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Elucidating the mechanism of peptides interaction with membranes using the intrinsic fluorescence of tryptophan: Perpendicular penetration of cecropin B-like peptides into *Pseudomonas aeruginosa*

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The importance of small molecular weight antimicrobial peptides as novel therapeutic agents stems from their ability to act against bacteria, viruses, and fungi. As part of the innate immune system, they are also capable of killing cancerous cells. Herein, we study the interaction between a synthetic cecropin B peptide and a target *Pseudomonas aeruginosa* membrane (PA) using steady-state and time-resolved fluorescence measurements in order to elucidate the mechanism of membrane rupture. The importance of synthetic cecropin B as a therapeutic peptide stems from its effect against a wide range of bacteria which is indistinguishable from that of naturally occuring cecropins. Fluorescence of cecropin B results from the sole tryptophan residue in the peptide. In order to understand the mechansim of peptide-membrane binding, we modified the original peptide (cecropin B1: **KWKVFKKIEKMGRNIRNGIV**) by attaching a terminal tryptophan residue (cecropin B2: **KWKVFKKIEKMGRNIRNGIVW**). Both peptides show a large inhibition effect against a wide range of bacteria, compared to naturally occurring peptides. The fluorescence results show an enhancement in the peak intensity of cecropin B1 upon mixing with the membrane, accompanied by a blue shift. For cecropin B2, a blue shift was observed upon mixing with the PA membrane, but no enhancement in intensity was observed. The results indicate perpendicular penetration of cecropins B1 and B2 from the Lys side where the Trp residue of cecropin B1 is immersed in the PA membrane. Partial quenching of the Trp fluorescence by acrylamide was observed and the values of the Stern-Volmer constants (K_{sv}) indicate that the Trp molecule penetrates into the membrane, but resides close to the interface region. Two fluorescence lifetimes were measured for the cecropin B1-PA complex which are for two rotamers of Trp. The results point to a degree of flexibility of the local environment around the Trp molecule. A mechanism of membrane disruption is proposed in which the cecropin peptide creates cracks through the negatively charged outer membrane of PA.

1 Introduction

Antimicrobial peptides (AMPs) are oligopeptides consisting of amino acid residues and can be found in both prokaryotes and eukaryotes.[1,2] To date, more than 5,000 AMPs have been discovered and characterized.[3] Cecropins are lytic antimicrobial peptides originally isolated from the haemolymph of the *Hylophora cecropia*.[4] Cecropins were first isolated in 1980 and usually are of 35 to 37 residues length.[5] Cecropin B was found to be not only effective against a range of gram positive and gram negative bacteria but also cytotoxic to a range of mammalian cancer and non-cancer cell-lines.[6] Attempts to generate recombinant and chemically synthesized cecropin B were successful and the results indicate that naturally produced and synthetic cecropins are indistinguishable in terms of their antibacterial activity.[7] Cecropin B and its analogs showed to penetrate bacterial cell membranes and this permeabilization depends on the liposome composition in the targeted cells.[8] The structure of cecropin A has been studied previously by NMR spectroscopy and shown to consist of an amphiapathic α-helical N-terminus (which plays the main role in the antibacterial activity of cecropins), a glycine-proline bend and a hydrophobic C-terminal α-helix.[9] Unlike other amphiapathic α-helical peptides such as maganin[10] and dermaseptin,[11] cecropins do not lyse erythrocytes. The mode of action of cecropin is membrane permeabilization via peptide-lipid interaction rather than receptor-mediated recognition.[12]

In the present study, we investigate a model of peptide-membrane interaction using synthetic cecropin B-like peptides against the outer membrane of *Pseudomonas aeruginosa*. The study is based on steady-state and time-resolved fluorescence measurements, utilizing the fluorescence change of the sole tryptophan residue in cecropin B. The peptide has 20 amino acids with the following sequence: **KWKVFKKIEKMGRNIRNGIV**. In order to better understand the mechanism of interaction, we modified the peptide and attached a second tryptophan residue to the C-terminal residue (**KWKVFKKIEKMGRNIRNGIVW**). Accordingly, we named the former cecropin B1 and the latter cecropin B2. Table 1 includes the physical and chemical characteristics of the peptides, compared with the natural peptide (cecropin B). From the fluorescence results using the two model peptides, there is clear evidence of perpendicular penetration of the active peptide (cecropin B1) into the bacterial membrane.

