Supporting Information

Fatty Acid Comprising Lysine Conjugates: Anti-MRSA Agents That Display In Vivo Efficacy by Disrupting Biofilms with No Resistance Development

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Details of experimental protocols

Bacterial strains and growth condition: Methicillin-resistant *S. aureus* (MRSA) ATCC33591 was obtained from the American Type Culture Collection (ATCC). Clinically isolates MRSA strains, MRSA-R3545, MRSA-R3889 and MRSA-R3890 were obtained from the Department of Neuromicrobiology, National Institute of Mental Health and Neuro Sciences, Hosur Road, Bangalore 560029, India. Bacterial identification was performed by the Vitek 2 Compact 60 system, bioMerieux, France. Methicillin-susceptible *S. aureus* (MSSA) MTCC737 was obtained from Microbial Type Culture Collection (Chandigarh, India). All bacterial strains were preserved in nutrient broth (NB) at -80 ºC supplemented with 15% (v/v) glycerol. Before using the bacteria from frozen stock, they were grown overnight on NB agar plate incubated at 37 ºC. Single bacterial colony was then grown for 6h (midlog phase) in 3 mL of nutrient broth. In order to achieve the stationary phase culture 3 µL of these midlog phase bacteria were added to 3 mL of fresh nutrient broth and allowed to grown for 16 h at 37 ºC. For determining the MIC values, 6 h grown midlog phase bacteria were suspended to ~10^5 CFU/mL and used for the assay. Similarly, stationary phase cultures were diluted to ~10^6 CFU/mL and assessed for activity test. For in vivo activity studies midlog phase bacterial culture was centrifuged and suspended in saline.

Antibacterial assay against growing planktonic bacteria: All the compounds and control antibiotics were serially diluted by 2-fold in 96-well plate in autoclaved millipore water. Single colony of the bacteria was grown for 6 h to reach mid-log phase in 3 mL of nutrient broth under shaking condition. The bacteria (~10^8 CFU/mL) were then diluted to ~10^5 CFU/mL in nutrient broth. 150 µL of these bacterial suspensions were then added to the 96 well plates containing 50 µL of compounds solution. After that the plates were incubated for 24 h at 37 °C under shaking condition. At the end of 24 h the optical density (O.D.) of the plates were measured at 600 nm using TECAN (Infinite series, M200 pro) Plate Reader. The

MIC values were determined by observing the O.D. values of the wells. MIC value was consider as the lowest concentration where the O.D. was lower than 0.1. The experiments were performed in a triplicate manner and performed thrice. The MIC values were expressed as the concentration range obtained in all the experiments.

Cytotoxicity assay: Cytotoxicity of the compounds was assessed by following the reported protocol with little modification. Briefly, the RAW cells (RAW 264.7 TIB-71) were grown in 96-well plate (in DMEM media supplemented with 10% fetal bovine serum and 5% penicillin-streptomycin) to the 70-80% confluency. The media was then discarded and the cells were treated with various concentration of compounds diluted in supplemented DMEM media. As the negative control, same volume of media was added (untreated) and as the positive control, the cells were treated with Triton-X 100 (10 vol % in media). After that the plate was incubated for 24 h at 37 °C under 5% CO_2 atmosphere. At the end, the medium was carefully removed and 100 µL of MTT solution (5 mg/mL concentration) was added to the wells. The plate was then incubated for 4 h and after that it was centrifuged at 1100 rpm for 5 min. The supernatant was removed and 100 µL of DMSO was added to solubilize formazan crystals. Finally, the O.D. of the plate was recorded at 570 nm to quantify the cell viability. The following equation was used to calculate the percentage of cell viability: Cell viability (%) = $(A_{treated} - A_{tritonX\text{-}treated})/(A_{untreated} - A_{tritonX\text{-}treated}) \times 100$. The average of triplicate O.D. was then plotted against concentration of the compounds and the EC_{50} values (concentration corresponding to 50% cell viability) were determined by fitted with sigmoidal plot.

Time-kill kinetics against growing planktonic MRSA: Mid-log phase (~10^8 CFU/mL) of all the MRSA culture was suspended to ~10^5 CFU/mL in fresh nutrient broth. These bacterial suspensions were then treated with 5 µg/mL (~1×MIC), 10 µg/mL (~2×MIC), 20 µg/mL (~4×MIC) and 40 µg/mL (~8×MIC) of D-LANA-14 and 0.8 µg/mL (~8×MIC) of fusidic acid and incubated at 37 ºC with shaking. As a negative control, the same volume of saline was

added to the bacterial suspensions instead of any antibacterial compounds. 20 µL of aliquots were then 10-fold serially diluted in saline at various time point such as 0, 20, 40, 60, 120 and 240 min. From these dilutions, 20 µL were spot plated on nutrient agar plate and incubated for 24 h in 37 ºC for counting viable cells. At the end of 24 h incubation, bacterial colonies were counted and average results are presented in logarithmic scale with standard deviation for each data point. The detection limit for this experiment is 50 CFU/mL.

