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## Lipidated cyclic $\gamma$ -AApeptides display both antimicrobial and anti-inflammatory activity

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### Abstract

Antimicrobial peptides (AMPs) are host-defense agents capable of both bacterial membrane disruption and immunomodulation. However, the development of natural AMPs as potential therapeutics is hampered by their moderate activity and susceptibility to protease degradation. Herein we report lipidated cyclic  $\gamma$ -AApeptides that have potent antibacterial activity against clinically relevant Gram-positive and Gram-negative bacteria, many of which are resistant to conventional antibiotics. We show that lipidated cyclic  $\gamma$ -AApeptides mimic the bactericidal mechanism of AMPs by disrupting bacterial membranes. Interestingly, they also harness the immune response and inhibit lipopolysaccharide (LPS) activated Toll-Like Receptor 4 (TLR4) signaling, suggesting that lipidated cyclic  $\gamma$ -AApeptides have dual roles as novel antimicrobial and anti-inflammatory agents.

### Keywords

$\gamma$ -AApeptides; peptidomimetics; antimicrobial activity; anti-inflammatory activity

### INTRODUCTION

Rapidly emerging resistance of antibiotics is a major public health concern.<sup>1</sup> As such, natural antibiotics including antimicrobial peptides have captured researchers' attention. Antimicrobial peptides (AMPs) are short cationic amphiphilic peptides present in almost every living organism.<sup>2</sup> Due to their broad-spectrum antimicrobial activity, AMPs are a promising new class of drug candidates.<sup>2</sup> AMPs are effective due to their ability to form a globally amphipathic structure with segregated hydrophobic and cationic regions, capable of disrupting bacterial membranes.<sup>2,3</sup> Such disruption is rooted in the physical properties of bacterial membranes, making the development of resistance difficult.<sup>4</sup> Additionally, AMPs are known as host defense peptides (HDPs) for their role in modulating host innate immunity and diminishing septic responses after bacterial infection.<sup>2,5</sup> The immunomodulatory activities of AMPs are speculated to be a crucial contributor to host

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Supporting Information

Supporting Information includes Figures S1–S3, Tables S1, experimental methods, HPLC traces and NMR data. This material is available free of charge via the Internet at <http://pubs.acs.org>

defense, since the direct antimicrobial activity of AMPs is often weak under physiological conditions.<sup>2,6,7</sup> One of the most important immune responses against bacterial infection is lipopolysaccharide (LPS)-activated Toll-like receptor 4 (TLR4) signaling. LPS is a characteristic component of Gram-negative bacterial cell walls that activates the immune response through TLR4. As such, AMPs may have dual roles as both antibiotic and anti-inflammation agents in the treatment of pathogen invasions. As host inflammatory responses to bacterial infection can lead to deadly septic shock, the current treatment for severe infections involves the administration of both anti-bacterial and anti-inflammatory drugs simultaneously. Therefore, the dual functional roles of AMPs have potential as new generation of anti-infective therapeutics.

Nonetheless, AMPs are susceptible to proteolytic cleavage,<sup>8</sup> hampering their development into novel therapeutics. Peptidomimetics as an alternative approach may circumvent these problems. This is because peptidomimetics, with modified peptide backbones, are more stable against protease degradation. Examples of successful antimicrobial peptidomimetics include  $\beta$ -peptides,<sup>9–11</sup> peptoids,<sup>12,13</sup> and others.<sup>4,8,14–24</sup> Similar to AMPs, these peptidomimetics are designed to form globally amphipathic structures upon interaction with lipid bilayers, disrupting bacterial membranes. Although many peptidomimetics have broad-spectrum antibacterial activity, the reports of immunomodulatory responses are very rare.<sup>8,24</sup> Tew et. al. identified that AMP mimics antagonize lipoteichoic acid (LTA)-activated Toll-like receptor 2 (TLR2) signaling.<sup>24</sup> Here we present a new class of immunomodulatory antimicrobial peptidomimetics, which inhibit TLR signaling without ligand interaction.

We recently developed a new class of peptidomimetics termed “ $\gamma$ -AApeptides” based on a chiral peptide nucleic acid (PNA) backbone.<sup>25,26</sup> Due to the versatility of  $\gamma$ -AApeptides, we were able to synthesize potent antimicrobials with broad-spectrum activities against both Gram-positive and Gram-negative bacteria.<sup>19,22,23,26</sup> The design of antimicrobial  $\gamma$ -AApeptides is straightforward, which is achieved through joining of amphipathic  $\gamma$ -AApeptide building blocks. Herein, we report the design and synthesis of a new generation: lipidated cyclic  $\gamma$ -AApeptides. We show that this class of compounds has potent and broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria, including community-acquired multidrug-resistant pathogens. Interestingly, several lipidated cyclic  $\gamma$ -AApeptides inhibit Toll-Like Receptor 4 (TLR4) signaling and block production of the proinflammatory cytokine tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), implying the potential for a new generation of antibiotic agents with dual functionality.

