

## Notes

Effects of Lys-linked Dimerization of an  $\alpha$ -Helical Leu/Lys-rich Model Antimicrobial Peptide on Salt Resistance and LPS-neutralizing ActivityYong Hai Nan, Jeong-Kyu Bang,<sup>†</sup> and Song Yub Shin<sup>\*</sup>

Department of Bio-Materials, Graduate School, and Department of Cellular &amp; Molecular Medicine, School of Medicine, Chosun University, Gwangju 501-759, Korea. \*E-mail: syshin@chosun.ac.kr

<sup>†</sup>Division of Magnetic Resonance, Korea Basic Science Institute, Chungbuk 363-883, Korea

Received July 4, 2011, Accepted September 2, 2011

**Key Words :**  $\alpha$ -Helical model antimicrobial peptide, LPS-neutralizing activity, Lys-linked dimerization, Salt resistance

Antimicrobial peptides (AMPs) exhibit potent antimicrobial activities against Gram-positive and Gram-negative bacteria, fungi and viruses as part of their innate immunity to pathogen invasion.<sup>1</sup> Their fast action and slow resistance development make promising lead compounds for new antibiotics. Although the mechanism of action of AMPs has not been clarified, it is believed that most AMPs kill bacteria by membrane disruption and/or by interaction with cytoplasmic membranes.<sup>2</sup>

The presence of salts (NaCl is the most predominant salt) in human body fluids is known to hinder antimicrobial activity of many cationic AMPs including  $\beta$ -defensins, cecropins, indolicidins, gramicidins, bactenecins and magainins.<sup>3-5</sup> This is one of the main obstacles preventing the widespread adoption of AMPs for systemic clinical use.<sup>6</sup>

Lipolysaccharide (LPS; known also as endotoxin) is an integral structural component of the outer membrane of Gram-negative bacteria, and is thought to be a major mediator of sepsis and septic shock.<sup>7</sup> Recent studies have demonstrated that in addition to their antimicrobial activities, some AMPs including human LL-37, rabbit CAP18, sheep SMAP-29, bactenecin, indolicidin and BMAP-27, have the potential to inhibit LPS-induced cellular cytokine and/or nitric oxide (NO) release by binding directly to LPS or by blocking the binding of LPS to LPS-binding protein (LBP).<sup>8,9</sup> These properties make these peptides attractive drug candidates for treatment of endotoxin shock and sepsis.

In the present study, to investigate the effect of Lys-linked

dimerization of  $\alpha$ -helical Leu/Lys-rich AMPs on salt resistance and LPS-neutralizing activity, we designed and synthesized an ideal amphipathic  $\alpha$ -helical 11-meric model peptide ( $K_6L_4W_1$ ) composed of 6 lysines, 4 leucines and 1 tryptophan (*i.e.* the hydrophobic and hydrophilic amino acids are localized in opposite faces of the helix) and its Lys-linked dimeric peptide ( $(K_6L_4W_1)_2$ -K (Table 1).

The hydrophobicity of peptides was assessed by measuring the reverse-phase high performance liquid chromatography (RP-HPLC) retention time (Table 1). The retention time of peptides on a reverse-phase matrix ( $C_{18}$ -column) was reported to be related to their hydrophobicity.<sup>10</sup> We examined the antimicrobial activities of the peptides against a representative set of bacterial strains, including three Gram-negative bacteria (*Escherichia coli* [KCTC 1682], *Pseudomonas aeruginosa* [KCTC 1637] and *Salmonella typhimurium* [KCTC 1926]) and three Gram-positive bacteria (*Bacillus subtilis* [KCTC 3068], *Staphylococcus epidermidis* [KCTC 1917] and *Staphylococcus aureus* [KCTC 1621]).