Antibacterial activity against stationary phase MRSA: Stationary phase culture of all the MRSA (~10^9 CFU/mL) were centrifuged at 9000 rpm for 2 min and suspended in minimum essential media (MEM). These bacterial suspensions containing high bacterial count were then serially diluted by 10-fold to ~10^6 CFU/mL in MEM. 150 µL of the bacterial suspensions were then added to the wells of 96-well plate containing 50 µL of various concentrations of D-LANA-14 and fusidic acid in MEM. As negative control, 50 µL of MEM were used instead of D-LANA-14 or fusidic acid. Now, this treated plate was incubated for 2 h at 37 ºC under shaking condition. At the end of 2 h, 20 µL of the treated bacterial suspensions were serially diluted by 10-fold in saline and 20 µL from each dilution were spot-plated on NB agar plate. The plates were then incubated for 24 h at 37 ºC for bacterial growth. The viable colonies were then counted and results are expressed in logarithmic scale. The data presented is the average of two individual experiments with standard deviations. The limit of detection for this experiment is 50 CFU/mL.

Biofilm eradication assay: In order to grow preformed biofilms, 100 µL of ~10^5 CFU/mL of mid-log phase MRSA (suspended in NB supplemented with 1% (w/v) NaCl and 1% (w/v) glucose) were placed into the wells of polystyrene 96-well plates. The plates containing the bacterial suspensions were then incubated for 24 h at 37 ºC under stationary condition. At the end of 24 h, media was discarded from the wells and preformed biofilms were washed with 1×PBS. 100 µL of various concentrations of LANAs and fusidic acid diluted in freshly

prepared complete biofilms medium (NB supplemented with 1% (w/v) NaCl and 1% (w/v) glucose) were added carefully to the wells containing preformed biofilms. The plates containing compound treated biofilms were incubated under stationary condition at 37 ºC. After 24 h, the media was discarded and the plates were washed with 1×PBS. 200 µL of fresh NB were then added and bacteria were allowed to regrow at 37 ºC under shaking condition. At the end of 24 h, the O.D. (at 600 nm) of regrown biofilm was measured by using TECAN (Infinite series, M200 pro) plate reader. This assay was performed twice in triplicate and the MBEC values were evaluated as the minimum concentration at which O.D. was found to be below 0.1.

Quantification of biofilm disruption and visualization through confocal microscopy: Glass coverslips with diameter of 18 mm were soaked into ethanol and placed in wells of 6-well plate after drying it in presence of flame. These sterile coverslips were then allowed to cool to room temperature for 10-15 minutes. After that, 2 mL of mid-log phase bacterial suspension (~10^5 CFU/mL suspended in NB supplemented with 1% (w/v) NaCl and 1% (w/v) glucose) was added to the wells containing coverslips. Biofilms were allowed to form on coverslips under stationary condition for 24h at 37 ºC. At the end of 24 h, biofilms containing coverslips were taken out carefully and washed with 1×PBS. After that, they were placed into the wells of 6-well plate containing 2 mL of various concentrations of D-LANA-14 and 40 µg/mL of fusidic acid. A negative control was prepared where 2 mL of complete medium was added instead of any antibacterial compound. The plate was then incubated for 24 h at 37 ºC under stationary condition. Post-incubation, the cover slips were carefully washed with 1×PBS and allowed to dry for 10-15 min. These dry disrupted biofilms were then stained with crystal-violet (2 mL of 0.1% w/v solution prepared in sterile milipore water) for 5-10 min. After that, the stained biofilms were carefully washed with 1×PBS. 2 mL of 95% ethanol in water was then added to these CV-stained biofilms and the O.D. (at

520 nm) of the solution was recorded by using plate reader. These experiments were performed twice and the average data with standard deviation were presented in the figure. In order to visualize the extent of biofilm disruption, assay was performed by following the same protocol. Here, the disrupted biofilms were stained with syto-9 dye and images were captured by using confocal laser scanning microscopy instead of staining with crystal-violet.

Membrane depolarization of both growing planktonic and stationary phase MRSA: Mid-log phase (6 h grown) culture or stationary phase of MRSA were harvested (9000 rpm, 2 min), washed in 5 mM HEPES buffer and suspended in 1:1:1 of 5 mM HEPES buffer, 5 mM glucose, and 100 mM KCl solution. In case of stationary phase, bacterial suspensions were 10-fold diluted in the same cocktail buffer media to achieve bacterial count of ~10^8 CFU/mL. To this bacterial suspension (~10^8 CFU/mL), 60 µL of 200 µM $DiSC_3$ (5) (3,3′-Dipropylthiadicarbocyanine iodide) was added to have a resulting concentration of 2 µM of dye in 6 mL of suspension. 190 µL of these dye containing bacterial suspension was then placed into the wells of black and clear bottom 96-well plate and incubated for 30 min in darkness. At the end of incubation, the fluorescence of dye containing bacterial suspensions was monitored for 4 min. The emission at 670 nm was recorded by exciting at a wavelength of 622 nm. After that, 10 µL of D-LANA-14 (diluted in sterile milipore water) of various concentrations were added to these dye containing bacterial suspension and fluorescence of the treated wells were monitored for 22 more minutes. As the negative control, 10 µL of milipore water was added instead of D-LANA-14. These experiments were performed in triplicates and the average data with standard deviation were presented in the figure.

