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Improved Antibacterial Activity of the Marine Peptide N6 against Intracellular *Salmonella* Typhimurium by Conjugating with the Cell-penetrating Peptide Tat_{11} via a Cleavable Linker

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ABSTRACT: The poor penetration ability of antimicrobial agents limits their use in the treatment of intracellular bacteria. In this study, the conjugate–CNC (**6**) was generated by connecting the cell-penetrating peptide–Tat_{11} (**1**) and marine peptide–N6 (**2**) via a cathepsin-cleavable linker, and the C-terminal aminated N6 (**7**) and CNC (**8**) were first designed and synthesized to eliminate intracellular *Salmonellae* Typhimurium. The cellular uptake of **6** and stability of **7** were higher than those of **2**, and conjugates–**6**/**8**/**7** had almost no hemolysis and cytotoxicity. The antibacterial activities of **6**/**8**/**7** against *S.* Typhimurium in RAW264.7 cells were increased by 67.2–76.2%, 98.6–98.9% and 96.3–97.6%, respectively. After treatment with 1–2 μmol/kg of **6**/**8**/**7**, the survival of the *S.* Typhimurium–infected mice was 66.7–100%, higher than that of **2** (33.4–66.7%). This result suggested that **6**/**8**/**7** may be excellent candidates for novel antimicrobial agents to treat intracellular pathogens.

Keywords: Cell-penetrating peptides; Tat_{11}; antimicrobial peptide; N6; amination; intracellular activity

INTRODUCTION

The high worldwide incidence of *Salmonella* infection and the rapid emergence of antibiotic-resistant strains highlight the urgent need for new approaches to therapeutic intervention.[1] Another challenge is that *Salmonellae* can invade and survive in a specialized membrane-bound compartment named the *Salmonella*-containing vacuole (SCV) in phagocytic cells, wherein pathogens can adapt to intracellular acidic conditions to evade host immune responses, and commonly used drugs such as aminoglycosides limit access to intracellular targets due to their poor cellular uptake.[2] Phagocytic cells become a reservoir for recurrence and reinfections of *S.* Typhimurium, resulting in a wide range of diseases in humans and animals, including gastroenteritis, bacteremia, enteric fever and focal infections.[3] Until now, there has been no effective way to eradicate intracellular *Salmonellae*.

Due to the advantages of the broad-spectrum activity, rapid-killing effect and low resistance potential, antimicrobial peptides (AMPs) may open a new avenue for the development of antibacterial agents.[4] Our preliminary study has demonstrated that N6 (**2**) exhibited potent antimicrobial activity against Gram-negative bacteria, especially *Salmonellae* and *Escherichia coli*.[5] However, **2** displayed poor ability to traverse eukaryotic cell membranes and kill intracellular *S.* Typhimurium, and it is very susceptible to acidic pH values between pH 2.0 and pH 6.0.[5] Recently, cell-penetrating peptides (CPPs) have been widely used as delivery vectors to deliver traditional antibiotics and drugs into cells to target intracellular pathogens.[6, 7] However, most of the current delivery vectors-CPPs cannot effectively release "cargoes" or lack a target, leading to a partial loss of efficacy. The human immunodeficiency virus type 1 (HIV-1) trans-activator of transcription Tat_{11} (**1**) peptide (residues 47–57), one of the most commonly used CPPs, can deliver various drug molecules into eukaryotic cells, including antibodies, oligonucleotides and peptides;[8, 9] thus, **1** is a promising tool to improve the non-invasive cellular

delivery of drug molecules. Additionally, it has been demonstrated that C-terminal amidation of some AMPs such as aurein and PMAP-23 can enhance their structural stability and antimicrobial activity or decrease cytotoxicity due to an increase in cationic charges and the helical content, facilitating translocation into the bacterial cell inner membrane and reducing interaction with animal cells.[10, 11]

In this study, **2** was first covalently linked to **1** via a cathepsin-cleavable linker (maleimidocaproyl-L-valine-L-citrulline- p-aminobenzylcarbonyl, MC-VC-PABC) containing a novel quaternary ammonium salt (Figure 1A) to improve penetration.[12, 13] The resulting CPP-linker-N6 (CNC, **6**) conjugate may have no distinct antibacterial activity until it enters the cells, in which intracellular proteases cleave the linker and readily release **2** in its active form (Figure 1D). Meanwhile, peptide **2** is amidated at the C-terminus (named as N6NH2, **7**) to enhance its stability within macrophages and its intracellular activity (Figures 1B and 1D). Moreover, **8** (CPP-linker-N6NH2, CNCNH2) was synthesized to combine the advantages of **2** and **7** (Figure 1C). Peptides **6**, **8** and **7** were first evaluated for their antimicrobial activity, cytotoxicity, and pharmacodynamics against *S.* Typhimurium *in vitro*. The potential antibacterial activities of **6**, **8** and **7** were then investigated against intracellular *S.* Typhimurium in RAW264.7 macrophages and mice, and a possible mechanism of the peptide uptake into macrophages were further elucidated.

