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# Supporting Information

## Polymyxin Derivatives as Broad-Spectrum Antibiotic Agents

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### 1. General information

2-Chlorotrityl chloride (CTC) resins (0.972 mmol/g, 100–200 mesh) were purchased from Chem-Impex Int'l Inc. Solvents and other chemicals were ordered from either Fisher Scientific or Sigma-Aldrich, and were used without further purification. The solid phase synthesis of all compounds was carried out in a peptide reaction vessel on a Burrell Wrist-Action shaker. All compounds were analyzed and purified using the Waters Breeze 2 HPLC system under 215 nm of UV detector equipped with both analytical and preparative modules. The desired fractions were lyophilized on a Labcono lyophilizer.

### 2. Synthesis of building block

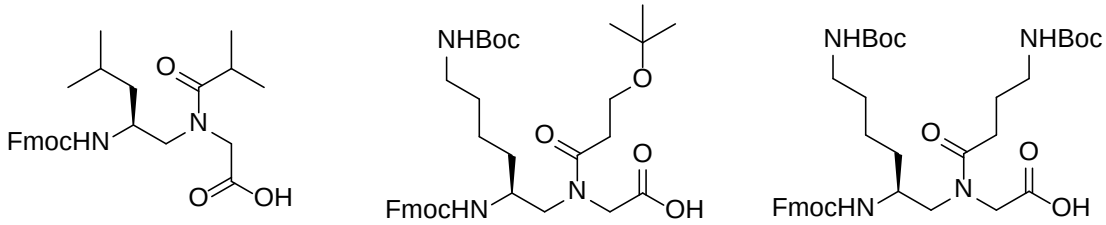
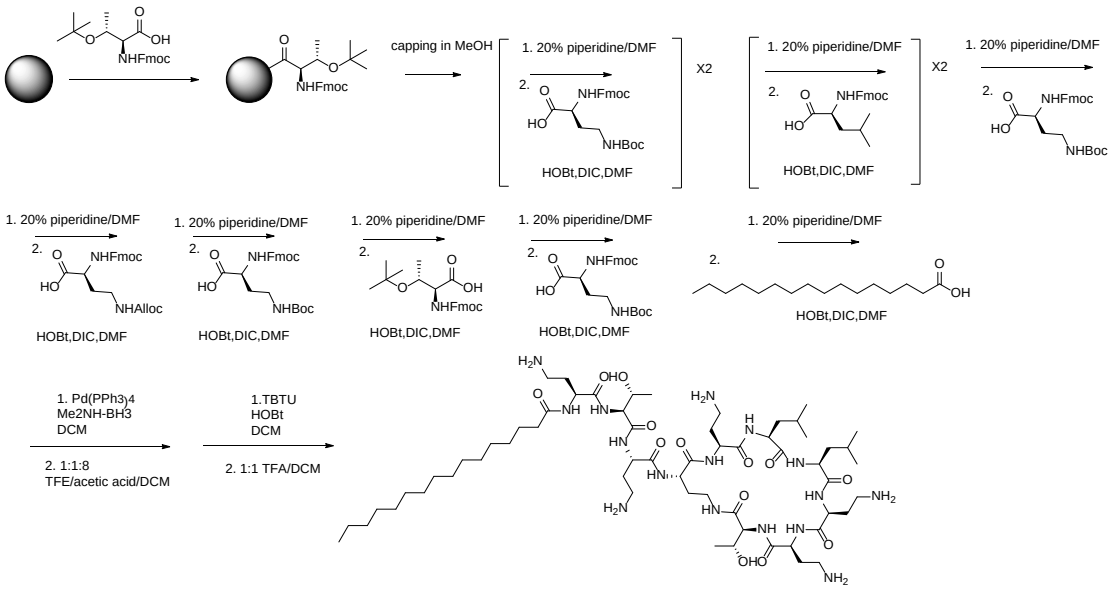



**Figure S1.** Structures of γ-AApeptide building blocks

All 3 γ-AApeptide building blocks were used in synthesis of compounds on solid phase, and procedure of synthesis was reported in previously paper.[1]