## RESULTS AND DISCUSSION

We have shown previously that antimicrobial AApeptides with potent and broad-spectrum activity can be designed and prepared easily by joining amphipathic AApeptide building blocks together.<sup>19,22,23</sup> This is because AApeptides formed by this approach are capable of adopting globally amphipathic structures, which are crucial for the disruption of bacterial membranes.<sup>19,22,23</sup> Moreover, cyclization<sup>22</sup> or lipidation<sup>23</sup> can increase their potency. Cyclization reduces structure motility and facilitates bacterial membrane disruption;<sup>22</sup> while lipidation encourages antimicrobial agent interactions with membranes.<sup>23</sup> We therefore speculated that  $\gamma$ -AApeptides that are both cyclized and lipidated would be more potent in killing bacterial pathogens. In fact, lipidated cyclic peptides polymyxin<sup>27</sup> and daptomycin<sup>28</sup> have been used as “last-resort” antibiotics to treat infections caused by Gram-negative and Gram-positive bacteria, respectively. However, the exploration of antimicrobial peptidomimetics with lipidated cyclic structural motifs is scarce. To test our hypothesis, a series of lipidated cyclic  $\gamma$ -AApeptides (Figure 1) containing different numbers of amphiphilic  $\gamma$ -AApeptide building blocks were designed and synthesized on the solid phase.

Their antibacterial activity was then measured against a range of multi-drug resistant Gram-negative and Gram-positive bacteria (Table 1).

We initially designed and synthesized lipidated cyclic  $\gamma$ -AApeptides (**HW-B-73**, **-77** and **-78**, Figure 1) in which the lipid tail C16 is directly connected to the ring structure. Unfortunately, these sequences only possess weak activity against bacteria. We hypothesize that this is because the lipid tail on the ring structure has limited orientation, therefore cannot position itself for membrane insertion even after the amphipathic ring contacts the bacterial membranes. Indeed, a trend was apparent in other membrane-active lipidated cyclic peptide antibiotics, including daptomycin and polymyxin B, which contain at least two amino acid residues connecting both lipid tails and ring structures.<sup>27,28</sup> To test our hypothesis, we moved the lipid tail outside the cyclic ring, leading to the sequence **YL-1**. As expected, **YL-1** showed strong activity against Gram-positive bacteria, although it only contains three amphiphilic building blocks. To determine how ring size affects antibacterial activity, we then included more amphiphilic building blocks into the ring structure. However, an additional amphiphilic building block (**YL-4**) did not boost activity. A similar phenomenon has been observed in cyclic peptoids<sup>12</sup> and other cyclic peptide antimicrobial agents,<sup>30,31</sup> which normally contain 6–8 residues in their ring structures. In fact, **YL-4** has weaker antimicrobial activity than **YL-1**, which may be due to the increased flexibility of the larger ring. This trend is also seen in **HW-B-73**, **HW-B-77**, and **HW-B-78**.

We have previously observed that the exact amphiphilic structures can affect the antimicrobial activity of a sequence.<sup>20</sup> The reversal of cationic and hydrophobic groups results in antimicrobial agents with different potency.<sup>20</sup> It appears that while the overall amphipathicity dominates antimicrobial activity, the precise distribution of functional groups in molecules will affect the strength of peptidomimetic interaction with bacterial membranes. As such, **YL-12**, containing one reversed amphiphilic building block in relation to **YL-1**, was prepared. This sequence has improved antimicrobial activity against both Gram-negative and Gram-positive bacteria. The reversal of additional building blocks leads to the most potent lipidated cyclic  $\gamma$ -AApeptides **YL-34** and **YL-36**. Particularly, **YL-36**, with a small amphipathic ring and a C16 lipid tail, shows very potent activity against all tested drug-resistant Gram-positive and Gram-negative strains. It not only has much improved antimicrobial activity over the AMP Pexiganan, but is also superior when compared with the previously reported cyclic  $\gamma$ -AApeptide **HW-B-13** of much larger ring size, especially against Gram-negative pathogens.

We hypothesize that lipidated cyclic  $\gamma$ -AApeptides are able to kill bacteria through membrane disruption analogous to AMPs, as they possess globally cationic amphipathic structures and broad-spectrum activity against both Gram-positive and Gram-negative bacteria. As such, a fluorescence microscopy experiment was carried out to study the ability of the most potent lead **YL-36** to affect the membranes of *S. aureus* (Gram-positive) and *E. coli* (Gram-negative). Briefly, both bacteria were stained with the membrane permeable dye 4',6-diamidino-2-phenylindole (DAPI) and the non-permeable dye propidium iodide (PI) in the absence or presence of **YL-36** (Figure 2). **YL-36** treatment resulted in PI becoming visible using fluorescence microscopy, suggesting bacterial membranes of both *S. aureus* and *E. coli* were damaged. Aggregation of *S. aureus* after treatment with **YL-36** is observed, which is generally believed to arise from the loss of membrane potential after the disruption of membranes.<sup>19–23,26</sup>

In addition to bacterial membrane disruption, some AMPs are speculated to modulate the immune system through TLR signaling.<sup>32</sup> Since lipidated cyclic  $\gamma$ -AApeptides were designed to mimic AMPs, we anticipated that they may be also capable of harnessing immune responses. As such, **YL-1**, **YL-12**, **YL-29**, **YL-34**, and **YL-36** were also assessed

for the ability to modulate the LPS-induced TLR4 signaling response by measuring nitric oxide production (Figure 3a).<sup>33</sup> Nitric oxide is produced as a downstream inflammatory factor in all TLR signaling, and is part of the global inflammatory response. Figure 3a shows that lipidated cyclic  $\gamma$ -AApeptides are a potent class of anti-TLR4 signaling agents, capable of reducing nitric oxide production. To further investigate the anti-inflammatory capabilities of lipidated cyclic  $\gamma$ -AApeptides, we chose the most potent antimicrobial agent **YL-36** for further investigation. The  $IC_{50}$  of **YL-36** was obtained by treating RAW 264.7 cells with varying concentrations of **YL-36** in concert with 20 ng/mL LPS. The resulting inflammatory response was monitored through measurement of nitric oxide production.