The MIC values of the peptides are shown in Table 2. Lys-linked dimeric peptide ( $(K_6L_4W_1)_2$ -K showed increased antimicrobial activity, as compared to monomeric peptide  $K_6L_4W_1$ . Similar to our result, the dimeric peptide halocidin showed a 4- to 20-fold increase in antimicrobial activity as compared to a monomeric peptide.<sup>11</sup> The disulfide-linked dimeric peptide of LLP1 derived from a lentivirus envelope protein possesses much greater antimicrobial activity against *S. aureus* than monomeric LLP1.<sup>12</sup> We next assessed the

**Table 1.** Amino acid sequences and calculated and observed molecular masses of the peptides

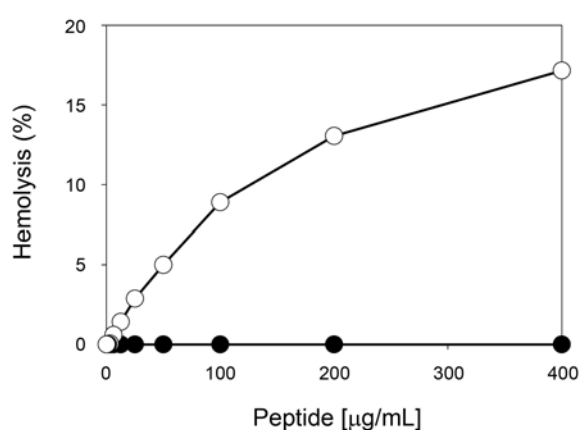
Peptide	Amino acid sequence	Molecular mass (Da)		Net positive charge	RP-HPLC retention time (min) <sup>b</sup>
		Calculated	Observed <sup>a</sup>		
$K_6L_4W_1$	KLKKLWKKLLK-NH <sub>2</sub>	1424.9	1424.8	+7	26.8
$(K_6L_4W_1)_2$ -K	$(KLKKLWKKLLK)_2$ K-NH <sub>2</sub>	2960.9	2960.2	+14	30.4

<sup>a</sup>Molecular masses were determined by MALDI-TOF-MS. <sup>b</sup>RP-HPLC retention time was measured using a  $C_{18}$  reverse-phase analytical column (5  $\mu$ m; 4.6 mm  $\times$  250 mm; Vydac). Peptides were eluted for 60 min, using a linear gradient of 0% to 90% (v/v) acetonitrile in water containing 0.05% (v/v) trifluoroacetic acid.

**Table 2.** Antimicrobial and hemolytic activities and cell selectivity of the designed peptides

Peptide	MIC <sup>a</sup> (μg/mL)						GM <sup>b</sup> (μg/mL)	MHC <sup>c</sup> (μg/mL)	Therapeutic Index <sup>d</sup> (MHC/GM)
	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. typhimurium</i>	<i>B. subtilis</i>	<i>S. epidermidis</i>	<i>S. aureus</i>			
K <sub>6</sub> L <sub>4</sub> W <sub>1</sub>	12.5	25.0	6.25	12.5	12.5	6.25	12.5	400 <	64
(K <sub>6</sub> L <sub>4</sub> W <sub>1</sub> ) <sub>2</sub> -K	12.5	12.5	6.25	6.25	12.5	6.25	9.4	126	13.4
Indolicidin	25	50	12.5	12.5	25	6.25	21.9	35	1.6

<sup>a</sup>MIC were determined in three independent experiments performed in triplicate. <sup>b</sup>The geometric mean (GM) of the MIC values from all six bacterial strains in this table. <sup>c</sup>The minimal peptide concentration (MHC) that produces 10% hemolysis. When no detectable hemolysis was observed at 400 μg/mL, we used a value of 800 μg/mL to calculate the therapeutic index. <sup>d</sup>The ratio of the MHC (μg/mL) over the geometric mean (GM) of the MIC (μg/mL).

