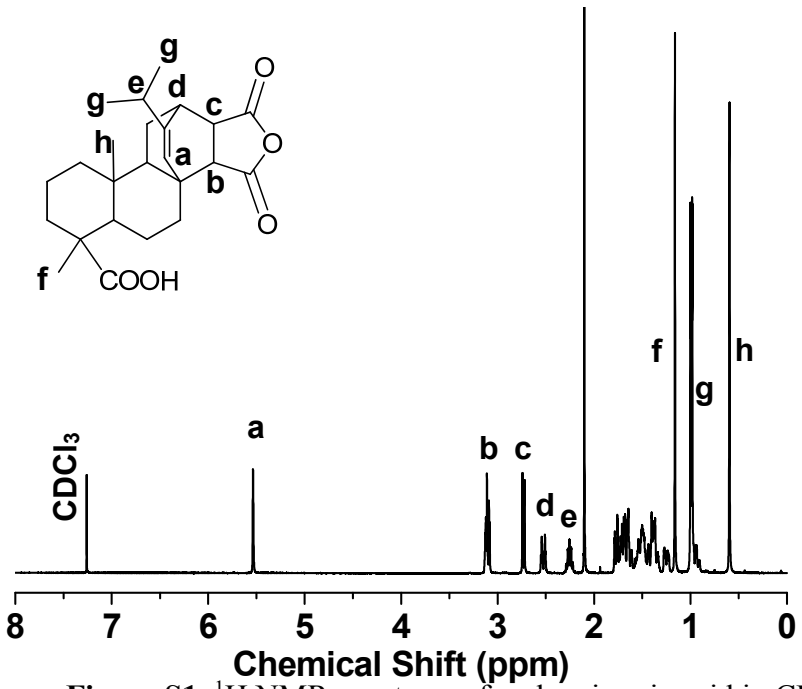


Figure S1. ^{1}H NMR spectrum of maleopimaric acid in $CDCl_3$.

Synthesis of compound A: Maleopimaric acid (50.0 g, 0.125 mol) was dissolved in ethanol (200.0 mL) followed by adding *N,N*-dimethylaminoethylamine (14.0 mL, 0.125 mol) and refluxed at 85 °C for 6 h. The product started to precipitate after about an hour. After 6 h the reaction was cooled using an ice bath to complete the precipitation, then filtrated and washed with cold ethanol and dried. The compound A appeared as a white powder (41.5 g, yield: 71%). ^{1}H NMR (Figure S2) (300 MHz, $CDCl_3$) δ (ppm): 5.52 (s, 1H, C*H*=C); 3.70 (t, 2H, NC*H*$_2$CH$_2$); 3.43 (d, 1H, C*H*C=O); 3.18 (d, 1H, C*H*C=O); 2.92 (t, 2H, C*H*$_2$N(CH$_3$)$_2$); 2.63 (d, 1H, CH$_2$C*H*C=CH); 2.35 (s, 6H, CH$_2$N(C*H*$_3$)$_2$); 2.21 (m, 1H, CH$_2$=CC*H*(CH$_3$)$_2$). ES-MS: m/z 471 (theoretical m/z: 470+H^+).

Synthesis of compound B: Compound A (35.0 g, 0.074 mol) was mixed with 50 mL of distilled thionyl chloride in a dry two neck round bottom flask under nitrogen at 0 °C. After adding two drops of dry DMF, it was refluxed for 2 h followed by vacuum distillation to remove unreacted thionyl chloride. Then the yellow colored viscous liquid containing the acid chloride was dissolved in minimum amount of dry DCM. Another dry round bottom flask was charged with dry propargyl alcohol (30 mL) and dry triethylamine (21 mL, 0.15 mol). Then the crude acid chloride was transferred slowly to the second flask and kept stirring for 24 h at 50 °C. The crude mixture was dissolved in THF/DCM mixture and stirred with 3 M NaOH solution for 3 h and extracted into DCM. The ester can be further purified by using a silica column and ethyl acetate

as the mobile phase. But this purification is not required. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): δ: 5.37 (s, 1H, C*H*=C); 4.65 (m, 2H, CH_2C≡CH); 3.44 (t, 2H, NCH_2CH$_2$); 3.00 (d, 1H, C*H*C=O); 2.77 (s, 1H, CH$_2$C≡C*H*); 2.49 (d, 1H,C*H*C=O); 2.44 (t, 2H, CH_2N(CH$_3$)$_2$); 2.00 (s, 6H, N(CH_3)$_2$); 1.16 (s, 3H, CCH_3); 0.98 (m, 6H, CCH(CH_3)$_2$); 0.59 (s, 3H, CCH_3).

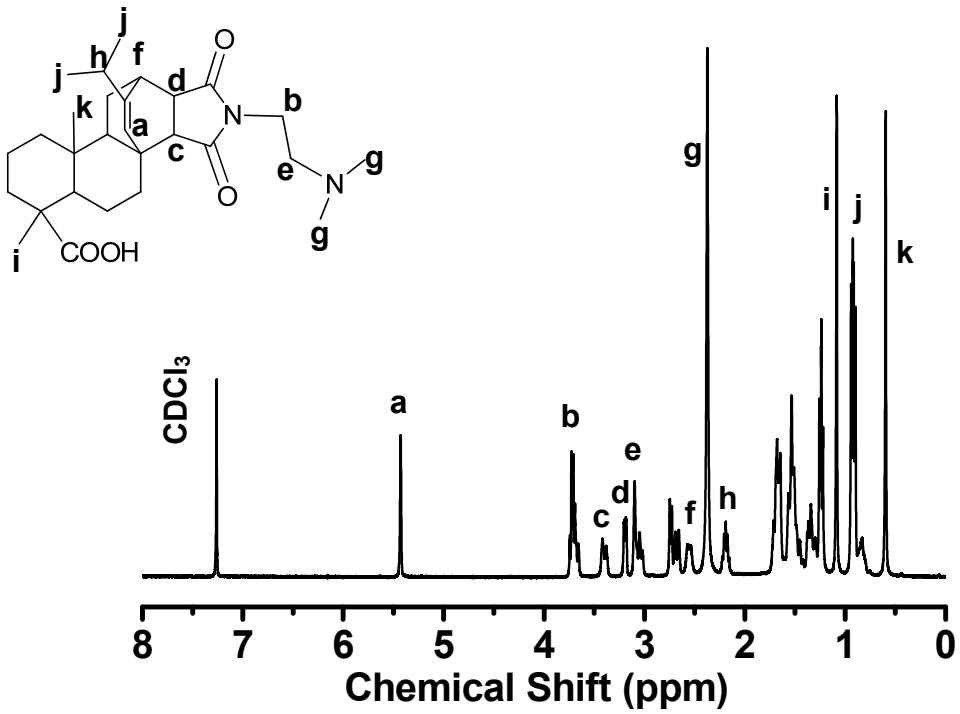


Figure S2. ^{1}H NMR spectrum of compound A in $CDCl_3$.