MRSA chronic skin infection model: Briefly, 6-8 weeks-old female BALB/c mice of body weight ranging from 18-22 g were randomly divided in groups of five mice in each. At first, neutropenia in mice was induced by injecting doses of 150 mg/kg and 100 mg/kg of cyclophosphamide intraperitoneally four days and one day before the experiment

respectively. After that, the mice were intraperitoneally injected with 150 µL of xylazine-ketamine cocktail to anesthetize them. Then the fur on the back of the mice was removed by using a trimmer followed by shaving with a razor. A wound that could be visually observed as ruptured and damaged area in the skin. The wound was then infected with 20 µL of freshly prepared bacterial suspension (~10^7 cells of MRSA per mice) in sterile saline. 24 h after bacterial infection, one group of mice (n = 5) were sacrificed in order to quantify the pretreatment bacterial count at the infection site. The other three groups of mice were treated with either 20 mg/kg or 40 mg/kg of D-LANA-14 or 40 mg/kg of fusidic acid, respectively. 40 µL of D-LANA-14 or fusidic acid of the required concentration prepared in 0.9% saline were applied in the infection site. All groups of infected mice were treated with 4 doses at 24 h intervals of the respective antibacterial agents (D-LANA-14 or fusidic acid). As a negative control, one group of mice was treated with saline instead of any antibacterial agents. On day 5, 24 h after the last dose of compound treatment, the mice were sacrificed using isofluorane. The skin-tissue from infected part were collected and placed into 10 mL of sterile saline and homogenized. This homogenized bacterial suspension was serially diluted 10-fold in sterile saline and each dilution was plated on NB agar plate. These plates were then incubated at 37 °C to check for colony appearance. At the end of 24 h, bacterial colonies were counted and results were expressed as Log CFU/g of weight of the tissue collected from mice.

Acute dermal toxicity: Ten 6-8 weeks-old female BALB/c mice of body weight 18-22 g were randomly divided in two different groups. The mice were anesthetized by intraperitoneally injecting 150 µL of xylazine-ketamine cocktail and the fur from the back of the mice (~$1/10^{th}$ of total body surface) was removed without any injury on the skin. 24 h after fur removal, 200 mg/kg of D-LANA-14 (40 µL of 100 mg/mL compound solution dissolved in saline) was applied to the shaved area of the skin of one group. The other group of the mice was left untreated as the control. The compound treated mice were kept under

continuous observation for 2 h to check for any abnormal behavior due to compound exposure. After that, both groups of mice were observed for a period of 14 days with specific attention towards changes in furs.

Resistance development studies: Initially, the MIC values of D-LANA-14 and fusidic acid were determined against two strains of MRSA (MRSA-ATCC33591 and MRSA-R3889). On the second day of the study, ~10^5 CFU/mL bacterial inoculum from the half-MIC concentration of the D-LANA-14 and fusidic acid was subjected to another MIC assay. 18 subsequent passages were repeated by following this same protocol and the fold of MIC increased for both D-LANA-14 and fusidic acid was plotted against the passages number. These experiments were performed in triplicate and all the MIC values were determined by visual observation. The fold of MIC was obtained by dividing first MIC value (corresponds to zero passage) from MIC values of every passages.