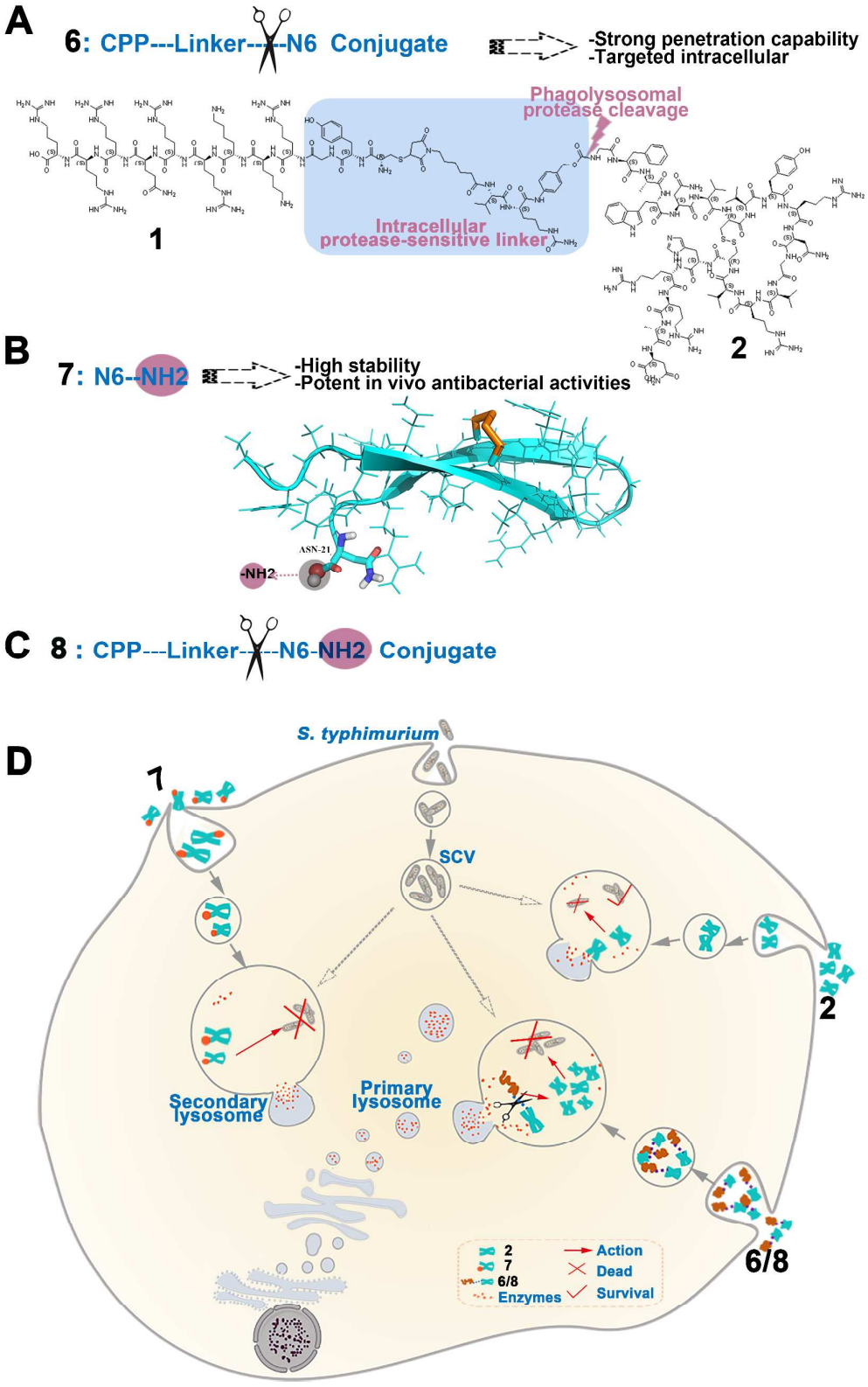


Figure 1. Design scheme of **6**, **7** and **8** and their possible modes of action against intracellular *S.* Typhimurium. (A) Design scheme of **6** by connecting **2** and **1** via a MC-VC-PABC linker. (B) Design

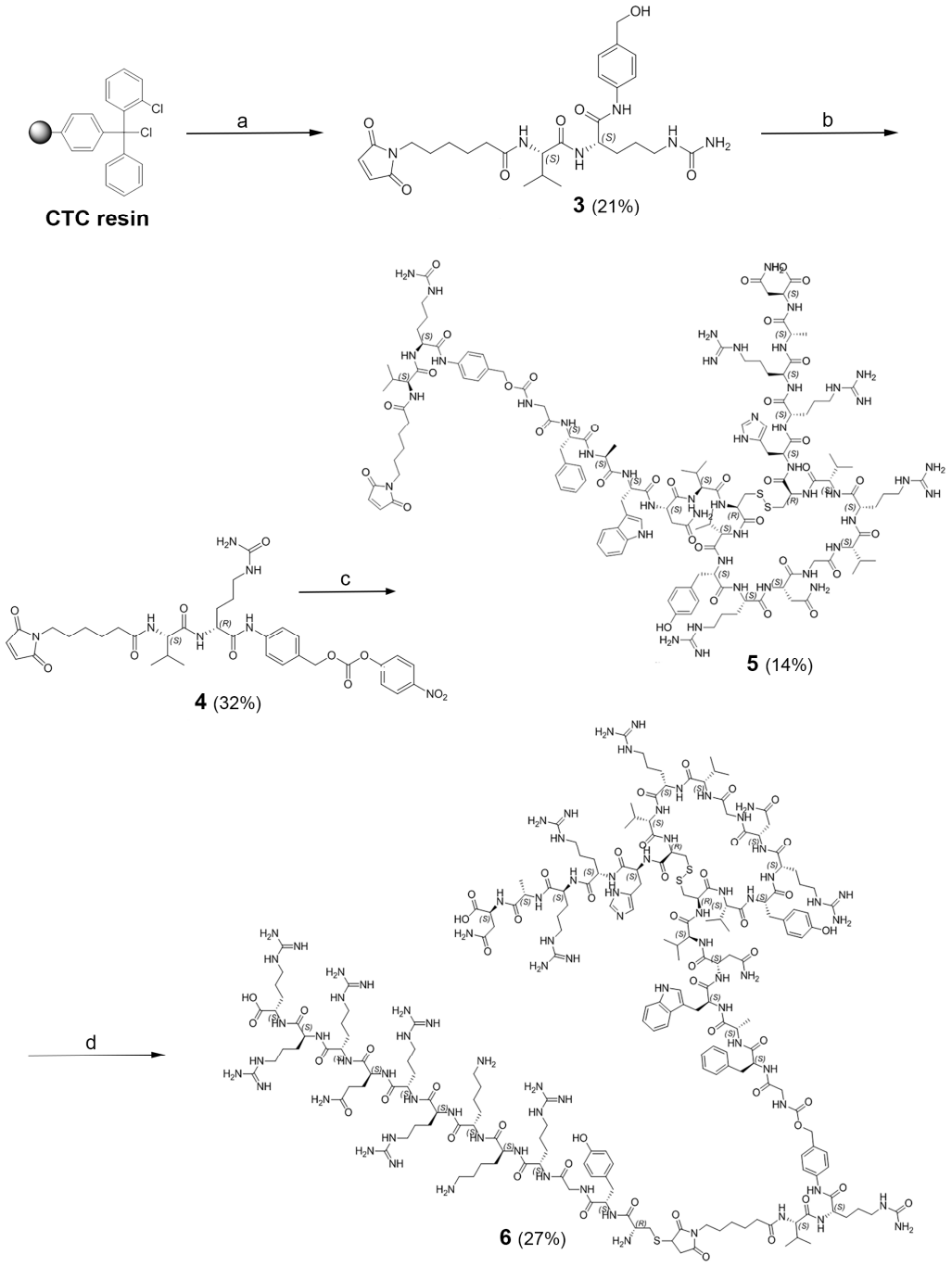
scheme of **7** with C-terminal amination. (C) Design scheme of **8** by connecting **7** and **1** via a MC-VC-PABC linker. (D) Possible modes of action of **6**, **7** and **8** against intracellular *S.* Typhimurium. Bacteria and **2**, **6**, **7** and **8** can enter the cells through phagocytosis and gradually form SCVs and vacuoles.[2] After entering the primary lysosomes, **2** and **7** are released from **6** and **8**, respectively and begin functioning immediately. Due to the resistance to low intracellular pH, **7** can promote antibacterial efficacy.

RESULTS AND DISCUSSION

Synthesis. Compound **2** was conjugated with **1** via a cathepsin-cleavable linker containing a novel quaternary ammonium salt to improve the ability to penetrate cells (Scheme 1). Initially, **1** (Supporting Information, Scheme S1) and **2** were synthesized through solid phase synthesis, and a Cys was connected to the N-terminal of **1** to facilitate the connection with the linker. Compound **3** was further coupled to bis (4-nitrophenyl) carbonate using N,N-diisopropylethylamine (DIEA) to obtain **4**, which was further coupled to **2** using DIEA combined with hydroxybenzotriazole (HOBt) to obtain **5**. A mixture of compound **5**, **1**, and NH_4HCO_3 in 30% ACN/H_2O was degassed and purged with N_2 three times to produce **6**. Compound **8** was comprised **1** and **7** and synthesized by the same way as compound **6**. All intermediate compounds were purified by high-performance liquid chromatography (HPLC) to >91% purity. The charges of **6** (+12), **7** (+5) and **8** (+13) were higher than that of the parent peptide **2** (Table S1). Mass spectrometry (MS) analysis showed that **6**, **7**, **8** and **2** were successfully synthesized as described previously (Supporting Information, Figures S1-S10).[13]

Antimicrobial Activity and Cytotoxicity. To evaluate the antimicrobial activities of **6** and **7**, the minimum inhibitory concentration (MIC) was measured under different conditions. The compounds **1**

Scheme 1. Synthesis of 6[a]