Synthesis of compound 1: The crude product from the previous step was used without chromatography purification. It was dissolved in dry THF and bromoethane (40 mL, 0.37 mol) was added. The mixture was heated at 38 °C for 48 h. The product precipitated in THF in the process of reaction. Then it was filtrated and then washed with THF. Pure product appeared as an off white powder. The crystals were prepared by dissolving 500 mg of compound 1 in 1 mL of DCM, adding 3 mL of THF and evaporating slowly over night. (41.7 g, yield: 91%) ^{1}H NMR (Figure S3) (300 MHz, $CDCl_3$) δ (ppm): 5.39 (s, 1H, C*H*=C); 4.65 (m, 2H, CH_2C≡CH); 3.76 (t, 2H, NCH_2CH$_2$); 3.73 (m, 2H, NCH$_2$CH_2); 3.67 (m, 2H, N+CH_2CH$_3$); 3.39 (s, 6H, N+(CH_3)$_2$); 3.00 (m, 1H, C*H*C=O); 2.96 (s, 1H, CH$_2$C≡C*H*); 2.64 (d, 1H, C*H*C=O); 2.49 (d, 1H, CH$_2$C*H*C=CH); 2.16 (m, 1H, CH=CC*H*(CH$_3$)$_2$); 1.16 (s, 3H, CCH_3); 0.92 (m, 6H, CCH(CH_3)$_2$); 0.58 (s, 3H, CCH_3). ^{13}C NMR (Figure S4) (75 MHz, methanol-d4) δ (ppm): 178.5 (C*C*=OO); 177.8-177.0 (O=*C*N*C*=O); 147.2 (*C*=CH); 124.5 (C=*C*H); 77.5 (CH$_2$*C*≡CH); 74.6 (CH$_2$C≡*C*H); 59.5-60.0 (CH$_2$*C*H$_2$N+ and CH$_3$*C*H$_2$N+). ES-MS (Figure S5): m/z 537 (theoretical m/z: 537+81(Br)).

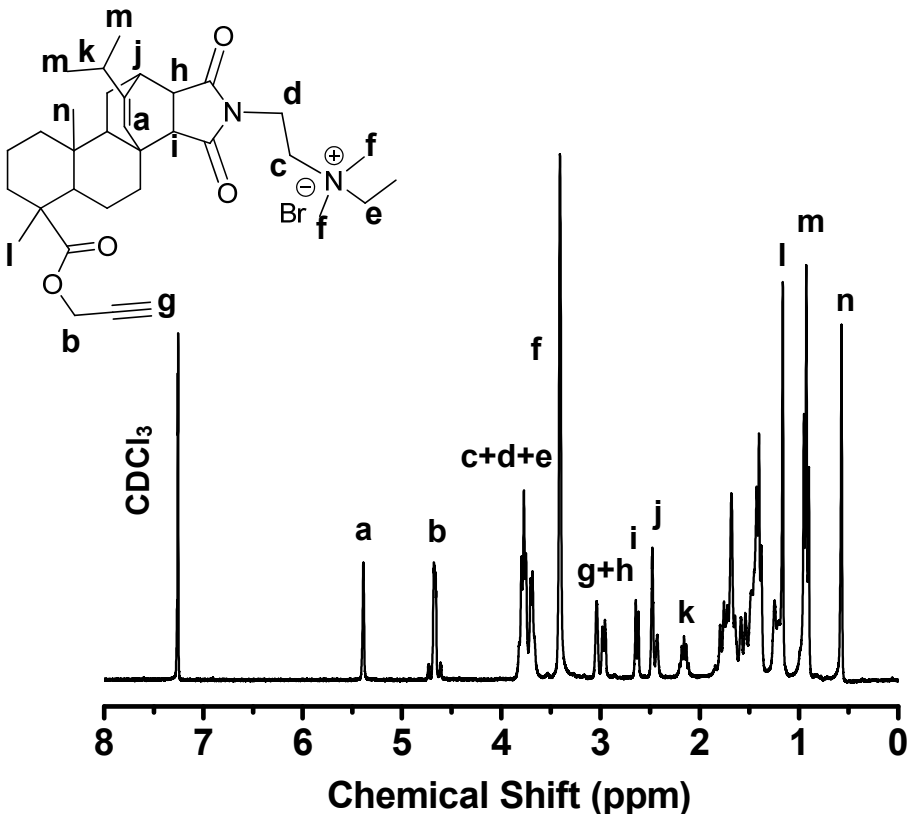


Figure S3. ^{1}H NMR spectrum of compound **1** in $CDCl_3$.

