Supporting Information for

Antibiotic-conjugated antimicrobial peptides for enhanced bacterial inhibition

Xingrao Peng,[a] Yong Luo,[a, b] Tianzhi Xu,[a] Zihan Chen,[a] Peiyao Chen,[c] Cong Hu [d] and Shuang Liu*[a]

a. School of Materials Science and Engineering, Wuhan University of Technology, 122 Luoshi Road, Wuhan, Hubei, 430070, China.

b. State Key Laboratory of Chemo/Biosensing and Chemometrics, Hunan University, Changsha 410082, China.

c. Key Laboratory of Fermentation Engineering (Ministry of Education), National "111" Center for Cellular Regulation and Molecular Pharmaceutics, Hubei Key Laboratory of Industrial Microbiology, School of Life and Health Sciences, Hubei University of Technology, Wuhan, Hubei, 430068 China.

d. Guangxi Key Laboratory of Automatic Detecting Technology and Instruments, Guilin University of Electronic Technology, Guilin 541004, China.

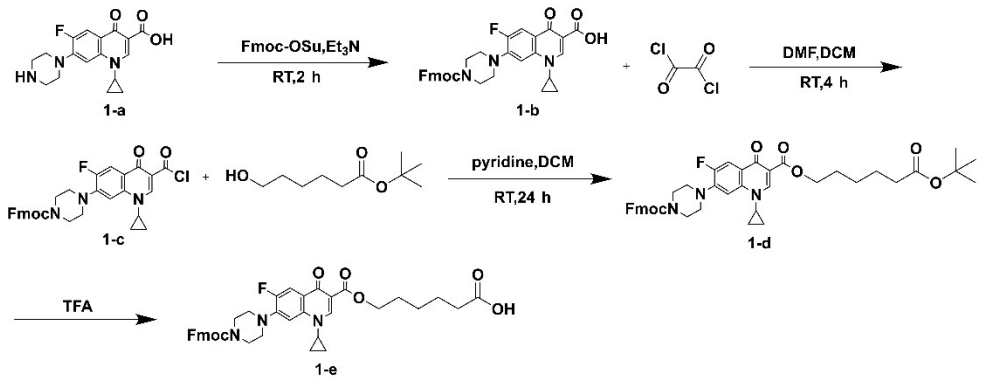
* Corresponding E-mail: shuangliu@whut.edu.cn

Content

S1. Materials and instruments

Rink Amide-AM resin (0.6-0.8 mmol/g), HOBt, HBTU, Fmoc-OSu, and other Fmoc-amino acids were purchased from GL Biochem (Shanghai, China). Other chemical reagents and solvents were purchased from Fisher Scientific. The gram-negative bacterium *E. coli* (ATCC 13706) and gram-positive bacteria *S. aureus* (ATCC 25923) were obtained from BNCC (Beijing, China). All peptides were purified using the Wufeng LC-100 High Performance Liquid Chromatography (HPLC) system equipped with an LC-UV100 ultraviolet detector and dual LC-P100 high-pressure constant-flow pumps. The LC-MS spectra were obtained with a Waters Acquity Ultra Performance LC with Waters MICROMASS detector, and ^{1}HNMR spectra on Varian Unity Inova 400. Circular dichroism (CD) spectra were obtained with a Jasco J-810 Spectropolarimeter. Zeta potential and size distribution were measured by a Malvern Zetasizer Nano ZST nanoparticle potentiometer. TEM images were obtained with a JEM-1400Plus transmission electron microscope. SEM images were captured by a JSM-IT300 scanning electron microscope. Sections of organs were scanned using a Olympus VS200 digital pathological section scanner.

S2. Synthesis and characterization of the precursors



Scheme S1. Synthetic route of **1-e**.

Synthesis of compound 1-b

Ciprofloxacin (1.0 g, 3.0 mmol, 1.0 equiv.) was suspended in deionized H_2O (40 mL) in a 150 mL round-bottom flask. The pH was adjusted to 8–9 with Et_3N, followed by dropwise addition of Fmoc-OSu (1.21 g, 3.6 mmol, 1.2 equiv.) in anhydrous MeCN (40 mL) over 10 min. After stirring at 25°C for 3 h, the mixture was concentrated under reduced pressure. Acidification with 1 M HCl (pH 3) precipitated the product, which was extracted with EtOAc (50 mL × 3). The combined organic layers were washed by 1 M HCl (50 mL × 2) and brine (50 mL × 1), dried by Na_2SO_4, filtered and concentrated by evaporation. Purification by silica gel chromatography (DCM/MeOH 20:1) yielded compound **1-b** as a white powder.

Synthesis of compound 1-c

A solution of **1-b** (1.11 g, 2.0 mmol, 1.0 equiv.) and catalytic DMF (15.5 μL, 0.1 equiv.) in anhydrous DCM (20 mL) was cooled to 0°C under N_2. Oxalyl chloride (0.51 g, 2.0 equiv.) was injected via syringe, and the reaction was warmed to 25°C in 4 h. Solvent removal afforded activated acid chloride **1-c** as a yellow solid, used directly

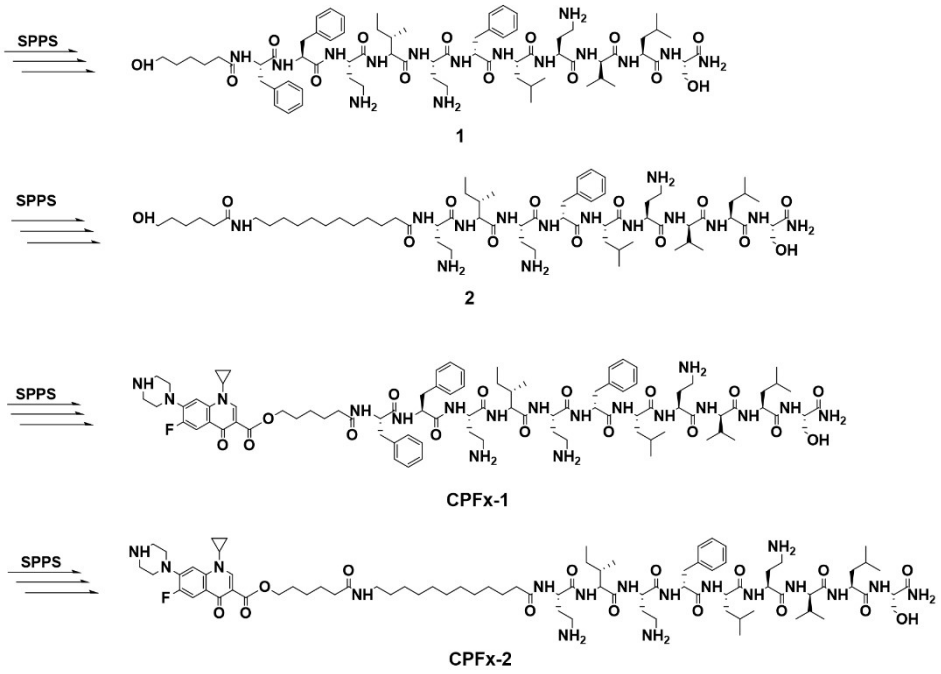
without purification.

Synthesis of compound 1-d

A mixture of pyridine (88 μL, 1.1 equiv.) and tert-butyl 6-hydroxyhexanoate (376 mg, 2.0 equiv.) in anhydrous DCM (20 mL) was cooled to 0°C under N_2. A solution of 1-c (572 mg, 1.0 equiv.) in DCM (5 mL) was added dropwise. After 1 h at 0°C, the reaction was stirred at 25°C for 24 h. Workup (DCM extraction, 1 M HCl/brine washes) and silica chromatography (DCM/MeOH 30:1) gave **1-d** as a pale-yellow powder.

