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Battacin (Octapeptin B5), a New Cyclic Lipopeptide Antibiotic from *Paenibacillus tianmuensis* Active against Multidrug-Resistant Gram-Negative Bacteria

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Hospital-acquired infections caused by drug-resistant bacteria are a significant challenge to patient safety. Numerous clinical isolates resistant to almost all commercially available antibiotics have emerged. Thus, novel antimicrobial agents, specifically those for multidrug-resistant Gram-negative bacteria, are urgently needed. In the current study, we report the isolation, structure elucidation, and preliminary biological characterization of a new cationic lipopeptide antibiotic, battacin or octapeptin B5, produced from a *Paenibacillus tianmuensis* soil isolate. Battacin kills bacteria *in vitro* and has potent activity against Gram-negative bacteria, including multidrug-resistant and extremely drug-resistant clinical isolates. Hospital strains of *Escherichia coli* and *Pseudomonas aeruginosa* are the pathogens most sensitive to battacin, with MICs of 2 to 4 μg/ml. The ability of battacin to disrupt the outer membrane of Gram-negative bacteria is comparable to that of polymyxin B, the last-line therapy for infections caused by antibiotic-resistant Gram-negative bacteria. However, the capacity of battacin to permeate bacterial plasma membranes is less extensive than that of polymyxin B. The bactericidal kinetics of battacin correlate with the depolarization of the cell membrane, suggesting that battacin kills bacteria by disrupting the cytoplasmic membrane. Other studies indicate that battacin is less acutely toxic than polymyxin B and has potent *in vivo* biological activity against *E. coli*. Based on the findings of the current study, battacin may be considered a potential therapeutic agent for the treatment of infections caused by antibiotic-resistant Gram-negative bacteria.

Hospital-acquired infections caused by multidrug-resistant (MDR) Gram-negative bacteria, including *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and extended-spectrum-resistant *Escherichia coli*, have progressively increased over the past decade (18, 20). For example, since the New Delhi metallo-β-lactamase was first identified in *K. pneumoniae* in Sweden in 2008, broad-spectrum β-lactamase has been extensively reported for *E. coli* and *K. pneumoniae* isolates from a number of countries worldwide (27). Clinical isolates with New Delhi metallo-β-lactamase usually exhibit resistance to a wide range of antibiotic classes and, in some cases, are highly multiresistant to almost all commercially available antibiotics. No novel antimicrobial agent has been specifically developed for these bacteria in recent years (8, 9). Thus, there is an urgent need for the discovery and development of new antibiotics against MDR Gram-negative bacteria.

Antimicrobial peptides have received considerable attention due to their broad spectrum of activity, novel antimicrobial targets, and low frequency of antibacterial resistance (1a, 31). Although numerous peptide antibiotics have been discovered in almost all known types of organisms, microbial peptides, especially lipopeptide antibiotics and their semisynthetic derivatives, appear to be more useful than other peptide antibiotics. These peptides represent a promising class of antibiotics against MDR pathogens (14, 30). Lipopeptide antibiotics generally consist of a fatty acyl side chain and a hydrophilic peptide portion. Some also contain nonprotein amino acids or other unusual components. These characteristics endow lipopeptide antibiotics with a wide variety of structures and biological functions (12). Peptide antibiotics are generally able to depolarize the cytoplasmic membrane of their target bacteria, resulting in cell death through the disruption of membrane integrity and/or interactions with intracellular targets (2, 23). An interesting family of peptidomimetic antibiotics, typified by L27-11 and POL7001, was recently shown to have a unique mechanism of action, which may include the perturbation of the critical lipopolysaccharide (LPS) transport function of LptD (37). Minor alterations in the structure of antimicrobial lipopeptides may produce significant differences in antimicrobial mechanisms (33, 37).

Paenibacillus strains are well known for their potential to produce lipopeptide antibiotics such as polymyxins and fusaricidins (19, 34). As "old" antibiotics, polymyxins are increasingly being used as a last-line therapy to treat infections caused by MDR Gram-negative bacteria (20, 43). Although the level of toxicity of polymyxins may not be as high as that observed in early clinical applications, it may still complicate patient therapy or even lead to treatment failure (42). During a screening program for new anti-

Received 22 August 2011 **Returned for modification** 10 October 2011
Accepted 10 December 2011
Published ahead of print 19 December 2011
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Supplemental material for this article may be found at http://aac.asm.org/.

doi:10.1128/AAC.05580-11

biotics produced by the genus, we found a novel analog of polymyxins with less acute toxicity from a *Paenibacillus tianmuensis* strain. In the present paper, we report the isolation, purification, and structural elucidation of the active compound battacin. We demonstrate its biological activity *in vitro* and *in vivo* as well as propose its antibacterial mechanism.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The producer strain *P. tianmuensis* F6-B70 was isolated from a soil sample (44). Nutrition broth was used for routine culture. Synthetic Katznelson and Lochhead (KL) medium, used for the production of secondary metabolites, had the following composition: glucose at 5 g/liter, $(NH_4)_2SO_4$ at 1.5 g/liter, $MgSO_4 \cdot 7H_2O$ at 0.2 g/liter, NaCl at 0.1 g/liter, $CaC1_2$ at 0.1 g/liter, $FeSO_4 \cdot 7H_2O$ at 0.01 g/liter, $ZnSO_4$ at 0.01 g/liter, $MnSO_4 \cdot H_2O$ at 0.0075 g/liter, and KH_2PO_4 at 2.7 g/liter. The pH was 7.5. *E. coli* ATCC 35218, *P. aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 43300, and *Enterococcus faecalis* ATCC 29212 were purchased from the American Type Culture Collection. Clinical isolates were obtained from patients at The Fourth People's Hospital of Wuxi, Wuxi, China. All bacterial strains used for the activity assay were grown routinely at 37°C on nutrition agar or broth. *P. aeruginosa* ATCC 27853 was used as the standard sensitive strain. For long-term storage, all strains were stored in 20% (vol/vol) glycerol at −80°C.