## 3. Synthesis of polymyxin mimic peptides



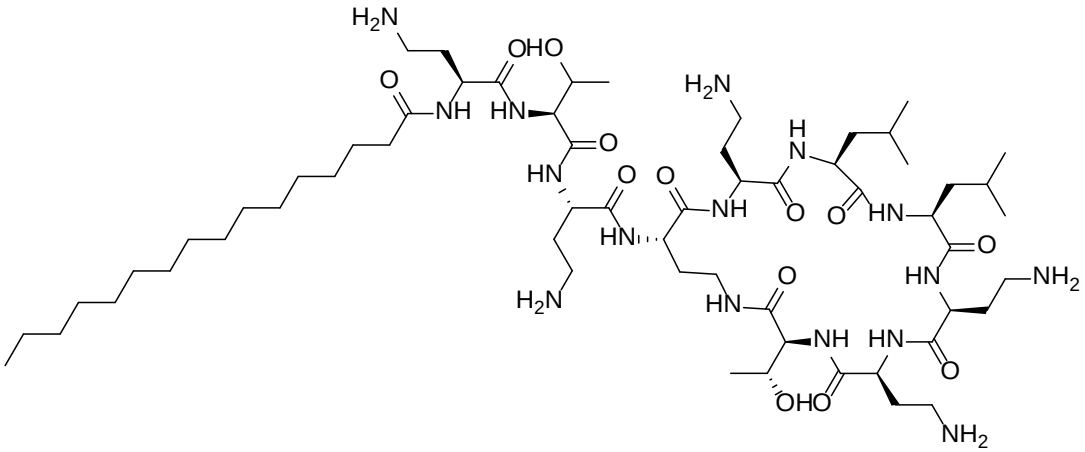

**Scheme S1.** Synthetic procedure of the compound **P1**.

200 mg CTC resin (0.196 mmol) was reacted with Fmoc-Thr(tBu)-OH (0.23 g, 0.588 mmol) and DIPEA (50 μL, 0.287 mmol) in DCM solution for 3 h, followed by DMF (2 mL × 3) and DCM (2 mL × 3) wash. Then resin beads were capped in methanol for 30 min. The attachment of the Fmoc-Dab(Boc)-OH to the resin was achieved by adding Fmoc-Dab(Boc)-OH (176 mg, 0.4 mmol), DIC (100 μL, 0.7 mmol), and HOBt (0.1 g, 0.8 mmol) in 3 mL DMF to the reaction vessel, and the reaction was allowed to shake at room temperature for 3 h. Then the solution was drained, and the beads were washed with DCM (3 mL × 3) and DMF (3 mL × 3). After that, beads were treated with 3 mL 20% piperidine/DMF (v/v) solution for 15 min (× 2) to remove the Fmoc protection group, followed by DMF (2 mL × 3) and DCM (2 mL × 3) wash. This step was repeated once, and Fmoc-Dab(Boc)-OH was added to the resin again . Next, Fmoc-L-leucine (141mg, 0.4 mmol), DIC (100 μL, 0.7 mmol), and HOBt (0.1 g, 0.8 mmol) in 3 mL DMF were added to the resin and allowed to react for 3 h at room temperature. The step was repeated until the desired building blocks were attached. Then, after Fmoc group was removed, palmitic acid ( 0.1g, 0.4 mmol) DIC (100 μL, 0.7 mmol), and HOBt (0.1 g, 0.8 mmol) in 3 mL DMF was added to the reaction vessel. The beads were with $Pd(PPh_3)_4$ (24 mg, 0.02 mmol) and $Me_2NH.BH_3$ (70 mg, 1.2 mmol) in 3 mL DCM for 10 min (×2) to remove the alloc protein group, then washed with DCM (3 mL x3) and DMF (3 mL ×3).followed by the

incubation with 4 mL cocktail of 1:1:8 TFE: acetic acid: DCM 1:1 (v/v/v) for 2 h to achieve cleavage of the compound. After the solvent was removed in vacuo, the residue was added with HOBt (73mg, 0.588 mmol), TBTU (188mg, 0.588 mmol) and DMAP (120mg, 0.98 mmol) in 500 mL DCM solvent for overnight to allow cyclization. Finally, all remained protecting groups were removed by incubation with 20 mL cocktail of 1:1 TFA: DCM 1:1 (v/v) for 2 h, and the resulted solution was collected and solvent was evaporated. Then the compound was analyzed and purified on the Waters HPLC system, and the desired fraction was lyophilized to give the pure product compound **P1.**