Synthesis of compound 1-e(Fmoc-CPFx-C6-OH)

Compound **1-d** was treated with 50% TFA/DCM (15 mL, 3 h, 25°C). After solvent evaporation, the residue was dissolved in DCM (5 mL) and precipitated into ice-cold Et_2O (45 mL). The product was collected by centrifugation (8000 rpm, 0°C), then yielded **1-e** as a light-yellow powder.



Scheme S2. Synthetic route of **1**, **2**, **CPFx-1** and **CPFx-2**.

Synthesis of 1, 2, CPFx-1 and CPFx-2

1, **2**, **CPFx-1** and **CPFx-2** were synthesized by solid phase peptide synthesis with Rink Amide-AM resin, Fmoc-$_L$-Ser(tBu)-OH, Fmoc-$_L$-Leu-OH, Fmoc-$_D$-Val-OH, Fmoc-Dab(Boc)-OH, Fmoc-$_D$-Phe-OH, Fmoc-$_L$-lle-OH, Fmoc-$_L$-Phe-OH, 6-Hydroxycaproic Acid, Fmoc-12-Ado-OH, Fmoc-CPFx-C6-OH, HOBt and HBTU. Purification with HPLC provided **1**, **2**, **CPFx-1** and **CPFx-2** as white powder.

^{1}H NMR of **1** (400 MHz, D_2O) δ 7.39-7.00 (m, 15H), 4.59 (t, J = 8.0 Hz, 1H), 4.51-4.42 (m, 2H), 4.38-4.24 (m, 5H), 4.13 (s, 1H), 4.03-3.73 (m, 3H), 3.39 (t, J = 6.7 Hz, 2H), 3.02-2.80 (m, 9H), 2.70 (s, 2H), 2.01 (dt, J = 12.8, 7.1 Hz, 5H), 1.66 (d, J = 109.6 Hz, 6H), 1.41-0.90 (m, 12H),

0.86-0.63 (m, 27H). MS of **1**: calc. $[M+H]^+$ = 1398.85, obsvd. MALDI-TOF-MS: M/Z = 1399.33.

^{1}H NMR of **2** (400 MHz, D_2O) δ 7.51-7.02 (m, 5H), 4.66 (s, 1H), 4.36 (d, J = 5.1 Hz, 5H), 4.09 (d, J = 3.6 Hz, 1H), 3.84 (s, 2H), 3.54 (s, 2H), 3.05-2.99 (m, 6H), 2.29-2.16 (m, 7H), 1.52 (dd, J = 15.0, 7.4 Hz, 7H), 1.23 (s, 17H), 1.17 (d, J = 5.2 Hz, 6H), 0.93-0.81 (m, 37H). MS of **2**: calc. $[M+H]^+$ = 1301.89, obsvd. ESI-MS: M/Z = 1301.89.

^{1}H NMR of **CPFx-1** (400 MHz, D_2O) δ 8.62 (d, J = 24.9 Hz, 1H), 7.50-6.74 (m, 17H), 4.57 (t, J = 8.1 Hz, 1H), 4.32 (d, J = 28.1 Hz, 8H), 3.99 (d, J = 6.9 Hz, 2H), 3.76 (s, 4H), 3.40 (s, 6H), 2.81-2.50 (m, 7H), 2.04-1.77 (m, 9H), 1.42-1.02 (m, 14H), 0.84-0.59 (m, 37H). MS of **CPFx-1**: calc. $[M+H]^+$ = 1711.97, obsvd. ESI-MS: M/Z = 1711.98.

^{1}H NMR of **CPFx-2** (400 MHz, D_2O) δ 8.64 (s, 1H), 7.35-6.97 (m, 7H), 4.55 (t, J = 7.8 Hz, 1H), 4.40-4.22 (m, 8H), 4.00 (d, J = 8.9 Hz, 2H), 3.76 (d, J = 5.4 Hz, 3H), 3.47 (s, 11H), 2.95 (s, 10H), 2.13-1.92 (m, 9H), 1.68-1.52 (m, 10H), 1.17-0.97 (m, 17H), 0.80-0.66 (m, 33H). MS of **CPFx-2**: calc. $[M+H]^+$ = 1615.01, obsvd. ESI-MS: M/Z = 1615.02.

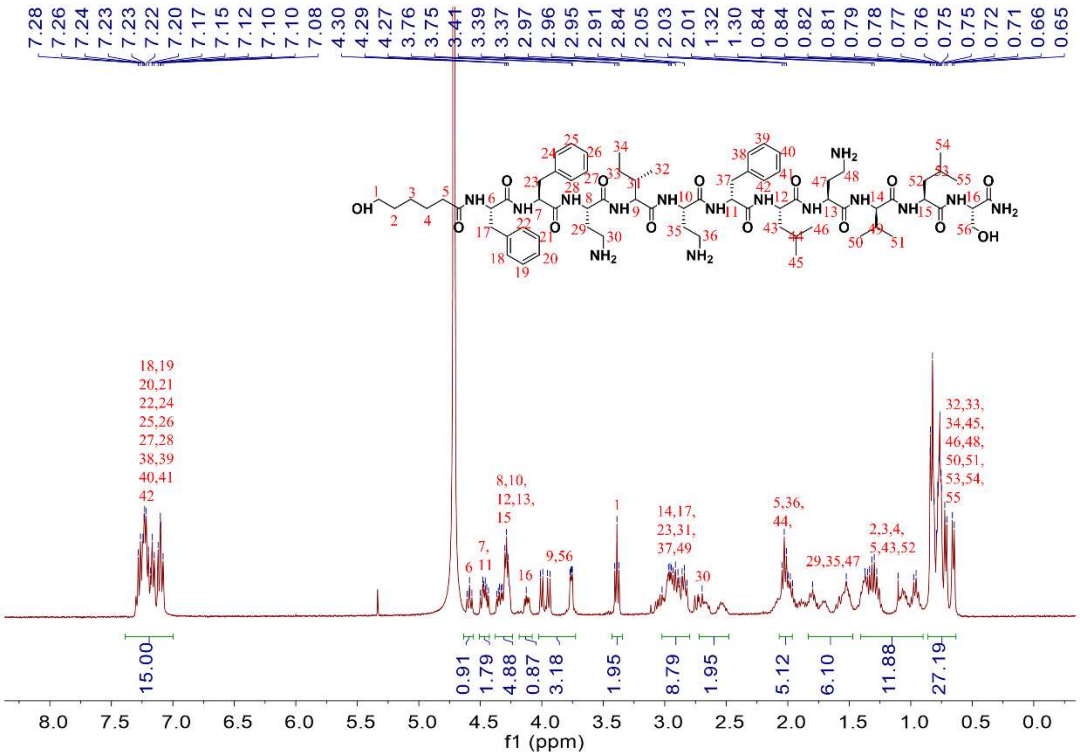


Figure S1. ^{1}H NMR spectrum of **1**.