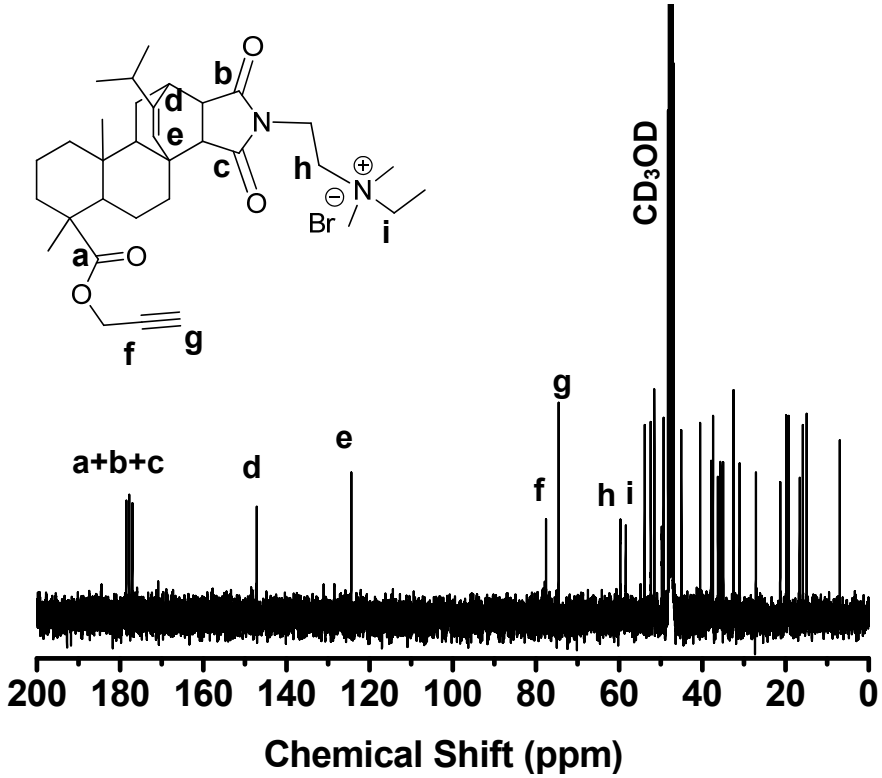


Figure S4. ^{13}C NMR spectrum of compound **1** in CD_3OD.

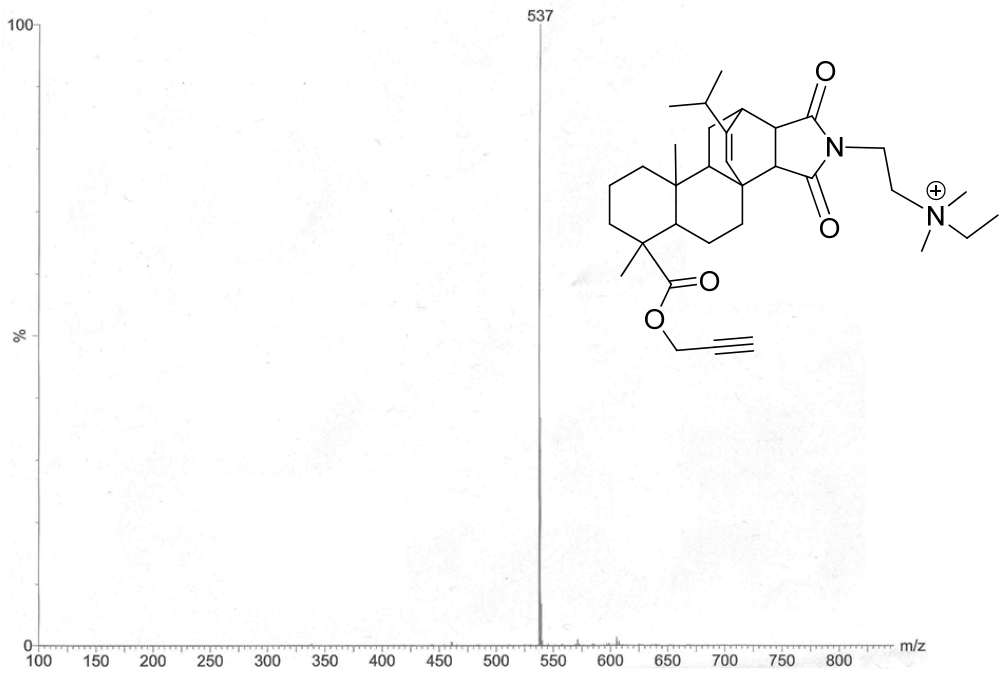
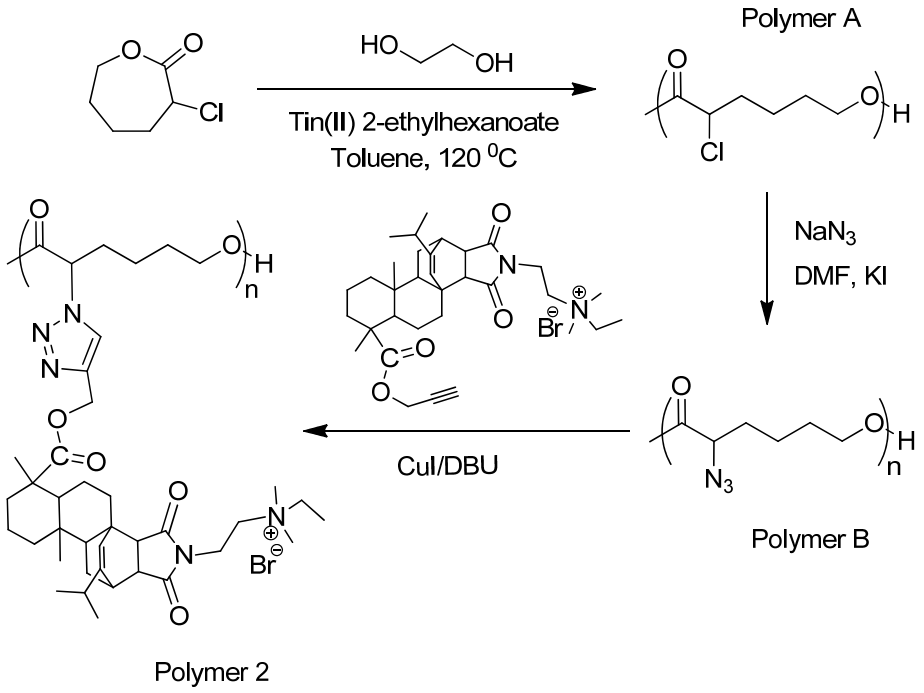


Figure S5. ES-Mass spectrum of compound **1**.

Synthesis of resin acid containing polymer **2**: The polymer **2** was prepared following our previous work as shown in scheme S2.[1]



Scheme S2. Synthesis of resin acid containing polymer **2**.