**Figure 1.** Concentration-response curves of percent hemolysis of the peptides against human red blood cells. Symbols: K<sub>6</sub>L<sub>4</sub>W<sub>1</sub> (●); (K<sub>6</sub>L<sub>4</sub>W<sub>1</sub>)<sub>2</sub>-K (○).

hemolysis of the peptides against mammalian cells by measuring their abilities to cause lysis of human erythrocytes. Concentration-response curves for the hemolytic activity of the peptides are shown in Figure 1. For a quantitative measure of the hemolytic activity of the peptides, we introduced the minimal hemolytic concentration (MHC) defined as the lowest peptide concentration that produced 10% hemolysis (Table 2). The MHC value for (K<sub>6</sub>L<sub>4</sub>W<sub>1</sub>)<sub>2</sub>-K was 126 μg/mL. In contrast, K<sub>6</sub>L<sub>4</sub>W<sub>1</sub> did not cause hemolysis at the highest peptide concentration tested (400 μg/mL).

The therapeutic potential of peptide antimicrobial drugs lies in the ability of peptide to effectively kill bacterial cells without exhibiting significant cytotoxicity toward mammalian cells. This property is defined by the concept of the therapeutic index (TI) as a measure of the relative safety of the drug.<sup>13</sup> The TI of each peptide was calculated as the ratio of the MHC value to the GM (geometric mean of MICs against 6 selected microorganisms). When there was significant no hemolysis at the highest concentration tested (400 μg/mL),

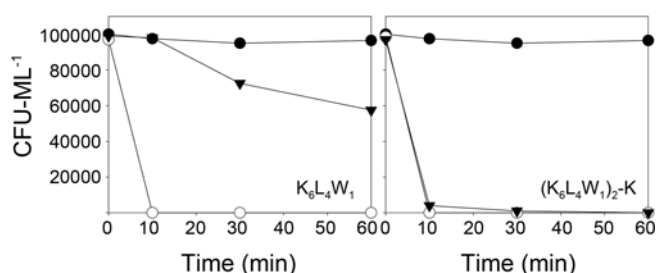
800 μg/mL was used for the TI calculation, since the test was carried out by two-fold serial dilution. A high therapeutic index is thus an indication of two preferred characteristics of the peptide: a high MHC (low hemolytic activity) and a low MIC (high antimicrobial activity). As shown in Table 2, our designed model peptides K<sub>6</sub>L<sub>4</sub>W<sub>1</sub> and (K<sub>6</sub>L<sub>4</sub>W<sub>1</sub>)<sub>2</sub>-K showed much higher TI value compared to the naturally-occurring AMP indolicidin. In addition, (K<sub>6</sub>L<sub>4</sub>W<sub>1</sub>)<sub>2</sub>-K had lower therapeutic index than K<sub>6</sub>L<sub>4</sub>W<sub>1</sub> because of its increased hemolytic activity.

NaCl was found to influence the MIC values of peptides against Gram-negative and Gram-positive bacteria similarly. This indicates that the inhibitory effects of monovalent Na<sup>+</sup> rely solely on interruption of the electrostatic attraction between the positively charged peptides and the negatively charged bacterial membranes.<sup>14</sup> To evaluate the effect of a Lys-linked dimerization of α-helical Leu/Lys-rich AMP on salt resistance, we examined the MICs of the peptides against six microorganisms and the bactericidal kinetics at MIC × 2 toward *E. coli* in the presence of 150 mM NaCl or absence. When judged in terms of MIC value (Table 3), the monomeric peptide, K<sub>6</sub>L<sub>4</sub>W<sub>1</sub> displayed 4- to 16-fold reduced antimicrobial activity in the presence of 150 mM NaCl, as compared in the absence of NaCl. In contrast, (K<sub>6</sub>L<sub>4</sub>W<sub>1</sub>)<sub>2</sub>-K exhibited nearly unaltered antimicrobial activity against both Gram-positive and Gram-negative bacteria in the presence of 150 mM NaCl. As observed in Figure 2, the bactericidal kinetics of (K<sub>6</sub>L<sub>4</sub>W<sub>1</sub>)<sub>2</sub>-K was not suppressed at 150 mM NaCl. In contrast, the bactericidal kinetics of K<sub>6</sub>L<sub>4</sub>W<sub>1</sub> was significantly inhibited at high salt concentration. Improving in salt resistance of (K<sub>6</sub>L<sub>4</sub>W<sub>1</sub>)<sub>2</sub>-K may be due to increase its net positive charge by Lys-linked dimerization. Our result indicates that a Lys-linked dimerization of α-helical AMP is a very useful tool in designing salt-resistant α-helical dimeric AMPs with antimicrobial activity.