Supplementary figures and tables

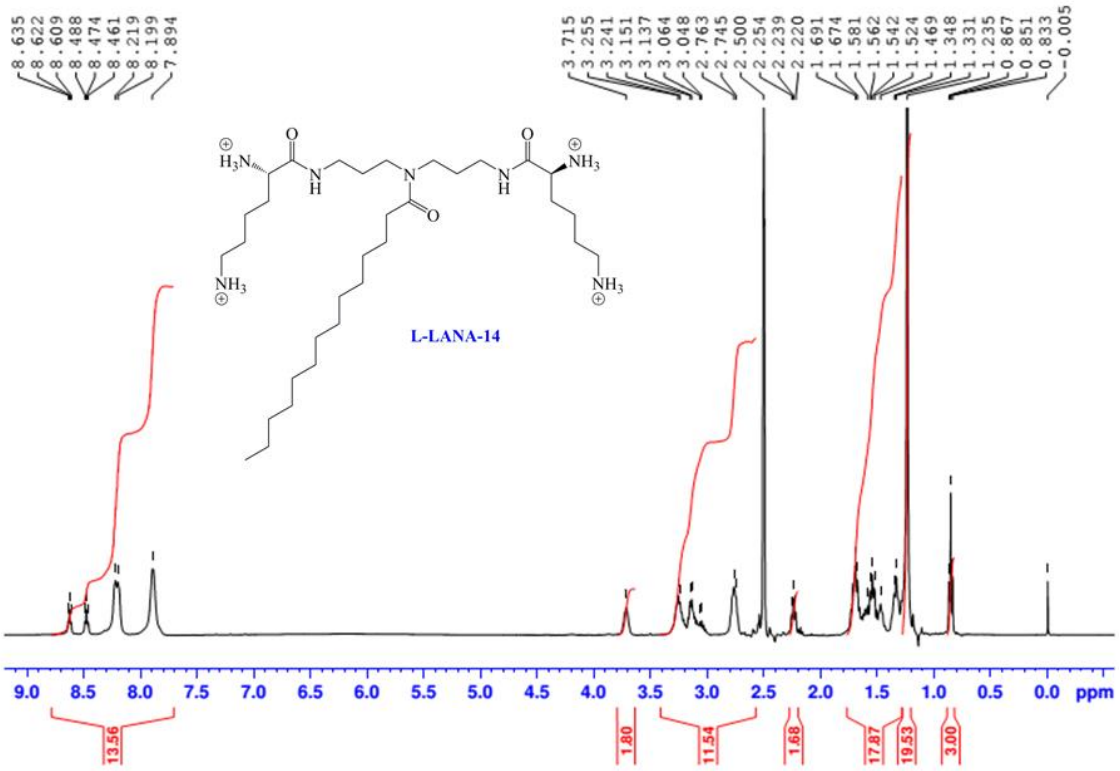

Figure S1: ^{1}H NMR of L-LANA-14. The NMR was taken in DMSO-d_6 and the solvent peak was calibrated at δ value of 2.500 ppm.

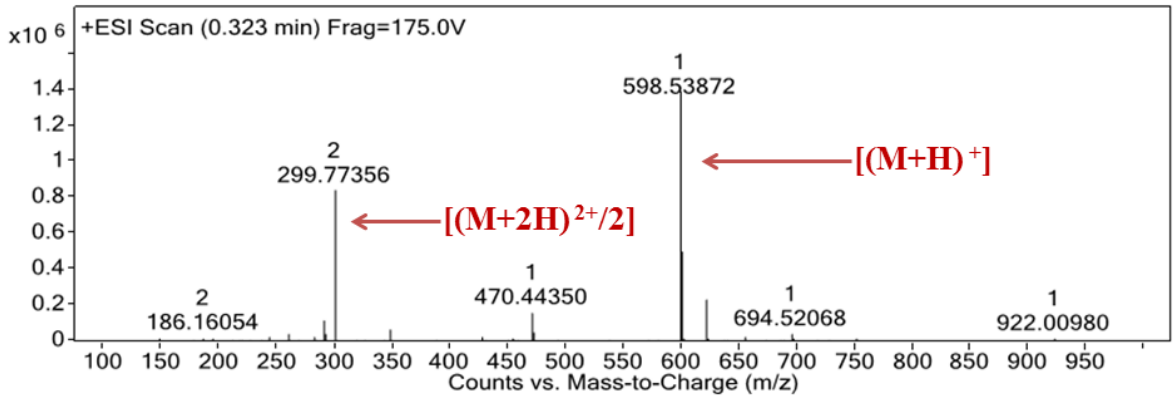

Figure S2: HRMS spectra of compound L-LANA-14.