**YL-36** shows an inhibitory effect of the nitric oxide signaling with an  $IC_{50}$  value of 1.98  $\mu$ M (Figure 3b). **HW-C-22-2** and **HW-C-22-4** demonstrate that lipidation increases the effectiveness of  $\gamma$ -AApeptides as TLR signaling antagonists, as neither construct is active under the tested concentrations (Figure 3a and 3b). Nitric oxide is produced by TLR activation, and correlates to the level of inflammatory response. Additionally, **YL-36** shows little cytotoxicity up to 100  $\mu$ M (Figure S3), suggesting the inhibition of nitric oxide is not due to the toxicity of YL-36. As such, we further explored the ability of **YL-36** to inhibit downstream NF- $\kappa$ B activation and the production of pro-inflammatory cytokines. Inhibition of the NF- $\kappa$ B activation (Figure 4a) was determined by a previously developed secreted embryonic alkaline phosphatase (SEAP) assay.<sup>34</sup> Furthermore, the pro-inflammatory cytokine tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is also strongly inhibited ( $IC_{50}$  = 2.05  $\mu$ M) (Figure 4b).<sup>35</sup> Both NF- $\kappa$ B and TNF- $\alpha$  are produced due to TLR activation. Their inhibition by **YL-36** shows that TLR signaling is being inhibited in a dose dependent fashion. Neither **HW-C-22-2** nor **HW-C-22-4** shows inhibitory activities, further demonstrating anti-inflammatory activity depends on the lipidation of  $\gamma$ -AApeptides.

Tew et. al. has previously elegantly identified antimicrobial agents that reduce TLR-induced inflammatory response.<sup>8,24</sup> Interestingly, our lipidated cyclic  $\gamma$ -AApeptides appear to have a different mechanism of inhibition, not interacting with the TLR ligands. If  $\gamma$ -AApeptides interacted with LPS, pre-treatment with **YL-36** prior to LPS addition would abolish its anti-TLR4 activities. Our data though demonstrates that both pre-treatment and co-treatment with  $\gamma$ -AApeptides and LPS results in complete antagonism of nitric oxide production (Figure 5), indicating that  $\gamma$ -AApeptides do not bind to LPS directly.

## CONCLUSION

As a new class of antimicrobial peptidomimetics that mimic AMPs, lipidated cyclic  $\gamma$ -AApeptides exhibit potent and broad-spectrum activity against a range of multi-drug resistant Gram-negative and Gram-positive bacteria. Maybe even more importantly, lipidated cyclic  $\gamma$ -AApeptides also imitate AMPs in their immunomodulatory capabilities. These  $\gamma$ -AApeptides have been shown to antagonize the LPS activated NF- $\kappa$ B signaling response, and to potently suppress release of harmful pro-inflammatory cytokines including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). Additionally, lipidated cyclic  $\gamma$ -AApeptides do not bind to TLR ligands, suggesting a novel mechanism for immunomodulation. These peptidomimetics provide an exciting new approach to treat bacterial infections by exerting dual-functional roles: they are a new generation of antibiotic agents that kill both multi-drug resistant Gram-positive and Gram-negative bacteria directly, as well as anti-inflammatory agents through harnessing immune responses. Furthermore, as deregulation of TNF- $\alpha$  is also related to diseases such as cancer and lupus erythematosus,<sup>36,37</sup> lipidated cyclic  $\gamma$ -AApeptides may also provide other therapeutic applications in the future.

## EXPERIMENTAL SECTION

### General experimental methods

Rink amide MBHA resins (200–400 mesh, 0.7 mmol/g) were purchased from Chem-Impex Int'l Inc. Other chemicals were ordered from either Sigma-Aldrich or Fisher Scientific, and used without further purification.  $^1\text{H}$  NMR spectra of the building blocks were obtained on an Agilent DD800 instrument. The solid phase syntheses of the lipidated cyclic  $\gamma$ -AApeptides were carried out in a peptide reaction vessel on a Burrell Wrist-Action shaker, were analyzed and purified using an analytical and preparative Waters HPLC system, respectively. The final products were dried in a Labcono lyophilizer. Molar masses were identified using a Bruker AutoFlex MALDI-TOF mass spectrometer.

### Solid phase synthesis of lipidated cyclic $\gamma$ -AA peptides

The syntheses of lipidated cyclic  $\gamma$ -AApeptides were carried out on the solid phase as reported previously.<sup>19,22</sup> For each coupling cycle, 20% Piperidine in DMF was used to remove the Fmoc protecting group, followed by the coupling of 1.5 equiv of building blocks with 4 equiv of HOBt (1-hydroxybenzotriazole monohydrate)/DIC (diisopropylcarbodiimide) in DMF for 6 h. The allyl group was removed by 0.2 equiv of  $\text{Pd}(\text{PPh}_3)_4$  in the presence of 10 equiv of  $\text{PhSiH}_3/\text{CH}_2\text{Cl}_2$  (2 h for each, repeated twice). The exposed carboxyl group reacted with the N-terminus of the sequence to complete the cyclization using PyBOP/HOBt/DIPEA/DMF. The lipidated  $\gamma$ -peptides was cleaved from solid support in 50:48:2 TFA/ $\text{CH}_2\text{Cl}_2$ /TIS (triisopropylsilane) for 2 h. The solvent was evaporated and the sequences were analyzed and purified using a Waters HPLC system monitored at 215 nm. The desired fractions were collected and lyophilized, and their molecular weights were confirmed by the Bruker AutoFlex MALDITOF mass spectrometer.