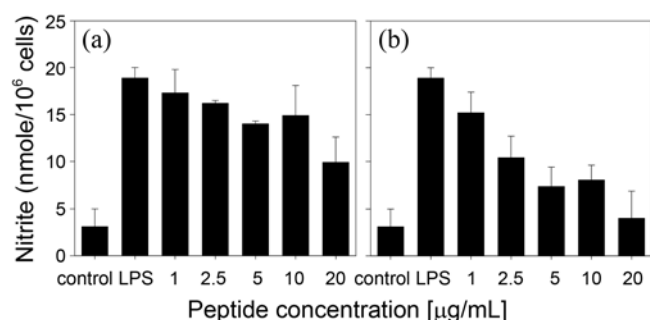
It had been demonstrated that release of LPS from antibiotic-treated Gram-negative bacteria can indeed enhance

**Table 3.** Antimicrobial activity of the designed model antimicrobial peptides in the presence of 150 mM NaCl

Peptide	MIC <sup>a</sup> (μg/mL)					
	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. typhimurium</i>	<i>B. subtilis</i>	<i>S. epidermidis</i>	<i>S. aureus</i>
K <sub>6</sub> L <sub>4</sub> W <sub>1</sub>	100	100	100	50	100	100
(K <sub>6</sub> L <sub>4</sub> W <sub>1</sub> ) <sub>2</sub> -K	12.5	12.5	6.25	6.25	12.5	6.25



**Figure 2.** Kinetics of the bactericidal activity of the peptides against *Escherichia coli*. Bacteria treated with the respective peptides ( $2 \times \text{MIC}$ ) were diluted at the indicated times and then plated on LB agar. The CFUs were then counted after 24 h of incubation at  $37^\circ\text{C}$ . Symbols: without peptide (●); 0 mM NaCl (○); 150 mM NaCl (▼).



**Figure 3.** Inhibitory activities of the peptides on LPS-stimulated nitric oxide (NO) production in RAW264.7 cells. RAW264.7 cells ( $5 \times 10^5$  cells/mL) were treated with 20 ng/mL LPS in the absence or presence of various concentrations (1.0  $\mu\text{g/mL}$ , 2.5  $\mu\text{g/mL}$ , 5  $\mu\text{g/mL}$ , 10  $\mu\text{g/mL}$  and 20  $\mu\text{g/mL}$ ) of  $\text{K}_6\text{L}_4\text{W}_1$  (a);  $(\text{K}_6\text{L}_4\text{W}_1)_2\text{-K}$  (b). The error bars represent standard deviations of the mean determined from three independent experiments.

sepsis.<sup>15</sup> Therefore, an effective antimicrobial agent should not only exert antimicrobial activity but also have the ability to neutralize LPS. To investigate whether  $\text{K}_6\text{L}_4\text{W}_1$  and its Lys-linked dimeric peptide  $(\text{K}_6\text{L}_4\text{W}_1)_2\text{-K}$  might have LPS-neutralizing activity, as well as potent antimicrobial activity, we assessed their ability to inhibit nitric oxide (NO) release in LPS-stimulated mouse macrophage RAW264.7 cells.  $\text{K}_6\text{L}_4\text{W}_1$  and  $(\text{K}_6\text{L}_4\text{W}_1)_2\text{-K}$  significantly inhibited NO production in LPS-stimulated mouse macrophage RAW264.7 cells at a concentration of 20  $\mu\text{g/mL}$  and 2.5  $\mu\text{g/mL}$ , respectively (Fig. 3). Rosenfeld *et al.* reported that the appropriate hydrophobicity of AMPs is important in LPS-neutralizing

**Table 4.** Percent  $\alpha$ -helical contents of the peptides in 50% TFE or 30 mM SDS

Peptide	50% TFE		30 mM SDS	
	$[\theta]_{222}$	% $\alpha$ -helical content	$[\theta]_{222}$	% $\alpha$ -helical content
$\text{K}_6\text{L}_4\text{W}_1$	-13303.7	37.7	-4599.5	8.7
$(\text{K}_6\text{L}_4\text{W}_1)_2\text{-K}$	-14449.3	41.5	-4708.4	9.0

