Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B

Electronic Supplementary Information

Synthetic amphiphiles as therapeutic antibacterials: Lessons on bactericidal efficacy and cytotoxicity and potential application as an adjuvant in antimicrobial chemotherapy

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EXPERIMENTAL METHODS

1.0 Synthesis of Amphiphiles

Compound 3 *[N-((pyridin-2-yl)methyl)dodecan-1-amine]*

100 mg of 2-pyridinealdehyde was refluxed with 190 mg (1.1 eqv.) of dodecylamine in methanol to afford the Schiff base N-((pyridin-2-yl)methylene)dodecan-1-amine in 70% yield. The Schiff base was reduced by gradual addition of 0.75 equivalent of $NaBH_4$ in its methanolic solution to obtain N-((pyridin-2-yl)methyl)dodecan-1-amine or **compound 3**. Crude product was purified by column chromatography with 62% yield.

Compound 4 *[N-((methylpyridinium-2-yl)methyl)dodecan-1-amine]*

Compound 3 was methylated by refluxing it in presence of 1.5 eqv. of methyl iodide in dioxane solvent to obtain the desired product N-((methylpyridinium-2-yl)methyl)dodecan-1-amine or **compound 4**. Crude product was purified by column chromatography with 49% yield.

Compound 5 *[N,N-bis((pyridin-2-yl)methyl) dodecan-1-amine]*

Compound 5 was synthesized from compound 3 by refluxing it with 1.2 equivalent of 1-chloromethyl (pyridine hydrochloride) in the presence of K_2CO_3 in CH_3CN for 48 hours. Crude product was purified by column chromatography with 59% yield.

Compound 6 *[N,N-bis((methylpyridinium-2-yl)methyl) dodecan-1-amine]*

Compound 6 was synthesized from **compound 5** by refluxing it with 3 equivalent of methyl iodide. The crude product was purified with column chromatography with 23% yield.

2.0 Minimum Inhibitory Concentration (MIC) and Minimum Killing Concentration (MKC) of Amphiphiles

Target bacterial cultures were inoculated at 1% level in microtitre wells (approximately 5×10^5 CFU/well) having 100 µL of the requisite growth medium and propagated overnight at 37ºC and 180 rpm in presence of varying concentrations of the amphiphiles (**compound 3**, **4**, **5** and **6**). The growth of the bacterial strains was verified by measuring absorbance at 600 nm in a microtitre plate reader (Infinite M200, TECAN, Switzerland). MIC of the amphiphile was defined as the minimum amphiphile concentration that resulted in growth inhibition of the target pathogens (A_{600}<0.1). An aliquot (1% v/v) from all the wells that indicated a lack of cell growth (A_{600}<0.1) was re-inoculated into separate microtitre wells with fresh growth medium in the absence of amphiphile and incubated overnight at 37^{o}C and 180 rpm. MKC of the amphiphile was defined as the lowest amphiphile concentration that inhibited the growth of the target bacterial cells following re-inoculation (A_{600}<0.1). MIC and MKC values were expressed as average values of six independent experiments.

3.0 Structure-Function Studies

The structure-function studies of the amphiphiles were conducted with equimolar amphiphile concentration and consisted of the following experiments:

5 (and 6)-carboxyfluorescein diacetate succinimidyl ester (cFDA-SE) leakage assay

A stock solution of cFDA-SE (500 µM) was prepared in ethanol and stored at -20^{o}C. Overnight grown cells of *S. aureus* MTCC 96 and *E. coli* MTCC 433 were harvested by centrifugation at 3,000 x g for 10 min. The cell pellet was washed twice with sterile phosphate buffer, resuspended in the same and labelled with cFDA-SE (final concentration of 50 µM) at 37°C for 20 min. The labelling reaction was terminated by pelleting the cells followed by washing twice with phosphate buffer to remove excess dye molecules. Equimolar concentration of **compound 3**, **4**, **5** and **6** (10, 20 and 40 µM) were added to 10^6

CFU/mL cFDA-SE labelled target bacteria and incubated at 37^{o}C and 180 rpm for 3 h. In case of control experiments, only DMSO solution devoid of amphiphile was added to labelled cells and incubated under the same conditions. Following incubation, cells were pelleted by centrifugation and leakage of carboxyfluorescein from amphiphile-treated cells was determined by measuring fluorescence of the cell free supernatant at an excitation wavelength of 488 nm and emission wavelength of 518 nm in a spectrofluorimeter (FluoroMax-3, HORIBA). The fluorescence measurements were recorded after subtracting the fluorescence of effluxed dye from control samples. For every amphiphile concentration and control sample, fluorescence measurements were acquired from three independent experimental samples.

PI uptake assay

A stock solution of PI (1.5 mM) was prepared in sterile MilliQ water and stored at 4^{o}C. Target bacterial cultures of *S. aureus* MTCC 96 and *E. coli* MTCC 433 (10^6 CFU/mL) were treated with equimolar concentration of **compound 3**, **4**, **5** and **6** (10, 20 and 40 μM) at 37^{o}C and 180 rpm for 3 h. As mentioned before, in case of control sample, only DMSO was added to cells and incubated under the same conditions. Following incubation, cells were washed with phosphate buffered saline (PBS), resuspended in the same and incubated with PI (final concentration of 30 μM) for 30 min at 37°C in a circulating water bath incubator (Amersham, USA). Subsequently, the cells were centrifuged and washed in distilled water to remove excess dye. The cells were then resuspended in PBS and fluorescence was measured in a spectrofluorimeter (FluoroMax-3, HORIBA) at an excitation wavelength of 535 nm and emission wavelength of 617 nm. The values obtained for untreated cells were subtracted from all experimental values. For every amphiphile concentration and control sample, fluorescence measurements were acquired from three independent experimental samples.

Fluorescence microscopy

Cell suspensions of *S. aureus* MTCC 96 and *E. coli* MTCC 433 in PBS (10^6 CFU/mL) were treated with 40 µM of amphiphile (**compound 3**, **4**, **5** and **6**) at 37^{o}C and 180 rpm for 3 h. Control sample consisted of DMSO treated cells incubated under the same conditions. Following incubation, both the treated and control cells were washed twice with PBS, resuspended in the same and labelled with cFDA-SE and PI as mentioned before. The stained samples were fixed in 2.5% glutaraldehyde and subsequently washed twice with PBS. A 10 µl aliquot of the stained sample was gently spotted on a clean glass slide, air dried and observed under a fluorescence microscope (Eclipse Ti-U, Nikon, USA) with a filter that allowed blue light excitation for cFDA-SE and green light excitation in case of PI stained cells. Images of the control and treated cell were recorded.

4.0 Mode of action of compound 6

To ascertain the mode of action of **compound 6**, the following experiments were performed:

Time- kill curve

Target cells of Gram-positive *S. aureus* MTCC 96 and Gram-negative *E. coli* MTCC 433 (approximately 10^6 CFU/mL each suspended in PBS) were treated with varying concentration (3 - 15 µM) of **compound 6** at 37^{o}C and 180 rpm. Samples were withdrawn at regular intervals of 3 h, 6 h, 12 h and 24 h and subjected to serial dilution and plating to the ascertain the viable cell number (Log_{10} CFU/mL). The viable cell count was also determined at the specified intervals for cells suspended in PBS as well as cells treated with DMSO alone.