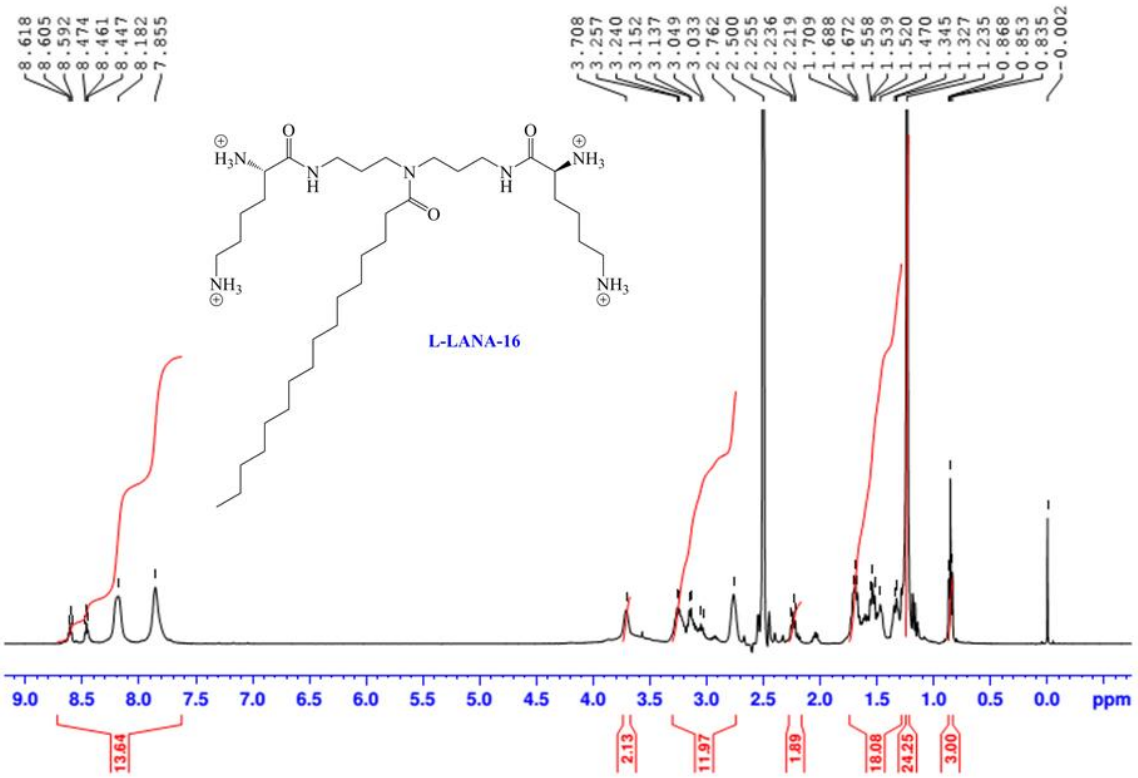

Figure S3: ^{1}H NMR of L-LANA-16. The NMR was taken in DMSO-d_6 and the solvent peak was calibrated at δ value of 2.500 ppm.

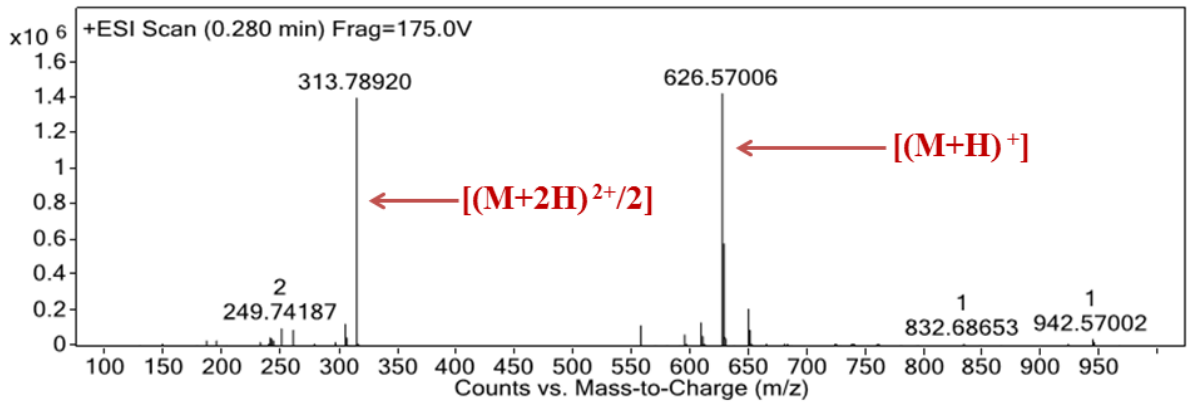

Figure S4: HRMS spectra of compound L-LANA-16.