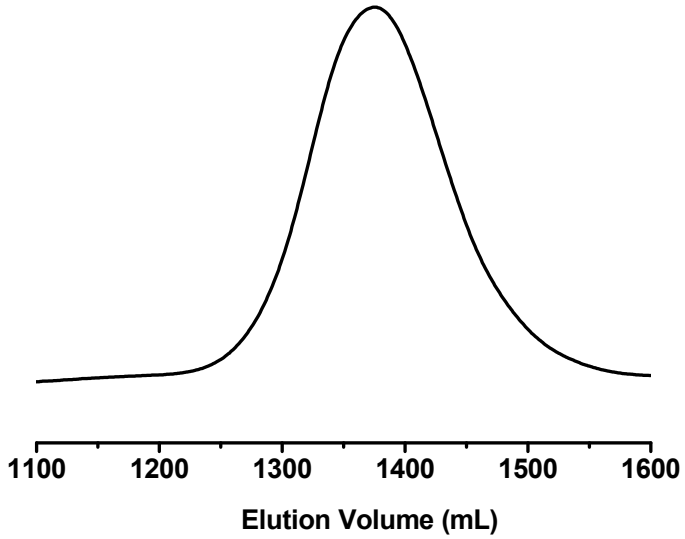


Figure S6. GPC trace of polymer A (M_n (GPC) = 27,900 gmol^{-1}, PDI = 1.59).

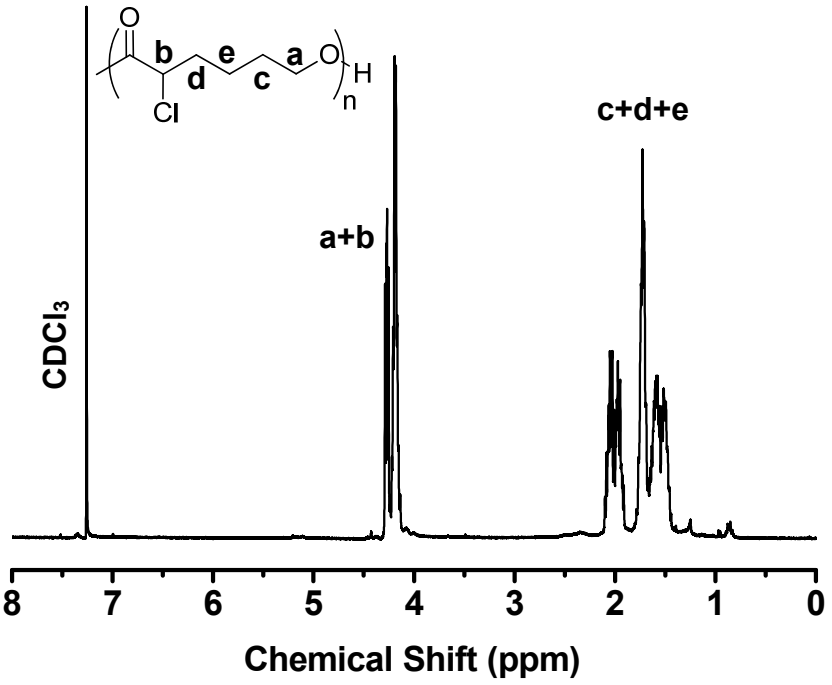


Figure S7. ^{1}H NMR spectrum of Polymer A in $CDCl_3$.

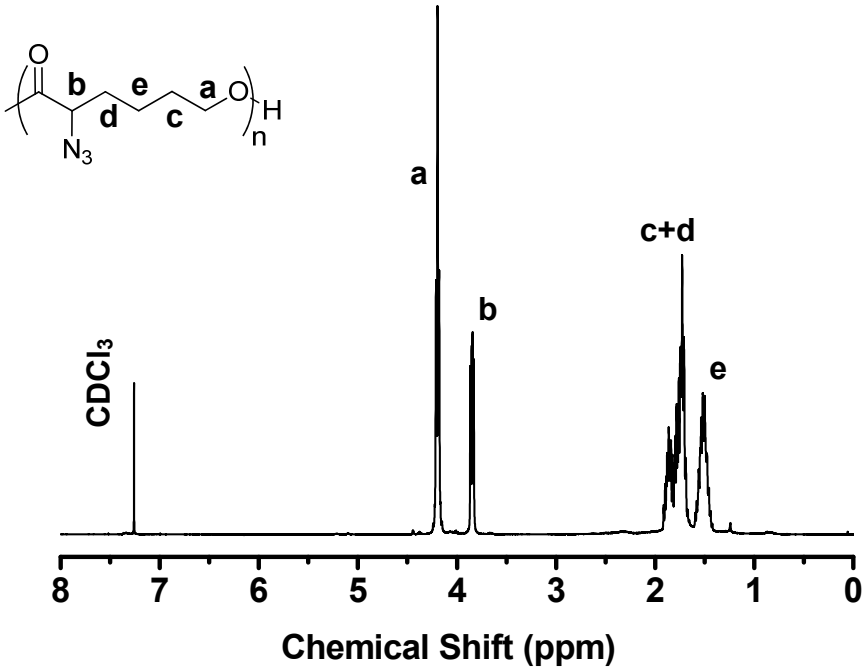


Figure S8. ^{1}H NMR spectrum of Polymer B in $CDCl_3$. (M_n(NMR) = 15,000 gmol^{-1})

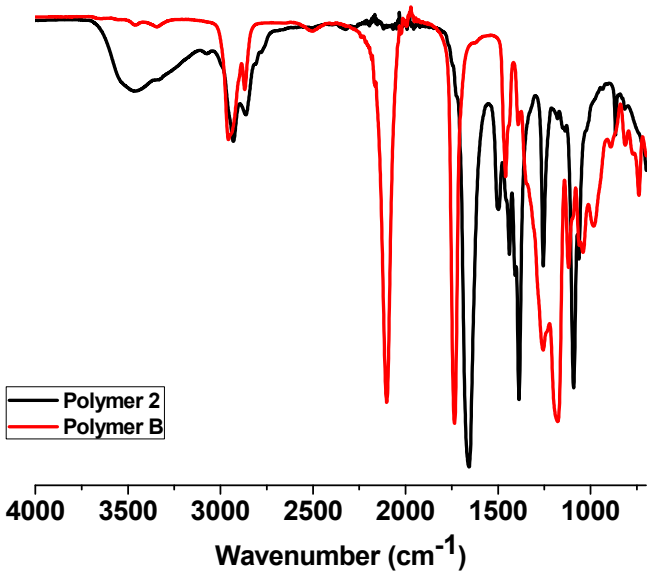


Figure S9. FT-IR spectra of polymer B and polymer **2**. The completion of CuAAC click reaction was confirmed by the disappearance of azide peak at 2100 cm^{-1}. Based on the quantitative click reaction, the molecular weight of polymer **2** was calculated to be: M_n(NMR) = 74,000 gmol^{-1}.