### Antimicrobial assays.<sup>19,22</sup>

The lipidated cyclic  $\gamma$ -AApeptides were tested for their antimicrobial activity against various microbial organisms including *E. coli* (ATCC 25922), *K. pneumonia* (ATCC 13383), multi-drug resistant *P. aeruginosa* (ATCC 27853), Methicillin-resistant *S. epidermidis* (MRSE, RP62A), Vancomycin-resistant *E. faecalis* (ATCC 700802), Methicillin-resistant *S. aureus* (ATCC 33592). The highest concentration of the tested AA-peptides was 25  $\mu\text{g}/\text{mL}$ . The bacteria in 5 mL of medium were grown at 37 °C overnight and then diluted to make a suspension of approximate  $1 \times 10^6$  CFU/mL. Aliquots of 50  $\mu\text{L}$  of bacterial suspension were mixed with 50  $\mu\text{L}$  of medium containing different concentrations of lipidated cyclic  $\gamma$ -AA-peptides. The plate was incubated at 37 °C overnight with cell growth monitored by a Biotek Synergy HT microtiter plate reader under the 600 nm wavelength. MIC was determined when the lowest concentration of the compounds inhibit the cell growth completely in 24 h. The results were repeated at least three times with duplicates for each time.

### Hemolysis assay.<sup>19,22</sup>

Freshly drawn,  $\text{K}_2$  EDTA treated human red blood cells (hRBCs) were washed with PBS buffer twice and centrifuged at 1000g for 10 min. After the clear supernatant was removed, the cell pellets were mixed with serial diluted lipo-cyclic  $\gamma$ -AApeptides in a 96-well plate. The plate was incubated at 37 °C for 1 h and centrifuged at 3500 rpm for 10 min. The supernatant was separated and diluted in PBS, and the absorbance was detected at 360 nm using a Biotek Synergy TH plate reader. % hemolysis =  $(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{PBS}})/(\text{Abs}_{\text{Triton}} - \text{Abs}_{\text{PBS}}) \times 100\%$ . 0% hemolysis (negative control) was determined by mixing blood with PBS and 100% hemolysis (positive control) was determined by mixing blood with Triton



X-100 (final concentration 0.1%). The results were repeated at least three times with duplicates for each time.

### Fluorescence microscopy.<sup>19,22</sup>

DAPI (4', 6-Diamidino-2-phenylindole dihydrochloride, Sigma, >98%) and PI (Propidium iodide, Sigma) were used to stain the bacteria cells of *E. coli* or *S. aureus*. DAPI is a DNA binding dye staining all bacterial cells regardless of their viabilities, and PI is an ethidium derivative which only can pass through damaged bacterial membranes and intercalates with their nucleic acids. Briefly, bacteria in mid-logarithmic phase were incubated with lipidated cyclic  $\gamma$ -AA peptides ( $2 \times \text{MIC}$ ) for 2 h, and then were centrifuged at 3000g for 15 min. The bacteria cell pellets were separated then incubated with PI, followed by washing and incubation with DAPI (each dye incubation was performed at 0 °C for 15 minutes in dark). Controls were bacteria culture without peptides following the same procedure described above. The stained bacteria cells were observed under Zeiss Axio Imager Zloptical microscope using the 100X oil-immersion objective.

### Fluorescent Detection of Nitric Oxide.<sup>34,35</sup>

Raw 264.7 (Mouse leukaemic monocyte macrophage cell line) cells were grown in RPMI 1640 medium containing 1% L-glutamine, 1% Penicillin/streptomycin and 10% fetal bovine serum (FBS). Cells were plated in a 96-well plate at 75,000 cells/well in complete RPMI 1640 medium, and allowed to grow overnight at 37°C and 5% CO<sub>2</sub> in a humidified incubator. The media was removed, and cells were placed in unsupplemented RPMI 1640 medium. 20 ng/mL LPS and the appropriate concentration of lipidated cyclic  $\gamma$ -AApeptides (20 mM stock solutions in PBS) were added to a final volume of 200  $\mu$ L. PBS controls were included in each experiment. Plates were then incubated for 24 h, and then 100  $\mu$ L of media was transferred to a flat black 96-well microfluor plate (Thermo Scientific, MA, USA). Following that, 10  $\mu$ L of 0.05 mg/mL 2,3-diaminonamthalene in 0.62 M HCl was added to the media and incubated for 20 minutes in the dark. The reaction was quenched with 5  $\mu$ L of 3.0 M NaOH, and the plate was read on a Beckman Coulter DTX880 plate reader (Beckman Coulter, CA, USA). Data was collected with excitation at 360 nm and emission at 430 nm. Data was normalized with the ligand only control as 100% activation, and the untreated cells as 0% activation. Fold inhibition = [(Sample<sub>430 nm</sub> - Untreated cells<sub>430 nm</sub>)/(Ligand Control<sub>430 nm</sub> - Untreated cells<sub>430 nm</sub>)]. The EC<sub>50</sub> values were calculated graphically using OriginPro v8.6 software.

