

The Activity of Small Urea- γ -AApeptides Toward Gram-Positive Bacteria

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Host Defense Peptides (HDPs) have gained considerable interest due to the omnipresent threat of bacterial infection as a serious public health concern. However, development of HDPs is impeded by several drawbacks, such as poor selectivity, susceptibility to proteolytic degradation, low-to-moderate activity and requiring complex syntheses. Herein we report a class of lipo-linear α /urea- γ -AApeptides with a hybrid backbone and low molecular weight. The heterogeneous backbone not only enhances chemodiversity, but also shows effective antimicrobial activity against Gram-positive bacteria and is capable of disrupting bacterial membranes and killing bacteria rapidly. Given their low molecular weight and ease of access via facile synthesis, they could be practical antibiotic agents.

Due to the omnipresent threat of bacterial infection which poses a serious public health concern, Host Defense Peptides (HDPs) have gained considerable interest.^[1] HDPs are peptides produced by immune system to protect the body from bacteria, fungi and viruses and can be found in all forms of life in variable sequences. Based on their different action modes, HDPs have been subdivided into two classes: membrane permeabilization and intracellular targeting. The primary mechanism of HDPs is the electrostatic and hydrophobic interaction and kill bacteria by damaging their cell membranes.^[2] Various models have been proposed, such as barrel stave model, toroidal pore model and carpet model. However, development of HDPs is impeded by their intrinsic drawbacks, such as poor selectivity, susceptibility to proteolytic degradation, and low-to-moderate activity. Furthermore, most HDPs have large molecular weight (> 1000 Da), and synthesis is challenging for large

production. Recently, antimicrobial peptidomimetics attracted attentions, as they could be smaller in size but still retain potential broad-spectrum activity.^[3] We reported a class of peptidomimetics " γ -AApeptide" which show resistance to proteolytic degradation, and various side chain acylating agents can create almost limitless chemical diversity.^[4] These advantages make γ -AApeptides promising candidates for paralleling function and structure of HDPs.^[5] Indeed, a variety of γ -AApeptides have been developed and displayed potent and broad-spectrum antimicrobial activity.^[6] However, most lead compounds are still large sequences which require multi-step synthesis, limiting their practical application. In the effort of searching for antimicrobial peptidomimetics with smaller molecular weight, we have developed a class of lipo-linear α / γ -AApeptides that utilize a hybrid backbone of α -peptide with γ -AApeptides.^[7] This class of peptides display broad-spectrum antimicrobial activity and excellent selectivity. Therefore, in this study we further proved that the heterogeneous backbone may enhance the chemodiversity for future optimization and development. It is also previously known that urea-containing compounds exhibited antibacterial activity.^[8] As such, we hypothesized that lipidated heterogeneous antimicrobial urea-AApeptides should also exhibit antimicrobial activity.

As amphipathic property is essential for HDPs and their derivatives, we designed a series of small linear urea- γ -AApeptides bearing different hydrophobic groups and cationic charged groups. They could be synthesized on the solid in a very straightforward manner (Scheme 1), which allowed derivatization and optimization rapidly in the future.

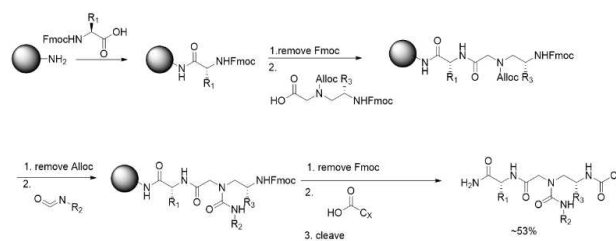
As shown in Table 1, compounds 1–23 are composed by one amino acid, one γ -AApeptide building block with urea side chain and lipid tail of variable length. All the molecules were tested against a panel of multi-drug resistant Gram-positive bacteria for their antimicrobial activity (Table 2). As shown in Table 2, although Compound 1–5 have the same R₁, R₂ and R₃

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Scheme 1. General approach of small linear molecules synthesis on solid phase.

Table 1. Structure-based design of compound 1–23.

Compd	R ₁	R ₂	R ₃	C _x
1				C ₅ H ₁₃
2				C ₉ H ₁₉
3				C ₁₁ H ₂₃
4				C ₁₃ H ₂₇
5				C ₁₅ H ₃₁
6				C ₁₁ H ₂₃
7				C ₁₁ H ₂₃
8				C ₁₁ H ₂₃
9	H			C ₁₁ H ₂₃
10				C ₁₁ H ₂₃
11				C ₁₃ H ₂₇
12				C ₁₅ H ₃₁
13				C ₁₁ H ₂₃
14				C ₁₁ H ₂₃
15				C ₁₁ H ₂₃
16				C ₁₁ H ₂₃
17				C ₁₁ H ₂₃
18				C ₁₁ H ₂₃
19				C ₁₁ H ₂₃
20				C ₁₁ H ₂₃

Table 1. continued



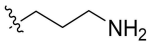

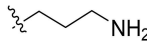
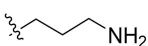

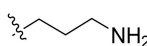
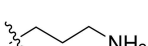

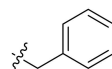
Compd	R ₁	R ₂	R ₃	C _x
21				C ₁₅ H ₃₁
22				C ₁₅ H ₃₁
23				C ₁₁ H ₂₃

Table 2. Antibiotic activity and selectivity of linear small molecule compounds. (NT = not tested).

