Robust Antimicrobial Compounds and Polymers Derived from Natural Resin Acids

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Supplementary Information

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I. Experimental and Characterization

Materials Maleic anhydride (99%), furan (99%), ethyl acetate (99%), ethanol, toluene, *N,N*-dimethylaminoethylamine (97%), abietic acid (85%), acetic acid, oxalyl chloride, propargyl alcohol, triethylamine (TEA), dichloromethane (DCM), *p*-toluene sulfonic acid (PTS), tetrahydrofuran (THF), hexane, methanol, bromoethane, 2-chlorocyclohexanone, *m*-chloroperoxybenzoic acid (mCPBA), Sn(II) 2-ethylhexanoate ($Sn(Oct)_2$), *N, N*-dimethylformamide (DMF), sodium azide, copper iodine and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) were purchased from Sigma Aldrich and used as received. α-Chloro-ε-caprolactone (αClεCL) and 2-hydroxyethyl 2-bromoisobutyrate (HEBIB) were prepared according to reported literatures.[1, 2]

Characterization Fourier transform infrared (FTIR) analysis was performed using a Perkin-Elmer spectrum by Attenuated Total Reflectance (ATR) method. Samples were analyzed as power or film on a ZnSn window. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Varian Mercury 300 MHz spectrometer with tetramethylsilane (TMS) as an internal reference. Gel permeation chromatography (GPC) was performed in DMF (containing 0.1% LiBr) 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.

II. Synthesis of Resin acid-Derived Antimicrobial Compounds and Polymers

Resin acids are mainly composed of diterpene resin acids (about 90%) (Figure S1) such as abietic, levopimaric, neoabietic, dehydroabietic acid and other non-abietane compounds (about 10%).[3, 4]

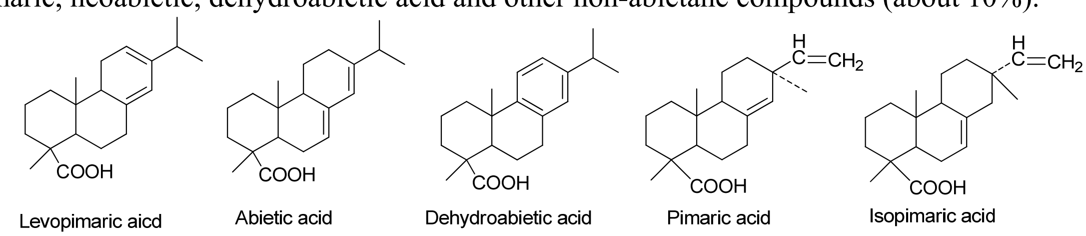
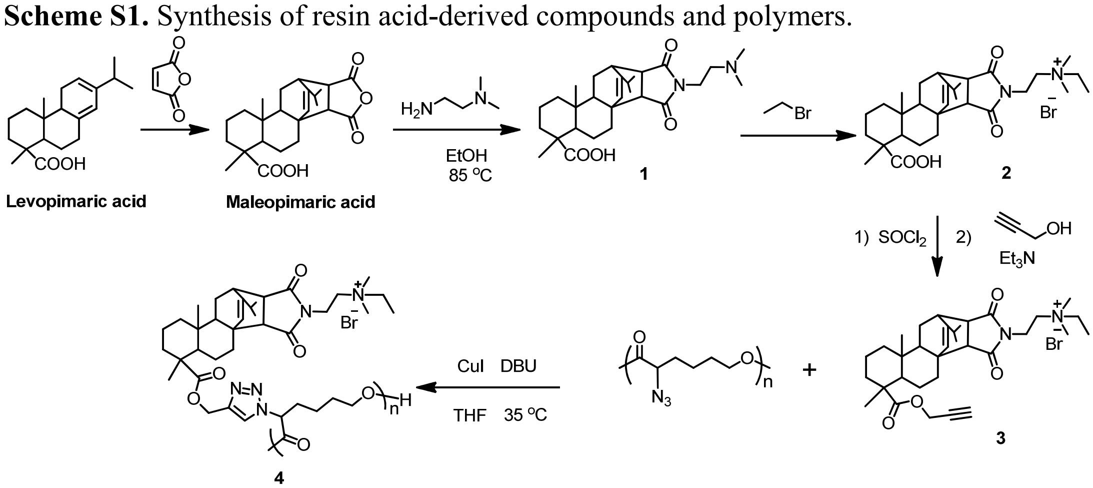


Figure S1. Structures of representative resin acids.

Scheme S1. Synthesis of resin acid-derived compounds and polymers.



Synthesis of maleopimaric acid Maleopimaric acid was prepared according to a reported procedure.[4] Abietic acid (100.0 g, 0.28 mol) was heated to 180 °C under a nitrogen atmosphere and maintained for 3h. After cooling the reaction to 120 °C, maleic anhydride (27.5 g, 0.28 mol), acetic acid (400.0 mL), and PTS (0.5 g, 0.0028 mol) were added. The reaction was refluxed at 120 °C for 12h and yellow crystals were observed. The product was obtained as white crystals after recrystallization from acetic acid twice (85.0 g, yield: 76%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 5.54 (s, 1H, C*H*=C); 3.10 (d, 1H, C*H*C=O); 2.73 (d, 1H, C*H*C=O); 2.5 (d, 1H, C*H*C=CH); 2.27 (m, 1H, CC*H*(CH_3)$_2$). ^{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).