### Secreted Embryonic Alkaline Phosphatase (SEAP) Reporter of NF- $\kappa$ B Transcription.<sup>34,35</sup>

HEK293 (Human Embryonic Kidney 293) cells were grown in Dulbecco's Modified Eagle Medium (DMEM) in the presence of 1% Penicillin/streptomycin, 10% fetal bovine serum (FBS), and 1% L-glutamine. HEK293 cells are stably transfected with human TLR4, as well as the required accessory proteins MD-2 and CD14. Moreover, the cells also possess an optimized alkaline phosphatase reporter gene under the control of a NF- $\kappa$ B inducible promoter.<sup>38</sup> Cells were first plated in a 96-well plate at 40,000 cells/well in complete DMEM medium and allow to grow overnight at 37°C and 5% CO<sub>2</sub> in a humidified incubator. Then the media was removed, and cells were placed in Optimem + 0.5% FBS medium. 20 ng/mL LPS and the appropriate concentration of lipidated cyclic  $\gamma$ -AApeptides were added to a final volume of 200  $\mu$ L. PBS buffer was included as control. Plates were then incubated for 24 h, and then medium was assayed per the instructions of the Phospha-Light™ SEAP Reporter Gene Assay System (Applied Biosystems, NY, USA). The plate was read on a Beckman Coulter DTX880 plate reader (Beckman Coulter, CA, USA). Data was collected with luminescence at 430 nm. Data was normalized with the ligand only control as 100% activation, and the untreated cells as 0% activation. Fold inhibition = [(Sample<sub>430 nm</sub>

– Untreated cells<sub>430 nm</sub>)/(Ligand Control<sub>430 nm</sub> – Untreated cells<sub>430 nm</sub>)]. The EC<sub>50</sub> values were calculated graphically using OriginPro v8.6 software.

### Enzyme-Linked Immunosorbent Assay (ELISA) Detection of TNF- $\alpha$ .<sup>34,35</sup>

Raw 264.7 (Mouse leukaemic monocyte macrophage cell line) cells were grown in RPMI 1640 medium with 10% fetal bovine serum (FBS), 1% Penicillin/streptomycin, and 1% L-glutamine. Cells were plated in a 96-well plate at 75,000 cells/well in complete RPMI 1640 medium and allowed to grow overnight at 37°C and 5% CO<sub>2</sub> in a humidified incubator. Then media was removed, and cells were placed in unsupplemented RPMI 1640 medium. 20 ng/mL LPS and the appropriate concentration of lipidated cyclic  $\gamma$ -AApeptides (20 mM stock solutions) were added to a final volume of 200  $\mu$ L. PBS was included as the control. Plates were then incubated for 24 hours, and then samples were assayed for TNF- $\alpha$  per the method outlined in the BD Biosciences Mouse TNF (Mono/Mono) ELISA Set (BD Biosciences, CA, USA). The plate was read on a Beckman Coulter DTX880 plate reader (Beckman Coulter, CA, USA). Data was collected with absorbance at 450 nm. Data was normalized with the ligand only control as 100% activation, and the untreated cells as 0% activation. Fold inhibition = [(Sample<sub>450 nm</sub> – Untreated cells<sub>450 nm</sub>)/(Ligand Control<sub>450 nm</sub> – Untreated cells<sub>450 nm</sub>)]. The EC<sub>50</sub> values were calculated graphically using OriginPro v8.6 software.

### Crystal Violet Toxicity Assay.<sup>34,35</sup>

Cells which were treated with compound for nitric oxide experimentation were also tested for compound toxicity using crystal violet stain. Cells were fixed for 20 minutes in 4% paraformaldehyde after the media was removed. After fixing, formaldehyde was removed and cells were incubated for 1h with 0.05% crystal violet stain. After incubation, cells were rinsed with deionized water and reconstituted in 100% methanol for 10 minutes. The plate was read on a Beckman Coulter DTX880 plate reader (Beckman Coulter, CA, USA). Data was collected with absorbance at 535 nm. Data was normalized with the untreated cells control as 100% survival, and the blank wells as 0% survival. Fold inhibition = [(Sample<sub>535 nm</sub> – Blank<sub>535 nm</sub>)/(Untreated cells<sub>535 nm</sub> – Blank<sub>535 nm</sub>)].

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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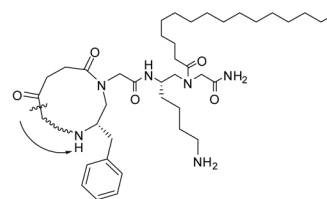
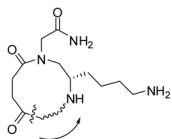
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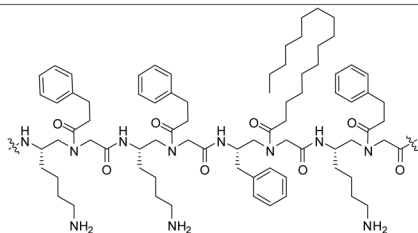


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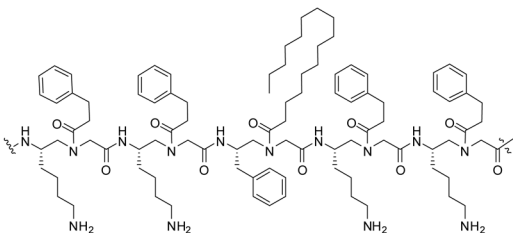


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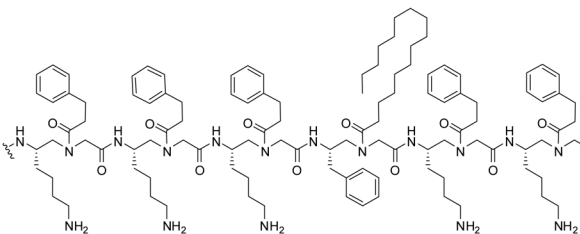
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**HW-B-77**