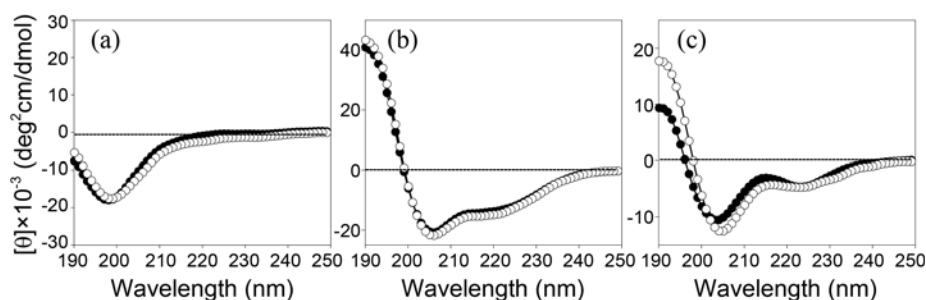
activity.<sup>16</sup> Therefore, improving in LPS-neutralizing activity of  $(\text{K}_6\text{L}_4\text{W}_1)_2\text{-K}$  may be due to increase its hydrophobicity by Lys-linked dimerization. Taken together, our results will be useful to design novel dimeric salt-resistant AMPs with potent antimicrobial and LPS-neutralizing activities.

To investigate the effect of a Lys-linked dimerization of  $\alpha$ -helical Leu/Lys-rich AMP,  $\text{K}_6\text{L}_4\text{W}_1$  on the secondary structure, the CD spectra of these peptides in the membrane-mimicking environments such as TFE or SDS were examined (Fig. 4). In aqueous buffer, CD spectra of  $\text{K}_6\text{L}_4\text{W}_1$  and  $(\text{K}_6\text{L}_4\text{W}_1)_2\text{-K}$  were characteristic of unordered structure. However, in the presence of TFE or SDS, their spectra had the typical appearance of  $\alpha$ -helical structures, with dichroic minimal values at 208 and 222 nm and a maximum near 194 nm. In membrane-mimicking environments,  $\text{K}_6\text{L}_4\text{W}_1$  and  $(\text{K}_6\text{L}_4\text{W}_1)_2\text{-K}$  had virtually indistinguishable CD spectra (Fig. 4 and Table 4), indicating that the helical content of the membrane-bound monomer was unaffected by Lys-linked dimerization.

In summary, we successfully developed a novel salt-resistant  $\alpha$ -helical dimeric AMP,  $(\text{K}_6\text{L}_4\text{W}_1)_2\text{-K}$  with potent antimicrobial and *anti*-inflammatory activities. Therefore, a Lys-linked dimeric peptide,  $(\text{K}_6\text{L}_4\text{W}_1)_2\text{-K}$  can potentially serve as a promising therapeutic agent for the treatment of microbial infection. Taken together, our results will help in designing of salt-resistant  $\alpha$ -helical dimeric AMPs with potent LPS-neutralizing and antimicrobial activities.

## Experimental Section

**Peptide Synthesis.** Peptides listed in Table 1 were synthesized by Fmoc-based solid phase method. DCC and HOBt were used as coupling reagents, and 10-fold excess of Fmoc-amino acids was added during every coupling cycle. After cleavage and deprotection with a mixture of trifluoroacetic acid/ $\text{H}_2\text{O}$ /thioanisole/phenol/ethanedithiol/triisopropylsilane



**Figure 4.** CD spectra of the peptides in 10 mM sodium phosphate buffer (pH 7.2) (a), 50% TFE (b) or 30 mM SDS (c). Symbols:  $\text{K}_6\text{L}_4\text{W}_1$  (●);  $(\text{K}_6\text{L}_4\text{W}_1)_2\text{-K}$  (○).