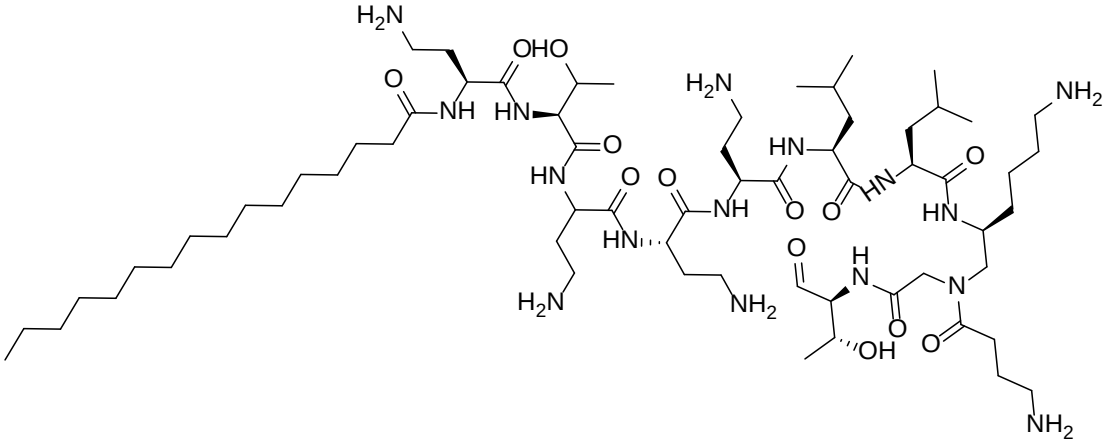
Compound **P2, P3** and **P4** were synthesized with similar methods.

**Compound P1**



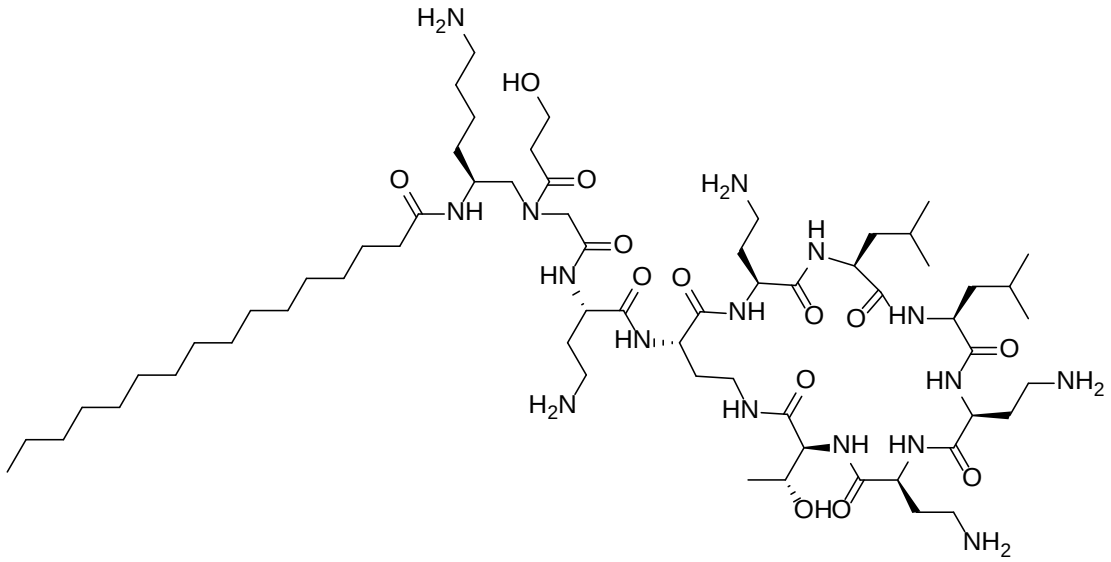
HRMS (ESI) $C_{60}H_{114}N_{16}O_{13}$ [M+H]+ calc'd = 1267.8824; found = 1268.0142.