[a]Reagents and conditions: (a) solid phase synthesis; CTC resin, Fmoc-AA-OH, MeOH, HBTU, DIEA, DMF, stir, 16 h; (b) bis (4-nitrophenyl) carbonate, DIEA, DMF, N_2, stir, RT, 2 h; (c) **2**, HOBt, DIEA, DMF, N_2, stir, RT, 1 h; (d) **1**, NH_4HCO_3, ACN/H_2O, N_2, stir, RT, 1 h.

and **6** showed no antibacterial activity at pH 7.2 (MIC >82.1 μM and MIC >27.2 μM, respectively) against extracellular planktonic *S.* Typhimurium ATCC14028 *in vitro*, but **8** showed a low antibacterial activity (MIC=13.6 μM). Similar to our previous result, the MIC of **2** at pH 7.2 (0.81 μM) was lower than that at pH 5.0 (12.96 μM), indicating lower activity under acidic conditions. It may be attributed to the C-terminal free Asn degradation of peptide **2** at acidic pH due to its deamidation and *O*-protonation of the amide carbonyl group, leading to increased electrophilicity of the carbon center and nucleophilic attack by water.[5, 14] This phenomenon also occurs in other peptides such as AF2 and glucagon 22-29 at acidic pH, leading to attenuated activity.[14, 15] After treatment with cathepsin B, the MIC of **6** and **8** was 1.7 μM at pH 7.2, similar to that of **2** and **7** (1.62 μM) (Table 1), indicating that free peptide **2** and **7** is released from the conjugate and can combat bacteria. Additionally, after the enzyme digestion, the MS spectra of **6** was almost the same as that of **2** (Figures S4-S6), and the MS spectra of **8** was almost the same as that of **7** (Figures S7-S10). These data suggest that the MC-VC-PABC linker is susceptible to esterase cleavage, and **2** and **7** can largely function in the CPP-delivered system, in agreement with

Table 1 MIC values of **1**, **2**, **6**, **7** and **8** against *S.* Typhimurium ATCC14028

S.	**1**		**2**			**6**			**7**			**8**	
Typhimurium	pH	pH	Cut[b]	pH	pH	Cut	pH	pH	Cut	pH	pH	Cut	pH
ATCC14028[a]	7.2	7.2		5.0[c]	7.2		5.0[c]	7.2		5.0	7.2		5.0[c]
MIC (μg/mL)	>128	2	4	32	>128	8	64	2	4	8	64	8	16
MIC (μmol/L)	>82.1	0.81	1.62	12.96	>27.2	1.7	13.6	0.81	1.62	3.2	13.6	1.7	3.4

[a] American Type Culture Collection (ATCC); [b] peptides treated by cathepsin B for 1 h at pH 5.0; [c] peptides treated by cathepsin B for 1 h and then the MIC was measured at pH 5.0.

previous results.[12] At pH=5.0, similar to the intracellular acidity of macrophages, the MIC of **7** (3.24 μM) and **8** (3.4 μM) was 3-fold lower than that of **2** (12.96 μM) and **6** (13.6 μM), respectively (Table 1). This may be associated with the C-terminal amidation of **2**, enhancing the acid tolerance and antibacterial activity.[16] Although compound **7** (0.81 μM) had the same MIC of **2** at pH=7.2, its inhibition zones were larger than those of **2** at 20–100 °C (Figure 2A).

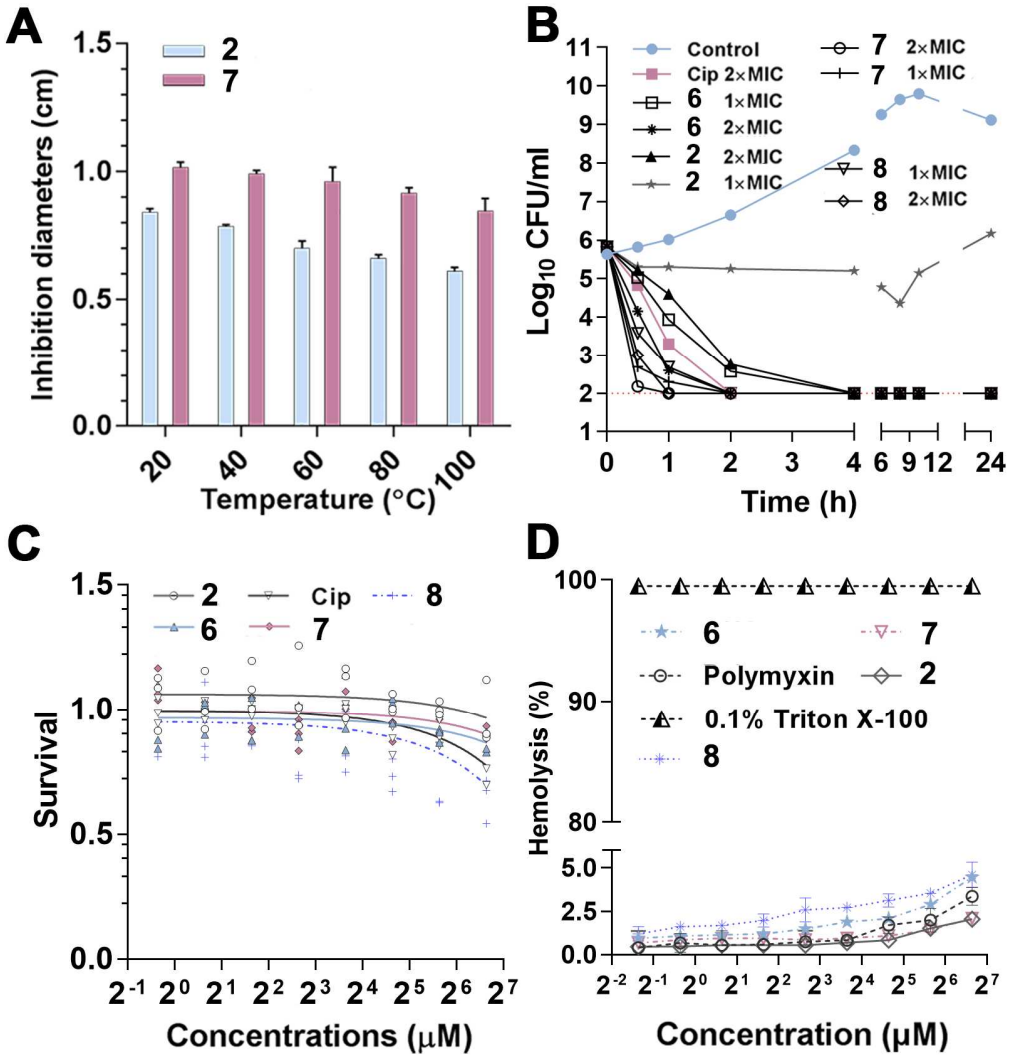


Figure 2. Stability, time-kill curve and toxicity of **6, 7** and **8**. (A) Effects of temperature on the activity of **7** against *S.* Typhimurium. (B) Time-kill curves of **6, 7** and **8** against *S.* Typhimurium *in vitro*. (C) Cytotoxicity of **6, 7** and **8** toward RAW264.7 cells. (D) Hemolytic activity of **6, 7** and **8** against mRBCs. Both 0.1% Triton X-100 and polymyxin were used as controls.