Synthesis of Compound 1 Maleopimaric acid (10.0 g, 0.025 mol) was dissolved in ethanol (250.0 mL) followed by adding *N,N*-dimethylaminoethylamine (2.8 mL, 0.025 mol) and refluxed at 85 °C for 5 h. When the reaction cooled to room temperature, compound **1** precipitated out, then filtrated and washed with ethanol and dried (9.4 g, yield: 80%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 5.37 (s, 1H, C*H*=C); 3.59 (t, 2H, NC*H*$_2$CH$_2$); 3.38 (d, 1H, C*H*C=O); 3.18 (d, 1H, C*H*C=O); 2.69 (t, 2H, C*H*$_2$N(CH_3)$_2$); 2.5 (d, 1H, CH$_2$C*H*C=CH); 2.36 (s, 6H, CH$_2$N(C*H*$_3$)$_2$); 2.20 (m, 1H, CH$_2$=CC*H*(CH_3)$_2$). ^{13}C NMR(75 MHz, $CDCl_3$) δ: 181.9 (*C*=OOH); 178.2-177.1 (O=*C*N*C*=O); 145.2 (*C*=CH); 124.2 (C=*C*H); 55.9 (*C*H$_2$N(CH_3)$_2$); 50.8 (N*C*H$_2$CH$_2$N(CH_3)$_2$); 48.2 (C=O*C*H*C*HC=O); 46.2 (CH$_2$N(*C*H$_3$)$_2$); 45.7 (*C*C=O). ES-MS): m/z 471 (theoretical m/z: 470+H$^+$). FTIR (cm^{-1}): 2931, 2867, 1770, 1696, 1564, 1461, 1400, 1360, 1335, 1224, 1154, 1077, 1035, 1008.

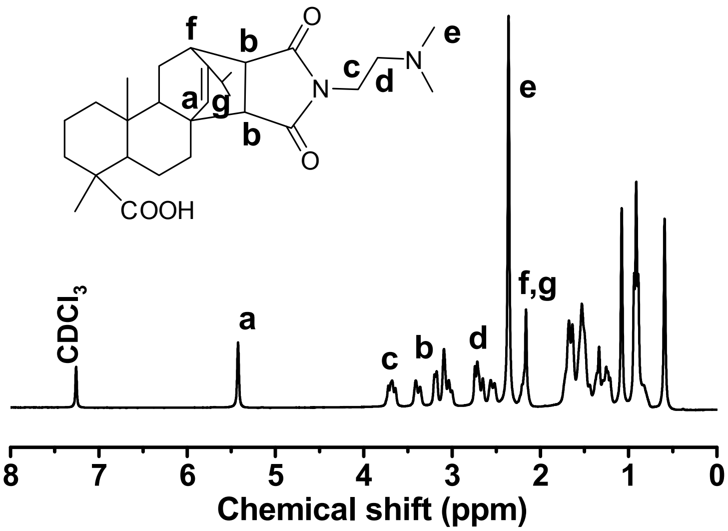


Figure S2. ^{1}H NMR spectrum of compound **1** in $CDCl_3$.

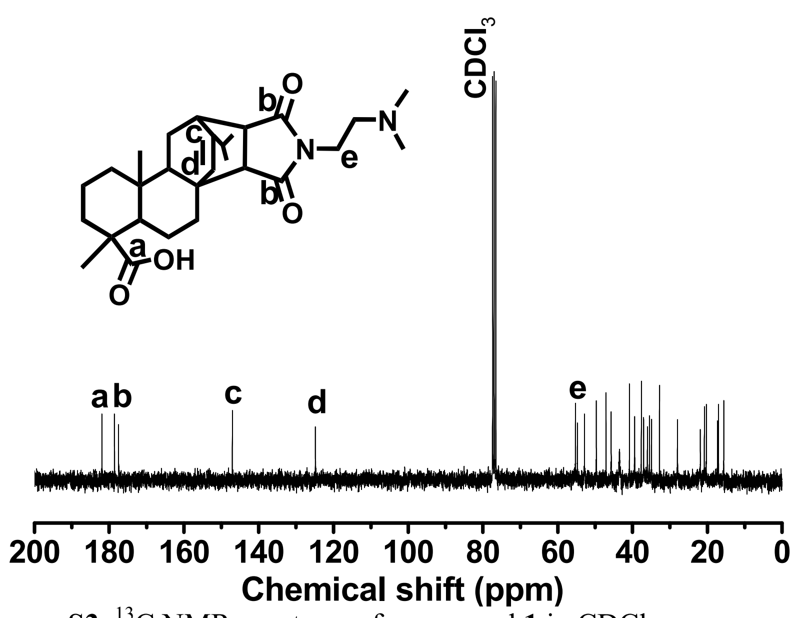


Figure S3. ^{13}C NMR spectrum of compound **1** in $CDCl_3$.