Flow cytometry analysis

The effect of **compound 6** on the viability of *S. aureus* MTCC 96 and *E. coli* MTCC 433 was studied by flow cytometry (FCM), which was performed on a FACS Calibur flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA). Target bacterial cells

were pre-labelled with cFDA-SE following the protocol as mentioned earlier. Labelled bacterial cells were treated with 50 µM of **compound 6** for 6 h at 37°C in circulating water bath incubator (Amersham, USA). Following treatment, treated cells were analysed along with unlabelled-untreated cells as well as labelled-untreated cells (control). For FCM analysis, approximately 10^8 cells per ml were analyzed at a low flow rate setting and the FACS instrument was adjusted to acquire 20,000 events. Before analysis of amphiphile-treated samples, appropriate voltage and threshold parameter was adjusted for unlabeled-untreated cells. The corresponding signal of unlabeled-untreated cells was set in the lower left quadrant in order to compensate for cellular autofluorescence. Green fluorescence of cFDA-SE stained cells was detected in the FL1 channel (band pass filter of 530 nm). Acquisition of fluorescence data was accomplished by setting a gate in the forward-angle light scatter (FSC) vs. sideward scatter (SSC) plot, which facilitated discrimination of bacterial cells from other artefacts. Data were acquired and analyzed with the CellQuest Pro software (BD CellQuest™ Pro Version 6.0, Becton-Dickinson, USA) and the WinMDI software program version 2.9 (http://en.bio-soft.net/other/WinMDI).

Fluorescence microscope analysis

Approximately 10^6 CFU/mL cells of *S. aureus* MTCC 96 and *E. coli* MTCC 433 were treated with varying concentrations (6-15 µM) of **compound 6** and incubated at 37^{o}C and 180 rpm for 3 h. DMSO treated cells incubated under the same conditions were designated as control sample. After amphiphile treatment, cells were washed and labelled with cFDA-SE and PI as mentioned before, fixed in 2.5% glutaraldehyde and washed twice with PBS. A 10 µl aliquot of the stained sample was spotted on a clean glass slide, air dried and observed under a fluorescence microscope (Eclipse Ti-U, Nikon, USA) with a filter that allowed blue light excitation for cFDA-SE and green light excitation in case of PI stained cells. Images of the control and treated cell were acquired.

Scanning electron microscope (SEM) analysis

Overnight grown cells of *S. aureus* MTCC 96 and *E. coli* MTCC 433 were washed twice in phosphate buffer saline (PBS) and resuspended in the same buffer. Approximately 10^6 CFU/mL cells were treated 50 µM of **compound 6** for 6 h at 37°C. Control samples consisted of untreated cells incubated in PBS under the same conditions. Treated as well as untreated cells were washed twice with PBS and fixed in 2.5% Glutaraldehyde for 90 mins at 4°C. Following fixation, cells were washed and resuspended in sterile MilliQ grade water. A 2.0 µl aliquot of each sample was spotted on SEM stubs and air dried in a laminar hood. Subsequently, the cells were coated with gold plasmon and observed under scanning electron microscope (Leo, 1430 vp) at 15 kV and the images were recorded.

Effect of membrane potential on antimicrobial activity of compound 6

Cells of *S. aureus* MTCC 96 and *E. coli* MTCC 433 were grown overnight in nutrient broth with 0.5% glucose and 20 µM CCCP as described in a previous report.[1] CCCP treated and untreated cells (control) were suspended in PBS (approximately 10^6 CFU/mL each) and incubated with varying concentration of **compound 6** (9 µM and 15 µM) for 6 h at 37°C. At regular time intervals of incubation period the bactericidal activity of the amphiphile on CCCP treated and untreated cells was compared by determining the viable cell count through conventional serial dilution and plating.

Membrane depolarization assay

The ability of **compound 6** to depolarize the transmembrane potential was determined by a fluorescence-based assay using 3,3'-dipropylthiadicarbocyanine iodide ($DiSC_35$), which is a membrane potential sensitive dye. Cells of *S. aureus* MTCC 96 and *E. coli* MTCC 433 were grown till mid-logarithmic phase (A_{600} = 0.4-0.5). The cells were harvested by centrifugation, washed and resuspended in HEPES buffer (5 mM HEPES, 20 mM glucose, pH 7.2) to achieve A_{600} of 0.05. The cell suspensions were incubated with 0.4 µM $DiSC_35$ for 1 h at

37°C followed by the addition of KCl (final concentration of 100 mM) and further incubated for 15 min.[2] The cell suspension was then placed in a cuvette to which varying concentrations of **compound 6** (6-15 μM) was added and the fluorescence emission intensity (λ_{Ex} = 622 nm and λ_{Em} = 670 nm) was monitored in short time intervals in a spectrofluorimeter (FluoroMax-3, HORIBA) with excitation and emission slit width set at 10 nm each. Cells treated with valinomycin (30 μM) were used as positive control. Fluorescence measurements were taken for three independent experimental samples.

5.0 Determination of Membrane Permeabilization by NPN Assay

A stock solution of NPN (500 μM) was made in acetone. Target cells of *E.coli* MTCC 433 were grown in nutrient broth (NB) medium at 37°C in a shaker incubator till mid-logarithmic phase (A_{600} of 0.5). The cells were centrifuged, washed twice with 5mM HEPES buffer (pH 7.4) and resuspended in the same buffer. A 1.0 ml aliquot of target cells was taken in a cuvette to which NPN (final concentration of 10 μM) was added. Enhancement in the fluorescence intensity of NPN was measured as a function of time following addition of varying concentrations of **compound 6** (9.0 and 15 μM). As a positive control sample, cells treated with polymixin B (1.0 μg/mL) was included. All fluorescence measurements were taken in a spectrofluorimeter (FluoroMax-3, HORIBA) at an excitation and emission wavelength of 350 nm and 420 nm, respectively. Fluorescence measurements were taken for three independent samples.

7.0 Reference

1. Mangoni, M. L.; Papo, N.; Mignogna, G.; Andreu, D.; Shai, Y.; Barra, D.; Simmaco, M. Ranacyclins, a new family of short cyclic antimicrobial peptides: biological function, mode of action, and parameters involved in target specificity. *Biochemistry* **2003**, *42*, 14023-14035.

2. Vooturi, S. K.; Cheung, C. M.; Rybak, M. J.; Firestine, S. M. Design, synthesis, and structure-activity relationships of benzophenone-based tetraamides as novel antibacterial agents. *J. Med. Chem.* **2009**, *52*, 5020-5031.

RESULTS

Characterization of Compound 3:

^{1}H NMR [400 MHz, $CDCl_3$, δ (ppm)]: 8.553 (1H, d, J=0.013), 7.640 (1H, t, J=0.020), 7.305 (1H, d, J=0.020), 7.158 (1H, t, J=0.013), 3.906 (2H, s), 2.651 (2H, t, J=0.019), 1.255 (20H, m), 0.0879 (3H, t, J=0.017), ^{13}C NMR [100 MHz, $CDCl_3$, $SiMe_4$, δ (ppm)]: 159.826, 149.294, 136.841, 122.379, 121.952, 55.258, 49.729, 31.974, 30.121, 29.686, 29.625, 29.404, 27.413, 22.738 14.158. ESI-MS (positive mode, m/z). Calcd for $C_{18}H_{33}N_2$: 276.26. Found: 277.26 ($M + H^+$).