After exposure to cathepsin-treated **6, 7** and **8**, *S.* Typhimurium showed an obvious decrease in growth within 2 h (Figure 2B, Figure S11), indicating faster bactericidal kinetics of peptides **6, 7** and **8** than that of peptide **2** and ciprofloxacin. It may be directly attributed to a higher positive net charge of **6** (+12), **7** (+5) and **8** (+13) than **2** (+4), consistent with the previous study that increasing the net charges enhanced the antibacterial activity of AMPs.[17]

The cytotoxicity of **6, 7** and **8** against mouse peritoneal RAW264.7 macrophages was determined via the 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide (MTT) assay. The results indicated that the survival rate of **6** (88.92%), **7** (93.54%) and **8** (64.31%) toward RAW264.7 cells was lower than that of **2** (94.63%) at 100 μM (Figure 2C), indicating some cytotoxicity of **6** and **8** to RAW264.7 cells at a high concentration after conjugation with **1**. This may be related to the membrane-permeable nature of CPPs.[19] Additionally, the peptides were assayed for potential toxicity against mouse red blood cells (mRBCs) *in vitro*. As shown in Figure 2D, the hemolytic rates of **6**, **7, 8** and **2** at 100 μM were 3.81%, 2.05%, 4.60% and 2.06%, respectively, indicating very low hemolysis at the concentrations needed to kill bacteria.

Uptake and Distribution in Cells. Eliminating intracellular *S.* Typhimurium by antimicrobials normally requires that drugs effectively enter into the host cells and directly combat with bacteria.[19, 20] Confocal microscopy was used to test whether **6** and **7** can enter macrophages and detect their

intracellular localization. As shown in Figure 3, fluorescein isothiocyanate (FITC)-labeled **1** and **7** were homogeneously distributed in the cytosol of RAW264.7 cells and appeared to be vesicles outside the nucleus, suggesting internalization by macropinocytosis or endocytosis pathways.[21] This result is in accordance with previous findings that CPPs (such as pro-rich VRLPPSip $(VRLPPP)_2$ and R_4W_4) and their conjugates (such as P14LRR-kanamycin and α1H/α2H-gentamicin) were localized to the cytosol.[6, 23–25] The fluorescence intensity of FITC-labeled **6** was higher than that of FITC-labeled **2** (Figure 3), indicating enhanced cell uptake after conjugation with **1**, which can be internalized through an endocytosis pathway.[25] However, the fluorescence intensity of FITC-labeled **7** was similar to that of FITC-labeled **2**, indicating that C-terminal amidation cannot enhance the cellular uptake of peptide.

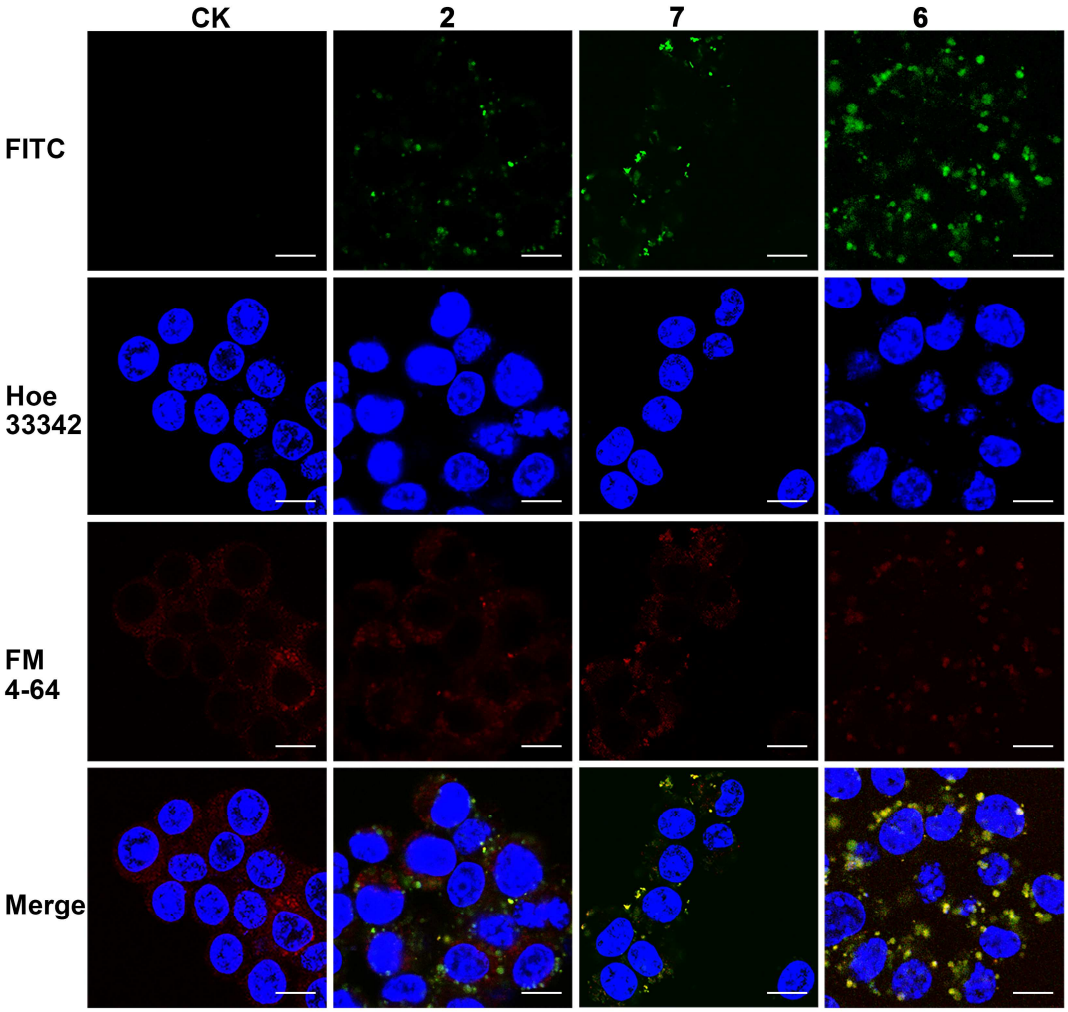


Figure 3. Localization of FITC-labeled **6**, **7** and **2** visualized by confocal microscopy. Scale bar = 5.0 μm. RAW264.7 cells were incubated with 5 μM FITC-labeled **6**, **7** and **2** (green). The cell nucleus and membrane were stained with the Hoe (Hoechst 33342) (blue) and FM 4-64 (red) dyes, respectively.

Additionally, the cell penetrating ability of FITC-labeled **1**, **7, 6** and **2** was measured using flow cytometry.[26] As shown in Figures S12A and S12B, all peptides entered the cells in a time-dependent manner. The cell internalization rates of 5 μM FITC-labeled **6** reached up to 82.1% and 95.8% at 0.5 h and 2 h, respectively, higher than that of **7** (30.4% and 75.2%), "**1**+**2**" (**1** and **2** combination, 1:1) (31.2% and 74.7%), and **2** (28.7% and 71.9%). This indicated that the internalization ability of **2** was enhanced by covalently coupling with **1**, which is in agreement with previous studies that peptide nucleic acids (PNAs) and antibiotics such as kanamycin, gentamicin and fosmidomycin failed to eliminate intracellular pathogens due to poor cell permeability, but their permeability was enhanced by coupling with CPPs.[6, 7, 27, 28] However, the cellular fluorescence of **7** was similar to that of **2**, consistent with the results of confocal microscopy. These data indicated that **6** and **7** may access pathogens that reside within cells.