**Compound P2**



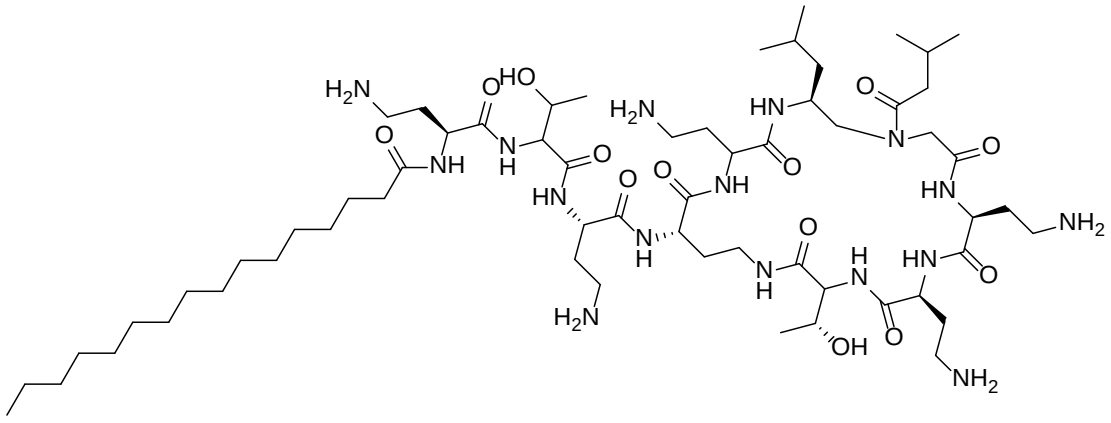
HRMS (ESI) $C_{64}H_{124}N_{16}O_{13}$ [M+H]+ calc'd = 1325.9607; found = 1326.0138.

**Compound P3**



HRMS (ESI) $C_{63}H_{120}N_{16}O_{13}$ [M+H]+ calc'd = 1309.9294; found = 1310.0184.

**Compound P4**



HRMS (ESI) $C_{61}H_{116}N_{16}O_{13}$ [M+H]+ calc'd = 1281.8981; found = 1282.0422.

**4. Minimum inhibitory concentrations (MICs) assays.**[2]

All compounds were tested against six different bacteria strains: Methicillin-resistant *S. aureus* (MRSA, ATCC 33591), Methicillin-resistant *S. epidermidis* (MRSE, RP62A), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853). One colony of each bacteria was inoculated in 4 mL TSB buffer overnight at 37 °C, then the suspension was diluted 100 times and incubated 6 hours to mid-logarithmic phase. All compounds were diluted in 96-wells plate with 50 μL 2-fold serial dilution, then 50 μL of diluted bacterial medium ($1 \times 10^6$ CFU/mL) was added to each well. After 18 hours incubation at 37 °C, absorption was read at 600 nm wavelength on a Biotek

Synergy HT microtiter plate reader. Minimum inhibitory concentrations were determined as the lowest concentrations that inhibit bacteria growth completely.

**5. Time kill assays.**[2]

Bacteria MRSA and *E. coli* suspensions were incubated at 37 °C to mid-logarithmic phase and diluted to $1 \times 10^6$ CFU/mL, then mixed with compound **P1** (50, 25, 12.5μg/mL). The mixtures were incubated at 37 °C for 10 min, 30 min, 1 h and 2 h respectively, then diluted by $10^2$ to $10^4$ folds and 100 μL was spread on TSB agar plates. Numbers of bacteria colonies were counted after 20 hours incubation at 37 °C.

**6. Drug resistance assays.** [3]

After MICs assay against *E. coli*, withdraw bacterial from the well which contained 1/2 MIC and diluted to $1 \times 10^6$ CFU/mL for next MIC measurement. The measurement was repeat for 12 passages.