Compd	MIC [$\mu\text{g mL}^{-1}$]			Hemolysis HC ₅₀ [$\mu\text{g mL}^{-1}$]	Selective Index (SI) [HC ₅₀ /MIC MRSA]
	Gram Positive MRSA	MRSE	VREF		
1	>50	>50	>50	NT	NT
2	25	25	>50	125	5
3	3.12	6.25	6.25	62.5	20
4	3.12	6.25	6.25	31.25	10
5	3.12	>50	25	31.25	10
6	6.25	3.12	>50	31.25	5
7	3.12	3.12	>50	45	14.4
8	3.12	3.12	>50	31.25	10
9	6.25	3.15	6.25	125	20
10	1.56	3.12	1.56	62.5	40
11	3.12	>50	6.25	125	40
12	25	>50	>50	125	5
13	12.5	12.5	25	125	10
14	25	25	>50	125	5
15	>50	>50	>50	NT	NT
16	>50	>50	>50	NT	NT
17	6.25	6.25	>50	62	10
18	6.25	3.12	>50	45	7
19	12.5	6.25	>50	250	16
20	>50	>50	>50	NT	NT
21	6.25	6.25	>50	31.25	5
22	1.56	1.56	12.5	15	10
23	>50	>50	>50	NT	NT

groups but different C_x groups, **3** and **4** have the best antimicrobial activity among them, suggesting that C₁₂ and C₁₄ are the lipid tails of optimal length for antibacterial activity. It is intuitive that short lipid tails (**1** and **2**) could not penetrate bacterial membranes. As to **5**, we hypothesized that long tails could lead to unwanted interactions such as non-specific hydrophobic aggregation which lead to deteriorated ability to bind to bacterial membranes. Therefore, appropriate length of lipid tail is essential to antimicrobial activity. In addition, Compound **10** exhibited the best overall antimicrobial activity among **3**, **6**, **7**, **8**, **9** and **10**, which have the same R₂, R₃ and C_x groups and but different side chains at R₁ position. It seems that a hydrophobic group at R₁ position boosted antibacterial activity. Similar to **5**, changing the length of C₁₂ lipid tail on **10** to C₁₄ and C₁₆, leading to **11** and **12** which have no activity improvement. It is also noted that in comparison compound **3**

to **13–20**, which have the same R₁, R₃ and C_x groups and different R₂ groups, compound **3** displayed the most potent antibacterial activity. It suggests that 1-chloro-3-isocyanatobenzene group provided optimal functionality. In addition to hydrophobicity, cationic charge is also crucial for antibacterial activity, as seen for compound **23** which is completely inactive.

Subsequently, hemolytic assay was carried out to assess the selectivity of these compounds. As shown in Table 2, compound **10** not only has the best antimicrobial activity but also has the best selectivity (compared to hemolytic activity) over MRSA among this series of small linear compounds.

We next conducted time-kill studies of compound **10** to study its bacteria killing kinetics. As shown in Figure 1, MRSA was treated with compound **10** at three different concentrations: 2×MIC, 4×MIC, and 8×MIC. Treatment **10** at 2×MIC, 4×MIC can control and slow down MRSA growing. Furthermore, at

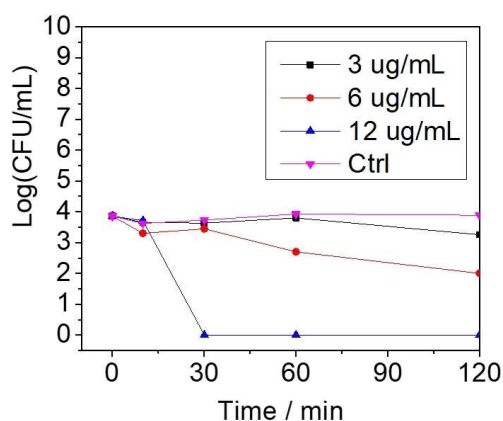


Figure 1. Time-kill plots of compound 10 against MRSA.