Uptake Mechanism. To investigate whether the conjugates enter the cells by endocytosis, low temperature (4 °C) and several inhibitors, including amiloride (an inhibitor of macropinocytosis), nocodazole (an inhibitor of the polymerization of tubulin into microtubules), methyl-β-cyclodextrin (MβCD) (a disruptor of lipid rafts) and chlorpromazine (an inhibitor of clathrin-mediated endocytosis), were used to pretreat the RAW264.7 cells.[7] As shown in Figure 4A, the uptake values of **1, 7** and **6** were decreased by 73.37%, 94.64% and 97.81%, respectively, at 4 °C compared with the uptake values at 37 °C, indicating that **1, 7** and **6** were internalized through endocytosis.[29]

The uptake of **1** was strongly reduced by chlorpromazine (48.35%), amiloride (32.36%) and MβCD (15.41%) but was only slightly reduced by nocodazole (1.82%) (Figure 4A). These data implied that FITC-labeled **1** could enter into cells preferentially by clathrin-mediated endocytosis, micropinocytosis and lipid rafts-dependent endocytosis.[24, 29] It seemed that FITC-labeled **1** can enter into cells through multiple endocytic pathways, consistent with other CPPs such as α1H, α2H, and KR_4W_4.[6,24] Additionally, the uptake of **7** was partially inhibited by amiloride (39.02%) and nocodazole (18.38%) and slightly reduced by chlorpromazine (3.79%) and MβCD (2.22%) (Figure 4A), indicating that micropinocytosis may be involved and the microtubule network is required for the delivery of peptides to late endocytic structures.[30, 31] The uptake of **6** was mainly inhibited by chlorpromazine (80.23%) and nocodazole (20.00%) and slightly reduced by amiloride (8.81%) and MβCD (2.32%), this implied that the FITC-labeled **6** could enter into cells preferentially by clathrin-mediated endocytosis and the microtubule network is also required for its delivery.[29, 30]

Bactericidal Activity against *S.* Typhimurium in RAW264.7 Cells. Some CPPs can not only improve non-invasive cellular delivery ability but also the enhance efficacy of therapeutic molecules.[6, 29] The ability of **2**, **6**, **7** and **8** to erase intracellular *S.* Typhimurium within RAW264.7 cells was investigated by an *in vitro* bacterial protection assay.[32] The intracellular time-kill curves showed that the number of *S.* Typhimurium cells continued to decline within 9 h after incubation with **6, 7** and **8** and that bacterial regrowth occurred after 9 h for **6** and **7**; however, for **2**, it was observed within 6 h (Figure S12C). Thus, 10 μM peptide **8** can eradicate the intracellular bacteria, but not for **6**, **7** and **2** unless their concentrations are elevated.

It has been demonstrated that intracellular bacteria, including *Salmonella*, can resist and survive in vacuoles.[6] After uptake into infected cells, **6, 7** and **8** also reached vacuoles, but how they combat

intracellular *S.* Typhimurium remains unclear. In the *in vitro* intracellular bactericidal experiment, RAW264.7 cells infected with *S.* Typhimurium were exposed to gentamicin for 2 h at 37 °C to kill extracellular bacteria and followed by treatment with peptides or ciprofloxacin for 10 h. The results showed that approximately 1.06 and 2.06 Log_{10} CFU reduction of intracellular *S.* Typhimurium was caused by 20 and 50 μM **6**, respectively (Figure 4B). The bactericidal activities of 20 and 50 μM **6** were increased by 67.2% and 76.2% compared with 20 and 50 μM **2**, respectively. However, compound **7** displayed relatively stronger activity than **6**, with a reduction of 2.01 and 3.13 Log_{10} CFU at 20 μM and 50 μM, respectively, corresponding to activities increased by 96.3% and 97.6%, respectively, compared with free **2** at the same concentrations. The cause may be ascribed to the C-terminal amidation of **7,** which can endow the peptide with more resistance to enzymatic degradation than free **2** in macrophages due to the enhanced structural stability.[33] This is further evidenced by compound **8** (connecting **7** and **1**), which showed the highest antibacterial activity, with a reduction of 2.54 and 3.29 Log_{10} CFU at 20 μM and 50 μM, respectively, corresponding to activities increased by 98.9% and 98.6%. However, the antimicrobial activity of "**1**+**2**" was not improved, even at 50 μM (Figure 4B).

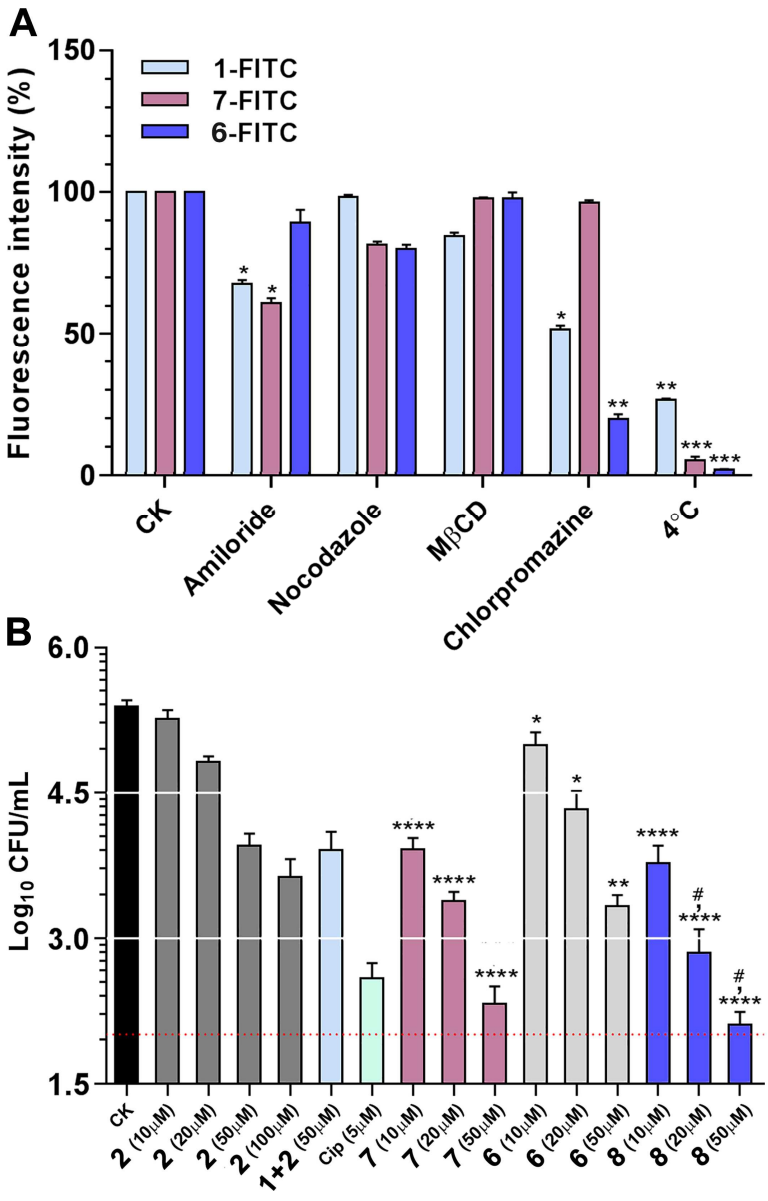


Figure 4. Effect of different endocytosis inhibitors and low temperature on the uptake of conjugates and their intracellular antibacterial activity. (A) Effect of different endocytosis inhibitors and low temperature on the uptake of FITC-labeled **1**, **6** and **7** in RAW264.7 cells. Cells were preincubated with different endocytosis inhibitors for 1 h before incubation with 2 μM FITC-labeled peptides for 2 h. External fluorescence was quenched with 0.4% Trypan blue, and the fluorescence intensity was then measured by flow cytometry. CK, the negative control. (B) Intracellular antibacterial activity of **6**, **7** and **8** in RAW264.7 cells infected with *S.* Typhimurium. After exposure to gentamicin for 2 h at 37 °C,

the infected cells were treated with peptides or ciprofloxacin before bacterial enumeration. The red dotted line at $y = 2$ indicates no bacteria observed on agar plates for enumeration. Statistical analysis was measured by one-way ANOVA, with Tukey's multiple comparisons test. P values <0.05 were considered significant. (*) indicates the significance between **6**, **7** or **8** and isodose **2**; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. (#) indicate significance of **8** compared to isodose **7**. #, $P < 0.05$. The results are expressed as the means from three replicates ± SD (n = 3). "**1**+**2**" refers to the combination of **1** and **2**.