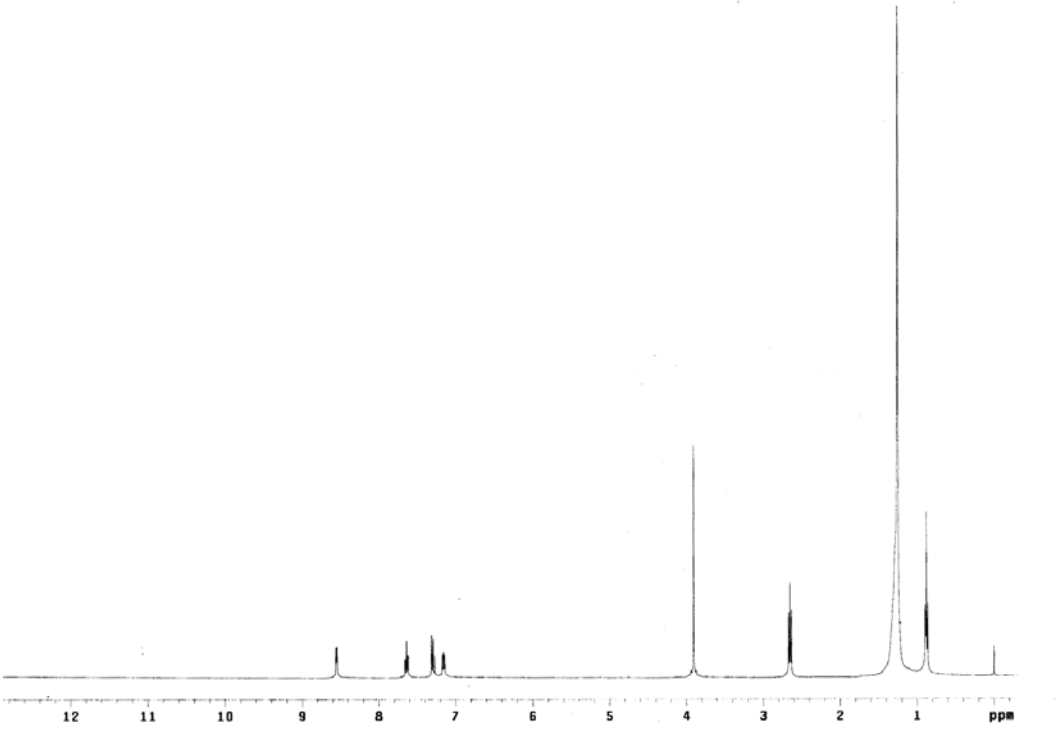

Fig. S1 ^{1}H NMR spectra of **compound 3** in $CDCl_3$ solution.

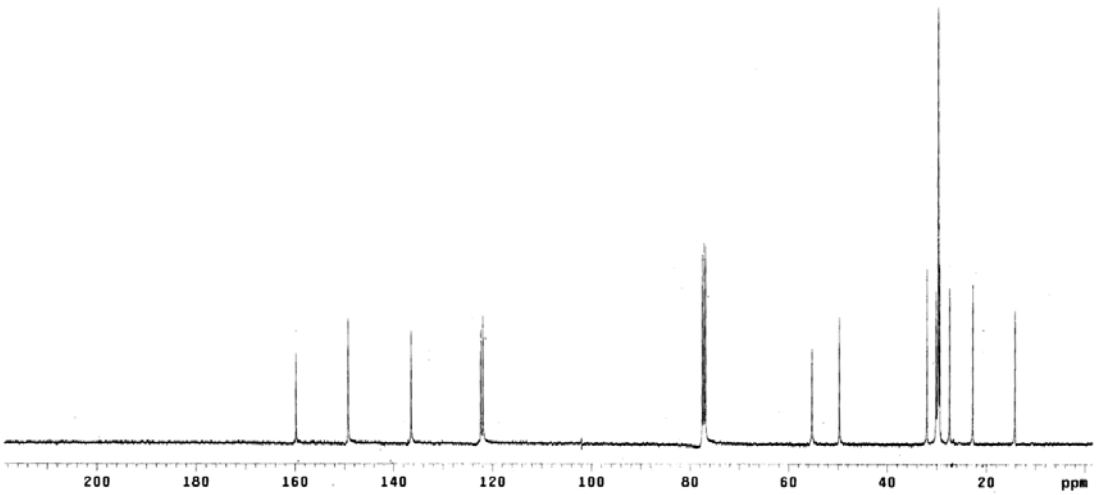

Fig. S2 ^{13}C NMR spectra of **compound 3** in $CDCl_3$ solution.

Characterization of Compound 4:

^{1}H NMR [400 MHz, DMSO-d_6, δ (ppm)]: 9.077 (1H, d, J=0.014), 8.739 (1H, t, J=0.020), 8.551 (1H, d, J=0.020), 8.152 (1H, t, J=0.017), 6.813 (3H, s), 5.458 (2H, s), 4.869 (2H, t, J=0.011), 4.381-4.544 (20H, m), 2.781 (3H, t, J=0.014), ^{13}C NMR [100 MHz, $CDCl_3$, $SiMe_4$, δ (ppm)]: 149.385, 148.714, 148043,147.723, 146.579, 51.185, 40.744, 31.356, 29.068, 28.786, 26.148, 25.644, 23.707, 22.128, 13.601. ESI-MS (neutral mode, m/z). Calcd for $C_{19}H_{36}IN_2$: 418.18. Found: 418.18.

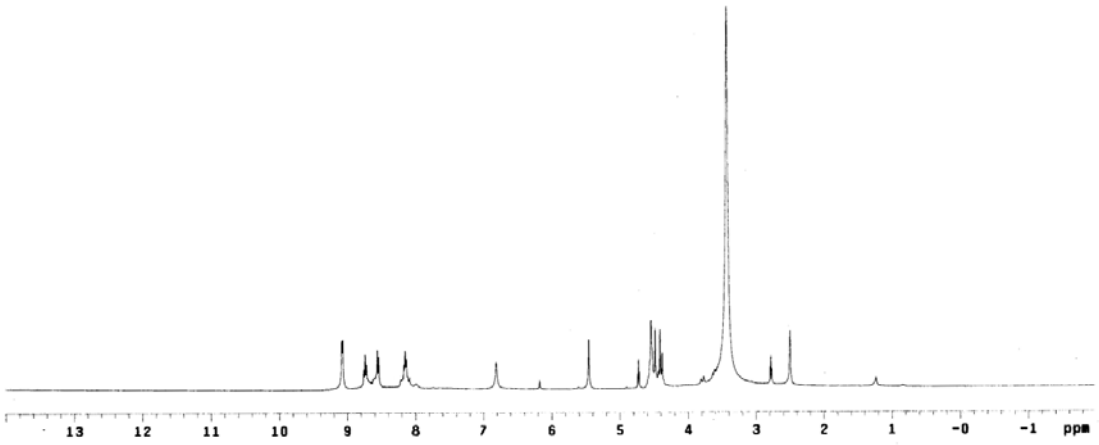

Fig. S3 ^{1}H NMR spectra of **compound 4** in DMSO-d_6 solution.