Antimicrobial assay. Antimicrobial activity was monitored during fermentation and purification by using the paper disc method. The MICs of the active compounds were determined by using an agar dilution method according to Clinical and Laboratory Standards Institute M7-A8 guidelines (3). Mueller-Hinton (MH) agar plates containing increasing concentrations (from 0.25 μg/ml to 128 μg/ml in 2-fold increments) of the test active compounds were used. The plates inoculated with 2×10^4 CFU per spot were incubated for 16 h to 20 h at 37°C. Polymyxin B and vancomycin, obtained from Sigma-Aldrich (Schnelldorf, Germany), were used as positive controls. The susceptibilities of the clinical isolates to various commonly used antibiotics were determined by using the Vitek 2 Compact system (bioMérieux, Marcy l'Étoile, France).

Production and purification of battacin. *P. tianmuensis* F6-B70 cells were cultured for 1 to 3 days in an agar slant and used to inoculate the nutrient broth. The seed culture was incubated at 30°C for 24 h on a rotary shaker (220 rpm). About 25 ml of the seed broth was transferred into a 2-liter Erlenmeyer flask containing 500 ml of KL medium. The fermentation was carried out on a rotary shaker at 30°C for 5 days. A total of 10 liters of culture was used for the isolation of the active compound. Cells were removed by centrifugation at 6,500 × *g* for 30 min, and the clear supernatant was passed through a column (60 cm by 5.5 cm) packed with 400 g of Amberlite XAD-16 resin (Sigma, St. Louis, MO). The resin was washed with 10 liters of distilled water and 3 liters of 25% methanol. The active compound was then eluted with 2.5 liters of 80% methanol. Each fraction was concentrated and assessed for activity by using the paper disc method described above. The active 80% methanol fraction was evaporated to dryness by rotary evaporation, and the yellow residue was redissolved in 180 ml of purified water (Milli-Q system; Millipore, Bedford, MA). The concentrated solution was then applied onto a cartridge column packed with 30 g of C_{18} silica. The column was washed with 300 ml of Milli-Q-purified water, followed by 90 ml of an acetonitrile-water mixture (20:80, vol/vol). The active fraction was eluted from the cartridge column by using 60 ml of an acetonitrile-water mixture (55:45, vol/vol) and dried. Further purification was performed by high-pressure liquid chromatography (HPLC) using a YMC-pack DOS-A C_{18} (5-μm, 250-mm by 20-mm) column. Eluent A was composed of Milli-Q-purified water containing 0.02% trifluoroacetic acid, and acetonitrile was selected as eluent B. HPLC was performed on an Elite system (Dalian Elite, Dalian, People's Republic of China) with a linear gradient from 20% to 60% acetonitrile at a flow rate of 10 ml/min over 40 min. UV detection was performed at 210 nm. Each peak was collected and tested for antimicrobial activity. The active fraction was evaporated to remove the acetonitrile and then freeze-dried with a lyophilizer. The ultrapure battacin used for the amino acid analysis was passed through the HPLC column three consecutive times.

Mass spectrometry. Electrospray ionization (ESI) mass spectra were recorded in the positive-ion mode on a Thermo Finnigan LCQ mass spectrometer (Thermo Electron Corporation, San Jose, CA). The ESI-mass spectrometry (MS) conditions included a capillary voltage of 35 V, a source voltage of 4.5 kV, and a capillary temperature of 300°C. Tandem MS (MS/MS) was also performed on this instrument, using helium as the collision gas.

Amino acid analysis. Approximately 200 μg of the ultrapure sample was hydrolyzed *in vacuo* at 110°C for 24 h with 0.2 ml of 6 M HCl and 0.1% phenol. Analysis of free amino acids was performed by using ion-exchange chromatography with a Hitachi (Tokyo, Japan) L-8900 amino acid analyzer according to the manufacturer's program for the analysis of protein hydrolysates. Amino acids were detected and quantified at 570 nm for primary amines or at 440 nm for proline after a postcolumn reaction with ninhydrin at 135°C. The system was calibrated with a Hitachi amino acid standard mixture to which L-2,4-diaminobutyric acid (Dab) had been added.

Fatty acid analyses. About 10 mg of the purified active compound was hydrolyzed with 6 M HCl at 110°C for 2 h in a sealed vial. The free fatty acid was obtained and converted to methyl esters as previously described (35). The methyl ester was analyzed with a Focus gas chromatography-MS system (Thermo Scientific) equipped with a DB5 capillary column (30 m by 0.25 mm, 0.25 μm; Agilent). The injector temperature was 250°C, and the helium carrier gas flow rate was 1 ml/min. The column temperature was maintained at 60°C for 2 min and then raised to a final temperature of 280°C at a rate of 10°C/min.

Stereochemical analysis of battacin. 2,4-Dinitrophenyl (DNP) derivatives of battacin and deacyl battacin were prepared as described previously by Kato and Shoji (15). The purified peptides were hydrolyzed with 6 N HCl at 110°C for 8 to 12 h. The absolute configuration of the amino acids was determined by using the advanced Marfey method with 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA) and 1-fluoro-2,4-dinitrophenyl-5-D-leucinamide (D-FDLA) as derivatizing agents (7). FDLA derivative analysis was performed by liquid chromatography/MS.