Similar to ciprofloxacin, **6** and **8** could effectively kill intracellular *S.* Typhimurium, suggesting that **1** can facilitate the uptake of **2** and that the MC-VC-PABC linker is effectively cleaved by cathepsin in the macrophages, leading to active **2** or **7** being released from conjugate **6** or **8**.[23] It is likely that, similar to R_4W_4, after endocytosis and subsequent endosomal trafficking, **6**, **7** and **8** fuse with *S.* Typhimurium-containing vacuoles and then exert their bactericidal activity,[6, 34] a finding that needs further investigations in future studies.

***In Vivo* Efficacy.** To determine the *in vivo* intracellular antibacterial activity, compounds **6**, **7** and **8** were tested in a mouse peritonitis model.[12] The 30-min period after infection with *S.* Typhimurium is sufficient to observe significant bacterial translocation into the intraperitoneal macrophages (Figure S13). The mice challenged with *S.* Typhimurium were treated with the isodoses of **8**, **7**, **6** and **2**, respectively, and the bacterial burden was monitored in the total peritoneal, total and intracellular fluids, respectively. The results showed that treatments of 1 μmol/kg **2** and "**1**+**2**" led to a slight decrease in

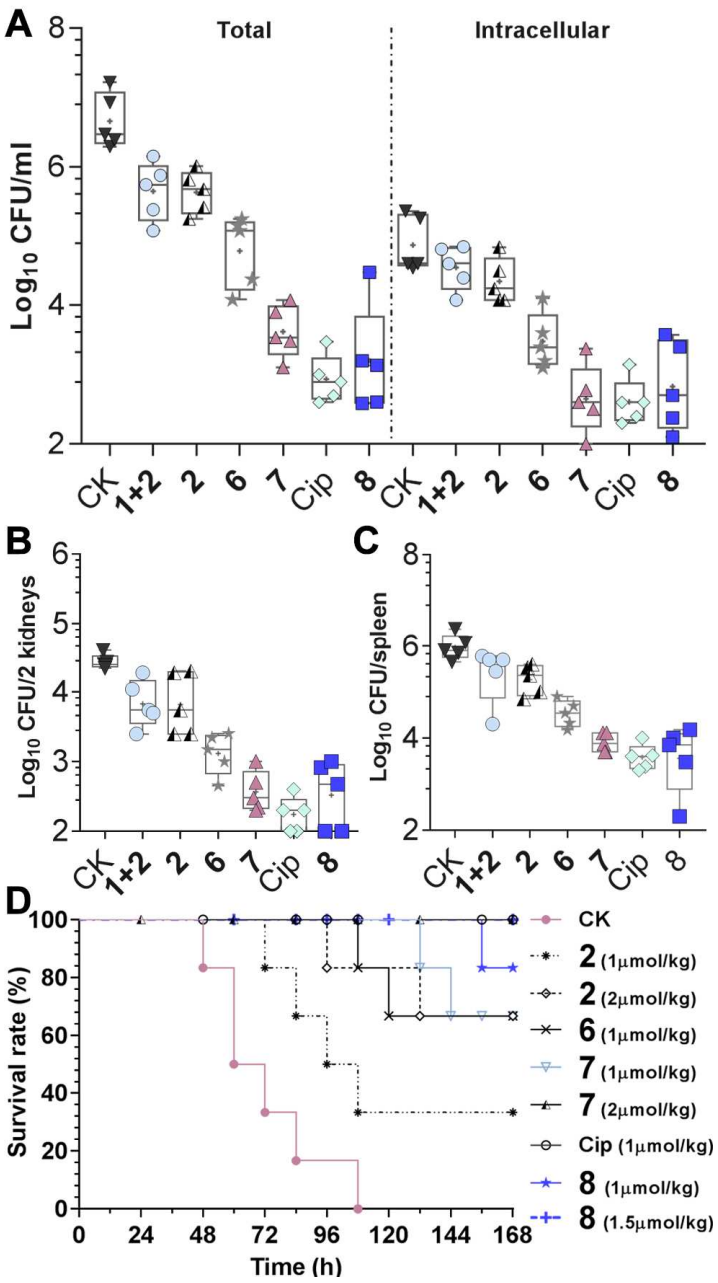


Figure 5. Intracellular antibacterial activities of conjugates in mice infected with *S.* Typhimurium. (A) Bacterial loads in total peritoneal and intracellular fluids. *S. typhimurium* was counted at 10 h after treatment with the peptides (**6**, **7**, **8**, "**1**+**2**", **2**) or ciprofloxacin. Each point represents data from a single mouse. Cip, ciprofloxacin. (B, C) Bacterial loads in the kidneys and spleens. (D) Survival of mice.

CFU in total (1.01 and 1.03 Log_{10} CFU reduction, respectively) and intracellular fluids (0.43 and 0.52

Log_{10} CFU reduction, respectively) (Figure 5A). However, a significant decrease in CFU was noted in total (1.87, 3.04 and 3.46 Log_{10} CFU reduction, respectively) and intracellular fluids (1.39, 2.22 and 2.04 Log_{10} CFU reduction) for **6, 7** and **8**. The efficacy of **7** and **8** was almost equal to that of ciprofloxacin. Additionally, for the total bacterial burden of organs, after treatment with **2** and "**1**+**2**", approximately 0.6 and 1.86–2.07 Log_{10} CFU reductions were observed in the kidneys and spleens, respectively (Figure 5B). The bactericidal activities of **6, 7** and **8** were increased by approximately 81%, 200% and 241%, respectively, indicating potent inhibition of the growth of *S.* Typhimurium *in vivo*.

Moreover, untreated mice began to die at 48 h after inoculation, and all were dead within 108 h. After treatment with 1 μmol/kg **6**, the survival rate of mice was 66.7%, which was higher than that with 1 μmol/kg **2** (33.4%) and lower than that with 1 μmol/kg ciprofloxacin (100%). The survival rates of 1 and 2 μmol/kg **7** reached 66.7% and 100%, respectively. Also, the survival rates of 1 and 1.5 μmol/kg **8** reached 83.3% and 100%, respectively. Thus, **6, 7** and **8** can protect mice from lethal *S.* Typhimurium challenge *in vivo*.

CONCLUSION

Synthetic compounds **6, 7** and **8** showed enhanced antimicrobial activities against intracellular *S.* Typhimurium *in vitro* and *in vivo*, indicating that "CPP-cleavable linker-AMP" may be a novel platform to efficiently deliver AMPs and that C-terminal modification is an effective strategy to largely improve the stability of peptides. Although further investigation of potential clinical applications of **6, 7** and **8** is needed, it will open a door for the exploration and development of other AMPs or antibacterials conjugated to CPPs and other chemical modifications.