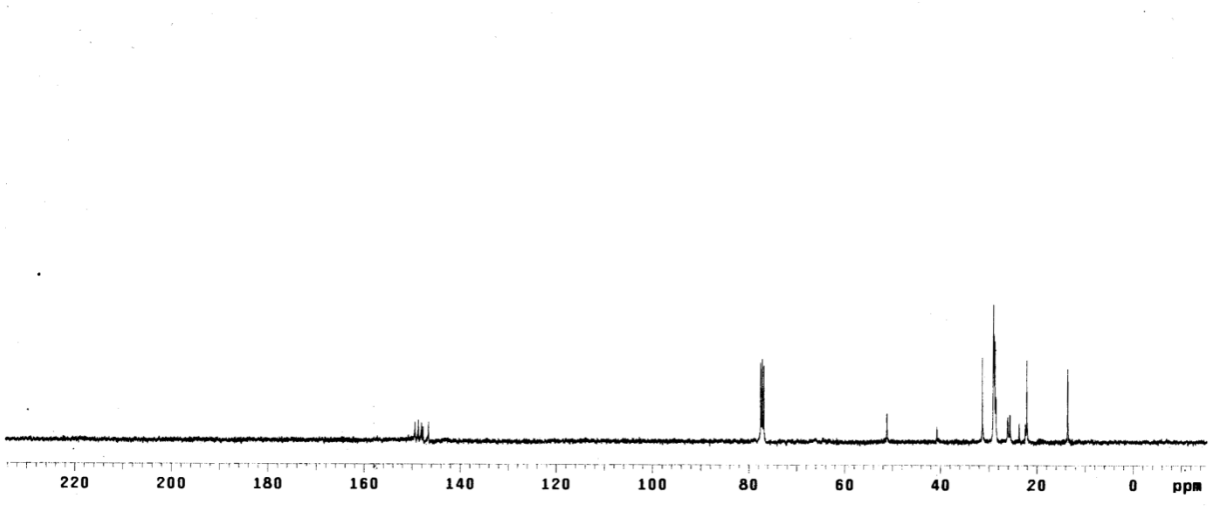

Fig. S4 ^{13}C NMR spectra of **compound 4** in $CDCl_3$ solution.

Characterization of Compound 5:

^{1}H NMR [400 MHz, $CDCl_3$, δ (ppm)]: 8.512 (2H, d, J=0.013), 7.646 (2H, t, J=0.019), 7.305 (2H, d, J=0.020), 7.13 (2H, t, J=0.015), 3.807 (4H, s), 2.529 (2H, d, J=0.019), 1.235 (20H, broad multiplate), 0.0878 (3H, t, J=0.018), ^{13}C NMR [100 MHz, $CDCl_3$, $SiMe_4$, δ (ppm)]: 160.184, 148.905, 136.344, 122.860, 121.838, 60.528, 54.549, 31.943, 29.655, 29.503, 29.373, 27.345, 27.108, 22.715, 14.151. ESI-MS (positive mode, m/z). Calcd for $C_{24}H_{37}N_3$: 367.2987. Found: 368.3066 ($M + H^+$).

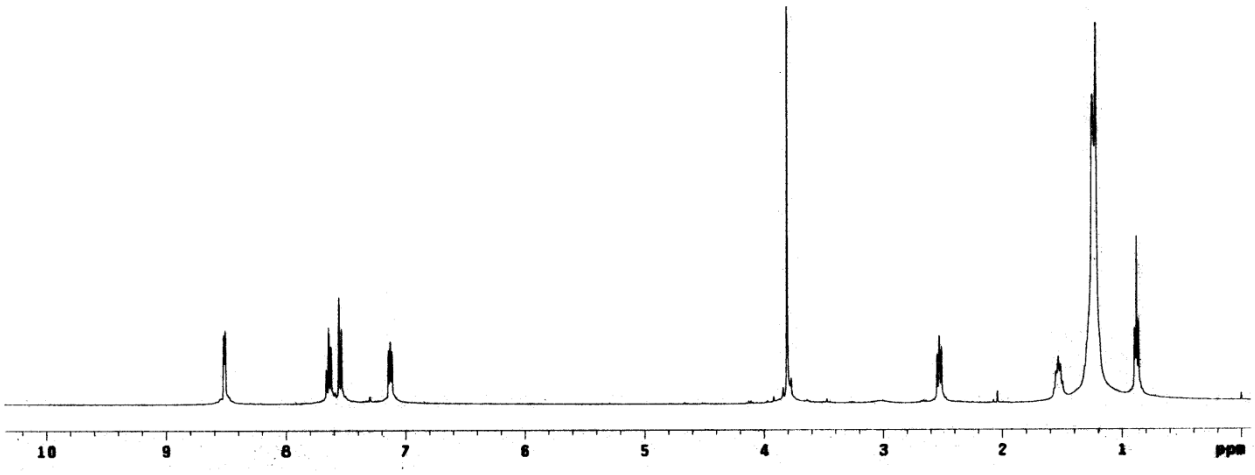

Fig. S5 ^{1}H NMR spectra of **compound 5** in $CDCl_3$ solution.

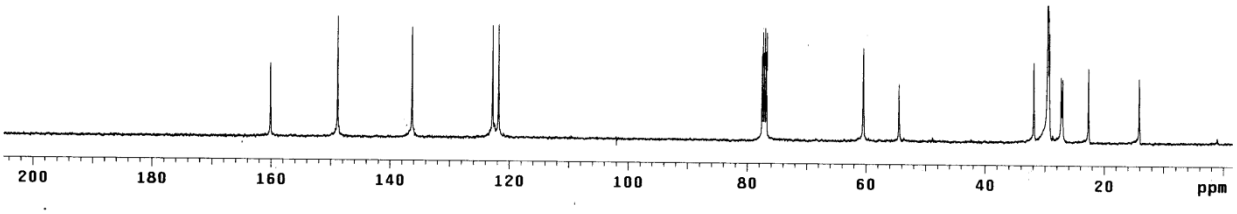

Fig. S6 ^{13}C NMR spectra of **compound 5** in $CDCl_3$ solution.

Characterization of Compound 6:

^{1}H NMR [400 MHz, DMSO-d_6, δ (ppm)]: 8.686 (2H, t, J=0.011), 8.604 (2H, t, J=0.020), 8.355 (2H, d, J=0.020), 8.232 (2H, d, J=0.009), 6.665 (6H, s), 5.690 (4H, s), 4.238-4.496 (22H, m), 2.788 (3H, t, J=0.015), ^{13}C NMR [100 MHz, $CDCl_3$, $SiMe_4$, δ (ppm)]: 157.719, 154.135, 146.744, 145.822, 144.838, 78.983, 65.888, 46.730, 46.471, 45.891, 37.288, 28.907. ESI-MS (positive mode, m/z). Calcd for $C_{26}H_{43}I_2N_3$: 651.15. Found: 325.58 (M/2)