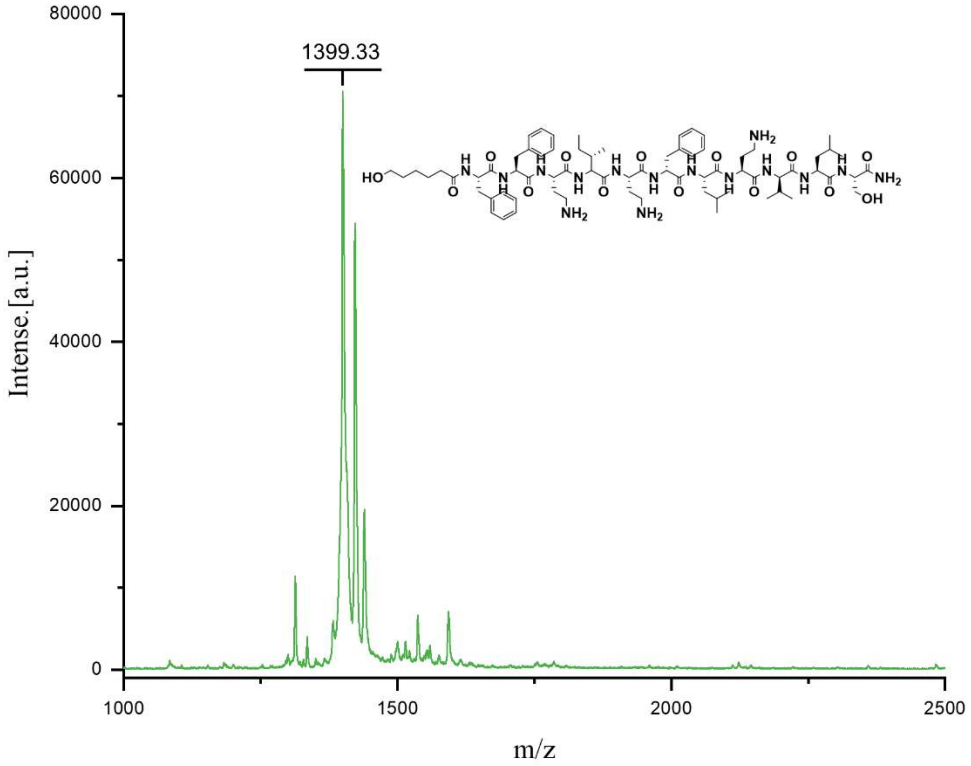


Figure S2. Mass spectrum of **1** (M/Z = 1399.33).

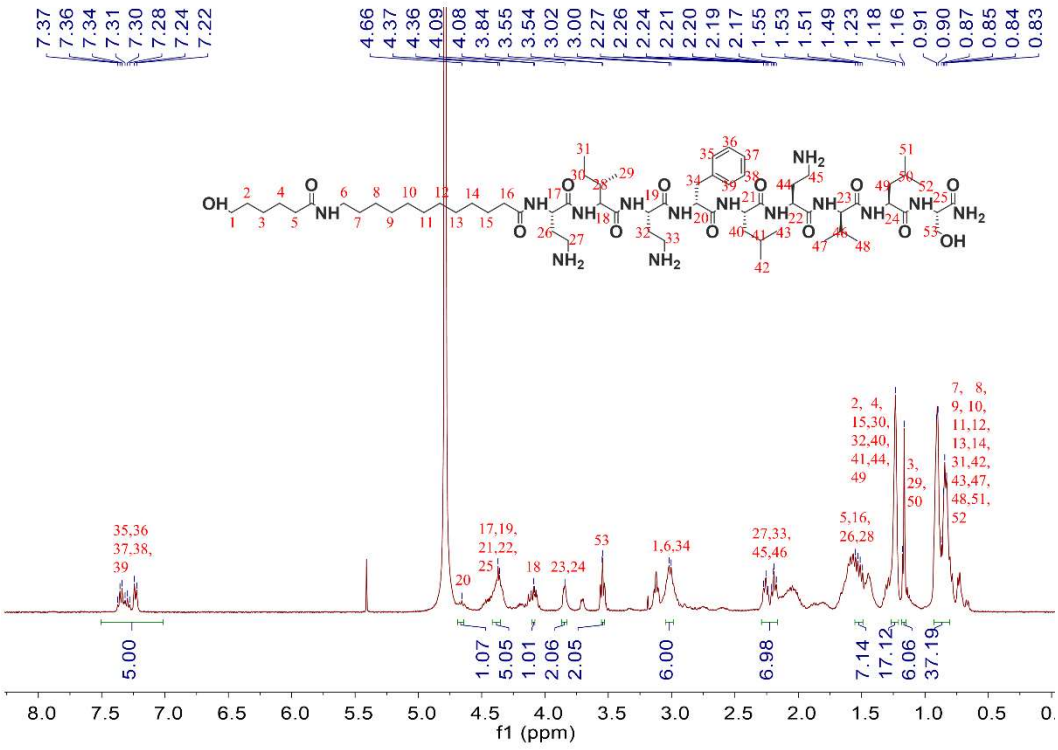


Figure S3. ^{1}H NMR spectrum of **2**.

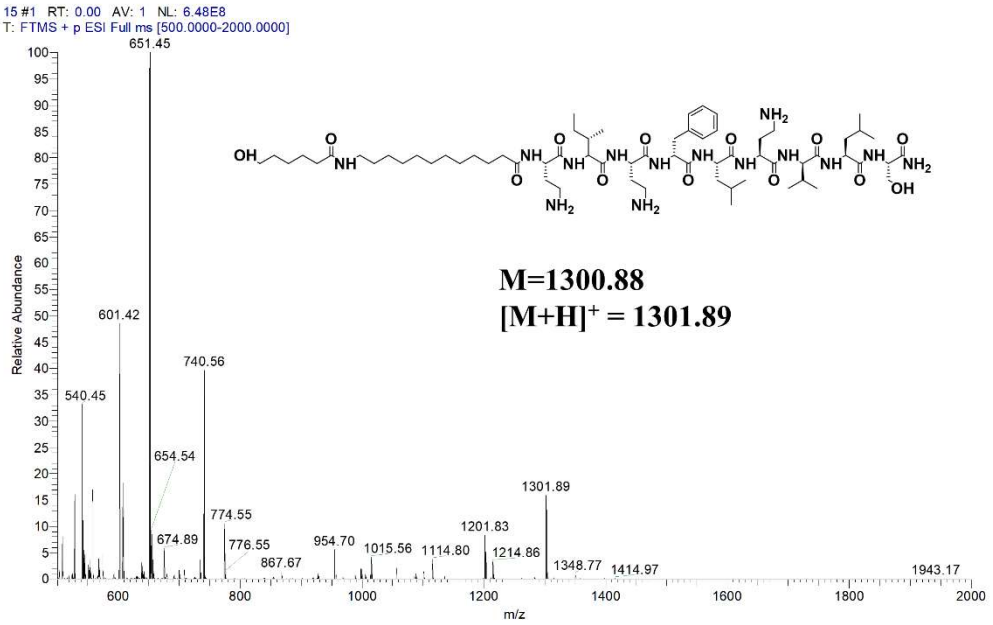


Figure S4. Mass spectrum of **2** (M/Z = 1301.89).

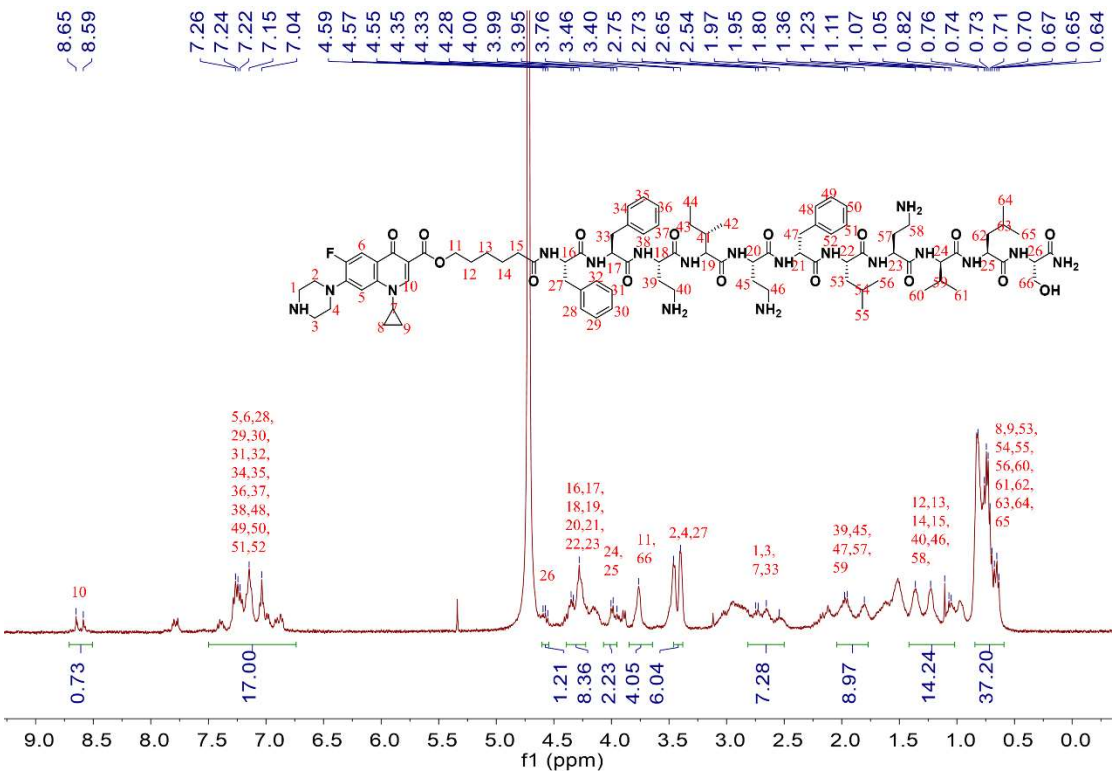


Figure S5. ^{1}H NMR spectrum of **CPFx-1**.