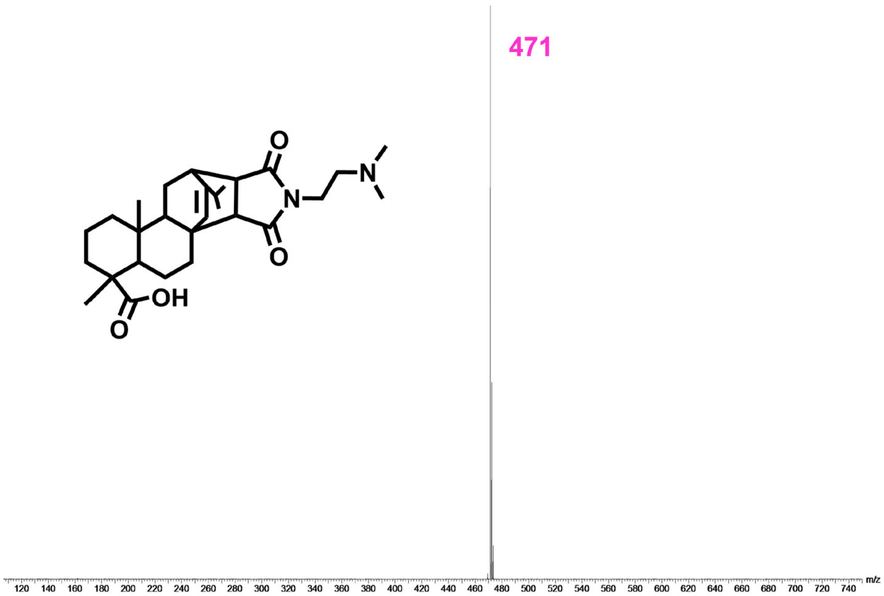


Figure S4. Mass spectrum of compound **1**.

Synthesis of Compound 2 Compound **1** (1.0 g, 0.0021 mol) and bromoethane (3.1 mL, 0.043 mol) were dissolved in dry THF (30.0 mL). The mixture was heated at 40 °C for 48 h. The crude product was precipitated in THF in the process of reaction. The product was filtrated and then washed with THF (0.9 g, yield: 75%). ^{1}H NMR (300 MHz, methanol-d$_4$) δ: 5.44 (s, 1H, C*H*=C); 3.77 (t, 2H, NCH_2CH$_2$); 3.46 (m, 2H, NCH$_2$CH_2); 3.30 (m, 2H, N$^+$CH_2CH$_3$); 3.13 (s, 6H, N$^+$(CH_3)$_2$); 2.98 (d, 1H, C*H*C=O); 2.63 (d, 1H, C*H*C=O); 2.49 (d, 1H, CH$_2$C*H*C=CH); 2.19 (m, 1H, CH=CC*H*(CH$_3$)$_2$). ^{13}C NMR (75 MHz, Methanol-d$_4$) δ: 180.3 (*C*=OOH); 178.8-178.0 (O=*C*N*C*=O); 148.7 (*C*=CH); 126.0 (C=*C*H); 61.0-60.4 (CH$_2$*C*H$_2$N$^+$ and CH$_3$*C*H$_2$N$^+$). ES-MS: m/z 499 (theoretical m/z: 499+79(Br)). FTIR (cm^{-1}): 2938, 2865, 1769, 1698, 1562, 1456, 1402, 1357, 1225, 1181, 1138, 1102, 1075, 1008.

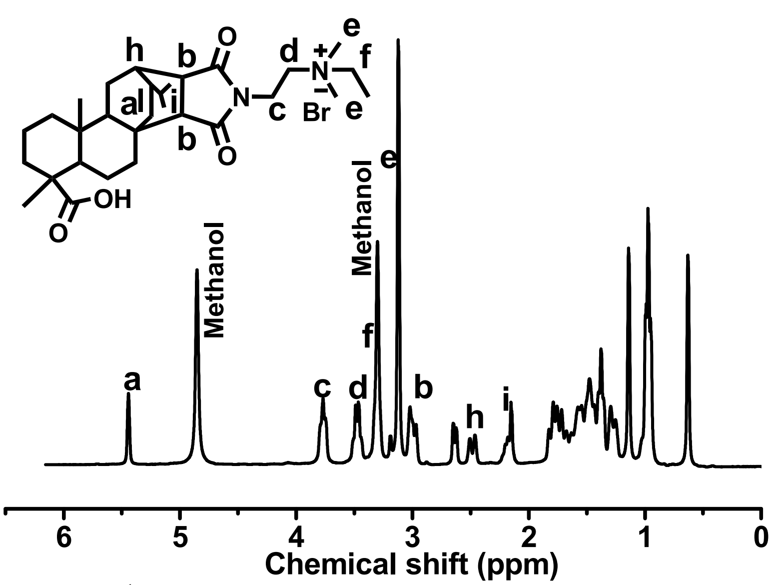


Figure S5. ^{1}H NMR spectrum of compound **2** in methanol-d$_4$.