Time-kill curves. To evaluate the antimicrobial characteristics of the newly isolated compound, the time-kill kinetics of the *E. coli* and *P. aeruginosa* strains were examined. The active compound was added to a logarithmic-phase broth culture of approximately 10^6 CFU/ml to yield concentrations of 0×, 0.5×, 1×, and 4× MIC. The culture was incubated with shaking (120 rpm) at 37°C for 24 h. Surviving bacteria were counted after 0, 0.5, 1, 3, 6, and 24 h of incubation by subculturing 100-μl serial dilutions of samples in 0.9% sodium chloride onto MH agar plates. A bactericidal effect was defined as a $\geq$3-$\log_{10}$ CFU/ml decrease compared with the CFU of the initial inoculum after 24 h of incubation.

OM permeabilization assay. The outer membrane (OM) permeation activity of battacin was determined by an *N*-phenyl-1-naphthylamine (NPN) assay as previously described (16). NPN is commonly used to evaluate the interaction of compounds with the OM (6, 22). The nutrient medium was inoculated with a fresh broth culture of *P. aeruginosa* ATCC 27853 grown overnight and incubated on a shaker (250 rpm) at 37°C for 3 to 6 h to an optical density at 600 nm of 0.5. The *P. aeruginosa* cells were harvested, washed several times, and resuspended at the same optical density in 5 mM HEPES (pH 7.2) containing 5 μM carbonyl cyanide *m*-chlorophenyl hydrazone. NPN (Sigma-Aldrich) was added to a final concentration of 10 μM in quartz cuvettes containing 1 ml of the cell suspension. The tested antibiotics were added, and the sample was mixed well. Changes in fluorescence were continuously recorded by using an RF5301 spectrofluorometer (Shimadzu Co., Ltd., Tokyo, Japan), with an excitation wavelength of 350 nm and an emission wavelength of 420 nm. The reported results are from three independent experiments.

Cytoplasmic membrane depolarization. A cytoplasmic membrane depolarization assay was performed with *P. aeruginosa* ATCC 27853 using

the membrane potential-sensitive dye 3,3′-dipropylthiadicarbocyanine iodide ($diSC_3$-5; Sigma-Aldrich) (46). *P. aeruginosa* cells in the mid-logarithmic phase were washed and resuspended in 5 mM HEPES buffer (pH 7.2) containing 0.2 mM EDTA to an optical density at 600 nm of 0.05. The cell suspension was incubated with 0.4 μM $diSC_3$-5 for about 30 min at room temperature. KCl (100 mM) was then added to equilibrate the external and cytoplasmic K^+ concentrations. The tested compound was added to 1 ml of the bacterial suspensions in a 1-cm cuvette. Changes in fluorescence were continuously recorded by using an RF5301 spectrofluorometer, with excitation and emission wavelengths of 622 nm and 670 nm, respectively. Cytoplasmic membrane depolarization assays were independently performed three times. Similar results were obtained.

Hemolytic activity. The ability of battacin to induce the hemolysis of human red blood cells was assessed as previously described (16). Red blood cells (2% hematocrit) were incubated for 2 h at 37°C in 0.9% saline with a series of concentrations of the active compound. The concentration required for complete lysis was visually determined. The hemoglobin released into the supernatant was measured at 570 nm with a microplate reader (Multiscan MK3; Thermo Labsystems). Red blood cells prepared in distilled water were used as a positive control.

Cytotoxicity assay on HEK293 cells. Cytotoxicity assays were performed by using the HEK293 human embryonic kidney cell line. The cells were seeded into 96-well plates at 1×10^4 cells/well. After incubation for 24 h, the medium was replaced with fresh medium containing battacin (1 μg/ml to 128 μg/ml, in 2-fold increments). Negative and positive controls consisted of plates with no battacin and plates with phenol (500 μg/ml), respectively. After incubation for another 48 h, cell growth was assayed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The relative MTT absorbance was recorded at 550 nm.

***In vivo* studies.** All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at Zhejiang University. Chinese Kun Ming mice (Slaccas Laboratory Animal Co., Ltd., Shanghai, People's Republic of China) between 6 and 8 weeks old and weighing 18 to 22 g were used to test acute toxicity. Solutions of test antibiotics (0.2 ml in sterile saline) were intravenously injected into the tail veins of each mouse. Each group consisted of 20 mice (10 males and 10 females). The number of dead mice was counted daily for 14 days, and the lethal dose for 50% of the animals (LD_{50}) was calculated from survival curves by using the Bliss method (1).

For mouse protection assays, adult female imprinting-control-region (ICR) mice weighing 18 to 22 g were used. The ability of battacin to protect mice from a lethal dose of *E. coli* (ATCC 35318 and clinical isolate 5539) was determined. ICR mice were challenged by the intraperitoneal administration of an exponential-phase culture of *E. coli* with 0.5 ml of the bacterial suspension. One hour after bacterial inoculation, the mice were injected intravenously with 0.9% saline, battacin, or reference compounds. On the following days (days 2 and 3), each group received the same treatment given on day 1. Survival was recorded daily for 7 days. Statistical analysis was performed by using Student's *t* test for two groups, and *P* values of <0.05 were considered significant.