(81.5:5:5:5:2.5:1, v/v) for 2 h at room temperature, the crude peptides were purified by reverse-phase high performance liquid chromatography (RP-HPLC) on a preparative Vydac C<sub>18</sub> column (15 μm, 20 × 250 mm) using an appropriate 0-90% H<sub>2</sub>O/CH<sub>3</sub>CN gradient in the presence of 0.05% TFA. The molecular masses of purified peptides were determined using MALDI-TOF MS (Shimadzu, Japan) (Table 1).

**Antimicrobial Activity.** The antimicrobial activity of the peptides was examined by using the broth microdilution method, as described in the previous study.<sup>17</sup> Aliquots (100 μL) of a bacterial suspension at 2 × 10<sup>6</sup> colony-forming units (CFU)/mL in 1% peptone with 0 or 150 mM NaCl were added to 100 μL of the peptide solution (serial 2-fold dilutions in 1% peptone). After incubation for 18-20 h at 37 °C, bacterial growth inhibition was determined by measuring the absorbance at 600 nm with a Microplate Autoreader EL 800 (Bio-Tek Instruments, VT). The minimal inhibitory concentration (MIC) was defined as the minimum peptide concentration inhibited bacteria growth. All bacterial strains were supplied from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology (KRIBB).

**Bactericidal Kinetics.** The kinetics of the peptides' bactericidal activity was assessed using *E. coli* [KCTC 1682] at MIC × 2 in the presence of 0 or 150 mM NaCl. The initial density of the cultures was approximately 1 × 10<sup>5</sup> CFU/mL. After 0, 10, 30 or 60 min of exposure to the peptides at 37 °C, 50 μL aliquots of serial 10-fold dilutions (up to 10<sup>-3</sup>) of the cultures were plated onto LB agar plates to obtain viability counts. Colonies were counted after incubation for 24 h at 37 °C.

**Hemolytic Activity.** Fresh human red blood cells (hRBCs) were washed 3 times with PBS (35 mM phosphate buffer, 150 mM NaCl, pH 7.4) by centrifugation for 7 min at 1000 × g, and resuspended in PBS. The peptide solutions (serial 2-fold dilutions in PBS) were added to 100 μL of hRBC suspension [4% (v/v) in final] in PBS to a final volume of 200 μL, and incubated for 1 h at 37 °C. Samples were centrifuged at 1000 × g for 5 min, and hemoglobin release was monitored by measuring the supernatant absorbance at 405 nm with a Microplate ELISA Reader (Bio-Tek Instruments, VT, USA). Minimal hemolytic concentration (MHC) was defined as the minimal peptide concentration that produced 10% hemolysis. hRBCs in PBS (A<sub>blank</sub>) or 0.1% Triton X-100 (A<sub>triton</sub>) were used as the negative and positive controls, respectively. The hemolysis percentage was calculated according to the equation:

$$\% \text{ hemolysis} = 100 \times [(A_{\text{sample}} - A_{\text{blank}}) / (A_{\text{triton}} - A_{\text{blank}})].$$

**Measurement of Nitric Oxide (NO) Release from LPS-induced RAW264.7 Cells.** RAW264.7 macrophages were cultured overnight in 96-wells plate (5 × 10<sup>5</sup> cells/well). The medium was then removed followed by the addition to each well of fresh DMEM supplemented with 5% of bovine serum. The cells were stimulated with LPS (20 ng/mL) in the presence or absence of peptides. Cells that were stimulated with LPS alone, and untreated cells served as controls.

After incubating for 24 h, the amount of NO in the supernatant was estimated from the accumulation of the stable NO metabolite nitrite with Griess reagent according to the manufacturers' instructions (1% sulfanilic acid, 0.1% N-1-Naphthylethylenediamine dihydrochloride, and 5% phosphoric acid). Absorbance was measured at 540 nm.