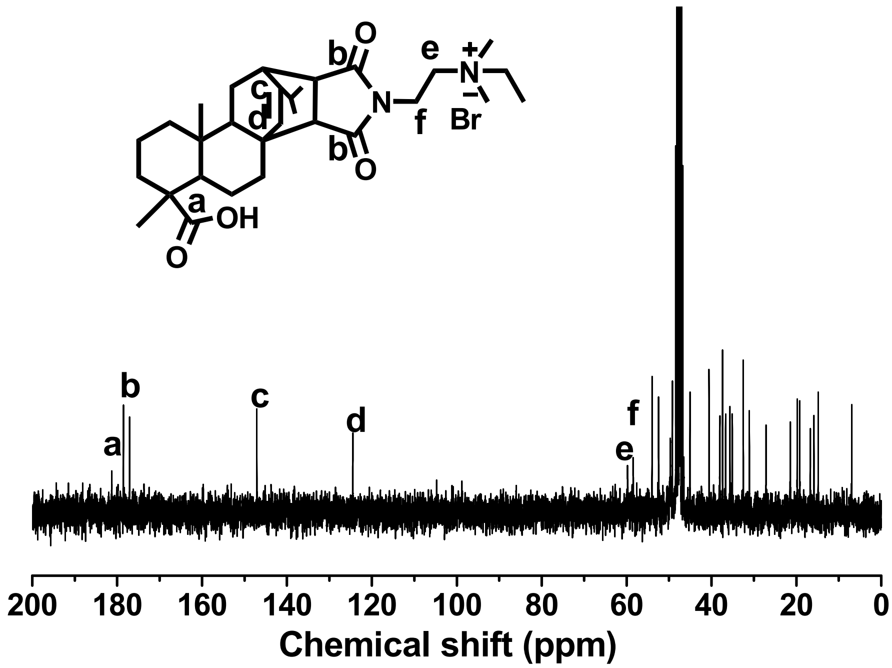


Figure S6. ^{13}C NMR spectrum of compound **2** in methanol-d_4.

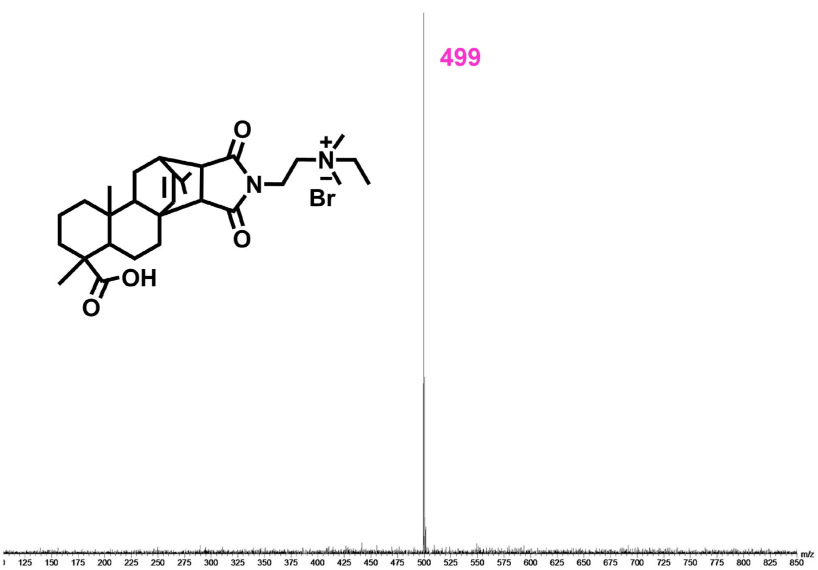


Figure S7. Mass spectrum of compound **2**.

Synthesis of Compound 3 Compound **2** (1.0 g, 0.0017 mol) was refluxed in thionyl chloride for 6 h followed by vacuum distillation to remove unreacted thionyl chloride. Propargyl alcohol (5.0 mL, 91.8 mmol) and sodium carbonate (0.2 g, 0.0017 mol) were added. The reaction was stirred at temperature overnight. The crude product was filtered, washed with hexane and dried in a vacuum oven (0.62 g, yield: 60%). ^{1}H NMR (300 MHz, methanol-d_4) δ: 5.44 (s, 1H, C*H*=C); 4.69 (m, 2H, CH_2C≡CH) [C*H*$_2$C≡CH]; 3.77 (t, 2H, NCH_2CH_2); 3.46 (m, 2H, NCH_2CH_2); 3.30 (m, 2H, $N^+CH_2CH_3$); 3.12 (s, 6H, $N^+(CH_3)_2$); 3.00 (m, 1H, C*H*C=O); 2.64 (d, 1H, C*H*C=O); 2.49 (d, 1H, CH_2C*H*C=CH); 2.19 (m, 1H, CH=CC*H*$(CH_3)_2$); 2.15 (s, 1H, CH_2C≡C*H*). ^{13}C NMR (75 MHz, methanol-d_4) δ: 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_2C≡*C*H); 59.5-60.0 ($CH_2CH_2N^+$ and $CH_3CH_2N^+$).