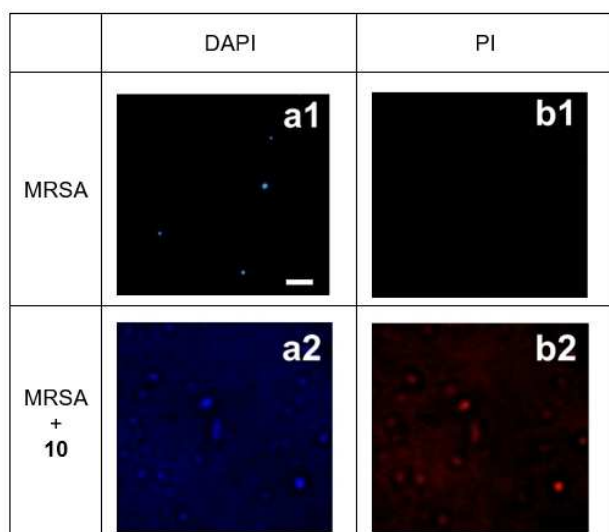


Figure 2. Fluorescence micrographs of MRSA treated and not treated with $2\times$ MIC. (a1) Control, no treatment, DAPI stained; (a2) control, no treatment, PI stained; (b1) MRSA treated with 10, DAPI stained; (b2) MRSA treated with 10, PI stained.

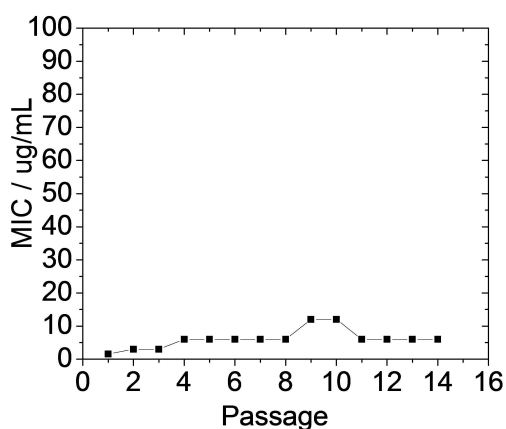


Figure 3. Drug resistance study for compound 10.

$8\times$ MIC, compound 10 can eradicate MRSA completely within 30 min. This indicates that 10 can kill MRSA rapidly.

Their impact on bacterial membranes was also subsequently tested by fluorescent microscopic studies. Compound 10 was tested against MRSA at $2\times$ MIC for 2 h. As shown in Figure 2, in the DAPI channel, MRSA cells emitted blue fluorescence with and without compound 10 treatment. However, MRSA cells only emitted red fluorescence with compound 10 treatment, indicating that their membranes were disrupted therefore stained by PI dye.

Since compounds are designed to disrupt bacteria cell membranes, we hypothesized that 10 can prevent the generation of resistance. Therefore, drug resistance study was conducted. MRSA was incubated with 10 at $1/2\times$ MIC and new MICs were tested continuously for 14 passages. Data from Figure 3 shows that MICs are relatively stable during 14 passages, suggesting that resistance of this series of compounds in bacteria is difficult to form.

In summary, we investigated a new class of small linear molecules as potential antibiotic agents against Gram-positive bacteria. They were structure-based designed with both cationic charged groups and hydrophobic groups. Our studies suggest that these compounds can disrupt bacteria membranes and kill bacteria rapidly. Due to their small molecular weight and facile synthesis approach, they could be potential antibiotic agents. Further characterization of lead compound is currently underway in our lab.

Experimental Section

General information: Rink amide MBHA resins (0.7 mmol/g, 200–400 mesh) were purchased from Chem-Impex Int'l Inc. The solid phase syntheses of all compounds were carried out in a peptide reaction vessel on a Burrell Wrist-Action shaker. Solvents and other chemicals were ordered from either Fisher Scientific or Sigma-Aldrich, and were used without further purification. 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 Labconco lyophilizer.

Synthesis of desired compounds: Synthetic procedure of the compound 10: 200 mg Rink-amide (MBHA) resin (0.14 mmol) was treated with 3 mL 20% piperidine/DMF (v/v) solution for 15 min ($\times 2$) to remove the Fmoc protection group, followed by DMF (2 mL $\times 3$) and DCM (2 mL $\times 3$) wash. The attachment of Fmoc-L-Phenylalanine to the resin was achieved by adding Fmoc-L-Phenylalanine (155 mg, 0.4 mmol), DIC (101 mg, 114 μ L, 0.8 mmol), and HOBT (122 mg, 0.8 mmol) in 3 mL DMF to the reaction vessel, and the reaction was allowed to shake at room temperature for 3 h. The solution was drained, and the beads were washed with DCM (3 mL $\times 3$) and DMF (3 mL $\times 3$). After that, beads were treated with 3 mL 20% piperidine/DMF (v/v) solution for 15 min ($\times 2$) to remove the Fmoc protection group, followed by DMF (2 mL $\times 3$) and DCM (2 mL $\times 3$) wash. Then γ -Lys-BB (238 mg, 0.4 mmol), DIC (101 mg, 114 μ L, 0.8 mmol), and HOBT (122 mg, 0.8 mmol) in 3 mL DMF was added to the reaction vessel, and the reaction was allowed to shake at room temperature for 3 h. The solution was drained, and the beads were washed with DCM (3 mL $\times 3$) and DMF (3 mL $\times 3$). After that, the resin was treated with Pd(PPh₃)₄ (24 mg, 0.02 mmol) and Me₂NH.BH₃ (70 mg, 1.2 mmol) in 3 mL DCM for 10 min ($\times 2$) to