RESULTS

Production and isolation of battacin. *P. tianmuensis* F6-B70, a rod-shaped, spore-forming bacterium, is a potential source of new bioactive natural products (45). A novel macrolide antibiotic, paenimacrolidin, which has potent activity against methicillin-resistant *S. aureus* (MRSA), was isolated from the culture medium of the strain in our previous work. To induce the strain to produce more active compounds, especially lipopeptide antibiotics, KL synthetic medium was selected (29). In preliminary experiments, secondary metabolites extracted from KL medium of F6-B70 using XAD-16 resin were separated by HPLC. More than 10 peaks were detected in this case. Among them, battacin, which eluted at 18.05 min, was found to be the major peak, with potent activity

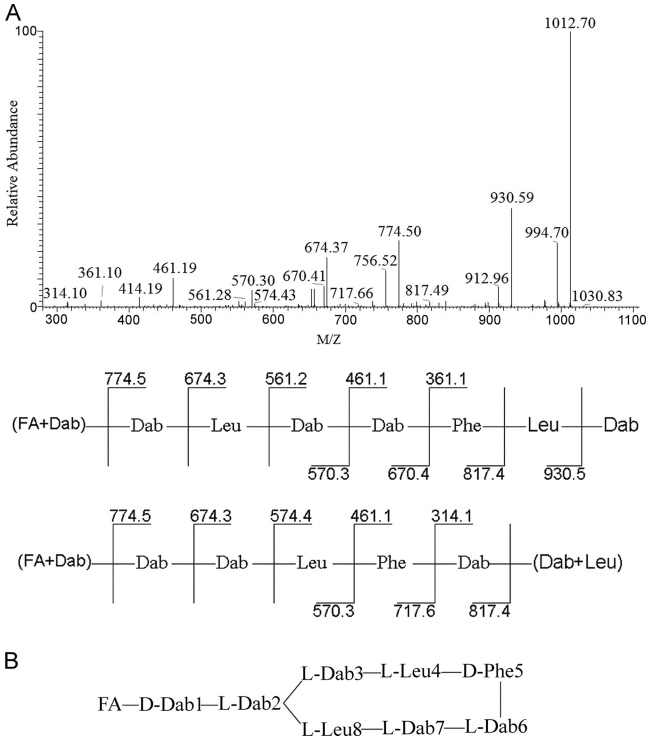

FIG 1 (A) MS/MS spectrum of a precursor ion of battacin at *m/z* 1,031.34 as well as the first and second linear acylium ions. (B) Proposed structure of battacin. Dab, 2,4-diaminobutyric acid; Phe, phenylalanine; Leu, leucine; FA, 3-hydroxy-6-methyloctanoic acid.

against *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 35218. Thus, in the current research, subsequent efforts were focused on the characterization of this active compound. Battacin was purified as described in Materials and Methods, with a yield of 332 mg per 10 liters of culture medium.

Battacin structure elucidation. Based on ion peaks at *m/z* 1,031.34 ($[M + H]^+$) and *m/z* 1,053.23 ($[M + Na]^+$) (see Fig. S1 in the supplemental material), the molecular mass of battacin was determined to be 1,030 Da. The low-mass fragment, which was found to be the immonium ion, at *m/z* 120.24, indicated the presence of phenylalanine (Phe) in the peptide antibiotic. Purified battacin was hydrolyzed with 6 M HCl, and amino acid analysis was performed. The results demonstrated that battacin contained the amino acid residues of Phe, leucine (Leu), and Dab, with molar ratios of 1:2:5, identical with the amino acid compositions of octapeptins B and C (35, 39). Thus, battacin is clearly an octapeptide probably belonging to the octapeptin family.

The $[M + H]^+$ ion was chosen as the precursor ion for further collision-induced dissociation analyses. As shown in the MS/MS spectrum (Fig. 1A), sets of fragment ions were observed, and two sequences were revealed, namely, Dab-Leu-Dab-Dab-Phe-Leu-Dab and Dab-Dab-Leu-Phe-Dab-(Dab + Leu). The results indicate that the active compound contains a peptide ring composed of seven amino acid residues, and the ring is further linked via an amide bond to the rest of the amino acid (Dab) (Fig. 1B). The cleavage of the bond between amino acids 2 and 3 produced the first linear acylium ion, and the ring opening at the bond between amino acids 2 and 8 produced a second linear peptide acylium (Fig. 1A). The ion at *m/z* 774.50 corresponded to the loss of 257 Da

TABLE 1 FDLA derivatives of the acid hydrolysates of battacin, DNP-battacin, and DNP-deacyl battacin using liquid chromatography-MS[a]

Sample	Retention time (min)		*m/z* ($[M + H]^+$)	FDLA derivative
	L-FDLA	D-FDLA		
Battacin	32.8	35.3	707	α,γ-Bis-FDLA-L-Dab
	35.2	32.8	707	α,γ-Bis-FDLA-D-Dab
	24.2	32.1	426	FDLA-L-Leu
	30.4	24.6	460	FDLA-D-Phe
DNP-battacin	33.2	35.9	707	α,γ-Bis-FDLA-L-Dab
	28.7	30.9	579	α-FDLA-γ-DNP-L-Dab
	31.0	28.9	579	α-FDLA-γ-DNP-D-Dab
	24.6	32.5	426	FDLA-L-Leu
	30.9	25.0	460	FDLA-D-Phe
DNP-deacyl battacin	33.1	35.6	707	α,γ-Bis-FDLA-L-Dab
	28.4	30.7	579	α-FDLA-γ-DNP-L-Dab
	24.4	32.3	426	FDLA-L-Leu
	30.6	24.8	460	FDLA-D-Phe

[a] Analysis was performed with a Zorbax SB-C_{18} column. The solvent was a gradient of acetonitrile (solvent A) and 20 mM ammonium acetate adjusted to pH 4.3 with formic acid (solvent B), with a linear increase from 10% solvent A to 20% solvent A within 5 min, followed by a linear increase to 50% solvent A in 45 min. The flow rate was 0.2 ml min^{-1}, and the wavelength was 340 nm.