ES-MS: m/z 537 (theoretical m/z: 537+79(Br)). FTIR (cm^{-1}): 3289, 2955, 2870, 1764, 1734, 1689, 1462, 1438, 1399, 1351, 1240, 1178, 1163, 1135, 1074, 1011.

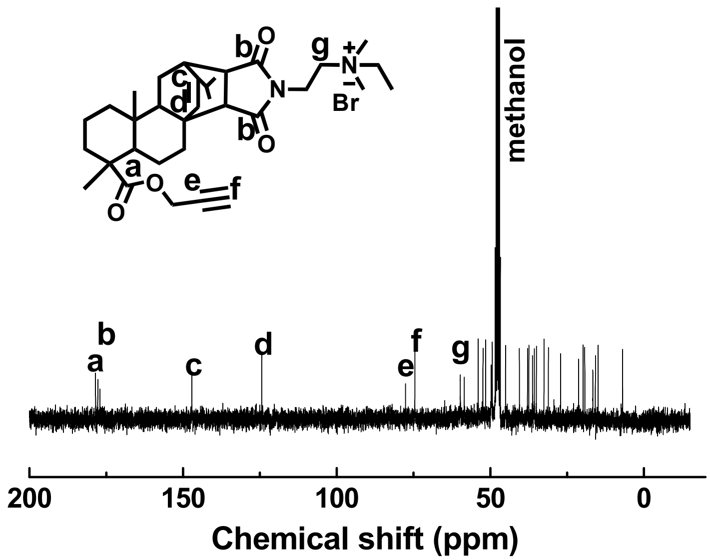


Figure S8. ^{13}C NMR spectrum of compound **3** in methanol-d$_4$.

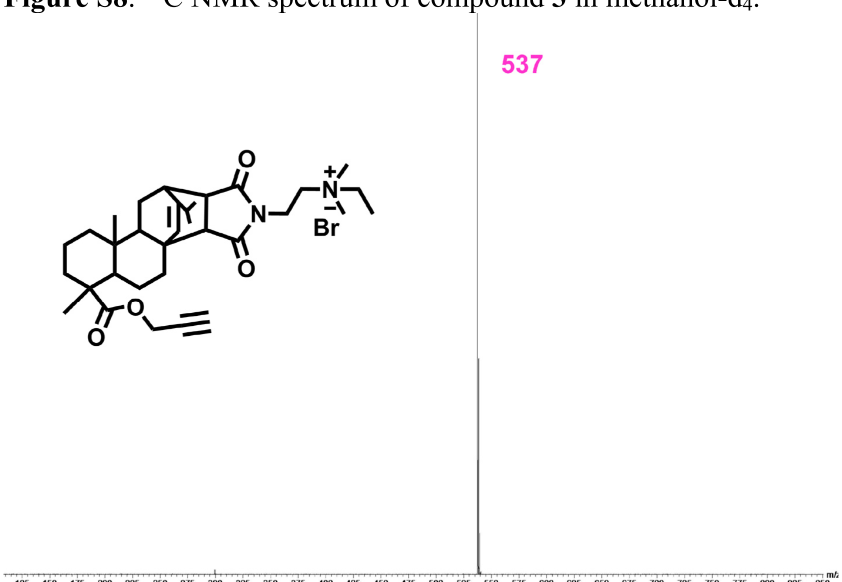


Figure S9. Mass spectrum of compound **9**.

Synthesis of Polymer 4 Azide-substituted PCL (poly(αN3εCL), M_n(GPC) = 26,400 g/mol; M_n(NMR) = 16,800 g/mol, M_w/M_n (GPC) = 1.15)) was prepared according to our previous report.[5] Compound **3** can be grafted onto the side chain of PCL via a click reaction. Degassed poly(αN_3εCL) (1g, 0.0062 (N_3) mol), compound **3** (4.56 g, 0.0074 mol) and CuI (0.12 g, 0.00062 mol) were dissolved in DMF and bubbled with nitrogen for 0.5 h. After DBU (0.094 g, 0.00062 mol) in deoxygenated THF was added, the solution was stirred at 35 °C overnight. At the conclusion of the click reaction, DMF was removed by distillation. The polymer was dissolved in DI water, and dialyzed against DI water to remove the excess compound **3**. The final product (Polymer **4**) was obtained by freeze dry. ^{1}H NMR (300 MHz, methanol-d_4) δ: 8.16 (s, C*H*=C, triazole); 5.49–5.14 (m, methlyene protons and methine proton next to the triazole and vinyl proton in resin acid moiety); 4.17 (s, OCH_2CH_2). Quantitative click reaction was further confirmed by

FTIR spectra (cm^{-1}). Based on the quantitative click reaction, the molecular weight of polymer **4** was calculated to be: M_n(NMR) = 71,000 g/mol.