**Circular Dichroism (CD) Spectroscopy.** CD spectra of the peptides were recorded using a Jasco J-720 spectropolarimeter (Japan). All peptide samples were maintained at 25 °C during analysis. Four scans per sample performed over wavelength range 190-240 nm at 0.1 nm intervals. The spectra were measured in 10 mM sodium phosphate buffer (pH 7.2), 50% TFE (trifluoroethanol) or 30mM SDS (sodium dodecyl sulfate) at 25 °C using a 1 mm pathlength cell. The peptide concentrations were 100 μg/mL. The mean residue ellipticity, [θ], is given in deg·cm<sup>2</sup>·dmol<sup>-1</sup>: [θ] = [θ]<sub>obs</sub> (MRW/10lc), where [θ]<sub>obs</sub> is the ellipticity measured in millidegree, MRW is the mean residue molecular weight of the peptide, c is the concentration of the sample in mg/mL, and l is the optical path length of the cell in cm. The spectra are expressed as molar ellipticity [θ] vs. wavelength. The percentage of α-helical structure was calculated as follows:

$$\% \alpha\text{-helical content} = ([\theta]_{222} - [\theta]_{222}^0) / ([\theta]_{222}^{100} - [\theta]_{222}^0) \times 100$$

where [θ]<sub>222</sub> is the experimentally observed mean residue ellipticity at 222 nm, and values for [θ]<sub>222</sub><sup>0</sup> and [θ]<sub>222</sub><sup>100</sup>, which correspond to 0% and 100% helix content at 222 nm, are estimated to be -2000 and -32000, respectively.<sup>18</sup>

## References

- Hancock, R. E.; Diamond, G. *Trends Microbiol.* **2000**, *8*, 402.
- Jenssen, H.; Hamill, P.; Hancock, R. E. *Clin. Microbiol.* **2006**, *19*, 491.
- Goldman, M. J.; Anderson, G. M.; Stolzenberg, E. D.; Kari, U. P.; Zasloff, M.; Wilson, J. M. *Cell* **1997**, *88*, 553.
- Wu, M.; Maier, E.; Benz, R.; Hancock, R. E. *Biochemistry* **1999**, *38*, 7235.
- Lee, I. H.; Cho, Y.; Lehrer, R. I. *Infect. Immun.* **1997**, *65*, 2898.
- Zasloff, M. *Nature* **2002**, *415*, 389.
- Raetz, C. R.; Whitfield, C. *Annu. Rev. Biochem.* **2002**, *71*, 635-700.
- Bowdish, D. M.; Davidson, D. J.; Scott, M. G.; Hancock, R. E. *Antimicrob. Agents Chemother.* **2005**, *49*, 1727.
- Rosenfeld, Y.; Papo, N.; Shai, Y. *J. Biol. Chem.* **2006**, *281*, 1636.
- Kim, S.; Kim, S. S.; Lee, B. J. *Peptides* **2005**, *26*, 2050.
- Jang, W. S.; Kim, C. H.; Kim, K. N.; Park, S. Y.; Lee, J. H.; Son, S. M.; Lee, I. H. *Antimicrob. Agents Chemother.* **2003**, *47*, 2481.
- Tencza, S. B.; Creighton, D. J.; Yuan, T.; Vogel, H. J.; Montelaro, R. C.; Mietzner, T. A. *J. Antimicrob. Chemother.* **1999**, *44*, 33.
- Chen, Y.; Mant, C. T.; Farmer, S. W.; Hancock, R. E.; Vasil, M. L.; Hodges, R. S. *J. Biol. Chem.* **2005**, *280*, 12316.
- Goldman, M. J.; Anderson, G. M.; Stolzenberg, E. D.; Kari, U. P.; Zasloff, M.; Wilson, J. M. *Cell* **1997**, *88*, 553.
- Hancock, R. E.; Scott, M. G. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 8856.
- Rosenfeld, Y.; Lev, N.; Shai, Y. *Biochemistry* **2010**, *49*, 853.
- Bang, J. K.; Nan, Y. H.; Lee, E. K.; Shin, S. Y. *Bull. Korean Chem. Soc.* **2010**, *31*, 2509.
- Wu, C. S.; Ikeda, K.; Yang, J. T. *Biochemistry* **1981**, *20*, 566.