**7. Hemolytic assays.**[2]

Fresh red blood cells (RBCs) was washed with 1× PBS buffer and centrifuged 10min at 3500rpm less than 3 times until the supernatant was clear, then RBCs was diluted into 5% v/v suspension in 1× PBS. 50 μL of compounds was diluted in 96-wells plate with 2-fold serial dilution and mixed with 50 μL RBCs suspension, then incubated for 1 hour at 37 °C. The mixture was then centrifuged for 10 min at 3500 rpm. 30 μL of the supernatant was added to 100 μL PBS, then absorbance of mixture was read on a Biotek Synergy HT plate reader at 540 nm. The hemolysis activity was calculated by the formula % hemolysis = $(Abs_{sample}-Abs_{PBS})/(Abs_{Triton}-Abs_{PBS})x100\%$. 1% and 5% Triton X-100 were used as positive control and 1× PBS buffer was used as negative control.

**8. Fluorescence microscopy.**[2]

Both propidium iodide (PI) and 4', 6'-diamidino-2-phenylindole dihydrochloride (DAPI) fluorescent dyes were used in the study. Bacteria MRSA and *E. coli* suspensions were incubated

at 37 °C to mid-logarithmic phase and diluted 100 folds, then incubated with compound **P1** for 2 hours at 37 °C. After centrifugation for 15 min at 5000 rpm, cell pellets were washed with 1× PBS buffer, and incubated with PI (5 μg/mL) for 15 min on ice in the dark, then washed 2 times with PBS. Then DAPI (10 μg/mL) was also applied the same way. The pellets were then diluted in 100 μL PBS and 10~20 μL was applied on chamber slides and observed under Zeiss Axio Image Zloptical microscope using 100× oil-immersion objective.

**9.In vivo study of mouse thigh burden infection model.[4]**

All protocols and methods associated with animal experiments were approved by University of South Florida (USF) Institutional Animal Care and Use Committee. The CD-1 female mice which were 6 to 8 weeks old and around 25 g in weights were used for the study. Neutropenic Mice were induced by injecting cyclophosphamide (150 mg/kg) intraperitoneally twice at 4 and 1 days before bacterial inoculation. One MRSA colony from TSA cultures was allowed to grow in TSB medium overnight at 37 °C, then 100 μL culture was withdrawn and diluted with TSB to a total volume of 4 mL, which was subsequently incubated at 37 °C for another 6 h. The bacterial culture was then diluted in sterile 1x PBS buffer. The thigh burden infection model was established by injecting both posterior thighs of mice with 100 μL of inoculums. Two doses of the compounds **P1** were given 1 h/ 13 h by i.v. bolus injection in the tail vein at 5 mg/kg per dose of drugs after bacterial infection. Thighs were harvested at 25 h for both groups after bacterial inoculation. Thigh muscles were collected in a sterile tared tube, to which 5 mL sterile PBS buffer was added. The mixture was then homogenized with a tissue homogenizer (BioSpec product tissue tearor 985-370) for approximately 30 sec. 100 mL of serial diluted aliquots were plated on TSA plates, which were incubated for 24 h at 37 °C. Then bacteria colonies were counted to calculate CFU per thigh.

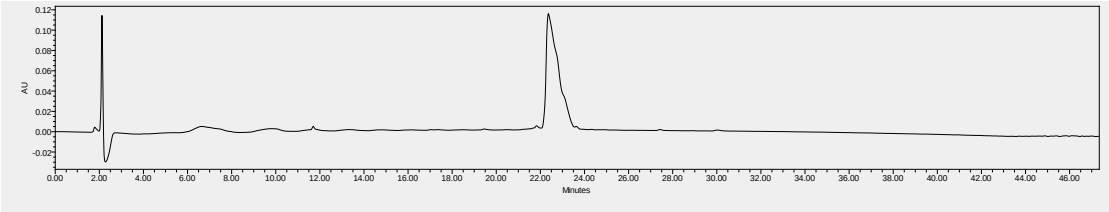
For statistical analysis, mean, standard deviation and P value of two groups were calculated. P value was 0.0286 using Kolmogorov-Smirnov test and Mann Whitney test for calculation.