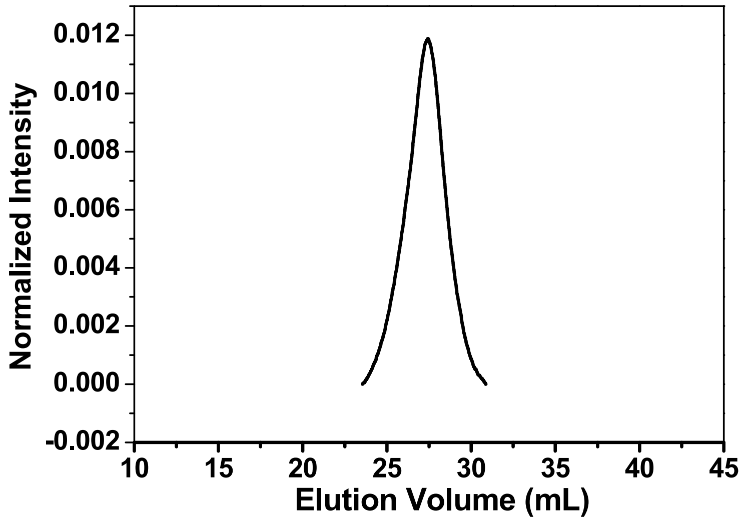


Figure S10. GPC trace of poly(αN3εCL).

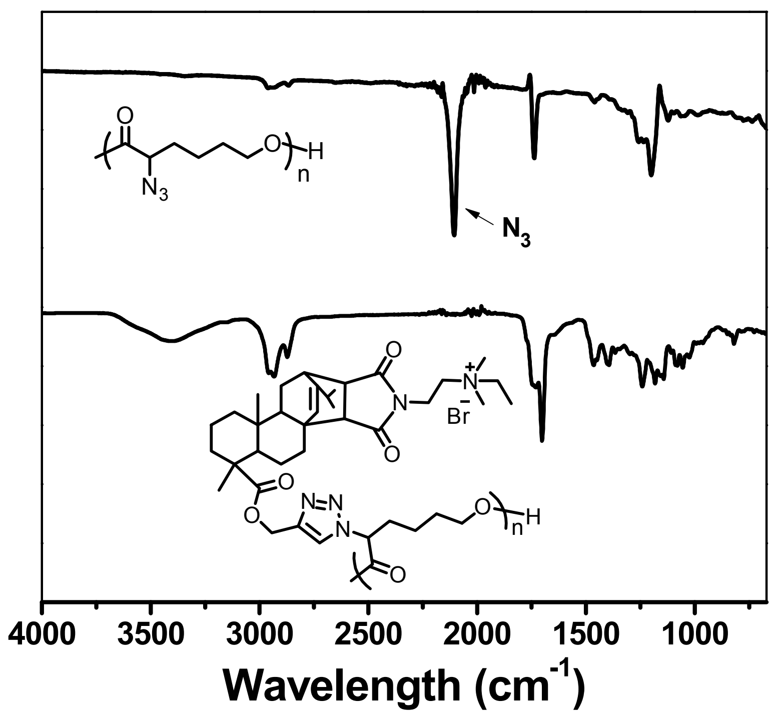


Figure S11. FTIR spectra of poly(αN3εCL) and Polymer **4**.

Synthesis of Tetraethylammonium Bromide (TEAB) Triethylamine (1.0 mL, 0.007 mol) and bromoethane (2.0 mL, 0.027 mol) were dissolved in dry THF (20.0 mL) and stirred at room temperature for 24 h. The crude product was precipitated from THF during the reaction. The precipitate was collected and washed with dry THF several times. A white solid was obtained after vacuum to drynes. ^{1}H NMR (300 MHz, D_2O) δ: 3.1 (m, 8H, NCH_2CH_3); 4.69 (m, 12H, NCH_2CH_3). ^{13}C NMR (75 MHz, D_2O) δ: 51.8 ($N^+CH_2CH_3$); 6.4 ($N^+CH_2CH_3$).

III. Antimicrobial Activity and Haemolysis Test

(1) Antimicrobial Activity

Antimicrobial activity tests of compound **2**, compound **3** and polymer **4** against *Staphylococcus aureus* (*S. aureus*) were carried out to determine minimum inhibitory concentration (MIC) by broth microdilution and disk-diffusion methods respectively. All other bacteria strains were tested using the disk-diffusion method.

Broth microdilution method. A 200 μL trypticase soy broth (TSB) medium solution was added to a 96 well microplate, and then inoculated 20 μL *S. aureus* (10^4 to 10^5 CFU/ mL). Different series of compound **2** (1-5 μg/mL), compound **3** (1-5 μg/mL) and polymer **4** (20-40 μg/mL) were added and placed in an incubator (37 °C for 18-24 h). These solutions were measured for absorbance at 660 nm by a microplate scanning spectrophotometer (powering wave 200™, Bio-Tek Instrument, Inc). 50 % (MIC50) and 90 % (MIC90) inhibition to bacterial cell growth were used to evaluate the antimicrobial activity.