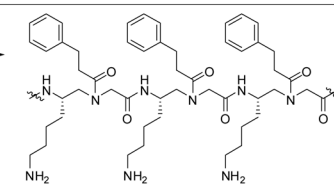


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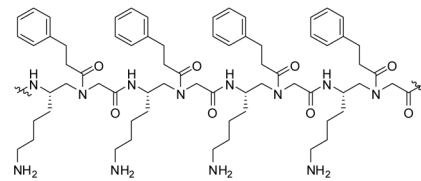


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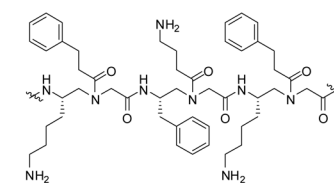
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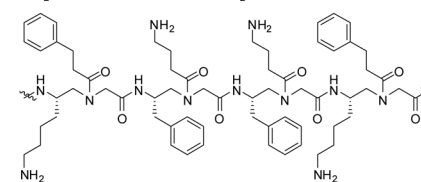
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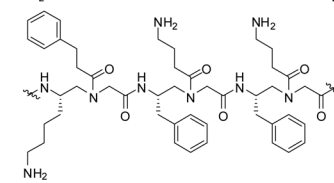
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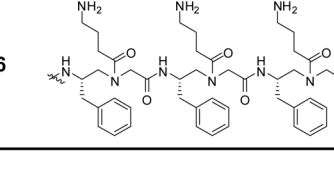
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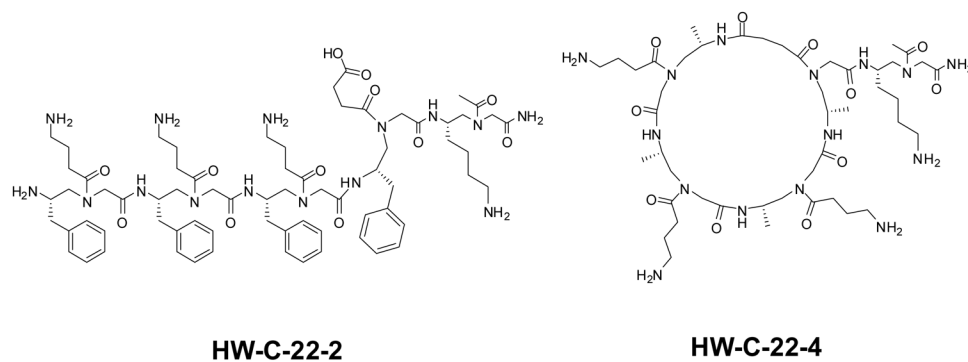