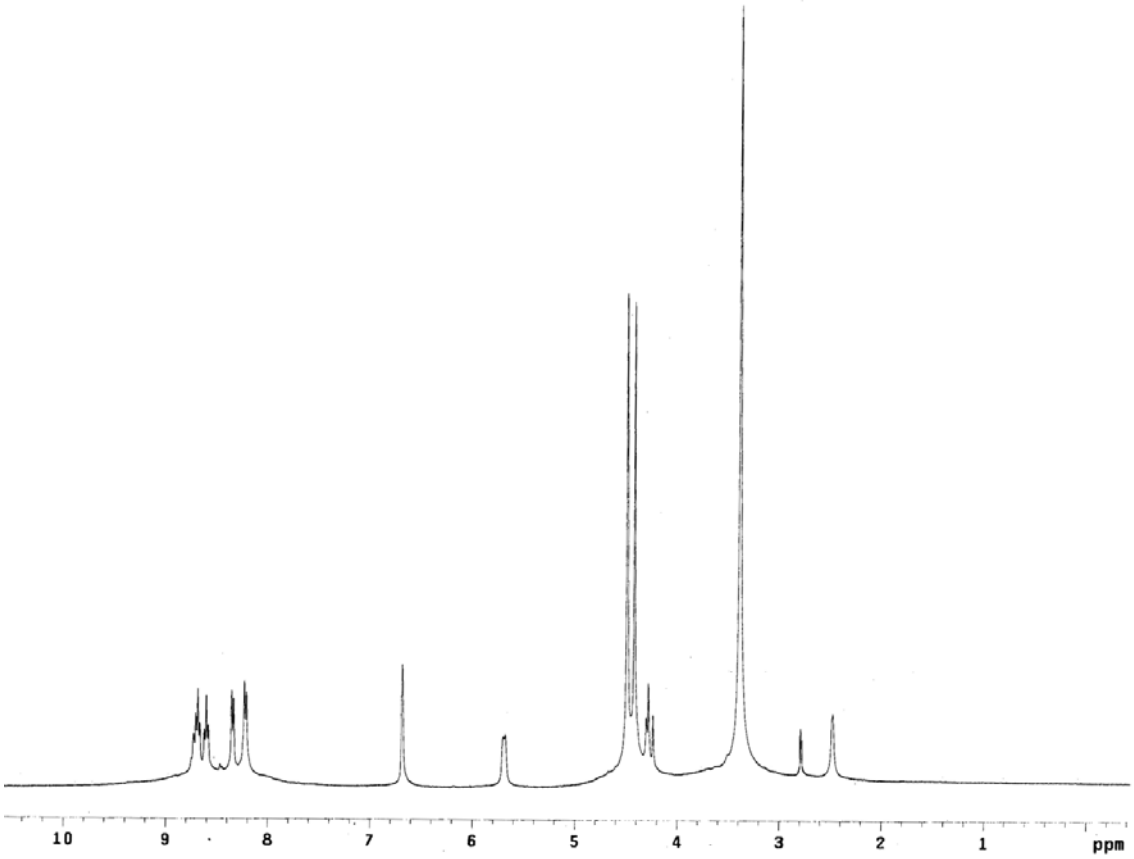

Fig. S7 ^{1}H NMR spectra of **compound 6** in DMSO-d_6 solution.

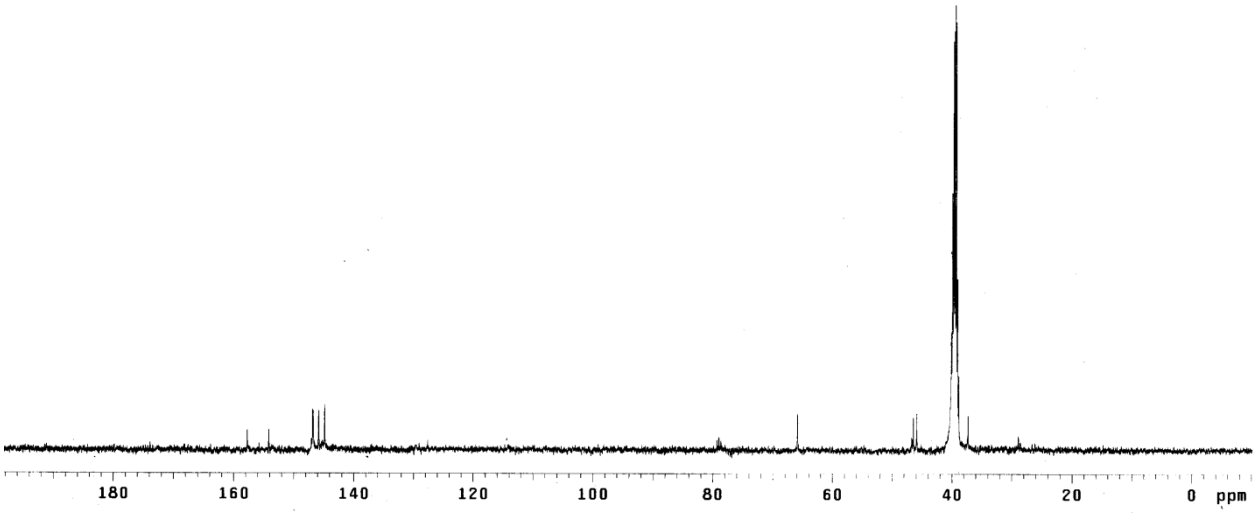

Fig. S8 ^{13}C NMR spectra of **compound 6** in $CDCl_3$ solution.

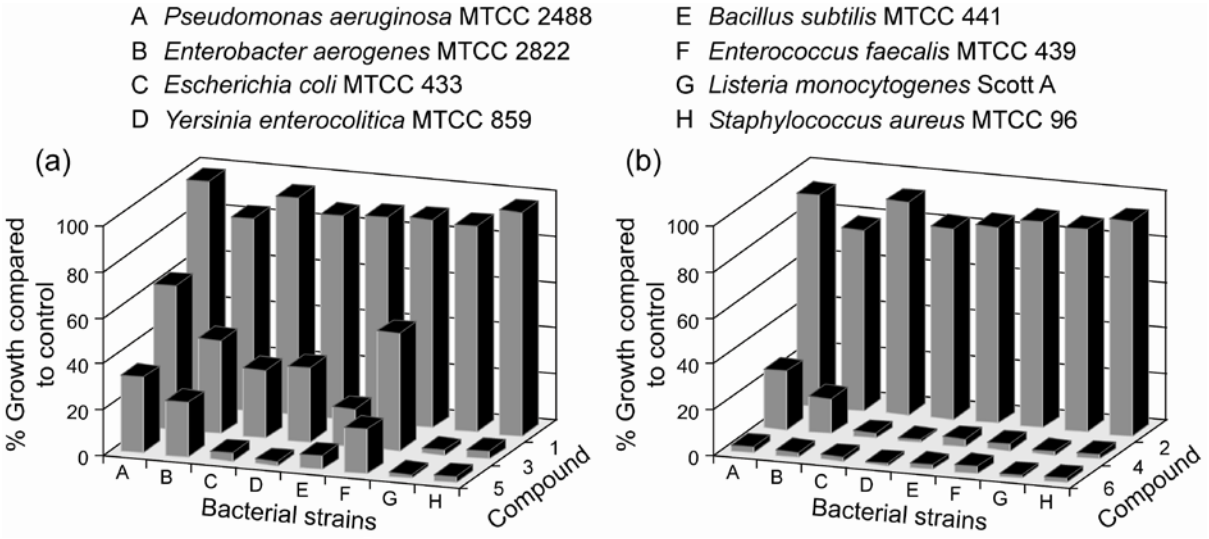

Fig. S9 Antimicrobial activity of (a) neutral (**compound 1**, **3** and **5**) and (b) charged amphiphiles (**compound 2**, **4** and **6**) against pathogenic bacterial strains. Bacterial cells were treated with 50 µg/ mL of amphiphile for 6 h.