TABLE 2 MICs of battacin compared to those of polymyxin and vancomycin

Indicator strain	MIC (μg/ml)[a]		
	Battacin	Polymyxin B	Vancomycin
Pseudomonas aeruginosa ATCC 27853	4	2	—
Pseudomonas aeruginosa 5215	2-4	2	—
Pseudomonas aeruginosa 5313	4	1-2	—
Pseudomonas aeruginosa 5585	4	2	—
Pseudomonas aeruginosa 5298	4	2	—
Pseudomonas aeruginosa 5510	4	2	—
Escherichia coli ATCC 35318	4	1	—
Escherichia coli 5237	4	1	—
Escherichia coli 5353	4	1	—
Escherichia coli 5364	4	0.5-1	—
Escherichia coli 5539	2	0.5	—
Acinetobacter baumannii 5064	8	1	—
Acinetobacter baumannii 5520	8-16	1	—
Acinetobacter baumannii 5383	8-16	1	—
Acinetobacter baumannii 5349	4	1	—
Acinetobacter baumannii 5075	8	1	—
Klebsiella pneumoniae 5405	2	1	—
Klebsiella pneumoniae 5147	4	1	—
Klebsiella pneumoniae 5614	8	1	—
Staphylococcus aureus ATCC 43300	64-128	100	1
Enterococcus faecalis ATCC 29212	64-128	100	0.5

[a] —, not determined.

from the $[M + H]^+$ ion. Examination of the neutral fragment lost suggested that it contains a fatty acyl moiety (157 Da) and a side-chain Dab residue. Attempts to partially hydrolyze the peptide antibiotic with a mild base (1 M KOH) failed to produce a new product. This finding indicates that no lactone is present in the active compound and that the fatty acyl moiety is linked to the Dab residue through an amide bond.

The fatty acid was cleaved from the active compound and derivatized as previously described (35). The fatty acid was then subjected to electron ionization using gas chromatography-MS (see Fig. S2 in the supplemental material). The peaks observed at *m/z* 74 and 103 (base peak) were characteristic of the methyl derivatives of β-hydroxy fatty acids. The molecular ion peak was not obtained, but peaks at *m/z* 170 (M-H_2O), *m/z* 141 (M-H_2O and C_2H_5), *m/z* 139 (M-H_2O and OCH_3), and so on were observed. When the mass spectrum of methyl fatty acid from battacin was compared with that of methyl β-hydroxy anteisononanoate from octapeptin C1 (35), a good agreement in the fragmentation pattern was observed. Thus, the free fatty acid of battacin is proposed to be a 3-hydroxy-6-methyloctanoic acid.

The absolute configuration of the amino acids in battacin was determined by chemical degradation. Acid hydrolysis of battacin followed by advanced Marfey's analysis of the resulting hydrolysate showed the presence of L-Leu, D-Phe, as well as both D- and L-Dab in the FDLA product mixture (Table 1 and see Fig. S3 in the supplemental material). To determine the absolute configuration of Dab at different positions, the DNP derivatives of battacin and deacyl battacin (DNP-battacin and DNP-deacyl battacin, respectively) were prepared. Analysis of the DNP-battacin hydrolysate (Table 1 and see Fig. S4 in the supplemental material) indicated that hydrolysis releases free L-Dab and the DNP derivatives of both D- and L-Dab (DNP-D/L-Dab). Thus, Dab2 was identified as the L-configured enantiomer. The hydrolysate of DNP-deacyl battacin contained peaks (*m/z* 579) matching only FDLA-DNP-L-Dab (Table 1 and see Fig. S5 in the supplemental material), indicating that Dab3, Dab6, and Dab7 are all L-amino acids. Consequently, Dab1 was assigned as the D-configured enantiomer. The proposed structure for battacin is shown in Fig. 1B.

***In vitro* antibacterial activity of battacin.** The new cationic peptide antibiotic was active against all tested Gram-negative and -positive bacteria *in vitro*, as shown in Table 2. However, battacin showed a higher order of activity against Gram-negative bacteria than against Gram-positive ones. Hospital strains of *E. coli* and *P. aeruginosa* were the pathogens most sensitive to battacin, with MICs of 2 to 4 μg/ml, slightly lower than those of polymyxin B (0.5 μg/ml to 2 μg/ml). For *K. pneumoniae*, the MICs of battacin ranged from 2 to 8 μg/ml, whereas for *A. baumannii*, the MICs ranged from 4 to 16 μg/ml. In contrast, *S. aureus* ATCC 43300 and *Enterococcus faecalis* ATCC 29212 were insensitive to less than 64 μg/ml battacin.

The susceptibilities of all clinical isolates to various commercial antibiotics were determined and are summarized in Table S1 in the supplemental material. Among the 17 clinical isolates, 8 were MDR strains, which were intermediate or resistant to at least three drugs in the following classes, according to Clinical and Laboratory Standards Institute breakpoints (4): broad-spectrum cephalosporins, aminoglycosides, fluoroquinolones, or carbapenems. Among them, three *A. baumannii* isolates (isolates 5383, 5064, and 5075) and one *P. aeruginosa* isolate (isolate 5215) were insensitive to all tested antibiotics except for polymyxin B.