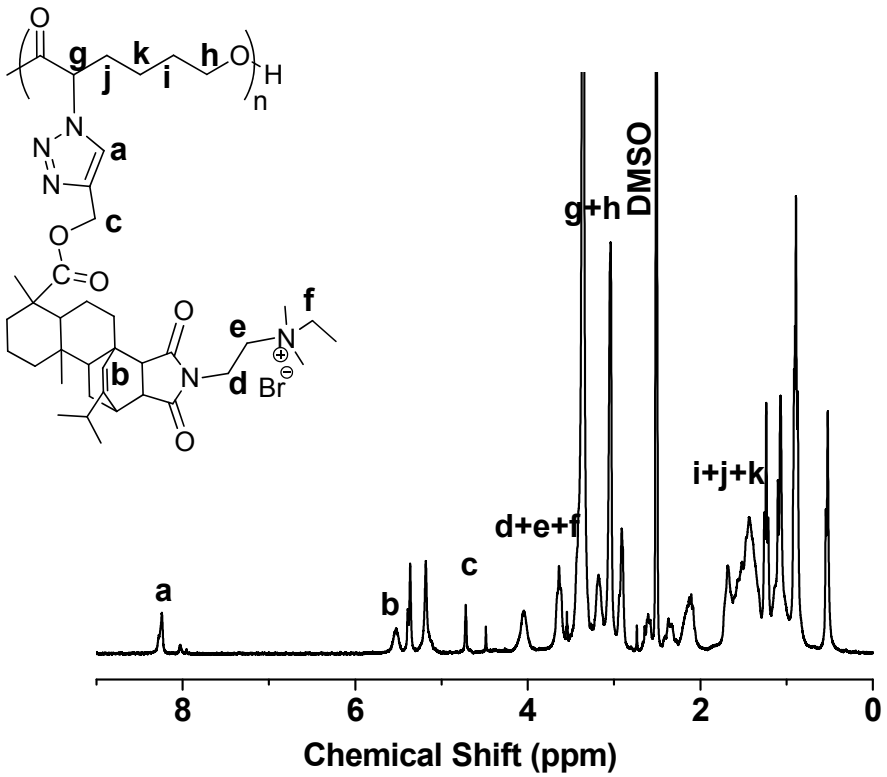


Figure S10. [1]H NMR spectrum of Polymer **2** in DMSO-d6.

Agar Disk-diffusion assays. To conduct the assays, actively-growing cultures of each bacterial strain on Mannitol salt agar (MSA) were inoculated on Tryptic Soy Broth (TSB) agar plates. The bacterial growth culture (cell concentrations were 1.0 x 10^6 CFU/mL) 10 µL was diluted to 1 mL in TSB and 100 µL of that was spread on TSB agar plates to form a bacterial lawn covering the plate surface. Then 6 mm (diameter) filter discs were added to the plate surface, then each compound at different concentrations in DMSO was added to disks, and the plates were incubated at 28 ºC for 18 h. The development of a clear zone around the disk was indicative of the ability of materials to kill bacteria. By quantifying the area (knowing its diameter and the depth of the agar) of inhibition, a minimum inhibitory concentration was calculated for each material/bacterial combination using established protocols.[2]

Hemolysis assay.[1] Fresh mouse red blood cells (RBC) were washed with PBS for three times. Next a suspension of 10x10^6 red blood cells in 50 µl PBS (4% in volume) was placed in each well of 96-well round bottom plates. Compound **1** and polymer **2** were dissolved in PBS added in individual wells at concentrations of 0, 100, 200, 300, 400, 500 µg/ml. PBS, 1% DMSO and 5% Triton solutions were accompanied in separate wells as negative or positive controls. Then all wells were adjusted with PBS to make the final volumes up to 200 µl. A humidified 5% CO_2 incubator was used to incubate the plates at 37 ºC for 1 h. After incubation the plates were centrifuged at 1000 g for 10 min. Then 100 µl of supernatants from each well were transferred to wells in a 96-well flat bottom plate. The absorbance at 576 nm for hemoglobin release from RBC was measured. Absorbance of supernatants from RBC lysed with 5% Triton was taken as 100%

hemolysis. Percentage hemolysis was calculated using the following formula: Hemolysis (%) = [(O.D.$_{576\ nm}$ in the resin acid material solution − O.D.$_{576\ nm}$ in PBS)/(O.D.$_{576\ nm}$ in 0.5% Triton X-100 − O.D.$_{576\ nm}$ in PBS)] × 100.

Cytotoxicity assays. *In vitro* cytotoxicity carried out using mouse splenocytes isolated from C57BL/6 mice and cultured in the presence of QA. Culture medium 200 μL was supplemented with 2 μL of DMSO containing different concentrations, up to 100 μg/mL of compound **1** or polymer **2**. DMSO 1% was used as the control. Fluorescence activated cell sorting (FACS) analysis of cell populations in mouse splenocytes after co-culture *in vitro* for two days was employed after staining with fluorochrome conjugated specific Abs to determine the cytotoxicity of compound **1** or polymer **2**. For *in vivo* tests the mice received intravenous injections of 10 mg/kg body weight of compound **1** or polymer **2**.