Disk-diffusion method. The various test microorganisms were stored in the laboratory and maintained at –70 °C in a 1: 1 mixture of glycerol and DMSO. To conduct the assays, a small volume of actively-growing cultures of each bacterial strain (100 μL) was spread on TSB agar plates, and incubated for 24 h to form a 'bacterial lawn' covering the plate surface. Then 6 mm (dia) filter discs were added to the plate surface, then each compound (30 μL) at different concentrations in DMSO was added to disks, and the plates were incubated. 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 (MIC) was calculated for each material/bacterial combination using established protocols.[6-8]

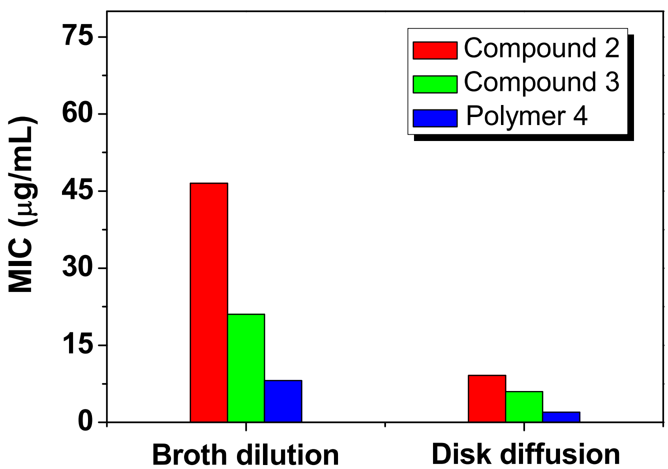


Figure S12. MIC of Compounds **2**, **3** and Polymer **4** against *Staphylococcus aureus* by broth dilution and disk diffusion method

Table S1. MIC values of resin acid-based antimicrobial materials determined by the broth microdilution method.

Microorganisms	Samples					
	Compound **2**		Compound **3**		Polymer **4**	
	MIC_{50} (μM)	MIC_{90} (μM)	MIC_{50} (μM)	MIC_{90} (μM)	MIC_{50} (μM)	MIC_{90} (μM)
S. aureus	25.9	46.6	9.7	21	3.5	8.1
P. aeruginosa	60.3	132.7	25.9	66.3	7.1	17.8
E. coli	37.9	79.3	16.2	38.8	9.9	18.4
K. pneumoniae	39.7	113.8	17.8	45.3	6.1	14.9

Table S2. MIC (μM) of resin acid-based antimicrobial materials determined by the disk diffusion method.

		TEAB[a]	Compound **1**[b]	Compound **2**	Compound **3**	Polymer **4**
Gram-positive	*S. aureus*	Non-toxic	Non-toxic	9.1	6	2
	B. cereus	Non-toxic	Non-toxic	9.3	7	1.4
	S. pyogenes	Non-toxic	Non-toxic	10.0	5.3	1.5
	M. luteus	Non-toxic	Non-toxic	10.1	3.1	0.8
	M. smegmatis	Non-toxic	Non-toxic	4.3	3.4	1
	C. xerosis	Non-toxic	Non-toxic	1.2	1.6	0.7
Gram-negative	*P. aeruginosa*	Non-toxic	Non-toxic	34.5	19.4	3.6
	E. coli	Non-toxic	Non-toxic	40	21	3.5
	K. pneumoniae	Non-toxic	Non-toxic	34.4	28.3	3.6
	P. vulgaris	Non-toxic	Non-toxic	32.8	17.8	4.9
	E. agglomerans	Non-toxic	Non-toxic	27.6	16.2	3.6
	S. typhimurium	Non-toxic	Non-toxic	24.1	19.4	3.3
	A. faecalis	Non-toxic	Non-toxic	22.4	17.8	3

[a]: MIC (TEAB) >> 2.3×10^4 μM; [b]: most of MIC (**1**) >> 6.4×10^3 μM.

Time-dependent efficiency of compound 3 and polymer 4 against *S. aureus*. *S. aureus* was incubated in a TSB solution until reaching O.D. value of 1.2. Cell culture medium (200 μL) was transferred into 96 well plates. Various samples of compound **3** (5 μL, a final concentration was 10 μg/mL) and polymer **4** (40 μL, a final concentration was 100 μg/mL) were added and incubated at 37 ^{o}C. At the designated intervals (1h, 3h and 6h), LIVE/DEAD assay reagent (5 μL, Invitrogen) was added and observed with a microplate fluorescence reader (PL 800, Bio-Tek Instrument, Inc). (**Figure S18**)

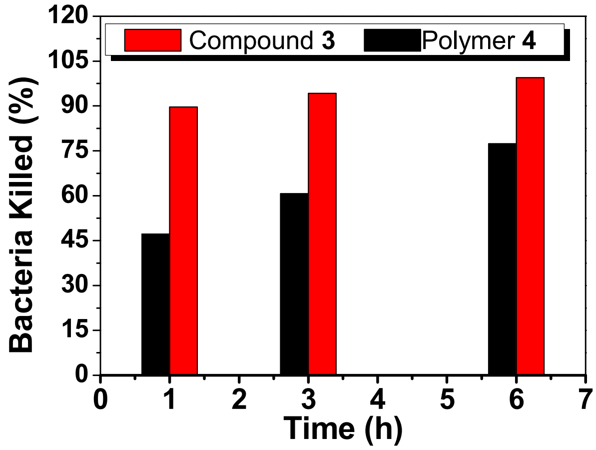


Figure S13. Time dependent antimicrobial activity of compound **3** and polymer **4** against *S. aureus*.