**YL-34**

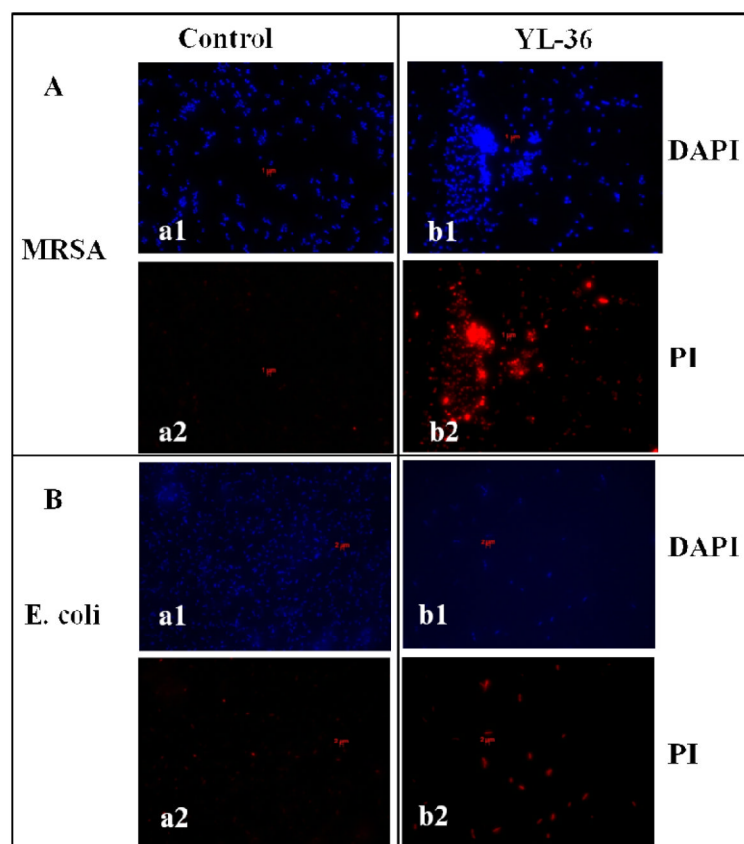


**YL-36**

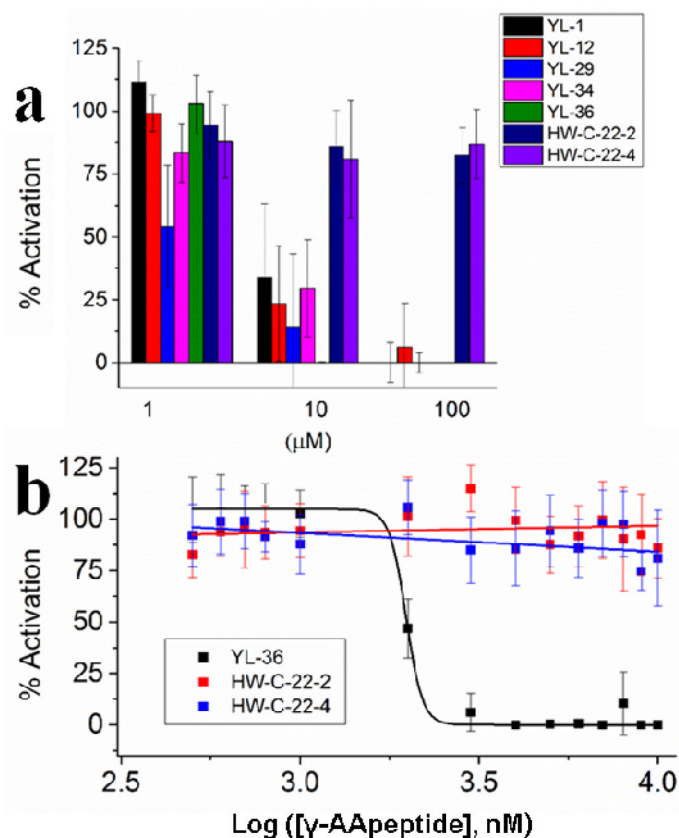




**Figure 1.** The structures of lipitated cyclic  $\gamma$ -AApeptides. Two  $\gamma$ -AApeptides, **HW-C-22-2** and **HW-C-22-4**, which is either linear or cyclic and do not contain lipid tails, were used for comparison. Compounds are named according to their notebook codes.

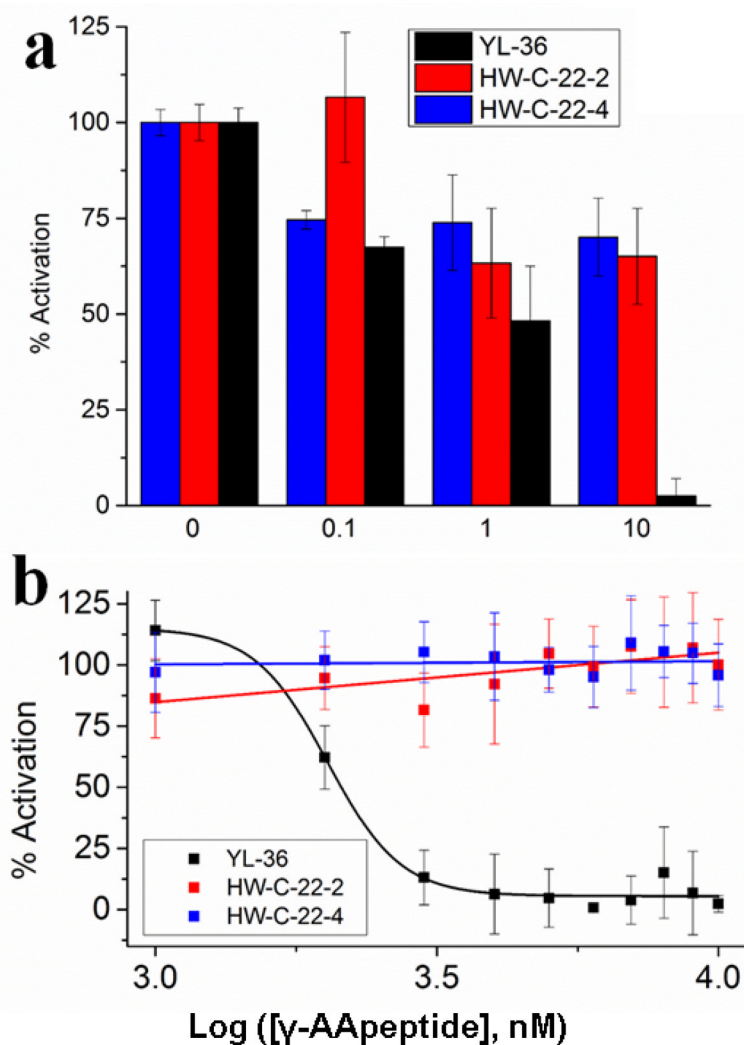


**Figure 2.** Fluorescence micrographs of *S. aureus* (A) and *E. coli* (B) that are treated/(or no treatment) with 5  $\mu\text{g}/\text{mL}$  lipidated cyclic  $\gamma$ -AApeptide **YL-36** for 2 h. **a1**, control, no treatment, DAPI stained; **a2**, control, no treatment, PI stained; **b1**, **YL-36** treatment, DAPI stained; **b2**, **YL-36** treatment, PI stained.



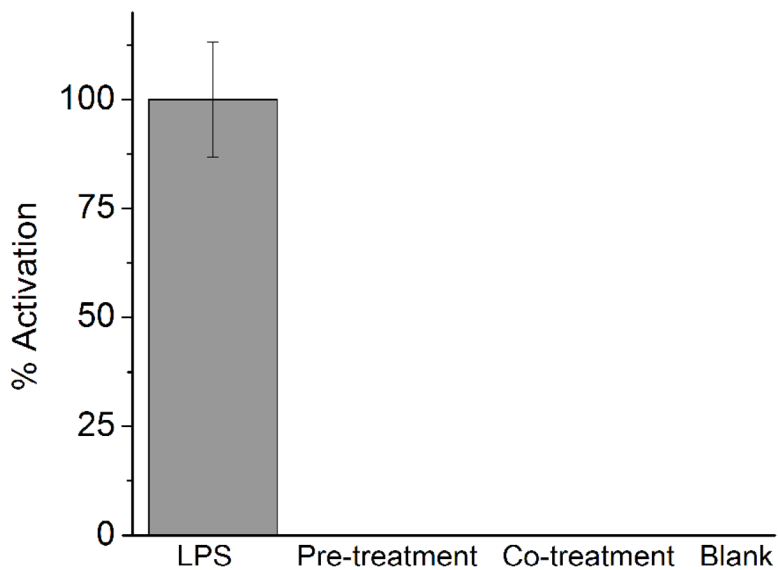
**Figure 3.**  
**a.** Nitric oxide production in the presence of the lipidated cyclic  $\gamma$ -AApeptides **YL-1**, **YL-12**, **YL-29**, and **YL-36**. Two  $\gamma$ -AApeptides lacking alkyl tails, either linear (**HW-C-22-2**) or cyclic (**HW-C-22-4**), were included to demonstrate the importance of  $\gamma$ -AApeptide lipidation on their TLR activities. RAW 264.7 cells/well were treated with 20 ng/mL LPS and varying concentrations of lipidated cyclic  $\gamma$ -AApeptides in a 96-well plate. Data is normalized to 20 ng/mL LPS as 100% activation, and untreated cells as 0% activation. **b.** Dose-dependent inhibition of nitric oxide production by **YL-36**. This experiment was performed as described in 3a, and a dose response curve was obtained for calculation of an  $IC_{50}$  value.