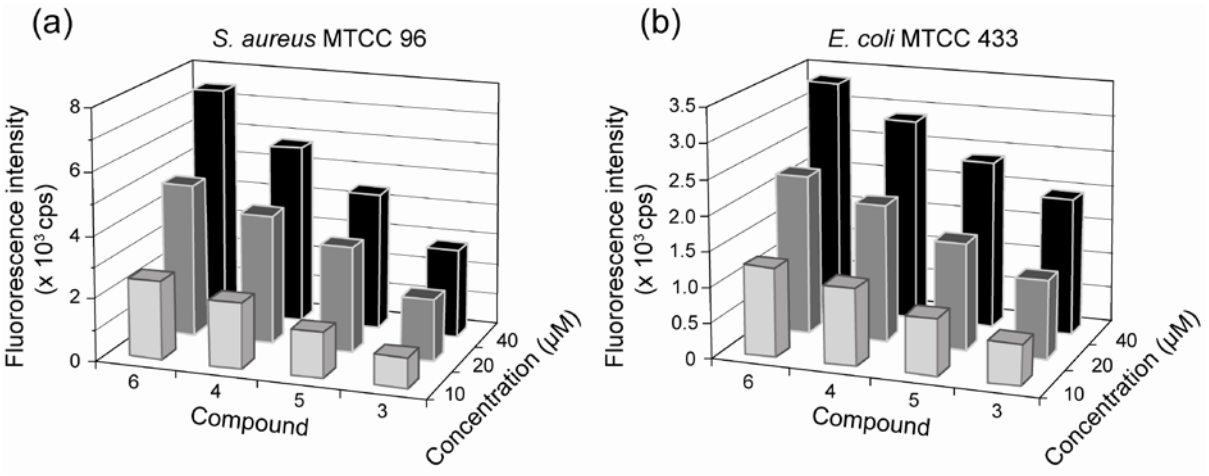

Fig. S10 Assessment of membrane damage in amphiphile-treated pathogenic bacteria by PI uptake assay.

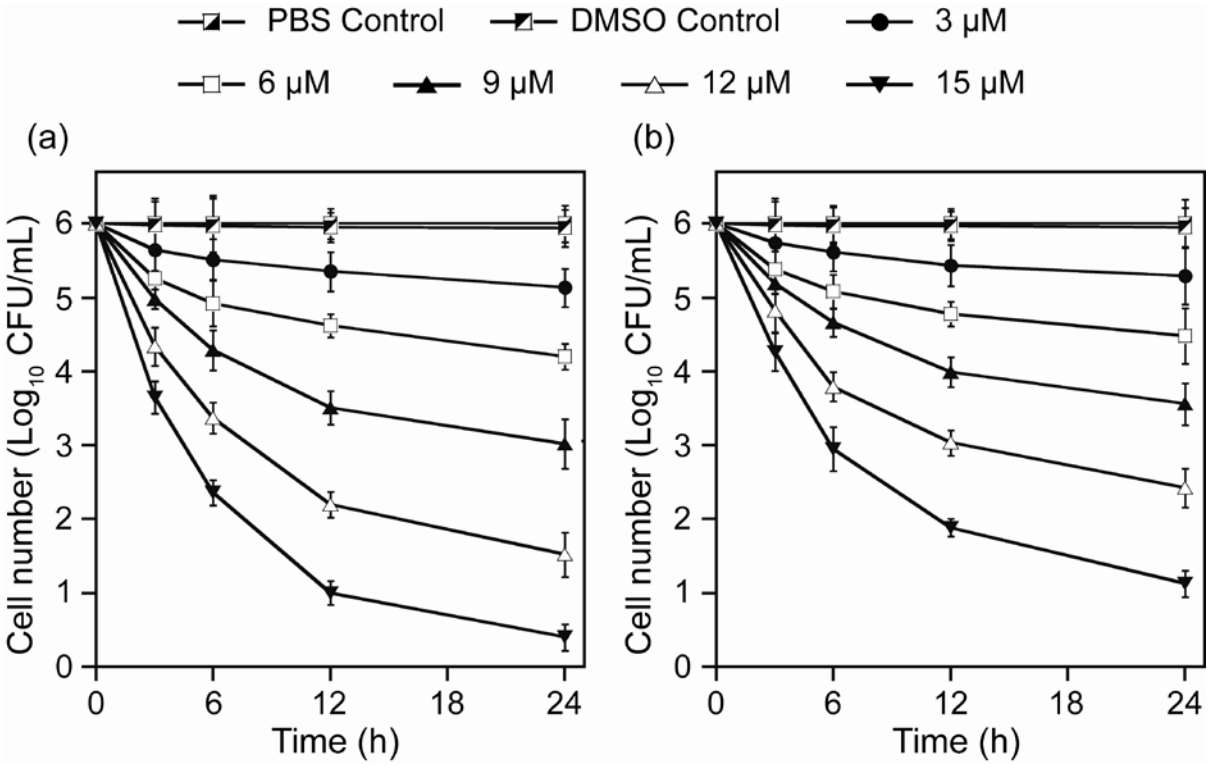

Fig. S11 Time-kill curves of **compound 6** against (a) *S. aureus* MTCC 96 and (b) *E. coli* MTCC 433.