LIVE/DEAD bacterial viability assays. *K. pneumoniae* and *S. aureus* cells were incubated in a TSB solution until reaching O.D. value of 1.2. Then, 200 μL of cell culture was transferred into 96 well plates. Compound **3** (5 μL, a final concentration was 10 μg/mL) was added into each well and incubated at 37 ^{o}C. After 12 h, LIVE/DEAD assay reagent (Invitrogen) was added and observed using CLSM.

Morphology of *S. aureus* and *E. coli* in contact with compound 3 and polymer 4. Morphology of *S. aureus* and *E. coli* with or without compound **3** and polymer **4** was carried out by SEM (Zeiss *Ultra Plus* Field Emission Scanning Electron Microscopy (FE-SEM)). *S. aureus* and *E. coli* were grown in a TSB medium. While reaching O.D. value of 1.2, 200 μL cell medium was transferred into 96 well plates. Compound **3** (5 μg/mL) and polymer **4** (40 μg/mL) were added and incubated at 37 °C for 1 h. The sample for SEM was fixed with 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (PH = 7.2) for 2-3 h at room temperature, followed by washing with 0.1 M cacodylate buffer (PH = 7.2) and post-fixed with 1 % osmium tetraoxide (1 h at 4 °C). Dehydration of the sample was carried out using a graded series of ethanol (50 %, 70 %, 80 %, 95 %) solutions. After dehydration with 100 % ethanol for 10 min twice and dried in the air, the sample was coated with gold in Denton Desk II Sputter Coater for 15 s and observed by FE-SEM.

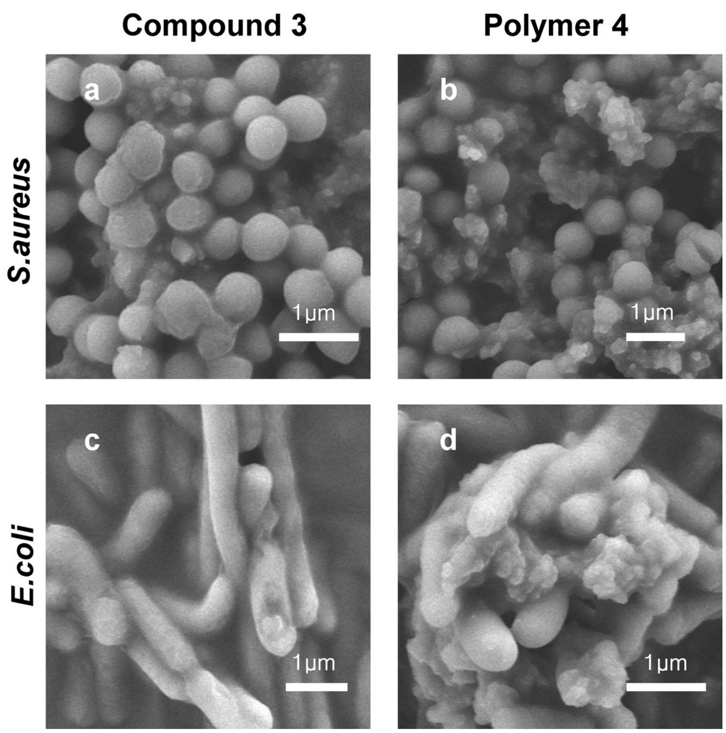


Figure S14. Morphology of *S.aureus* and *E.coli* in the presence of compound **3** and polymer **4**. (a) effects of compound **3** against *S.aureus;* (b) effects of polymer **4** against *S.aureus;* and (c) effects of compound **3** against *E.coli;* (d) effects of polymer **4** against *E.coli.*

(II) Haemolysis

Fresh mouse red blood cells were washed with phosphate buffered saline (PBS) solution for three times. 10×10^6 red blood cell suspension in 50 μL PBS (4% in volume) was placed in each well of 96-well round-bottom plates. Compounds **2**, **3** and polymer **4** were dissolved in PBS and added in individual wells at the concentrations of 0, 100, 200, 300, 400 and 500 μg/mL. PBS, 1% DMSO and 0.5% Triton were supplemented in separate wells as negative or positive controls. All wells were adjusted with PBS to make a final volume of 200 μL. Then, the plates were incubated at 37 °C for 1 h in a humidified 5% CO_2 incubator. After incubation, the plates were centrifuged. 100 μL supernatant in each well was transferred to 96-well flat-bottom plates. The absorbance at 576 nm for hemoglobin release from red blood cells was measured using a Wallac 1420 VICTOR^{2}™ Multilabel Counter (PerkinElmer, Shelton, CT). Absorbance of supernatants from red blood cells lysed with 0.5% Triton X-100 was taken as 100% haemolysis. Percentage of haemolysis was calculated using the following formula:

Haemolysis (%) = [(O.D.$_{576nm}$ in the resin acid material solution − O.D.$_{576nm}$ in PBS)/(O.D.$_{576nm}$ in 0.5% Triton X-100 − O.D.$_{576nm}$ in PBS)] × 100.

V. References

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