**Figure 4.**

**a.** NF- $\kappa$ B activation in HEK 293 cells in the presence of various  $\gamma$ -AApeptides. HEK 293 cells were stably transfected with TLR4 and its accessory proteins, as well as a secreted embryonic alkaline phosphatase (SEAP) reporter gene. Cells were plated in 96-well plate with 40,000 cells/well and treated with 20 ng/mL LPS and **YL-36**. Data is normalized to 20 ng/mL LPS as 100% activation, and untreated cells as 0% activation. Increasing concentrations of **YL-36** decrease transcription of SEAP in a dose-dependent fashion. **HW-C-22-2** and **HW-C-22-4** were included for comparison. **b.** **YL-36** reduces production of TNF- $\alpha$ . TNF- $\alpha$  production was measured with a monoclonal mouse ELISA. Data is normalized to 20 ng/mL LPS as 100% activation, and untreated cells as 0% activation. Increasing concentrations of **YL-36** result in decreased production of TNF- $\alpha$ . **HW-C-22-2** and **HW-C-22-4** were included for comparison.



**Figure 5.** **YL-36** inhibits TLR4 activation independent of LPS binding. Raw 264.7 cells were incubated for 30 minutes with 10  $\mu$ M **YL-36**. After incubation, pre-treatment cells were washed with medium and activated with 20 ng/mL LPS. Co-treatment cells were not washed, and were treated with 20 ng/mL LPS while **YL-36** remained in the medium. Antagonism for both cell treatments is 100%, suggesting that **YL-36** does not bind to the ligand as a mode of inhibition. Data is normalized to 20 ng/mL LPS as 100% activation, and untreated cells as 0% activation.

Table 1

The antimicrobial and hemolytic activities of lipidated cyclic  $\gamma$ -AApeptides. The microbial organisms used are *E. coli* (ATCC 25922), *K. pneumoniae* (ATCC 13383), *P. aeruginosa* (ATCC 27853), Methicillin-resistant *S. epidermidis* (RP62A), Vancomycin-resistant *E. faecalis* (ATCC 700802), and Methicillin-resistant *S. aureus* (ATCC 33591). The minimum inhibitory concentration (MIC) is the lowest concentration that completely inhibits growth after 24 h. HC<sub>50</sub> is the concentration causing 50% hemolysis. Pexiganan<sup>8,29</sup> and previously reported cyclic  $\gamma$ -AApeptide **HW-B-13**<sup>22</sup> are included for comparison. **YL-36**, the compound with the most potent and broad-spectrum activity, is shaded in grey.

Oligomers	MIC, $\mu\text{g/mL}$ ( $\mu\text{M}$ )						Hemolysis $\mu\text{g/mL}$ ( $\mu\text{M}$ )		
	Gram-negative			Gram-positive			HC <sub>50</sub>		
	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. aeruginosa</i>	<i>S. epidermidis</i>	<i>E. faecalis</i>	<i>S. aureus</i>			
<b>HW-B-73</b>	>25 (16)	20 (12)	>25 (16)	20 (12)	>25 (16)	20 (12)	-	-	-
<b>HW-B-77</b>	>25 (13)	20 (10)	>25 (13)	>25 (13)	>25 (13)	>25 (13)	-	-	-
<b>HW-B-78</b>	>25 (11)	20 (9)	>25 (11)	>25 (11)	>25 (11)	>25 (11)	-	-	-
<b>YL-1</b>	>25 (16)	8 (5)	20 (12)	2 (1)	5 (2)	5 (2)	250 (160)		
<b>YL-4</b>	>25 (13)	>25 (13)	>25 (13)	>25 (13)	>25 (13)	>25 (13)	250 (130)		
<b>YL-12</b>	5 (3)	5 (3)	10 (6)	5 (3)	5 (3)	10 (6)	180 (114)		
<b>YL-29</b>	>25 (14)	10 (5)	25 (14)	5 (3)	5 (3)	5 (3)	150 (82)		
<b>YL-34</b>	10 (6)	3 (2)	10 (6)	1 (0.6)	2 (1)	2 (1)	200 (129)		
<b>YL-36</b>	2 (1)	3 (2)	5 (3)	1 (0.6)	3 (2)	2 (1)	100 (65)		
<b>HW-B-13</b>	20 (10)	8 (4)	8 (4)	2 (1)	5 (3)	1 (0.5)	100 (50)		
<b>Pexiganan</b>	8 (3)	8 (3)	16 (6)	8 (3)	32 (12)	16 (6)	120 (45)		