Dye-Leakage Studies. Recent literature procedures were followed to prepare the dye-filled liposomes.[3] Chloroform solutions of the lipids (Figure S11) (total 15mg) were mixed in a 10 mL round bottom flask and chloroform was removed by a gentle nitrogen stream to form a uniform film. The flask was placed under vacuum for an additional 3 h at room temperature. The dried film was hydrated by addition of 1 mL of 40 mM calcein in phosphate buffer (10 mM) at pH 7.0. The suspension was stirred for 1 h. The suspension was sonicated briefly (5 min) in a bath-type sonicator at room temperature and freeze-thawed (liquid nitrogen/water at 35 ^{o}C temperature) after each sonication. This was repeated 4 times until the suspension changed from milky to nearly clear (i.e., only slightly hazy) in appearance. The non-trapped calcein was removed by eluting through a size exclusion Sephadex G-25-150 column (15 cm × 1 cm), using phosphate saline buffer (phosphate 10 mM, NaCl 90 mM) at pH 7.0 as the eluent. The vesicle solutions were stored in a vial at 4 °C up to 4 days, and diluted with the PBS buffer as needed. TEM, dynamic light scattering, zeta potential and fluorescence imaging was used to characterize the liposomes (Figure S12).

The dye-leakage from the calcein trapped vesicles was monitored by recording the increase of calcein fluorescence intensity at 515 nm (excit. = 490 nm, slit width 2.5) (Figure S13). The 5-fold diluted vesicles 30 μL was added to 2940 μL PBS buffer in a cuvette. To normalize data, a baseline of calcein fluorescence without compound addition was observed for each sample for 60 sec. Phospholipid vesicles that were suspended in PBS buffer (pH 7.0) were stable, and no increase of fluorescence was observed. Then 30 μL of compound **1** in DMSO was added at t = 60 sec and the solution was mixed using the pipette tip. Lysis was quantified by measuring the increase in fluorescence from solutions after the addition of the compound. Complete vesicle disruption was achieved by addition of 30 μL Triton X-100 (20% in DMSO) at t = 480 sec from the addition of the compound, into the 3 mL of vesicle suspension. The final fluorescence intensity was used as 100% leakage. Lysis caused by the compound was reported as a percentage, calculated as 100 × [(F-F_o)/(F_t-F_o)], where F is the fluorescence intensity after addition of the compound **1** and F_0 and F_t are fluorescence intensities without compound **1** and

with Triton X-100, respectively. For the bacterial cell wall mimic, peptidoglycan extract from *S. aureus* was dissolved in DMSO at varying concentrations and compound **1** at a constant concentration, stirred for 12 h at 25 ºC before addition to the solution containing dye-filled liposomes.

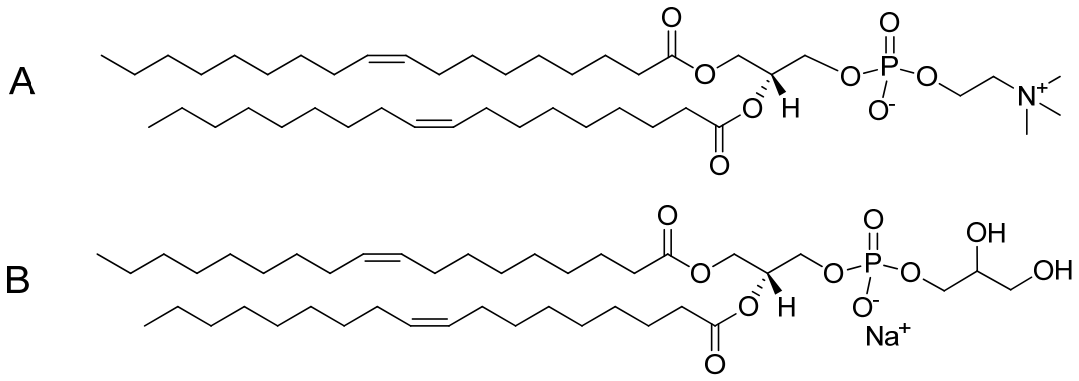


Figure S11. Lipids used for dye-leakage assays and molecular dynamics simulations. (A) 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC); (B) 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (DOPG).