To evaluate the growth inhibition effect of battacin, killing experiments with the new active compound against *E. coli* and *P. aeruginosa* were performed. As shown in Fig. 2, battacin was bactericidal in a dose-dependent manner. Interestingly, the bacterial killing of polymyxin B for *P. aeruginosa* 5215 was so rapid that at 8× MIC (16 μg/ml), no living cell could be detected within 60

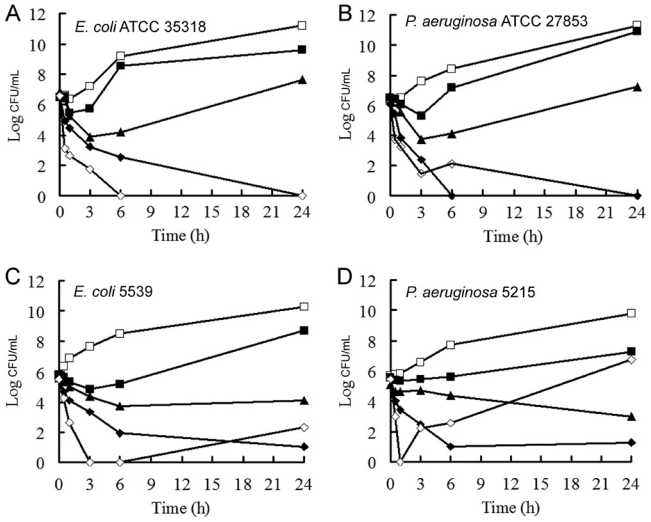

FIG 2 Growth curves of *E. coli* ATCC 35318 (A), *P. aeruginosa* ATCC 27853 (B), *E. coli* 5539 (C), and *P. aeruginosa* 5215 (D) cells treated with different concentrations of battacin. The curves are viable cell concentrations plotted against time. Open squares, nonbattacin control; closed squares, 0.5× MIC of battacin; closed triangles, 1× MIC of battacin; closed diamonds, 4× MIC of battacin; open diamonds, polymyxin B (16 mg/liter). For the four strains in the present study, time-kill assays were independently performed 2 to 4 times, and similar results were obtained. Mean values of the triplicate CFU/ml measurements from a single experiment are plotted.

min. However, regrowth was observed at as early as 3 h, and substantial regrowth occurred at 24 h. For *E. coli* 5539, regrowth occurred at 24 h even at 32× MIC of polymyxin B (16 μg/ml). In contrast, no significant regrowth was observed for all tested strains treated with 4× MIC of battacin.

Permeabilization of the outer membranes by NPN. Figure 3A shows that the addition of various concentrations of battacin to *P. aeruginosa* suspensions in the presence of NPN caused a concentration-dependent increase in fluorescence, indicating that the new cationic peptide antibiotic was capable of disrupting the OM of *P. aeruginosa*. The ability of battacin to facilitate NPN uptake was comparable to that of polymyxin B, a well-known OM-destabilizing agent (13). The uptake of NPN facilitated by battacin was dramatically inhibited by the addition of excess

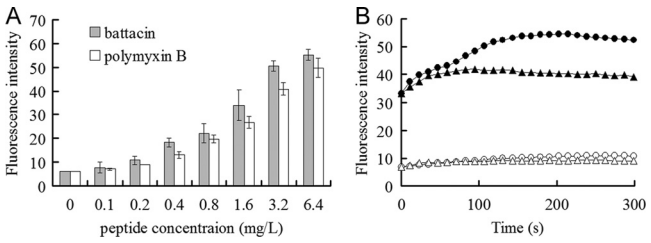

FIG 3 Peptide-mediated NPN uptake in *P. aeruginosa* ATCC 27853. (A) Effects of various concentrations of battacin and polymyxin B on NPN fluorescence. The corresponding value on the *y* axis is the maximum value of the fluorescence intensity. (B) Effect of added calcium ions on the permeation of the outer membrane of *P. aeruginosa* by peptide. The addition of calcium ions (10 mM) inhibited the increase in NPN fluorescence induced by 0.8, 1.6, 3.2, and 6.4 mg/liter peptide antibiotics. Only the NPN fluorescence caused by 3.2 mg/liter peptide antibiotics is presented. Filled circles, battacin with no Ca^{2+}; open circles, battacin with Ca^{2+}; filled triangles, polymyxin B with no Ca^{2+}; open triangles, polymyxin B with Ca^{2+}.

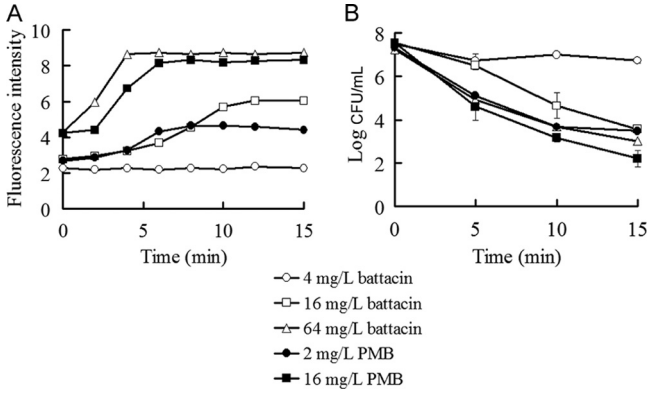

FIG 4 Cytoplasmic membrane depolarization assay using the fluorescent dye $diSC_3$-5 with *P. aeruginosa* ATCC 27853 (A) and analysis of cell killing under dye assay conditions (B). Open circles, battacin at 4 mg/liter (1× MIC); open squares, battacin at 16 mg/liter (4× MIC); open triangles, battacin at 64 mg/liter (16× MIC); filled circles, polymyxin B (PMB) at 2 mg/liter (1× MIC); filled squares, polymyxin B at 16 mg/liter (8× MIC).

$CaCl_2$ (Fig. 3B), suggesting that the new cationic peptide antibiotic competes for Ca^{2+} binding sites in the OM. This inference is consistent with those of previous reports (10, 32) and is supported by the fact that the MIC of battacin for *P. aeruginosa* increased from 4 to >128 μg/ml in MH medium with 10 mM $CaCl_2$. Battacin also showed the ability to permeabilize the OMs of *E. coli* ATCC 35318 and *A. baumannii* 5383 (data not shown), indicating its permeabilizing potency, as determined by its NPN uptake.