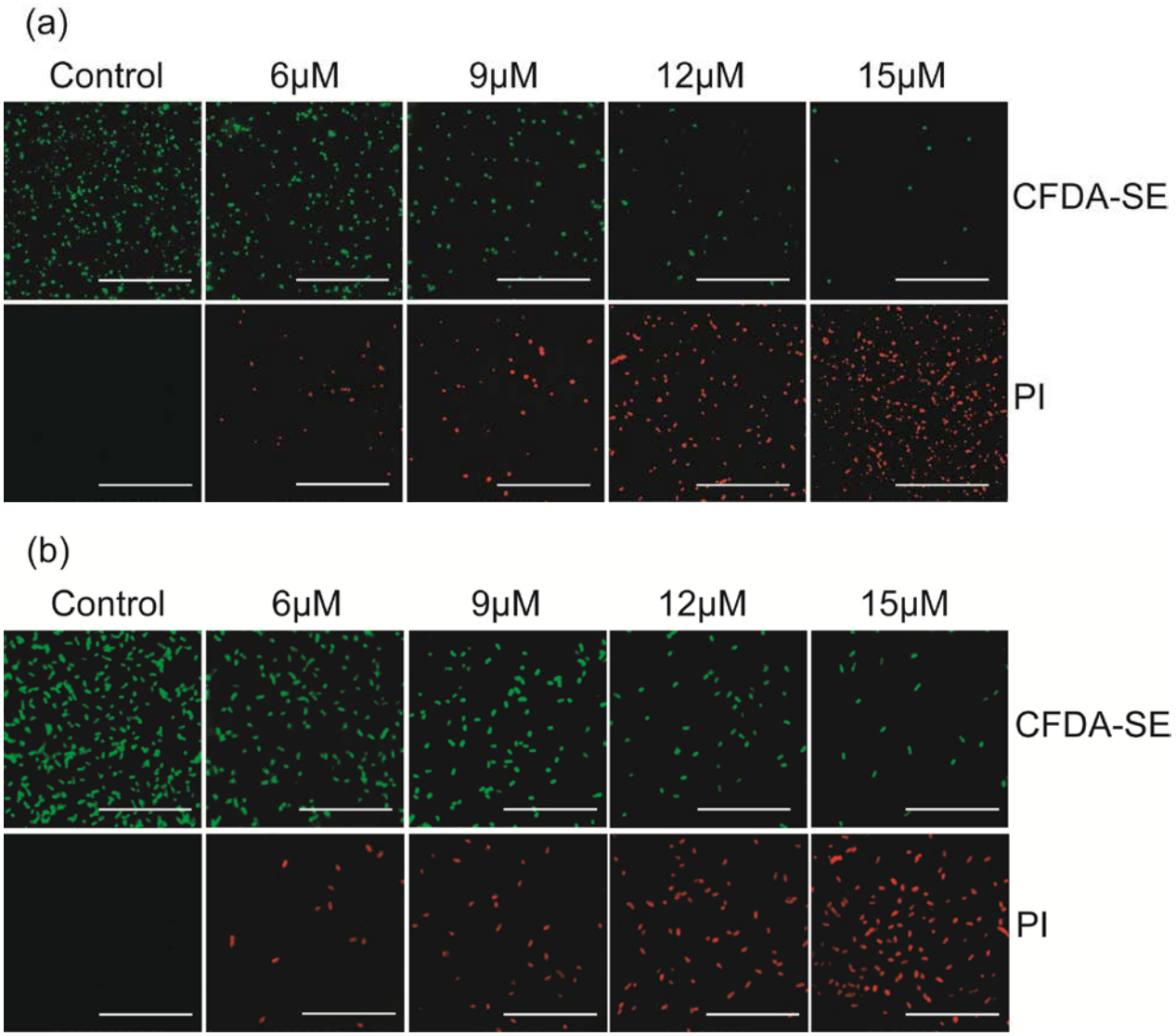

Fig. S12 Fluorescence microscopic images of (a) *S. aureus* MTCC 96 and (b) *E. coli* MTCC 433 treated with varying concentrations of **compound 6**. Scale bar for the images is 50 μm.

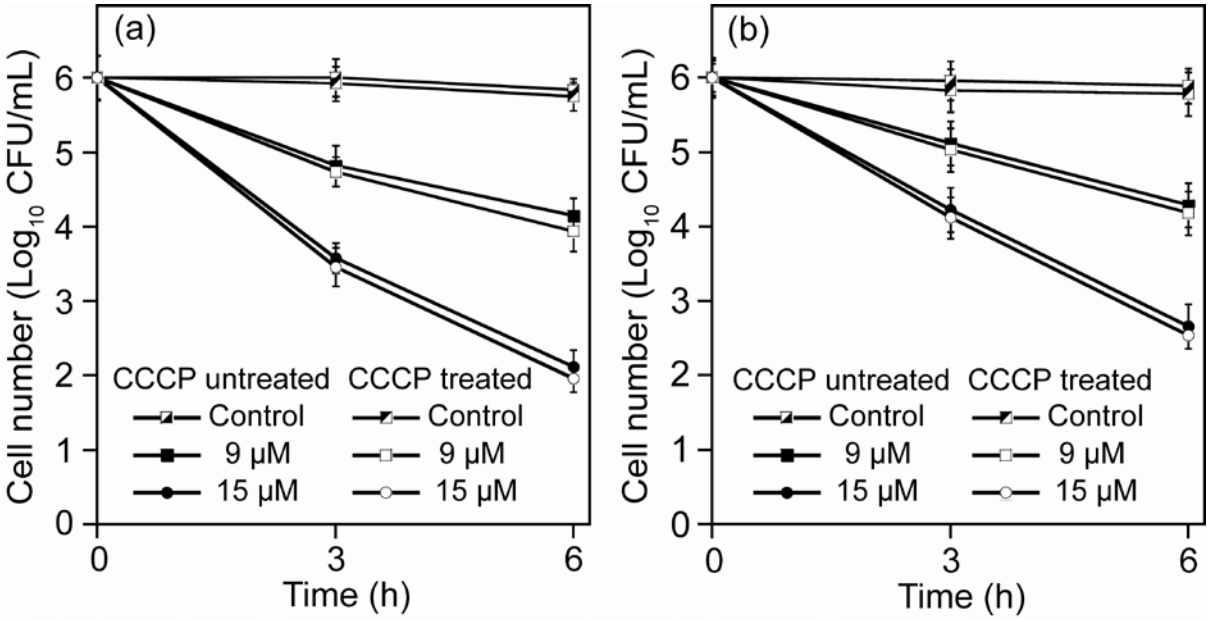

Fig. S13 Effect of membrane potential on the bactericidal activity of **compound 6** on (a) *S. aureus* MTCC 96 and (b) *E.coli* MTCC 433.

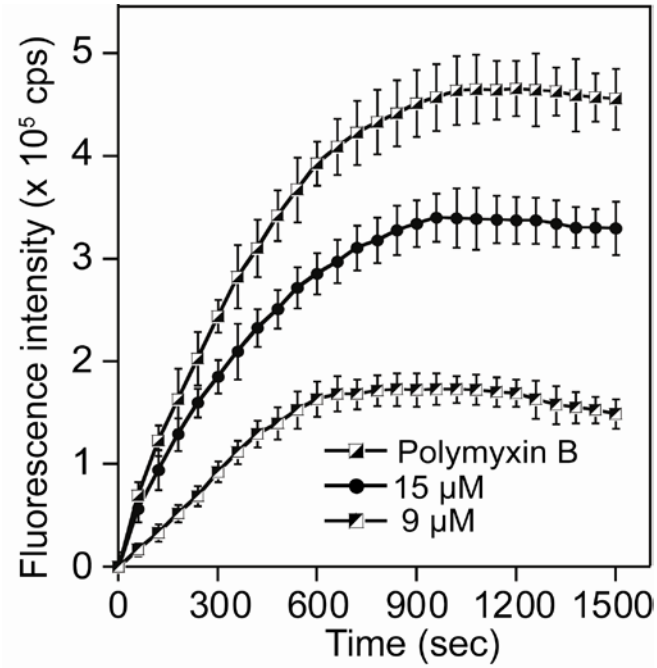

Fig. S14 NPN uptake assay for the assessment of membrane permeabilization in *E.coli* MTCC 433 treated with varying concentrations of **compound 6**. Cells treated with 1.0 μg/mL polymyxin B were used as positive control.