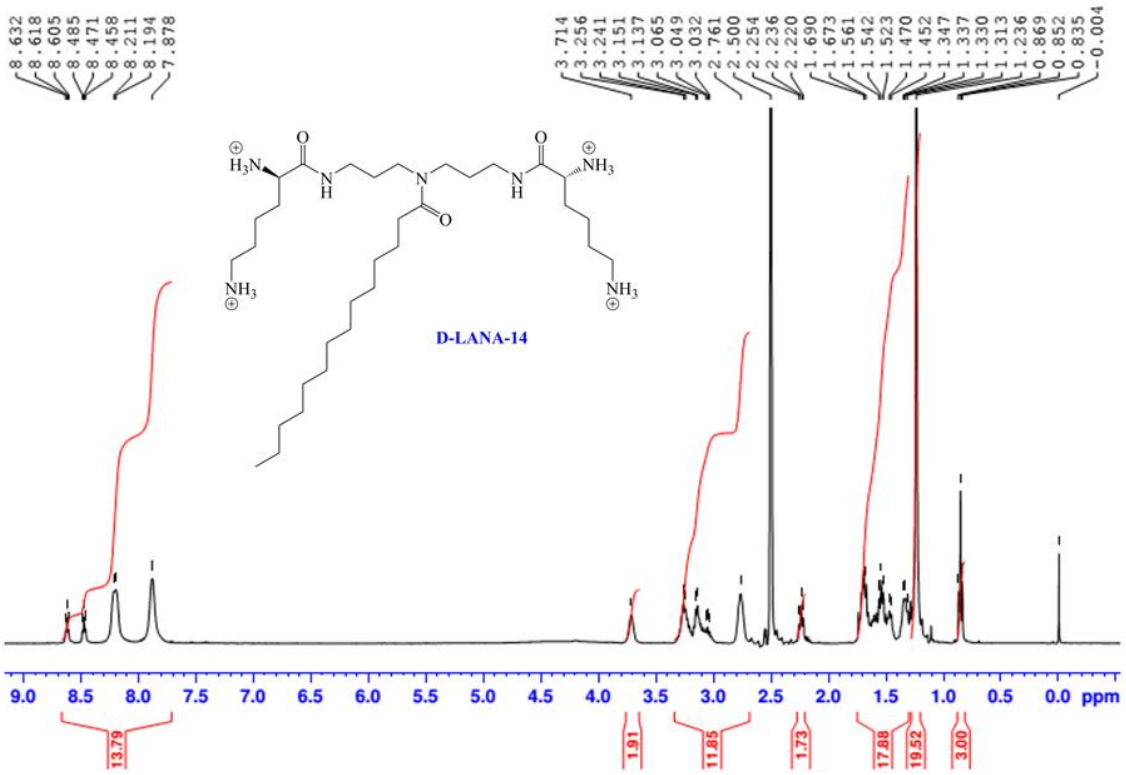

Figure S5: ^{1}H NMR of D-LANA-14. The NMR was taken in DMSO-d_6 and the solvent peak was calibrated at δ value of 2.500 ppm.

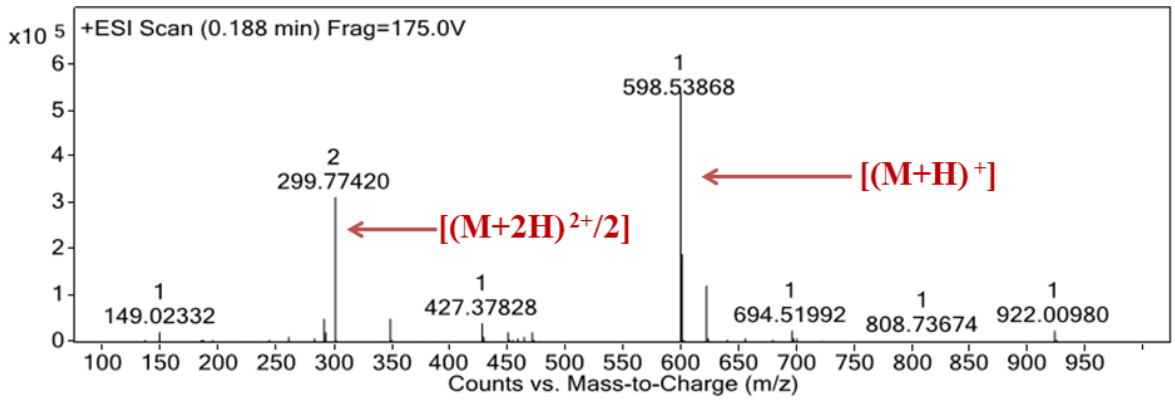

Figure S6: HRMS spectra of compound D-LANA-14.

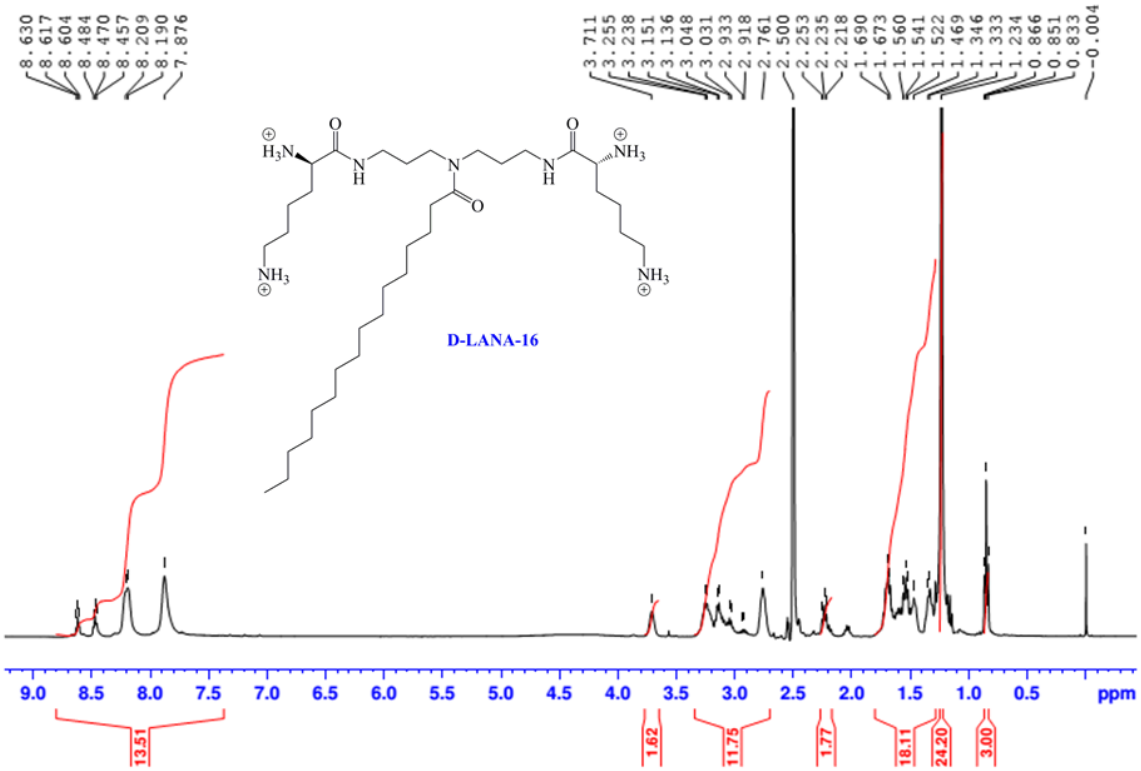

Figure S7: ^{1}H NMR of D-LANA-16. The NMR was taken in DMSO-d_6 and the solvent peak was calibrated at δ value of 2.500 ppm.