Depolarization of the cytoplasmic membrane. The ability of battacin to induce membrane depolarization was evaluated by using the fluorescent dye $diSC_3$-5 in *P. aeruginosa* cells permeabilized with EDTA. At the same time, bacterial cell viability was monitored by CFU counts performed on aliquots drawn from the $diSC_3$-5 assay mixture. As shown in Fig. 4A, battacin permeated the plasma membrane of *P. aeruginosa* in a dose-dependent manner. However, the effect was less than that of polymyxin B. Battacin at an MIC of 4 μg/ml caused the release of minimal amounts of $diSC_3$-5. Less than 1 logarithmic decrease in cell viability was observed after 15 min (Fig. 4B). When the cells were treated with a bactericidal concentration of battacin (4× MIC [16 μg/ml]), a visible amount of $diSC_3$-5 release was detected after a lag time of 5 min. At this point, 1 logarithmic order of bacterial killing was observed. After about 10 min, the level of depolarization appeared to reach a maximum, and more than 2.5 logarithmic units of killing by battacin were observed. The correlation between the concentration dependence of antibiotic activity and the effects on membrane depolarization suggests that battacin kills *P. aeruginosa* cells by damaging their cytoplasmic membranes. The activity of polymyxin B against *P. aeruginosa* was slightly more potent than that of battacin, consistent with the data that emerged from the $diSC_3$-5 assay (Fig. 4B).

Cytotoxicity. Although peptide antibiotics have attracted increasing attention over the last 2 decades, issues such as nonspecific cytotoxicity and hemolysis have limited their clinical utility (11, 26). The cytotoxicity of battacin against mammalian cells was tested by hemolysis and MTT assays. The concentration of battacin required for the complete lysis of human erythrocytes was more than 750 μg/ml. All tested concentrations of battacin (1 μg/ml to 128 μg/ml) showed no cytotoxicity against HEK293 cells.

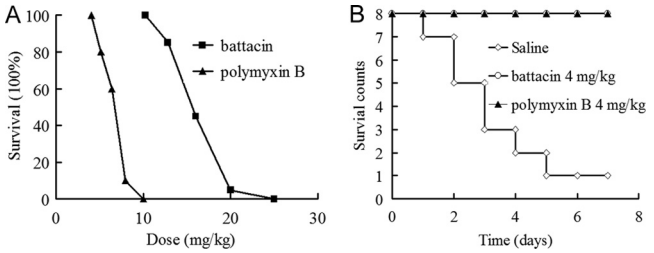

FIG 5 Properties of battacin *in vivo*. (A) Acute toxicities of battacin (filled squares) and polymyxin B (filled triangles) injected intravenously. Kun Ming mice ($n = 20$ per group) were injected with various doses of battacin and polymyxin B via the lateral tail vein. (B) Protective effects of battacin *in vivo*. ICR mice ($n = 8$ per group) were inoculated intraperitoneally with 0.5 ml of an MDR clinical isolate of *E. coli* 5539 (2×10^7 CFU/ml) and treated intravenously with three doses (1, 24, and 48 h after infection) of battacin at 4 mg/kg (open circles), polymyxin B at 4 mg/kg (filled triangles), or physiological saline (open diamonds). The infected mice were inspected every day for 7 days after infection.

Properties of battacin *in vivo*. The acute toxicity of battacin in mice was evaluated by intravenous administration. Toxic responses, such as respiratory distress, exophthalmos, jumping motions, and convulsions, were observed. All mice treated with the highest dose, 25 mg/kg of body weight, died within several minutes. Histopathological studies showed that no obvious change was found for the organs of dead mice. Figure 5A shows the dose-toxicity relationship observed on day 14. The LD_{50} of battacin for mice was 15.46 mg/kg (95% confidence interval, 14.47 mg/kg to 16.52 mg/kg), whereas that of polymyxin B was 6.52 mg/kg (95% confidence interval, 6.07 mg/kg to 7.01 mg/kg). Preliminary acute toxicity data showed no significant differences between male and female mice.

To assess the protective activity of battacin, mice were challenged with *E. coli* ATCC 35318 (Table 3). About 90% of the tested mice treated with 0.9% saline died 6 days after being challenged with ATCC 35318. In contrast, treatment with 2 or 4 mg/kg battacin protected 50% and 90% of mice challenged with the bacterium ($P < 0.05$), respectively. To confirm this finding, mice were challenged with an MDR clinical isolate, *E. coli* 5539. In this case, the drugs were administered intravenously 1 h after intraperitoneal infection. As shown in Fig. 5B, almost all untreated mice (7 out of 8) died 1 to 5 days after infection with the bacterium. In contrast, 100% of mice survived after 7 days in the groups treated with 4 mg/kg battacin or 4 mg/kg polymyxin B ($P < 0.05$). These results indicate that battacin has significant *in vivo* biological activity.