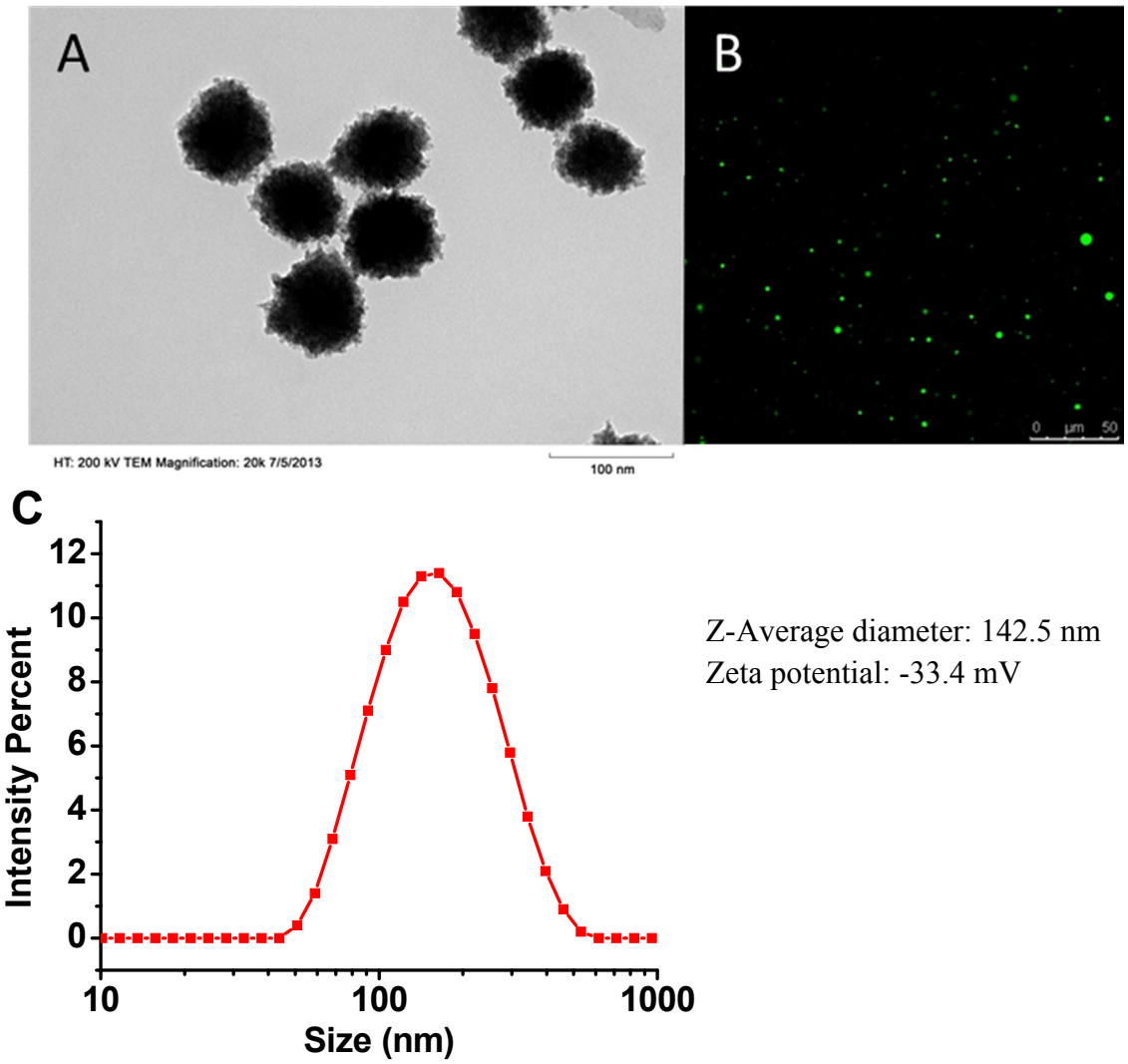


Figure S12. Dye-filled anionic large unilamellar liposomes. (A) Negatively stained electron microscopy image; (B) Fluorescence microscopy image; (C) Particle size distribution by dynamic light scattering and zeta potential data.

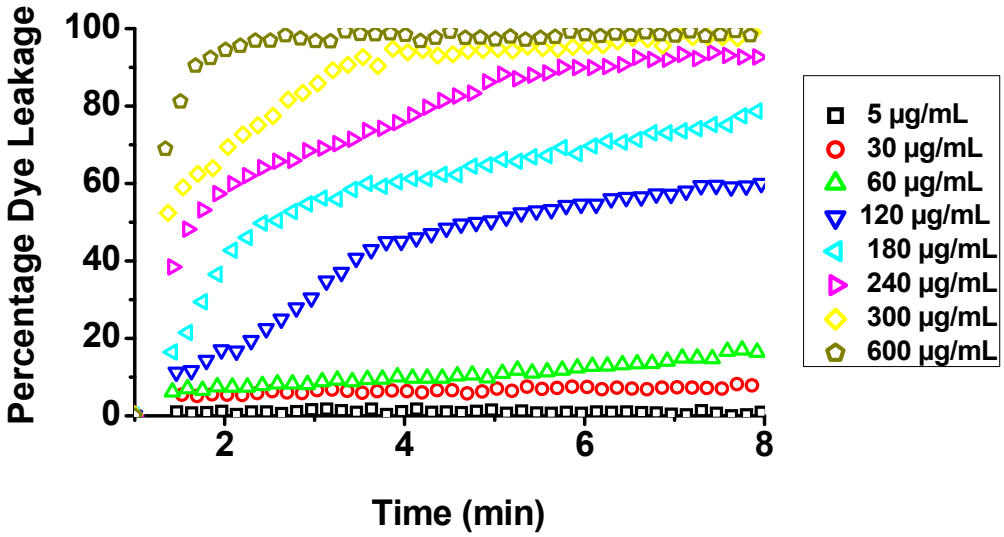


Figure S13. Dye-leakage from anionic lipid vesicles in the presence of different concentrations of compound **1**. Initial fluorescence was taken as 0% leakage and fluorescence after the addition of Triton X-100 was taken as 100%. Compound **1** was added at t=1 min and 20% Triton X-100 was added at t=9 min.

Molecular Dynamics Simulations. The anionic bacterial membrane was modeled using a 7:3 ratio mixture of dioleoylphosphatidylcholine (DOPC) and anionic dioleoylphosphatidylglycerol bilayer (DOPG)[4], while a quaternary ammonium-containing resin acid-derived agent (QAC) was used for modeling the natural resin acid-derived cationic compound. The system was constructed by placing QAC about 0.8 nm away from the surface of the upper leaflet of a pre-equilibrated DOPC-DOPG membrane. Overall, the equilibrated lipid bilayer contained 304 lipids. The CHARMM36 force field[5] parameter set was adopted for modeling DOPC and DOPG lipids. For modeling the cationic compounds, parameters were taken from the CHARMM General Force Field[6] or were derived from ab inito calculations, using the Gaussian09 software[7] performed at the MP2/6-31G* level of theory. All MDS were performed with the GROMACS program suite[8] version 4.6. The solvated bilayer-QAC system was subjected to stepwise energy minimization, equilibration followed by 600 ns of production MDS. A separate control experimental system was prepared by placing a long linear cationic alkyl chain near a similar DOPG-DOPC lipid bilayer under similar conditions (Figure S14). The MDS protocol used for the bilayer-QAC system was also repeated for the control-bilayer system.

The use of unconstrained MD to study the spontaneous insertion of a small compound into a bilayer is generally a difficult problem due to limited sampling. To improve the odds of capturing this event in our studies, we added 5 copies of either precursor of compound **1** or the control compound (alkyl ammonium) to the bulk solution. Using this procedure, two systems were setup. (A) QA-lipid system, consisting of five copies QAC, placed about 0.8 nanometers away from the surface of the upper leaflet of the pre-equilibrated DOPC-DOPG membrane. (B) AA-lipid system, consisting of five molecules of a long linear alkyl ammonium, randomly placed

0.8 nm near the charged surface of another copy of the lipid bilayer. The two complexes were then ionized with 0.1 M NaCl for electro neutrality, and stepwise energy minimization were performed using the GROMACS program.