**10. Biofilms Assay.**

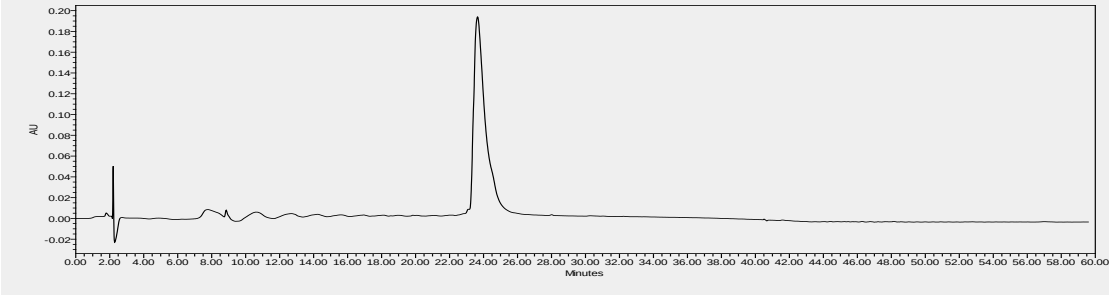
Compound **P1** were diluted in 96-wells plate with 50 μL 2-fold serial dilution, then 50 μL of diluted bacterial medium ($1 \times 10^6$ CFU/mL) was added to each well. After 48 hours incubation at 37 °C, the plate was washed gently and remained biofilm was stained with 125 μL 0.1% crystal violet solution for 10 min after dried. The washing and drying step was repeated for another time, and then 200 μL 30% acetic acid was added to all vials to dissolve stain for 10 min. The 125 μL solution was transferred to a new plate and the OD reading was recorded at 595 nm wavelength.

## 11. HPLC spectra of compounds P1− P4.

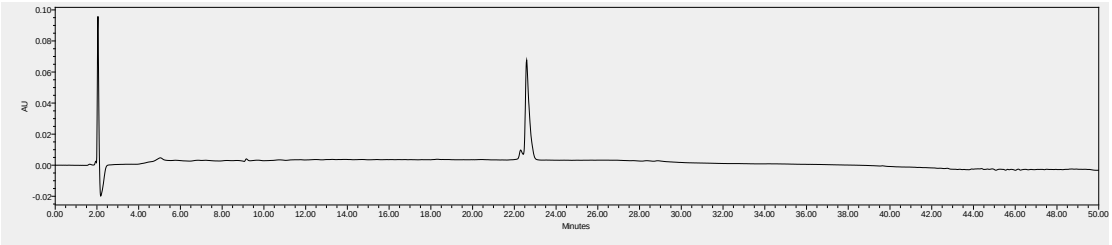
### Compound P1



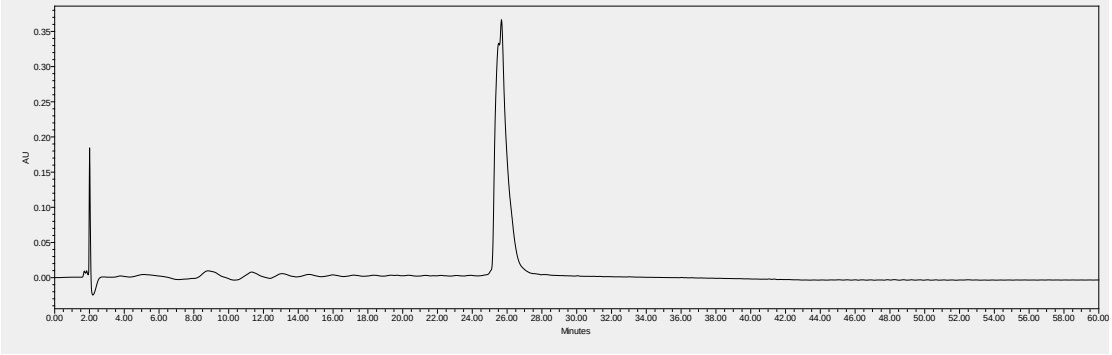

### Compound P2




### Compound P3




### Compound P4

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2. Li, Y.; Wu, H.; Teng, P.; Bai, G.; Lin, X.; Zuo, X.; Cao, C.; Cai, J., Helical Antimicrobial Sulfono-γ-AApeptides. *Journal of Medicinal Chemistry* **2015,** *58* (11), 4802-4811.

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