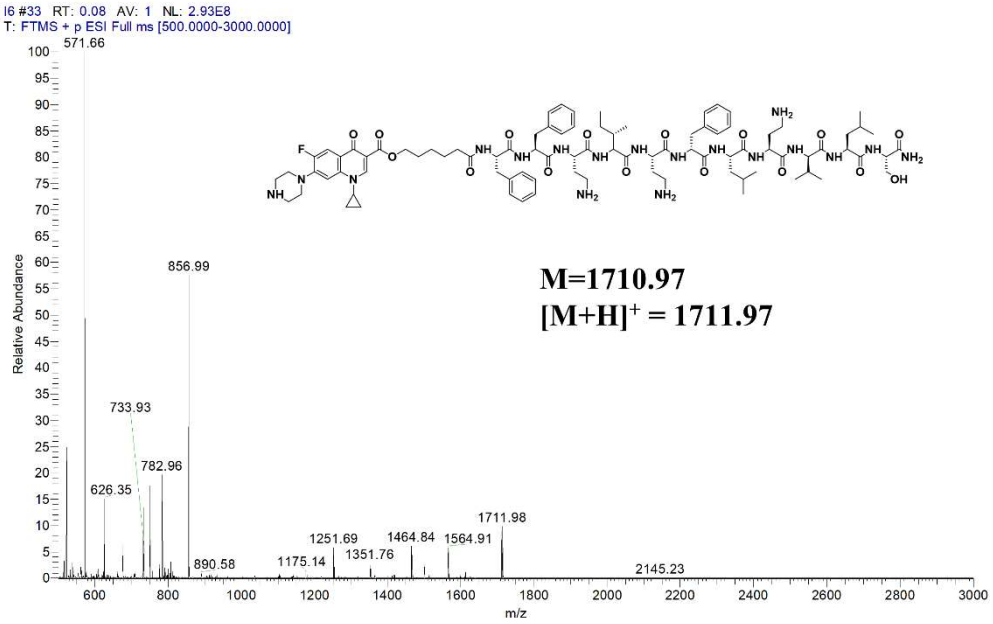


Figure S6. Mass spectrum of **CPFx-1** (M/Z = 1711.98).

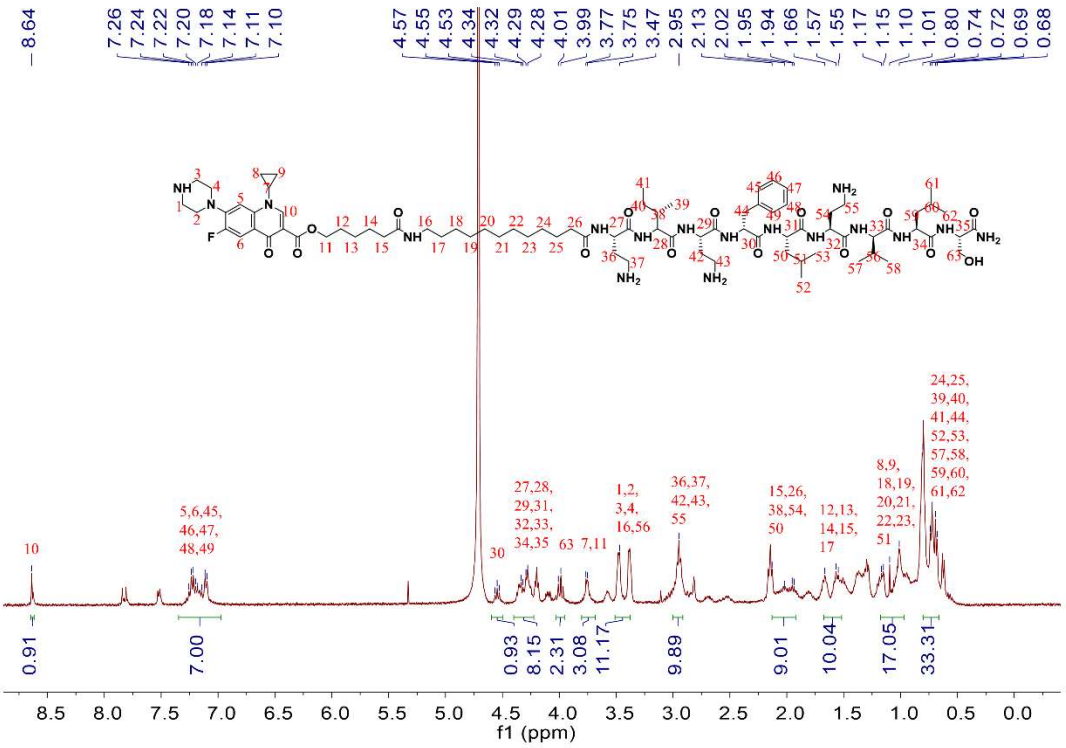


Figure S7. ^{1}H NMR spectrum of **CPFx-2**.

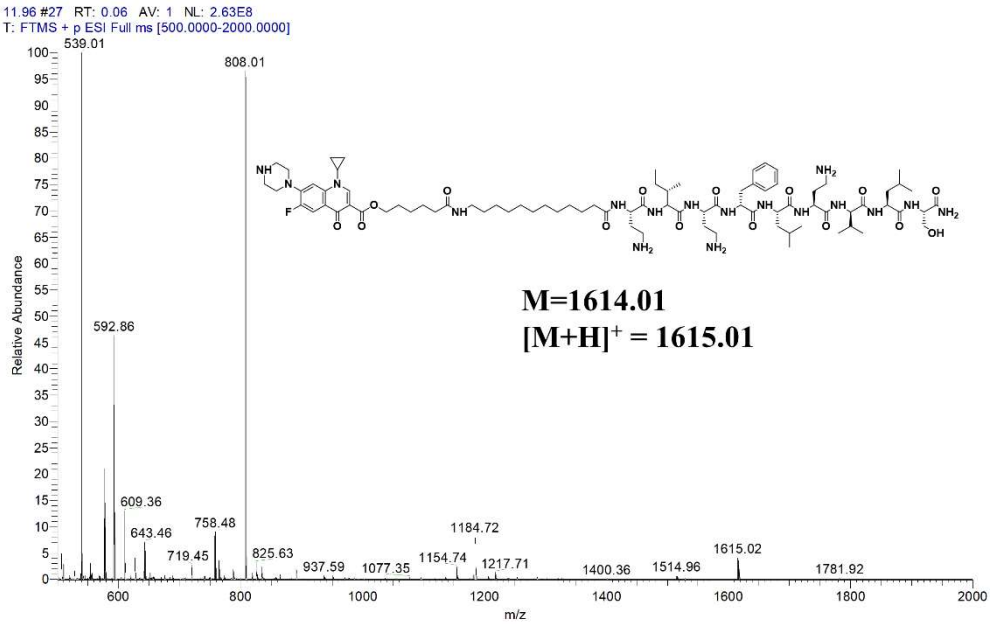


Figure S8. Mass spectrum of **CPFx-2** (M/Z = 1615.02).