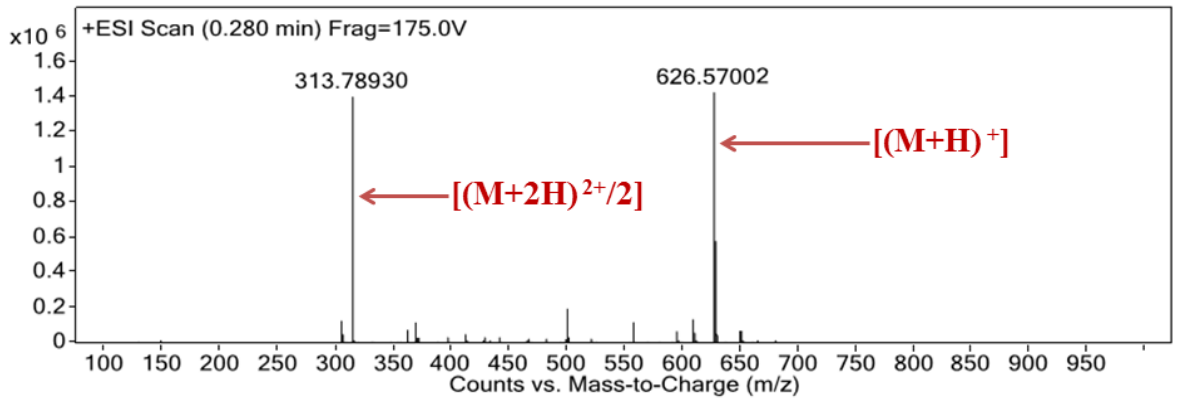

Figure S8: HRMS spectra of compound D-LANA-16.

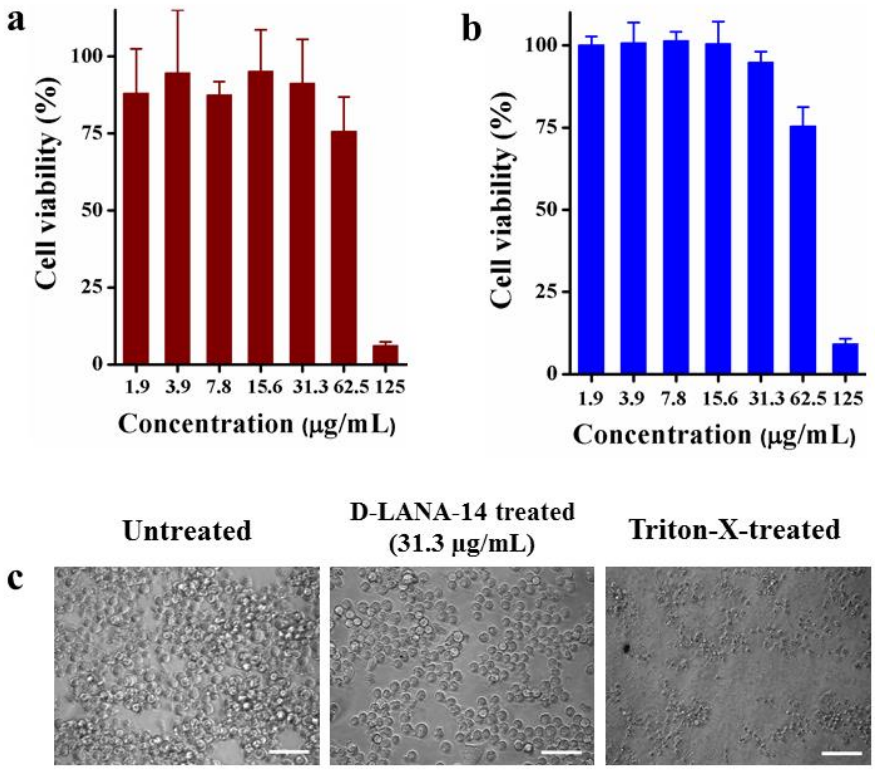

Figure S9: Cytotoxicity against Raw cell line: Cell viability after treatment with different concentrations of L-LANA-14 (a) and D-LANA-14 (b). (c) Bright field images of cells (scale bar = 100 μm)