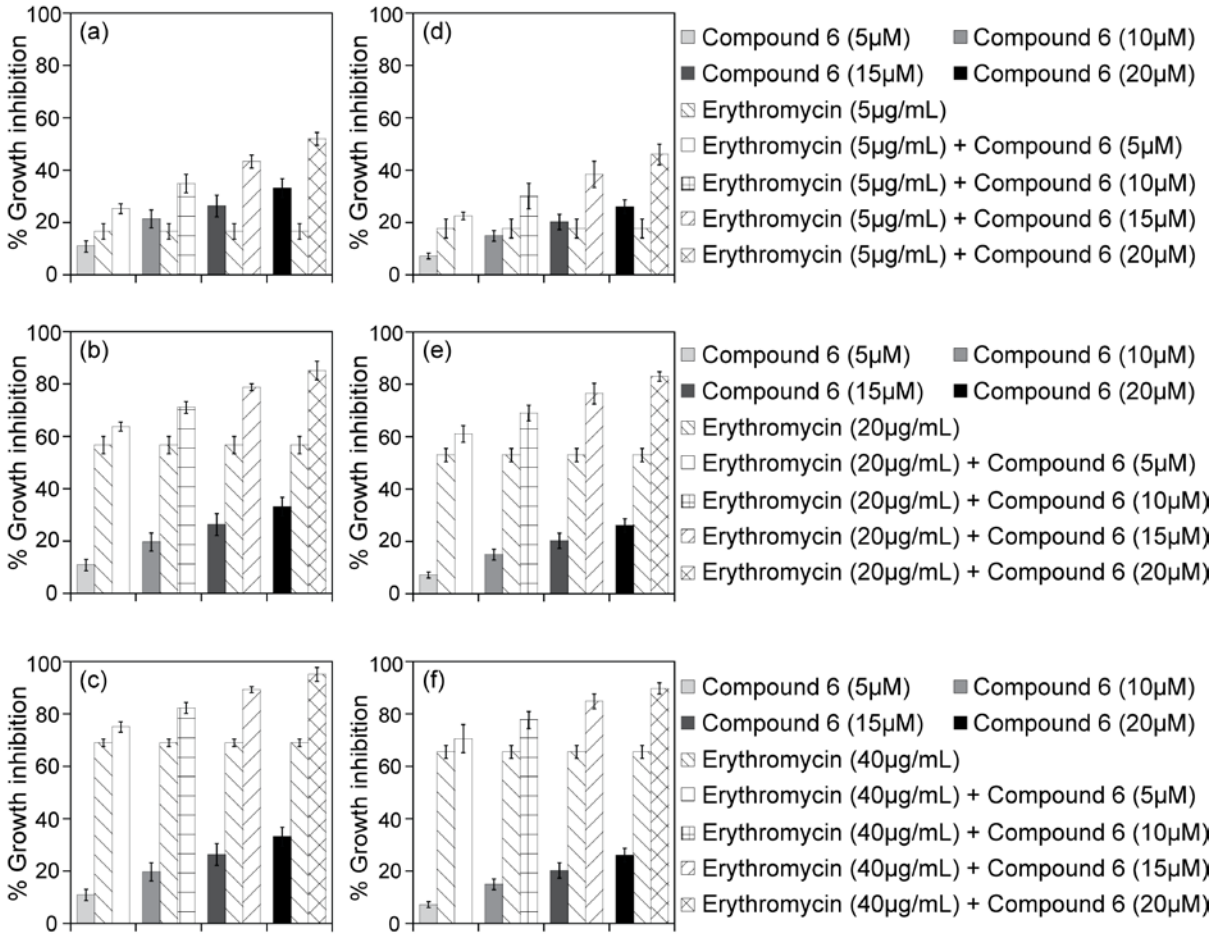

Fig. S15 Effect of combined treatment of **compound 6** and erythromycin on the growth of (a, b and c) *E. coli* MTCC 433 and (d, e and f) *E. aerogenes* MTCC 2488.

Table S1. Calculated hydrophobicity (LogP value) of neutral amphiphiles.

Synthetic Amphiphile	**Hydrophobicity* (LogP value)**
Compound 5	5.16
Compound 3	4.37
Compound 1	2.94

* Hydrophobicity of the amphiphiles was calculated using the software jlopP Version 1.1(Brian White, 2004) (http://www.vls3d.com/JME_EditorOK.dir/run_jlogp.html)

Table S2. MIC and MKC of antibacterial amphiphiles.

Amphiphile	*S. aureus* MTCC 96		*L. monocytogenes* Scott A		*E .coli* MTCC 433		*E. aerogenes* MTCC 2822	
	MIC (μM)/ OD_{600} ± standard deviation	MKC (μM)/ OD_{600} ± standard deviation	MIC (μM)/ OD_{600} ± standard deviation	MKC (μM)/ OD_{600} ± standard deviation	MIC (μM)/ OD_{600} ± standard deviation	MKC (μM)/ OD_{600} ± standard deviation	MIC (μM)/ OD_{600} ± standard deviation	MKC (μM)/ OD_{600} ± standard deviation
Compound 3	40/0.053 ± 0.003	100/0.057 ± 0.009	40/0.037 ± 0.005	100/0.049 ± 0.004	128/0.05 ± 0.004	200/0.037 ± 0.007	160/0.046 ± 0.006	>200/0.21 ± 0.018
Compound 4	20/0.063 ± 0.006	50/0.042 ± 0.003	20/0.043 ± 0.006	50/0.039 ± 0.004	80/0.056 ± 0.004	160/0.047 ± 0.004	100/0.041 ± 0.003	160/0.048 ± 0.006
Compound 5	25/0.086 ± 0.009	64/0.052 ± 0.003	25/0.050 ± 0.003	64/0.052 ± 0.009	100/0.066 ± 0.018	160/0.041 ± 0.007	128/0.048 ± 0.004	160/0.053 ± 0.004
Compound 6	16/0.056 ± 0.006	40/0.028 ± 0.009	16/0.053 ± 0.004	40/0.042 ± 0.002	64/0.054 ± 0.007	160/0.052 ± 0.003	80/0.041 ± 0.008	128/0.058 ± 0.009

Table S3. Comparative cytotoxic effect exerted by the bactericidal amphiphiles on human cell lines HeLa, MCF-7 and HT-29.

Sl. No.	**Comparison Group**	**Significant difference in % cell viability measured by MTT assay***	**Concentration of amphiphiles in MTT assay**
1.	**Compound 6** vs. **Compound 5**	No	Equivalent to 2X MIC and 3X MIC of *S. aureus* MTCC 96
2.	**Compound 6** vs. **Compound 4**	Yes	
3.	**Compound 6** vs. **Compound 3**	Yes	
4.	**Compound 5** vs. **Compound 4**	Yes	
5.	**Compound 5** vs. **Compound 3**	Yes	
6.	**Compound 4** vs. **Compound 3**	No	

* Significant difference implies p value < 0.001 based on analysis of variance (ANOVA) followed by all pair wise multiple comparisons (Holm-Sidak method) of % cell viability obtained in MTT assay.

Table S4. Comparative cytotoxic effect exerted by equimolar concentrations of bactericidal amphiphiles on human cell lines HeLa, MCF-7 and HT-29.

Sl. No.	Comparison Group	Significant difference in % cell viability measured by MTT assay*	Concentration of amphiphiles in MTT assay
1.	**Compound 6** vs. **Compound 5**	Yes	40 µM and 60 µM
2.	**Compound 6** vs. **Compound 4**	Yes	
3.	**Compound 6** vs. **Compound 3**	Yes	
4.	**Compound 5** vs. **Compound 4**	Yes	
5.	**Compound 5** vs. **Compound 3**	Yes	
6.	**Compound 4** vs. **Compound 3**	Yes	

* Significant difference implies *p* value < 0.001 based on analysis of variance (ANOVA) followed by all pair wise multiple comparisons (Holm-Sidak method) of % cell viability obtained in MTT assay.

Table S5. Selectivity of bactericidal amphiphiles based on IC_{50}/MIC values.

Amphiphiles	Selectivity for *S. aureus* MTCC 96 (IC_{50}/MIC)			Selectivity for *E. coli* MTCC 433 (IC_{50}/MIC)		
	HeLa	MCF-7	HT-29	HeLa	MCF-7	HT-29
Compound 3	2.5	2.5	2.45	0.78	0.78	0.76
Compound 4	4.3	4.3	4.1	1.0	1.0	1.0
Compound 5	3.2	3.2	3.2	0.8	0.8	0.8
Compound 6	3.12	3.12	3.12	0.78	0.78	0.78