S3. Stability measurement

In order to test the chemical stability, all antimicrobial peptides (50 μM) was incubated in phosphate buffer saline (PBS, pH 7.4) and 10% FBS for 24 hours. The absorbance of the antimicrobial peptides was measured at different time points using a Snyergy H1 microplate reader.

S4. Critical micelle concentration (CMC) measurement

A series of peptides solutions with different concentrations were prepared in pH 7.4 PBS buffer. The λ_{max} was determined by measuring the absorbance from 500 to 700 nm using a Snyergy H1 microplate reader.

S5. TEM sample preparation

After placing 5 μL samples on 400 mesh copper grids coated with continuous thick carbon film (~35 nm) which were glow-discharged, we washed the grids with ddH_2O. The residual liquid was absorbed with filter paper and then dried in air. TEM images were obtained with a JEM-1400Plus transmission electron microscope.

S6. Zeta-potential and size measurements

The zeta potential and size distribution of peptides solutions were determined by a Malvern Zetasizer Nano ZST nanoparticle potentiometer. The measurement temperature was set at 25.0 ± 0.1 °C. The averaged zeta-potential and diameter values were obtained by comparing the measured results at least 3 times for each sample.

S7. *In vitro* antibacterial activity assay

The bacterial survival rate was calculated by mixing 50 μL *E. coli* or *S. aureus* suspensions (OD_{600} ≈0.1) with equal volumes of antimicrobial peptides solutions at varying concentrations, followed by incubation at 37°C for 4 h and 12 h. The OD values of each group were measured using a microplate reader, and the bacterial survival rate was calculated according to the following equation:[1] Bacterial survival rate (%) = ($OD_{experimental\ group}$-$OD_{blank\ contral\ group}$)/($OD_{contral\ group}$-$OD_{blank\ contral\ group}$) × 100%, where the control group contained bacteria without antimicrobial peptides treatment, and the blank control group consisted of medium alone.

S8. Plate coating assay

Bacterial suspensions of *E. coli* and *S. aureus* with an initial OD_{600} ≈ 0.1 were mixed with an equal volume of antimicrobial peptides solution (100 μM) and incubated at 37°C for 6 hours. Subsequently, the bacterial mixture was diluted 1000-fold, and 100 μL of the diluted suspension was evenly spread onto LB agar plates. After incubation at 37°C for 12 hours, colony imaging analysis was performed.

S9. Agar well diffusion assay

To determine the antibacterial activity of the different peptides against *E. coli* and *S. aureus*, the agar well diffusion assay was performed. *E. coli* and *S. aureus* were cultured on LB agar plates at the density of 5×10^5 CFU mL^{-1}. The well (2 mm) was punched on LB agar, and peptides solution (100 μL, 50 μM) were poured into the respective well. The plates were then incubated overnight at 37°C. Then determination of growth inhibition's diameter was done with a ruler.

S10. Minimum inhibitory concentration (MIC) assay

E. coli and *S. aureus* were cultured and grown in LB medium for 10 h at 37 °C, and then the bacterial suspension was diluted in PBS to a cell density of 2×10^5 CFU mL^{-1}. Peptides were serially gradient diluted by PBS in a 96-well plate, and an identical volume of diluted bacterial suspension was dropwise added into each well, then incubated and grown at 37 °C for another 24 h, OD_{600} was monitored by a microplate reader and the MIC was obtained.

S11. Cytotoxicity assay of normal cells

An aliquot of the normal cell (BEAS-2B) suspension (100 μL) was seeded in each well of a 96-well plate (5000 cells/well, 100 μL medium), and the cells were incubated with antimicrobial peptides at different concentrations in a cell incubator for 24 h. Lastly, MTT assay was conducted according to the protocol.[2]

S12. Lactate dehydrogenase (LDH) assay

An aliquot of the normal cell (BEAS-2B) suspension (100 μL) was seeded in each well of a 96-well plate (5000 cells/well, 100 μL medium), The cells were then co-incubated with antimicrobial peptides at varying concentrations in a cell culture incubator for 24 h. Subsequently, the cell suspension was centrifuged at 400 × g for 5 min to collect the supernatant, and LDH release was measured using an LDH assay kit (Beyotime Biotechnology, Shanghai, China). The LDH release rate was calculated according to the following formula:

LDH release rate (%) = $(C - C_0)/(C_T - C_0) \times 100\%$

C and C_0 are the release of LDH from cells treated with various groups and untreated cells, respectively. C_T represents the total release of LDH from cells with 1% Triton X-100 treatment.

S13. SEM characterization of bacteria morphology

The morphologies of *E. coli* with different processing were performed by SEM. *E. coli* were cultured and grown in LB medium for 10 h at 37 °C, and then the bacterial suspension was diluted in PBS to 1×10^8 CFU mL^{-1} for later use. An aliquot of PBS (1 mL) containing peptides (50 μM) was incubated with an equal volume of the bacterial suspension at 37 °C for 4 h. The bacteria suspension was incubated with PBS only and was used as a control. After that, treated bacteria were soaked and fixed with 2.5% glutaraldehyde solution at room temperature for 12 h. The bacteria were collected and washed by PBS. Next, the collected bacteria were dehydrated in

ethanol solutions (30%, 50%, 70%, 80%, 90%, 95%, and 100%). Lastly, the samples were dried in a freeze dryer and sputter-coated with gold, followed by imaging with a scanning electron microscope.

S14. Membrane permeability assay using NPN

The membrane permeability assay was performed as follows: *E. coli* cells were cultured in LB medium until reaching mid-logarithmic growth phase (OD600 $\approx$ 0.5). Bacterial cells were then harvested by centrifugation, washed three times with PBS (pH 7.4), and resuspended in fresh PBS to a final concentration of 1×10^5 CFU mL^{-1}. Antimicrobial peptide solutions, prepared at varying concentrations, were introduced to the bacterial suspension and incubated at 37°C for 6 hours. Following incubation, the membrane-permeabilizing fluorescent probe NPN was added to achieve a final concentration of 20 μM, and the mixture was incubated in the dark for 30 minutes at room temperature. Fluorescence intensity was quantified using a microplate reader with excitation and emission wavelengths set at 350 nm and 420 nm, respectively.

S15. Murine wound infection model

All the following experimental mouse protocols were treated under the guidelines of the Institutional Animal Care and Use Committee, and operated under the permission of the Central China Normal University Administrative Panel on Laboratory Animal Care (No. CCNU-IACUC-2023-001). The experimental procedure was conducted in accordance with the NIH guidelines. And all operations were performed under 3% sodium pentobarbital anesthesia. Normal female Balb/c mice (6-8 weeks, 20 ± 4 g, Hunan SJA Laboratory Animal Co., Ltd.) were used as experimental mice to evaluate in vivo antibacterial properties. A total of 25 rats were randomly divided into five groups (Control, **1**, **2**, **CPFx-1** and **CPFx-2**). The infected mouse was created by cutting a full thickness wound (8 mm × 8 mm) on the dorsal skin of the mouse with surgical scissors and tweezers, then added *E. coli* bacterial suspension (20 μL, 1×10^6 CFU mL^{-1}) to the wound and preserved it on the wound for 2 h.

S16. Hemolysis assay

PBS was added to fresh murine blood to achieve a 4% blood concentration by diluting it 25 times. The red blood cell suspension of 100 μL was placed in tubes, and an equal volume (100 μL) of PBS solution containing peptides of different concentrations was added, including PBS as a negative control, while 1% ddH_2O as a positive control. After incubation at 37°C for 1 h to allow hemolysis to occur, the unhemolysed parts were subject to centrifugation at 100 g for 5 min. Then, each sample was photographed and supernatant was taken into a 96-well plate to measure the absorbance at 540 nm. Hemolysis Rate (%) = $(OD_{sample} - OD_{PBS})/(OD_{ddH2O} - OD_{PBS})\times100\%$.[3]

S17. Histological analysis

After 7 days, the wound tissues were excised and fixed in 10% formalin buffer at room temperature for 24 h. Finally, the tissues were embedded in paraffin to prepare the histological sections, and the sections were stained with H&E and Masson's Trichrome for better observation. The major organs were collected for H&E staining.