remove the Alloc protecting group, then washed with DCM (3 mL × 3) and DMF (3 mL × 3). Next, 3-chlorophenyl isocyanate (77 mg, 61 μL, 0.5 mmol) and DIPEA (65 mg, 87 μL, 0.5 mmol) in 3 mL DCM were added to the resin and allowed to react for 30 min at room temperature, and then the solution was drained. After DMF (2 mL × 3) and DCM (2 mL × 3) wash, beads were treated with 3 mL 20% piperidine/DMF (v/v) solution for 15 min (× 2) to remove the Fmoc protection group, followed by wash with DMF (2 mL × 3) and DCM (2 mL × 3). Subsequently, lauric acid (80 mg, 0.4 mmol), DIC (101 mg, 114 μL, 0.8 mmol), and HOBt (122 mg, 0.8 mmol) in 3 mL DMF were added to the reaction vessel and reacted for 3 h. After the solution was drained, the beads were washed with DMF (2 mL × 3) and DCM (2 mL × 3), followed by the incubation with 4 mL cocktail of 1:1 TFA:DCM 1:1 (v/v) for 2 h to achieve cleavage and global deprotection of the compound. After the solvent was removed in vacuo, the residue was analyzed and purified on the Waters HPLC system, and the desired fraction was lyophilized to give the pure product **10**. Synthesis of other compounds: The other compounds were synthesized following the similar procedure of compound **10**.

Minimum inhibitory concentrations (MICs) assays: all compounds were tested against three different bacteria strains: Methicillin-resistant *S. aureus* (MRSA, ATCC 33591), Methicillin-resistant *S. epidermidis* (MRSE, RP62A), vancomycin-resistant *Enterococcus faecalis* (ATCC 700802). One colony of each bacteria was incubated in 4 mL TSB buffer overnight at 37 °C, then diluted 100 times and incubated for another 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 (1×10^6 CFU/mL) was added to each well. After 20 hours incubation at 37 °C, absorption at 600 nm wavelength was read on a Biotek Synergy HT microtiter plate reader. Minimum inhibitory concentrations were determined as the lowest concentrations that inhibit bacteria growth completely.

Time kill assays: bacteria MRSA suspensions were incubated at 37 °C to mid-logarithmic phase and diluted to 1×10^6 CFU/mL, then mixed with compound **10** (12.5, 6.25, 3.125 μg mL⁻¹). The mixtures were incubated at 37 °C for 10 min, 30 min, 1 h and 2 h respectively, then diluted by 102 to 104 folds and 100 μL was spread on TSB agar plates. Numbers of bacteria colonies were counted after 20 hours incubation at 37 °C.

Drug resistance assays: after MICs assay against MRSA, bacteria from the well which contained 1/2 MIC were diluted to 1×10^6 CFU/mL for next MIC measurement. The measurement was repeat for 14 passages.

Hemolytic assays: fresh red blood cells (RBCs) was washed with 1 × PBS buffer and centrifuged 10 min at 3500 rpm less than 3 times until the supernatant was clear, then RBCs in the bottom layer was diluted into 5% v/v suspension in 1 × PBS. 50 μL of compounds were 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 410 nm and 540 nm. The hemolysis activity was calculated by the formula: % hemolysis = $(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{PBS}}) / (\text{Abs}_{\text{Triton}} - \text{Abs}_{\text{PBS}}) \times 100$. 1% and 5%

Triton X-100 were used as positive control and 1 × PBS buffer was used as negative control.

Fluorescence microscopy: both propidium iodide (PI) and 4', 6'-diamidino-2-phenylindole dihydrochloride (DAPI) fluorescent dyes were used in the study. Bacteria MRSA suspensions were incubated at 37 °C to mid-logarithmic phase and diluted 100 folds, then incubated with compound **10** 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⁻¹) for 15 min on ice in the dark, then washed 2 times with PBS. Then DAPI (10 μg mL⁻¹) 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.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: peptides · antibiotics · small molecule · drug resistance · MRSA

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