EXPERIMENTAL SECTION

General Chemistry. Tat_{11}-linker-N6 (CNC, **6**), composed of CPP (Tat_{11}, **1**: YGRKKRRQRRR), a

protease-cleavable linker MC-VC-PABC-OH moiety and target **2**, as well as Tat_{11}-linker-N6NH2 (CNCNH2, **8**) and FITC-labeled **6**, was designed and chemically synthetized by WuXi AppTec (Shanghai, China), and the purity was analyzed by HPLC.[11,36,37] The final purities of **2**, **6**, **7** and **8** are 94.41%, 91.76%, 93.48% and 94.89%, respectively (Supporting Information, Spectra S8-S11). The syntheses of other peptides, including **2**, **7**, and FITC-labeled **2**, **6** and **7**, were performed in Mimotopes Pty Ltd. (Wuxi, China) following the standard solid phase method.

MC-Val-Cit-PABOH (Compound 3). Dichloromethane (DCM) (10 mL) and N, N-diisopropylethylamine (DIEA) (4.0 equivalent (equiv)) were added dropwise into a mixture containing CTC resin (2.0 mmol, sub=1.0 mmol/g, 2000 mg) and Fmoc-Cit-OH (794 mg, 2.0 mmol, 1.0 equiv). After the addition of the resin and mixing for 2 h, MeOH (2.0 mL) was added and mixed for 30 min. The other amino acids were coupled with HBTU (2.85 equiv) and DIEA (6.0 equiv) for 30 min. Next, 20% piperidine in N, N-dimethylformamide (DMF) was used for deblocking. The coupling reaction was monitored by the ninhydrin color reaction. After washing with MeOH, the resin was dried under vacuum for 2 h. Next, 20 mL of cleavage buffer (1% trifluoroacetic acid (TFA)/99% DCM) was added into the flask containing the resin of peptide, mixed at room temperature for 10 min twice and evaporated to produce the crude peptide. The peptide was dissolved in DMF (10 mL), which was adjusted to pH 8 by DIEA. Finally, methanol (6.0 equiv), diisopropylcarbodiimide (DIC) (6.0 equiv) and 1-hydroxybenzotriazole (HOBT) (6.0 equiv) were added (4-aminophenyl), and the reaction was stirred for 16 h. The crude peptide was purified by Flash (A: H_2O, B: acetonitrile (ACN)) and was lyophilized to obtain the desired product (240 mg, 20.9% yield) (Supporting Information, Spectra S1).

MC-Val-Cit-PABC-P-nitrophenyl (Compound 4). A mixture of compound **3** (240 mg, 419.10 μmol, 1 equiv), bis (4-nitrophenyl) carbonate (764.98 mg, 2.51 mmol, 6 equiv) and DIEA (649.99 mg,

5.03 mmol, 876.00 μL, 12 equiv) in DMF (5 mL) was degassed and purged with N_2 three times. The mixture was then stirred at 15–25 °C for 2 h under N_2 atmosphere. When LCMS showed that the reaction was completed, the reaction was neutralized by TFA. The reaction was purified by Flash (A: H_2O, B: ACN), and the products were lyophilized to obtain compound **4** (100 mg, 135.55 μmol, 32.34% yield) (Supporting Information, Spectra S2).

MC-Val-Cit-PABC-2 (Compound 5). A mixture of compound **3** (50 mg, 67.77 μmol, 1.0 equiv), compound **4** (83.90 mg, 33.89 μmol, 0.5 equiv), HOBt (9.16 mg, 67.77 μmol, 1.0 equiv) and DIEA (8.76 mg, 67.77 μmol, 35.4 μL, 3.0 equiv) in DMF (5 mL) was degassed and purged with N_2 three times, and then the mixture was stirred at 15–25 °C for 1 h under N_2 atmosphere. The reaction was neutralized by TFA and purified by Flash (A: H_2O, B: ACN). Compound **5** was generated after lyophilization (30 mg, 9.76 μmol, 14.40% yield) (Supporting Information, Spectra S3).

1-MC-Val-Cit-PABC-2 (CNC, Compound 6) and 1-MC-Val-Cit-PABC-2-NH2 (CNC-NH2, Compound 8). A mixture of compound **5** (30 mg, 9.76 μmol, 1.0 equiv), compound **4** (16.23 mg, 9.76 μmol, 1 equiv) and NH_4HCO_3 (7.71 mg, 10 equiv) in 30% ACN/H_2O (45 mL) was degassed and purged with N_2 three times, and then the mixture was stirred at 15–25 °C for 1 h under N_2 atmosphere, lyophilized and purified by Pre_HPLC (A: 0.075% TFA in H_2O, B: ACN). Finally, the desired product **6** (12.4 mg, 26.82% yield) was obtained (Supporting Information, Spectra S4, S5 and S8). Compound **8** had a similar synthesis process in the Supporting Information (Supporting Information, Spectra S7-S9).

Antibacterial Tests. The *S.* Typhimurium ATCC14028 cells were grown to the semi-logarithmic period in MH broth (MHB) (pH 7.0) at 37 °C overnight and were centrifuged for 5 min at 4,000 rpm. The pellets were diluted to an optical density (OD_{600}) of 0.01 at 600 nm in MHB. The MIC values were determined using the broth microdilution method.[38] Briefly, 10 μL of peptides and 90 μL of bacterial

cells (5×10^5 CFU/mL) were added into a 96-well microplate and were incubated for 16–18 h at 37 °C. The MIC value was defined as the lowest concentration of peptides at which no growth was observed. The data were obtained in duplicate from at least two independent experiments. To test the sensitivity of **6** and **8** to cathepsin B (from bovine spleen, Sigma C7800) and its MIC after treatment, compound **6** or **8** was diluted to 640 μg/mL in cathepsin buffer (5 mM L-cysteine, 20 mM sodium acetate, 1 mM EDTA, pH 5). Next, 10 μg /mL cathepsin B was added into the **6** or **8** solution and incubated for 1 h at 37 °C. The reaction was stopped by the addition of 9 times the volumes of MHB (pH 7.2).[12] Compound **6** or **8** without cathepsin was used for the negative control. Additionally, the medium was adjusted to pH 5.0 to determine the MIC value in the acidic environment, which simulated the intracellular condition.

Stability, Extracellular Time-kill Curve and Toxicity. To determine the effect of temperature on the thermal stability, 25 μL each of **2** and **7** (32 μg/mL) was used as treatment for 1 h at 4, 20, 40, 60, 80, and 100 °C, respectively, and their antimicrobial activity against *S.* Typhimurium was tested using an inhibition zone assay.[39]

A time-kill curve assay was used to evaluate the pharmacodynamics of **6** and **8** treated with cathepsin B and **7** against *S.* Typhimurium *in vitro.* The mid-log phase cells (10^5 CFU/mL) were mixed with different concentrations of peptides (1 × and 2 × MIC) and were cultured at 37 °C (250 rpm). A 100-μL sample was taken from the mixture at an interval of 2 h, serially diluted and counted on plates. The cells treated with N6 and ciprofloxacin were used as controls.

The MTT assay was used to determine the dose-dependent effect of **6**, **7** and **8** on the viability of murine RAW264.7 cells.[40, 41] RAW264.7 cells were seeded into 96-well plates (2.5×10^4 cells/well), cultured for 24 h, and further incubated with **6**, **7** and **8** (a series of 2-fold dilutions from 100 to 0.625

μM) for 8 h. The untreated cells were used as a control. Following treatment, 20 μL of 5 mg/mL MTT was added and incubated for 4 h. Dimethyl sulfoxide (DMSO) (150 μL/well) was then added into the plates, and the absorbance was measured at 570 nm with a spectrophotometer. All samples were run in triplicate.