S18. Statistical analysis

All the data were expressed as the mean ± standard deviation. The sample number (n) expressed the number of each individual experiment samples. Statistical comparisons between two groups were performed using student t-test and more than three groups were determined by one-way ANOVA, analyzing by GraphPad Prism 9 software. Significance is indicated by P values (n.s. > 0.05, **P < 0.01, ***P < 0.001, ****P <0.0001).

S19. Supporting data

Table S1. Antibacterial activity of antimicrobial peptides against *E. coli* and *S.aureus*.

Peptide	MIC *E. coli* (μg/mL)	MIC *S. aureus* (μg/mL)	Reference
CPFx-2	2.52	5.04	This work
PapMA-3	16	32	4
KW-13	64	16	5
Chem. Syn. LR_{GG}	2	16	6
Mel	64	8	7
cathelicidin-BF	27	30	8

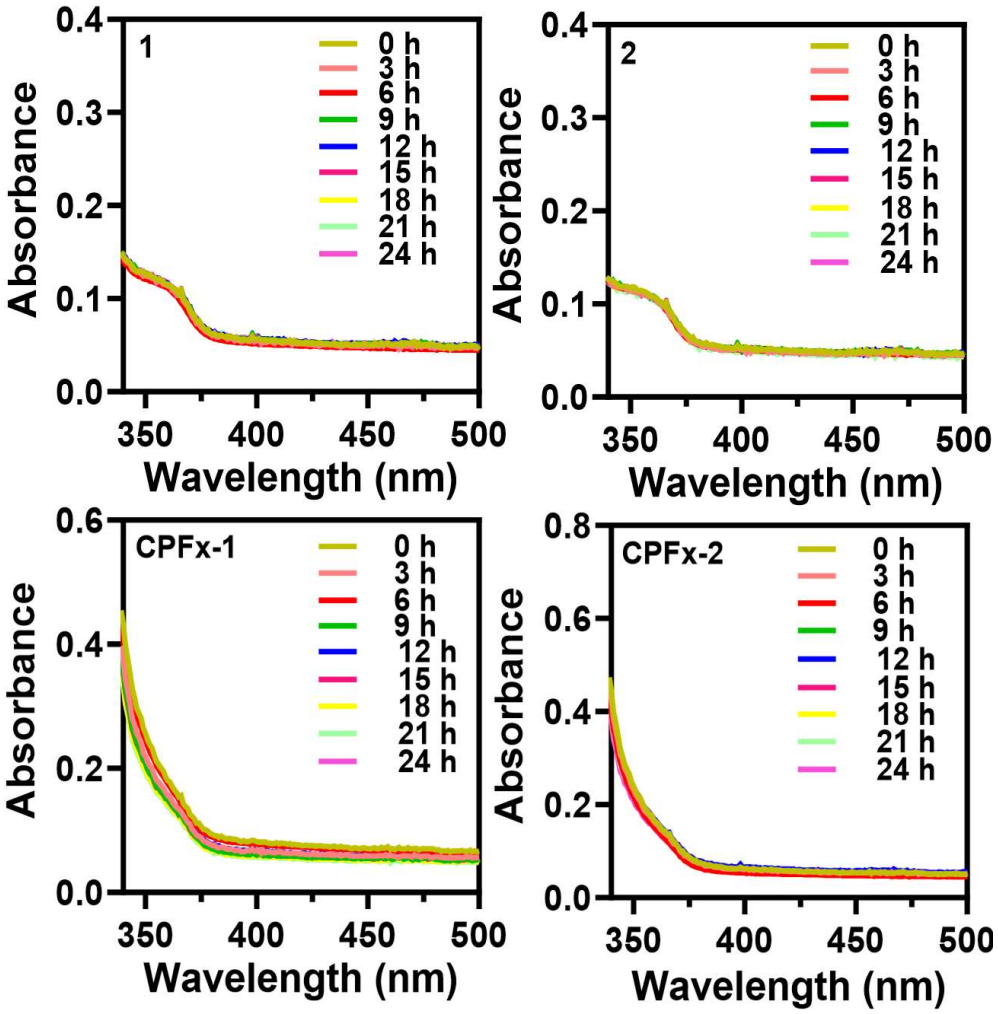


Figure S9. Absorption spectra of antimicrobial peptides (50 μM) in PBS at different time points.

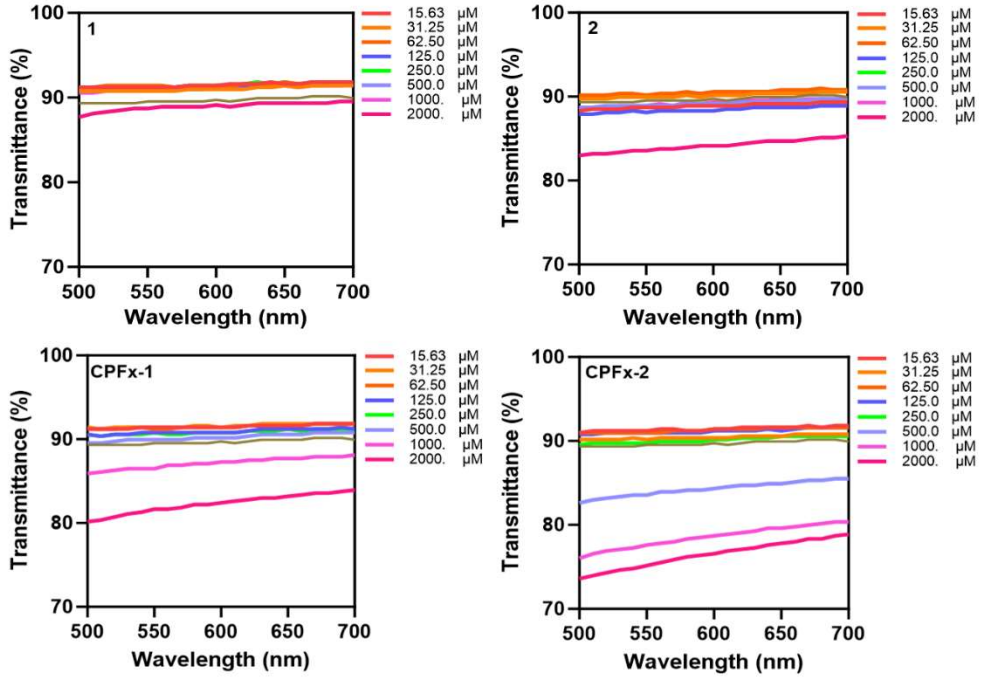


Figure S10. Changes in transmittance of antimicrobial peptides at different concentrations.