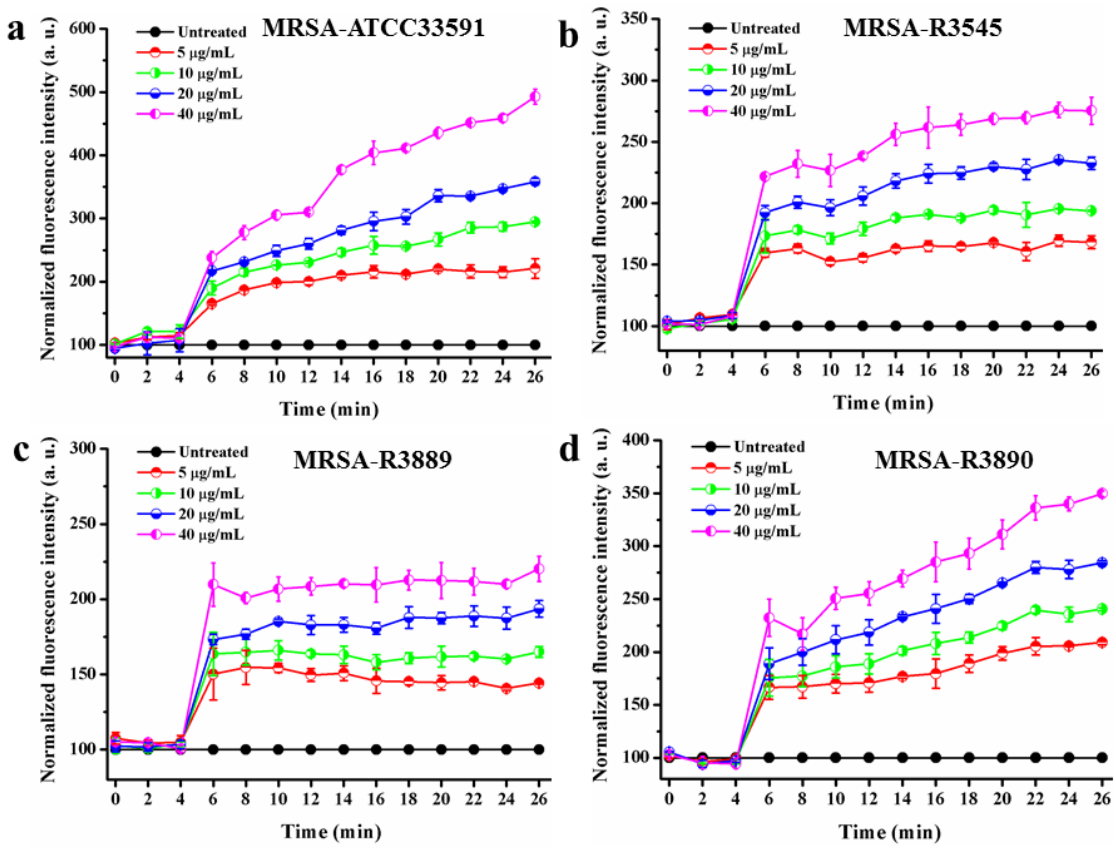

Figure S10. Membrane depolarization of growing planktonic MRSA by D-LANA-14. (a) MRSA-ATCC33591. (b) MRSA-R3545. (c) MRSA-R3889. (d) MRSA-R3890. The data demonstrated in the figure are the average of three different experiments and the average of normalized fluorescence intensity with standard deviation is plotted in the figure.

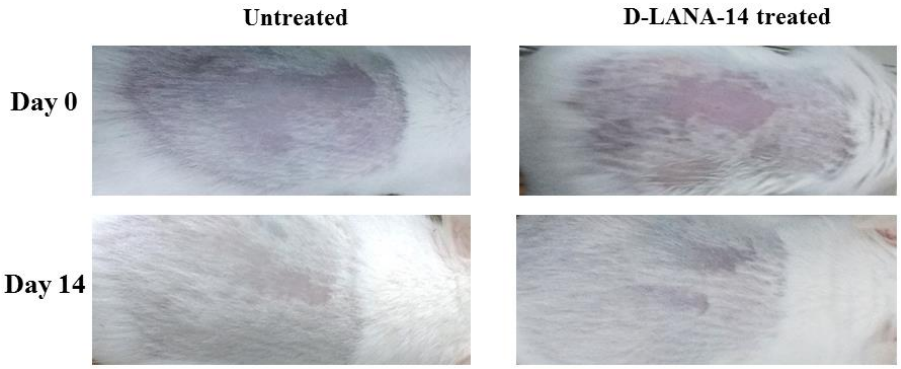

Figure S11. Photographical images indicated D-LANA-14 does not affect the normal growth of fur in mice.

Table S1. Antibacterial activity against fusidic acid-resistant (FAR) MRSA

antibacterial agent	MIC (μg/mL)	
	FAR-MRSA ATCC 33591	FAR-MRSA R3889
L-LANA-14	3.1-6.3	3.1-6.3
L-LANA-16	3.1-6.3	3.1-6.3
D-LANA-14	3.1-6.3	3.1-6.3
D-LANA-16	3.1-6.3	3.1-6.3
Fusidic Acid	3200	3200
Methicillin	>25	12.5-25