MD simulations were performed with GROMACS. Bond lengths were constrained with P-LINCS[9] allowing for a 2 fs time step. Long-range electrostatic interactions were calculated using the particle-mesh Ewald method. Lennard-Jones potential and forces were truncated at 1.2 nm. The systems were slowly heated to 300 K. The two heterogeneous lipid systems were equilibrated in three phases. The first phase used an isochoric-isothermal (NVT) ensemble, with temperature controlled using the Berendsen weak coupling algorithm.[10] The NVT ensemble was applied for 500 ps, during which the temperature of the system was maintained at 300 K with a coupling constant of 0.1 ps. Heavy atoms of both the lipid and QAC molecules restrained. Next the systems were equilibrated with respect to pressure under the isobaric-isothermal (NPT) ensemble for 5 ns, using Nose-Hoover thermostat and Parrinello–Rahman barostat. Coupling constants for temperature and pressure were 0.1 and 2.0 ps, respectively. Following this, all atomic restraints were removed, and the systems were further equilibrated for 10 ns under the NPT ensemble. Unconstrained production simulations were performed for 600 nanoseconds under similar conditions.

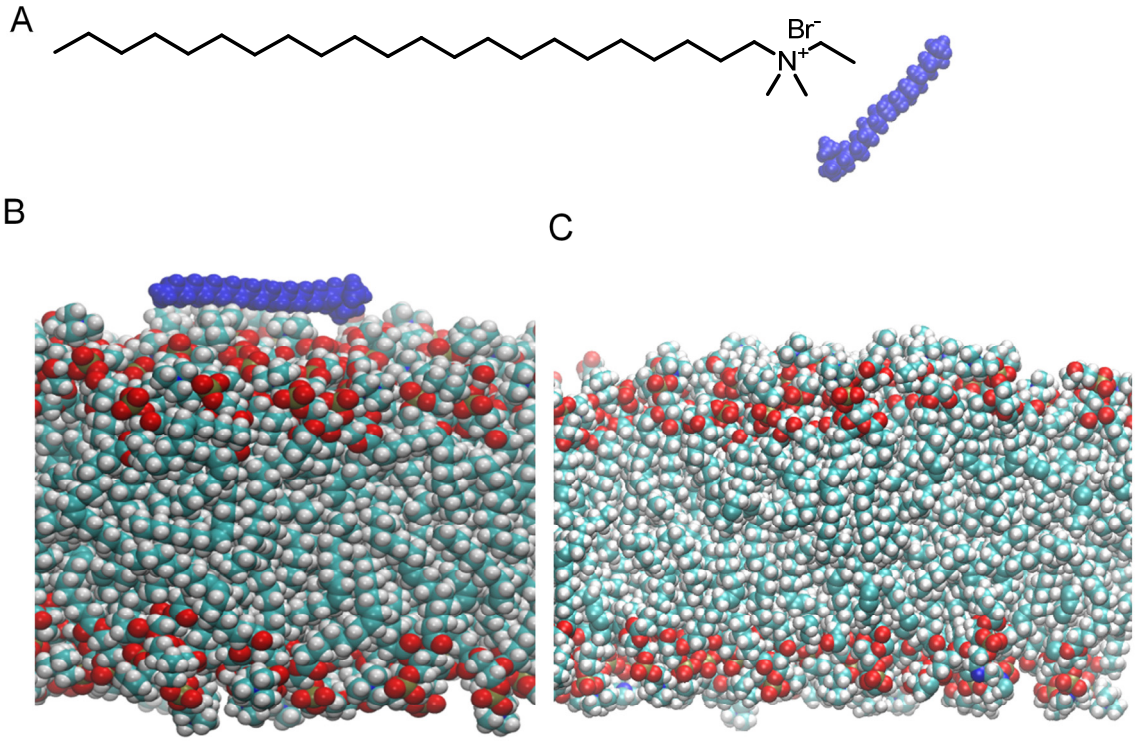


Figure S14. Control experiment for the molecular dynamics simulations. (A*)* Cationic linear alkyl chain used as the control (N-ethyl-N, N-dimethyldocosan-1-aminium bromide); (B*)* Start point of simulation with cationic alkyl chain placed near the surface of a lipid membrane; (C) As simulation progresses the cationic group drifts away from the membrane with its cationic head group oriented towards the polar head groups of the membrane.

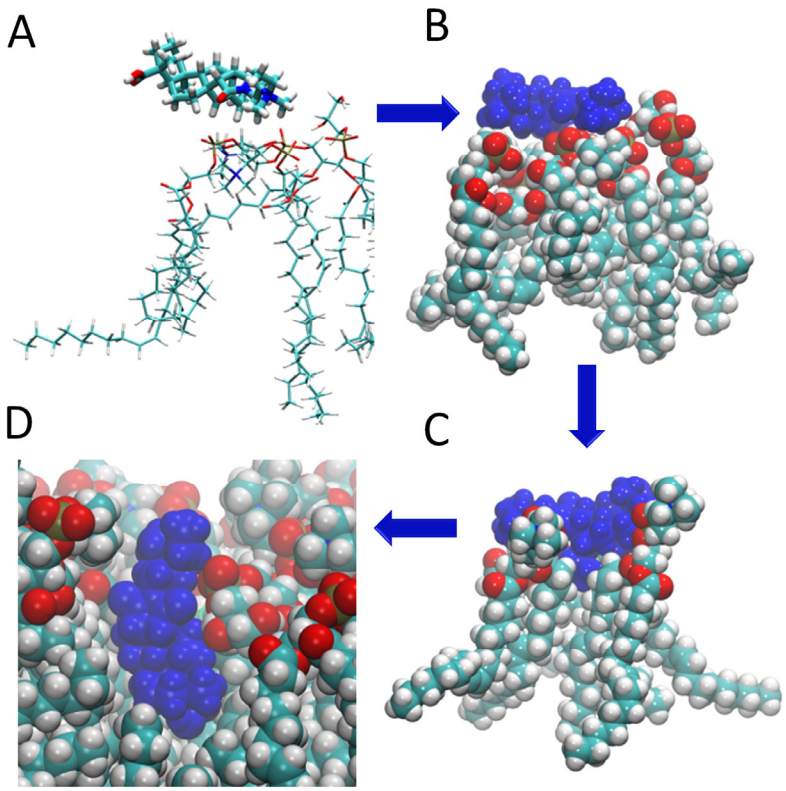


Figure S15. Molecular dynamics simulations of the acid format of compound 1 and model lipid bilayer. (A, B, C, D) Simulations snapshots (only neighboring lipid molecules and carboxylic acid containing the QA precursor of compound **1** are shown for clarity). The four stages depict QAC binding to lipid molecules in the model anionic membrane.

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