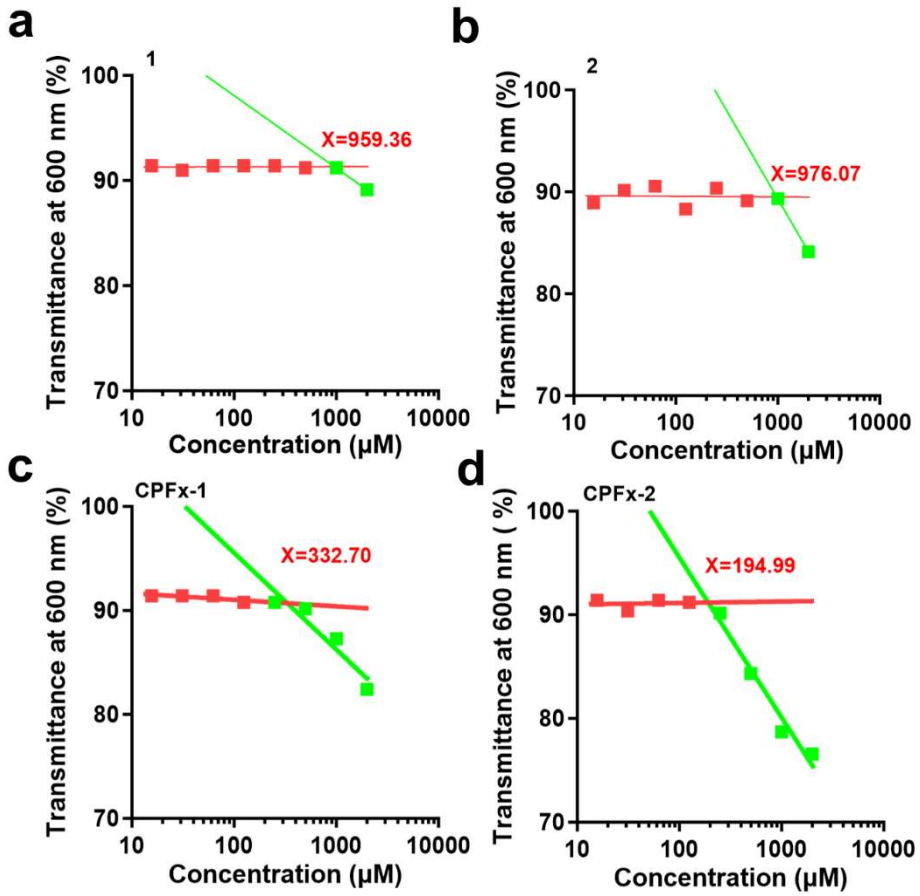


Figure S11. Critical micelle concentration (CMC) profiles of **1**, **2**, **CPFx-1** and **CPFx-2** (in PBS, pH = 7.4, n = 3, mean ± SD).

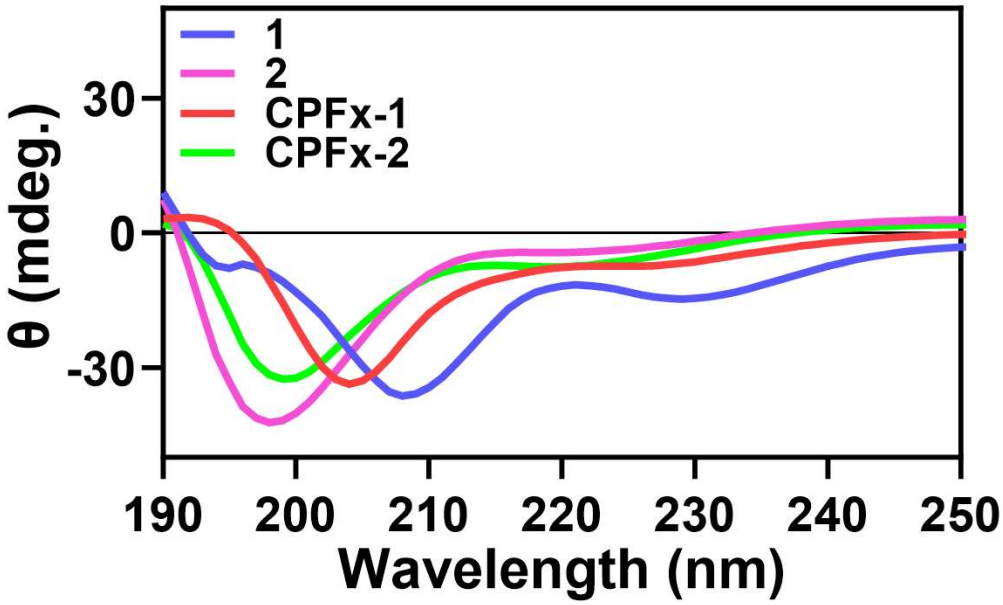


Figure S12. Far-UV circular dichroism (CD) spectra of antimicrobial peptides.

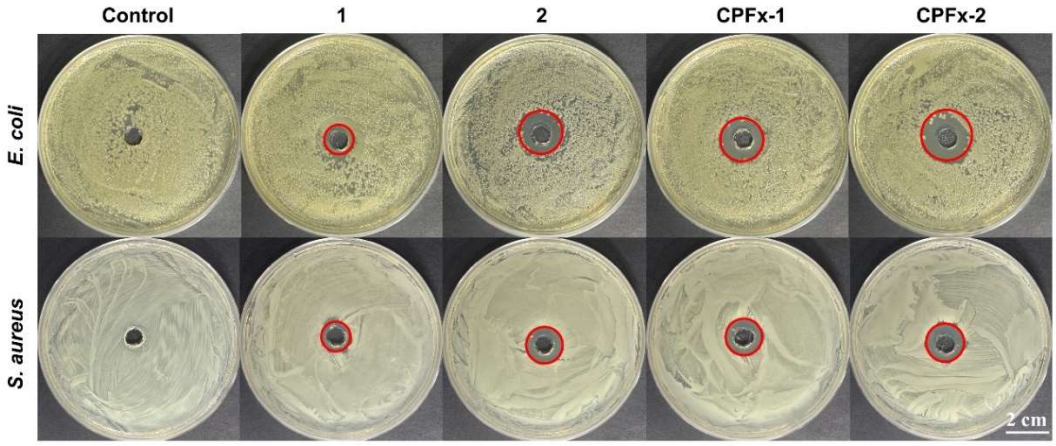


Figure S13. Photographs of inhibition zone plates after 12-hour co-incubation of bacteria (*E. coli* or *S. aureus*) with antimicrobial peptides, compared to a blank control group (PBS, pH 7.4).

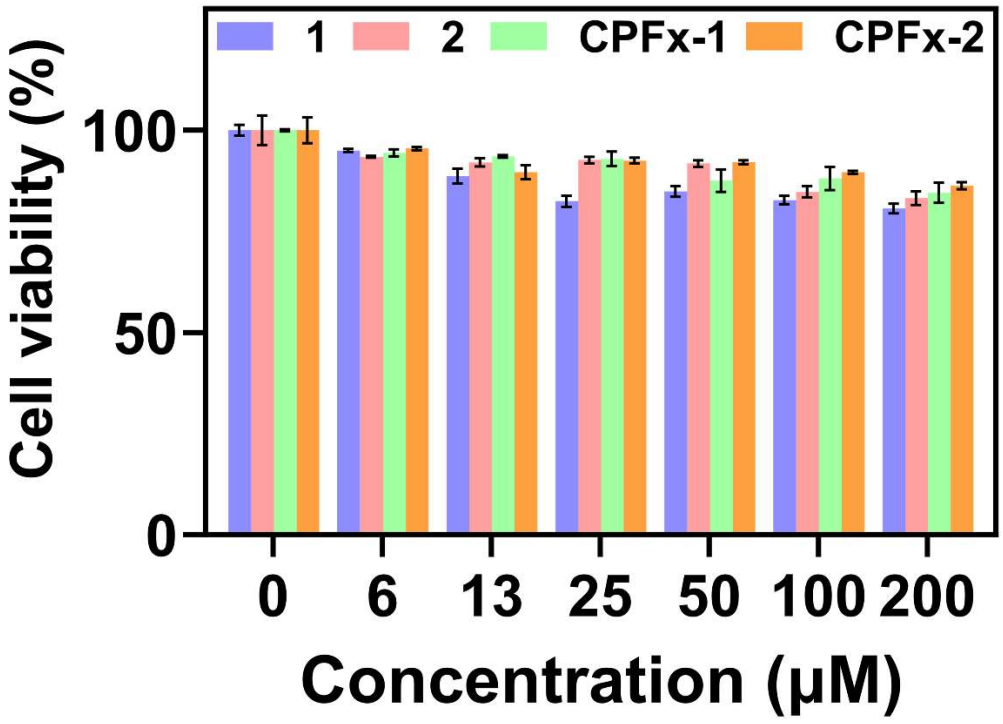


Figure S14. Cell viability of BEAS-2B cells after incubation of different concentration of antimicrobial peptides for 24 h (n = 3, mean ± SD).