The hemolytic ability of **6**, **7** and **8** (a series of 2-fold dilutions from 100 to 0.3125 μM) was evaluated by determining the amount of the released hemoglobin from fresh mRBCs at 540 nm.[42,43] Briefly, the mRBCs were centrifuged at 1,500 rpm for 10 min at 4 °C and were washed with 0.9% NaCl three times. The 8% mRBCs and peptides solutions were added into 96-well plates and incubated for 1 h at 37 °C. Maximum lysis (100%) was determined by analyzing the supernatant of erythrocytes that had been incubated with 1% Triton X-100. Polymyxin and NaCl were used as the positive and negative controls, respectively.

Location, Quantification and Internalization Mechanism in Macrophages. RAW264.7 cells were inoculated into culture dishes (2.5×10^4 cells/well) for 18 h at 37 °C. FITC (5 μM) or FITC-labeled peptides (5 μM) were added to cultures and incubated for 2 h. The cells were stained with 5 μg/mL Hoechst 33342 (ThermoFisher Scientific Inc., Waltham, MA, USA) for nuclear staining and with 5 μg/mL FM 4-64 (ThermoFisher Scientific, USA) for membrane staining at 37 °C for another 30 min, washed twice with PBS, and observed under a fluorescence microscope (Leica TCS SP 5, Germany).

RAW264.7 cells were grown to confluence and resuspended in PBS prior to incubation with 5 μM FITC-labeled **6** and **7** at 37 °C. During the ongoing incubation, samples were taken at 0.5 h and 2 h, respectively, and 0.4% Trypan blue (Invitrogen, UK) was added to quench extracellular fluorescence.[44] The FITC-labeled peptide uptake was analyzed by flow cytometry (Beckton Dickinson, CA, USA).

The cells treated with free FITC were used as a negative control. Data were analyzed using FlowJo V7.6 software.

To determine the internalization pathway of peptides, RAW264.7 cells were incubated with either of four inhibitors (3 mM amiloride, 20 μM nocodazole, 6 μg/mL chlorpromazine, or 5 mM MβCD) for 1 h at 37 °C), followed by 5 μM FITC-labeled **1, 7** or **6** for 2 h at 37 °C. The samples were mixed with 0.4% Trypan blue to quench the extracellular fluorescence, and the intracellular fluorescence was measured using flow cytometry. The effect of low temperature on the internalization of peptides was performed as described previously with some modifications.[6] Briefly, the cells were incubated with 5 μM FITC-labeled **1, 7** or **6** for 3 h at 4 °C prior to adding Trypan blue, and fluorescence was recorded using flow cytometry as described above.

Intracellular Time-kill Curves and Antibacterial Activity against *S.* Typhimurium. To investigate the potential of **6**, **7** and **8** to kill intracellular bacteria, RAW264.7 cells infected with *S.* Typhimurium ATCC14028 were exposed to gentamicin to kill extracellular bacteria. The cells were then treated with 10 μM ciprofloxacin, **6, 7** or **8** for 1, 3, 6, 9 and 12 h, respectively, followed by enumeration of the CFU of surviving intracellular bacteria as described above.

Macrophages were infected with *S.* Typhimurium as previously described.[45] Briefly, mid-log phase *S.* Typhimurium cells were centrifuged at 3,000 g for 10 min, washed twice in sterile PBS, and resuspended in antibiotic-free DMEM medium. The cells were then seeded into 48-well plates (5×10^4/well) (ThermoFisher Scientific; Cat. No. 150687) in DMEM media (containing 10% fetal bovine serum (FBS)), and incubated for 24 h at 37 °C. Next, RAW264.7 cells were infected with *S.* Typhimurium in DMEM and 10% FBS for 1 h at a multiplicity of infection (MOI) of 10. Following exposure to 100 μg/mL gentamicin for 2 h at 37 °C, the cells were thoroughly washed with prewarmed

PBS twice to remove extracellular bacteria. Subsequently, cells were treated with 100 μL of DMEM and 10% FBS-containing peptides at the desired concentrations for 10 h, washed three times with PBS and lysed with 0.1% Triton-X. The cell lysates containing the intracellular bacteria were serially diluted and plated for enumeration. Each drug treatment was performed with three replicates. Experiments were repeated independently twice. The cells treated with ciprofloxacin were used as a positive control, and the untreated cells were used as a negative control.

Mouse *in Vivo* Experiments. All mouse experiments were performed in accordance with the Animal Care and Use Committee of the Feed Research Institute of Chinese Academy of Agricultural Sciences (CAAS), and protocols were approved by the Laboratory Animal Ethical Committee and its Inspection of the Feed Research Institute of CAAS (AEC-CAAS-20090609).

To establish a peritonitis mouse model, six-week-old female ICR mice (six mice/group) were intraperitoneally injected with *S.* Typhimurium ATCC14028 (1×10^6 CFU/mL, 0.5 mL), and intracellular bacteria in intraperitoneal macrophages were determined by enumeration of the CFU at 15, 30 and 60 min after infection. The infected mice were followed by the intraperitoneal injection of **6**, **7**, **8** or **2** (1–2 μmol/kg of body weight, 0.4 mL) at 0.5 h and 8 h post-infection, respectively.[46–48] Mice injected with only bacteria or PBS were used as negative or blank controls. The survival of mice was recorded daily for seven days.

Similarly, mice were intraperitoneally injected with **2**, **1**+**2**, **7, 6 or 8** (1, 1.5 or 2 μmol/kg) or PBS at 2 h post-intraperitoneal infection of *S.* Typhimurium ATCC14028 (5×10^5 CFU, 0.5 mL). The mice were killed at 10 h after treatment, and peritoneal fluids were obtained by washing with 5 mL of ice-cold PBS. The total number of *S.* Typhimurium in the fluids was determined before any further procedures. For intracellular bacteria quantification, the other fraction was centrifuged for 5 min at

4 °C (1,000 rpm). The cells were collected and treated with 100 μg/mL gentamicin for 1 h at 37 °C to kill extracellular bacteria. The cells were washed with ice-cold PBS twice to remove extracellular gentamicin. Subsequently, cells were lysed and counted as described above for the *in vitro* experiment.[11] Kidneys and spleens were then harvested and homogenized in sterile PBS for the CFU assay to evaluate *S.* Typhimurium translocation. The mice treated with ciprofloxacin or PBS were used as a positive or negative control, respectively.

Statistical Analysis. GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA) was used for all statistical analyses, and $p < 0.05$ was considered to be statistically significant.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Materials and Methods section. Sequences and physicochemical properties of **2**, **6**, **7** and **8** (Table S1). Details for the mass spectrometry of **6**, **7**, **8** and **2** (Figures S1-S10). Details for the results of cellular internalization and time-kill curves of **2**, **6**, **7** or **8** in RAW264.7 cells and those of bacterial translocation into the intraperitoneal macrophages in mice (Figures S11-S13) (PDF)

Molecular formula strings (CSV)

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§ These authors contributed equally to this paper. Z.Z.L. and X.W. conducted the experiments. Z.Z.L., D.T., X.M.W., and J.H.W. designed experiments. R.Y.M. performed the HPLC analysis. Z.Z.L., X.M.W., and J.H.W. wrote the manuscript. All authors read and approved the final manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

CPPs, cell-penetrating peptides; AMPs, antimicrobial peptides; HIV-1, human immunodeficiency virus type 1; MC, Monte Carlo; 3D, three-dimension; MHB, Mueller-Hinton broth; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; MβCD, methyl-β-cyclodextrin; MOI, multiplicity of infection; MTT, the 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide; mRBCs, mouse red blood cells; DCM, dichloromethane; DIEA, N,N-diisopropylethylamine; equiv, equivalent; DMF, N,N-dimethylformamide; TFA, trifluoroacetic acid; DIC, diisopropylcarbodiimide; HOBT, 1-hydroxybenzotriazole; ACN, acetonitrile

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