DISCUSSION

Octapeptins, a group of polypeptide antibiotics characterized by a high percentage of Dab, have a general structure composed of a cyclic heptapeptide moiety and a side chain. This side chain consists of only one amino acid covalently bound to a β-hydroxy fatty acid via an acyl group (25). To date, 17 octapeptins have been extensively described (15, 39, 40). These antibiotics are highly similar in structure and differ in only one or two amino acid units or fatty acid moieties or both. This class of antibiotics is categorized into four subgroups (octapeptins A, B, C, and D) based on the amino acids in the peptide moieties. Octapeptin A contains no Phe in its peptide structure, octapeptin B has one Phe at position 5 (Fig. 1B), and octapeptin C contains a Phe at position 4. Except for the lack of Phe, octapeptin D has a serine rather than a Dab moiety at position 1. Each subgroup of octapeptins contains four to five components differing in the sizes of their fatty acid moieties. Although the amino acid compositions of battacin and octapeptins B (B1 to B4) are the same, the numbers of CH_2 groups in the fatty acid moieties differ (25, 40). The other members of the octapeptins contain D-amino acids at positions 1 and 4, whereas D-amino acids in battacin are located at positions 1 and 5. From a structural perspective, the major difference between octapeptin B and battacin is their fatty acid moieties. In the current work, battacin is also ascribed to the octapeptin B family and is therefore named octapeptin B5. All octapeptin group antibiotics, including battacin, exhibit broad-spectrum antimicrobial activities against many Gram-negative and -positive bacteria (17, 24, 36). EM49 antibiotics, a mixture of octapeptins A and B1 to B4, were also reported to show activities against yeasts, filamentous fungi, and protozoa (24, 25). All octapeptins are produced by *Bacillus* strains, except battacin, which is obtained from a *Paenibacillus* species. To the best of our knowledge, the present study is the first to report the isolation of a member of the octapeptins from paenibacilli.

Battacin showed potent activity against all tested Gram-negative bacteria, including *P. aeruginosa* ATCC 27853, *E. coli* ATCC 35318, and 17 clinical isolates (see Table S1 in the supplemental material). As noted above, 47% of these clinical isolates were MDR strains, and 23% were extremely resistant to almost all commonly used antibiotics. No clinical isolate was resistant to polymyxin B, due in part to its uncommon clinical use in China and to the small number of clinical strains included in our study. The numbers of clinical infections caused by drug-resistant and extremely MDR strains of Gram-negative bacteria have progressively increased over the past decade (18, 20). Polymyxins have become the last line of treatment for infections caused by these strains (5, 43). In spite of their potent activities, the use of polymyxins is compromised by their toxicity, especially nephrotoxicity (5, 42). Despite the similarity in their structures, battacin and polymyxin B differ significantly in their acute toxicity in mice. The LD_{50} of battacin by intravenous administration was 2.3-fold higher than that of polymyxin B, indicating that battacin is less toxic than polymyxin B. Several studies have indicated that the reduction of positive charges renders polymyxin less toxic (41, 42). This suggestion may explain the lower toxicity of battacin (containing four positive charges) than that of polymyxin B (possessing five positive charges).

Although octapeptins were discovered more than 40 years ago, their precise mode of action remains unknown. Battacin exhibits a

TABLE 3 Protection of mice from *E. coli* ATCC 35318 by battacin and reference compounds[a]

Drug	Single intravenous dose (mg/kg)/day for 1 day to 3 days	No. of surviving mice/total no. of mice	*P* value vs saline
Saline		1/10	
Cefodizime sodium	800	10/10	<0.05
Battacin	1	2/10	>0.05
Battacin	2	5/10	<0.05
Battacin	4	9/10	<0.05

[a] ICR mice ($n = 10$ per group) were inoculated intraperitoneally with 0.5 ml of *E. coli* ATCC 35318 cells (3.6×10^6 CFU/ml) and treated intravenously with three doses (1, 24, and 48 h after infection) of physiological saline, battacin, or a reference compound.

dose-dependent bactericidal activity, consistent with other cationic peptide antibiotics (21, 28). The bactericidal kinetics of battacin were correlated with the depolarization of the cell membrane under the specific experimental conditions of the $diSC_3$-5 assay, thus supporting the notion that battacin kills bacteria by disrupting the cytoplasmic membrane. The ability of battacin to disrupt the OM was comparable with that of polymyxin B. However, its capacity to damage the bacterial plasma membrane was somewhat less extensive than that of polymyxin B, which is probably why the antibacterial potency of battacin is slightly less than that of polymyxin B.

Based on our results as well as on previously reported data on structure-related peptide antibiotic polymyxins and EM49 (5, 32, 43), we hypothesize that the initial step of the active mode of battacin involves the interaction of positively charged battacin Dab residues with negatively charged lipid A head groups, which are normally stabilized by divalent cations (Ca^{2+} and Mg^{2+}). This conclusion is supported by the fact that the addition of excess $CaCl_2$ inhibited the uptake of NPN promoted by battacin and reduced the susceptibility of *P. aeruginosa* to the new cationic peptide. Initial electrostatic interactions facilitate the insertion of the fatty acid chain of battacin into the hydrophobic core of the OM of Gram-negative bacteria. After transiting through the OM through self-promoted uptake, battacin inserts into and disrupts the inner membrane, leading to cell death.

In conclusion, a new cationic lipopeptide antibiotic, battacin (octapeptin B5), was isolated and characterized in the present study. Battacin showed potent activity against Gram-negative bacteria, including MDR and extremely drug-resistant clinical isolates. The antimicrobial activity of battacin was correlated with its ability to disrupt the outer and inner membranes of bacterial cells. *In vivo* activity was assessed by using a murine peritonitis-sepsis model, and battacin was found to protect mice effectively from bacterial challenge. Presently, polymyxins have reemerged as a "no-choice" alternative for the treatment of infections caused by MDR Gram-negative pathogens. Although the incidence of resistance to polymyxins is currently relatively low, polymyxin-resistant strains from hospital patients are increasingly being reported (43). Some polymyxin-resistant bacteria are susceptible to octapeptins (38). However, whether polymyxins and battacin are cross resistant remains to be determined.

ACKNOWLEDGMENTS

The current work was supported by the Major State Basic Research Development Program (973 Program 2010CB833803).

We thank X. H. Jiang of the College of Life Sciences, Zhejiang University, for providing the MS measurements.

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