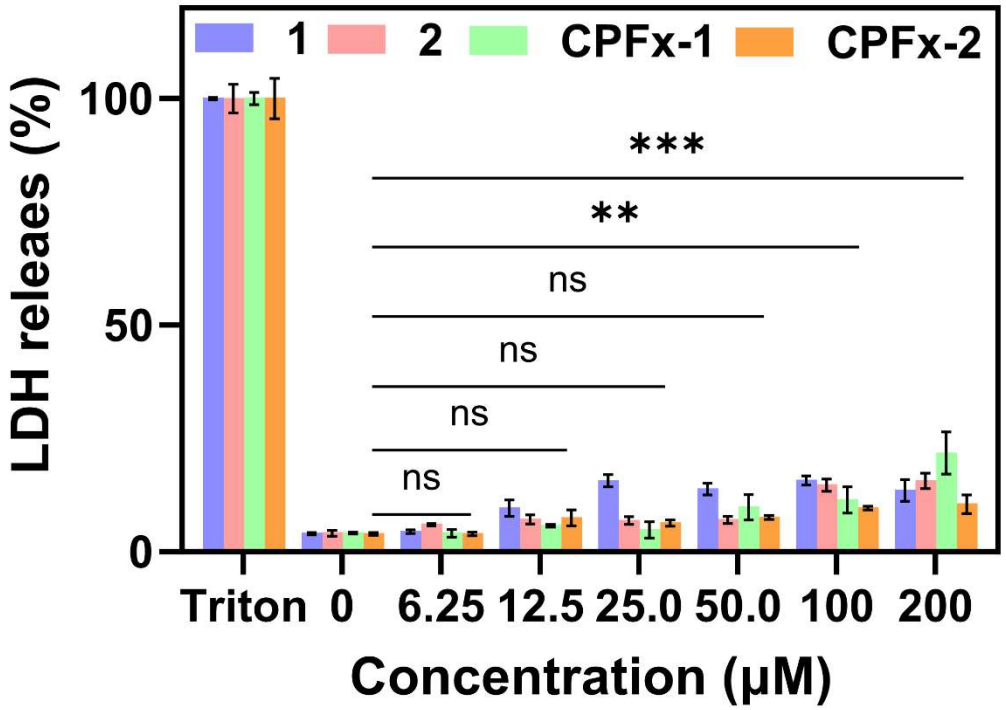


Figure S15. Release of LDH after 24-hour exposure to various concentrations of antimicrobial peptides (n = 3, mean ± SD, n.s. > 0.05, **P < 0.01, ***P < 0.001).

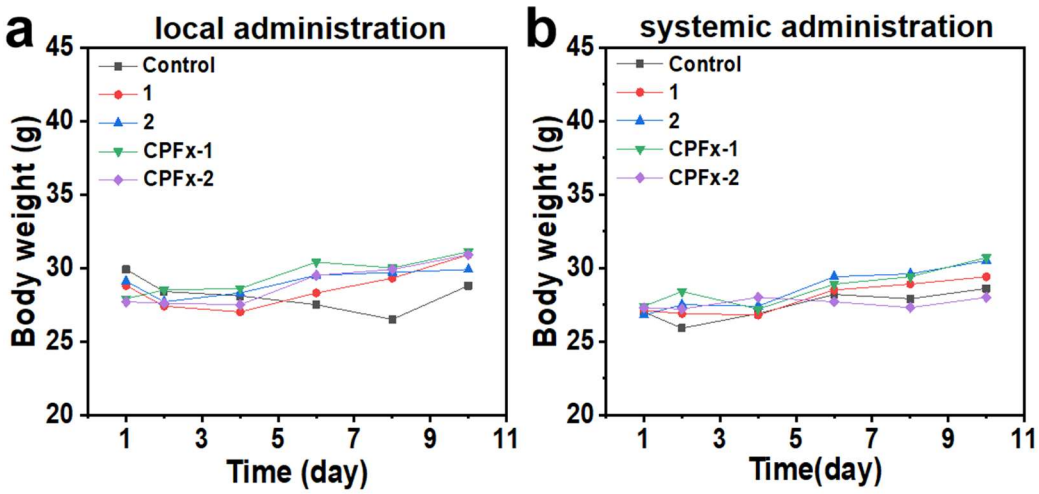


Figure S16. Body weight changes in wound-infected murine models after (a) local and (b) systemic administration, compared to a blank control group (PBS, pH 7.4).

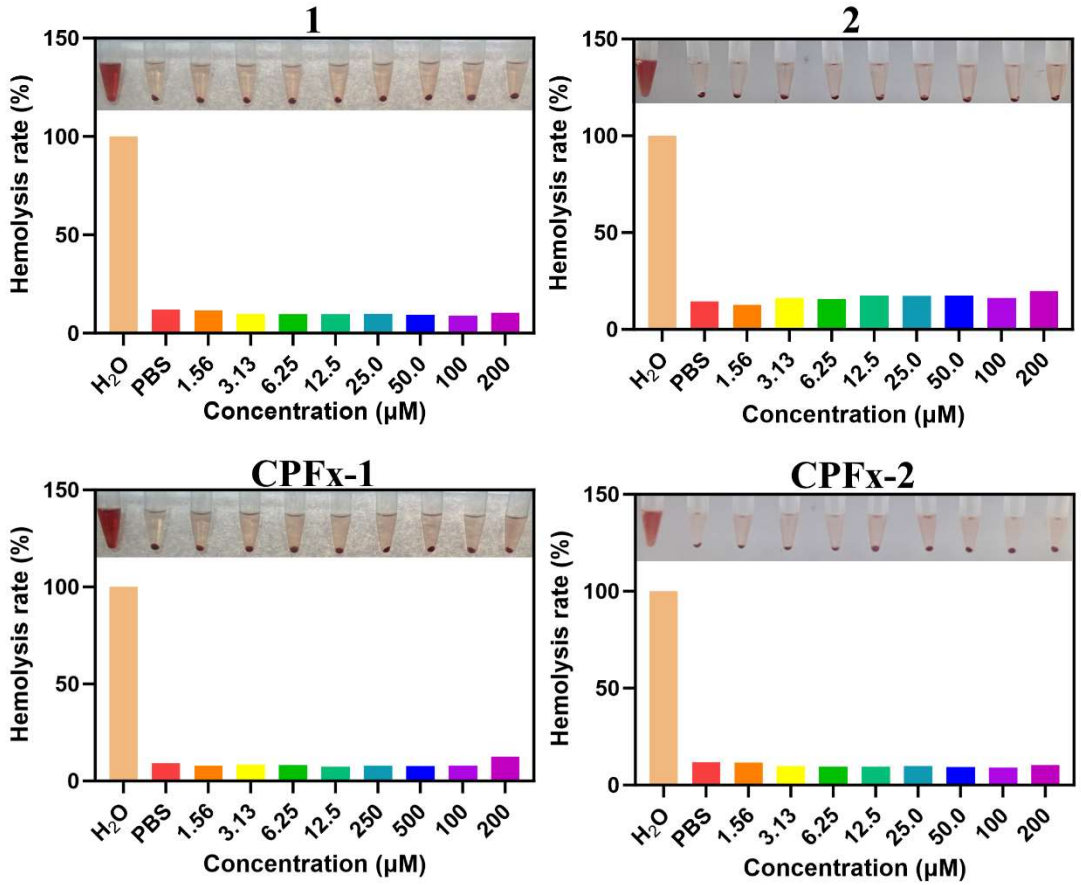


Figure S17. Hemolysis rate of red blood cells at varying antimicrobial peptides concentrations, compared to a blank control group (PBS, pH 7.4).

References

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