One specific target membrane was isolated from *Pseudomonas aeruginosa* to represent gram negative group of bacteria that was thinner than the gram positive ones to investigate the action of cecropins B1 and B2. The binding model of the cecropin B1 peptide to the outer membrane of the *Pseudomonas aeruginosa* membrane can be applied with clarity to many other bacterial membrane disruptions rather than just relying on the DNA fluorescence variation by the permeabilization assay used previously.[13] The mechanism of action of the cecropins is proposed and illustrated in this paper

based on the inhibitory results from this study and past models mentioned in the literature.

2 Experimental and theoretical methods

2.1 Peptides synthesis

The cecropins B1 and B2 were synthesized at Mimotopes Pty Ltd, Australia using the 20 and 21 amino acid sequences namely KWKVFKKIEKMGRNIRNGIV (20 residues with a molecular weight of 2444.6 Da) and KWKVFKKIEKMGRNIRNGIVW (21 residues, with a molecular weight of 2630.6 Da), respectively. The purity of both peptides was checked by HPLC and was found to be ≥ 98%.

2.2 Peptides: Minimal inhibitory concentration (MIC) and bactericidal inhibitory concentration (MBC)

Minimal inhibitory concentration for Ampicillin, Penicillin G and Tetracyclin was performed according to Clinical and Laboratory Standards Institute (CLSI) using the broth microdilution method. MIC test of cationic antimicrobial peptides (synthetic cecropins B1 & B2 and nisin) was performed following the R.E.W. Hancock Laboratory protocol (Minimal Inhibitory Concentration Determination for Cationic Antimicrobial Peptides by Modified Microtitre Broth Dilution Method) with some modifications. Briefly, indicator strains namely clinical isolates *Pseudomonas aeruginosa* PA7, *Escherichia coli* UT181, *Staphylococcus aureus* RF122, *Corynebacterium spp* GH17 and *Bacillus cereus* ATCC14579 were subcultured on MHA and incubated for 18 h at 37°C. Cultures were diluted with MHB to give 7 x 10^5 colony forming units/ml. 100 µl of each bacterial suspension was transferred to 10 polypropylene Eppendorf tubes and one tube was filled with MHB as a blank without bacteria. Serial dilutions of test cationic peptides were prepared at 10 times the required test concentrations in 0.01% acetic acid, 0.2% BSA in polypropylene Eppendorf tubes and 11 µl of 10X test peptide was added to the first nine bacterial tubes mentioned above. The tenth tube with bacterial growth was used as control. The tubes were incubated for 18 h at 37°C. MIC was taken as the lowest concentration of the peptide that reduces growth by more than 50%.

2.3 Isolation of outer membrane of *Pseudomonas aeruginosa*

Cells from a 250 ml culture of *Pseudomonas aeruginosa* were resuspended in 7 ml of 10 mM HEPES buffer (pH 7.4) and lysed by sonication for 20 s and then left at 4˚C for 20 s. The step was repeated 4 times. The suspension was then centrifuged at 10,000 rpm for 20 minutes at 4˚C to remove the cell debris. The membranes in the supernatant were collected by ultracentrifugation at 100,000 g for 1 h at 4˚C (Beckman Coulter, SW 41 Ti Ultracentrifuge Rotor Swinging Bucket). The pellets were resuspended in 2 ml of 10 mM HEPES at pH 7.4 and washed twice in 10 ml of 10 mM HEPES at pH 7.4. After final ultracentrifugation, the pellets were resuspended in 2 ml of 10 mM HEPES at pH 7.4 to concentrate the membranes.

2.4 Instrumentation for fluorescence measurements

Fluorescence spectra were recorded on a Shimadzu RF-5301 PC spectrofluorophotometer. Lifetime measurements were performed using a TimeMaster fluorescence lifetime spectrometer obtained from Photon Technology International. Excitation was done at 295 nm using a light-emitting diode and emission was detected using a Schott WG-320 nm cut-off filter. The instrument response function (IRF) was measured from the scattered light and estimated to be approximately 1.5 ns (full width at half-maximum). The measured transients were fitted to multiexponential functions convoluted with the system response function. The fit was judged by the value of the reduced chi-squared ($\chi 2$). The experimental time resolution (after deconvolution) was approximately 100 ps, using stroboscopic detection.[14] In all the experiments, samples were measured in a 1 cm path-length quartz cell at 23 ± 1 °C. The concentration of all species (*Pseudomonas aeruginosa* membrane, cecropins B1 and B2) was 0.05 mM in a phosphate buffer (10 mM) of pH 7.4. The reported values are the average of three measurements.

3 Results

Both synthetic cecropins B1 and B2 showed no inhibitory activity toward *B. cereus* in this test. On the other hand, significant activity was achieved when *Corynebacterium* spp was used as an indicator strain with MIC values of 10.9 and 26.5 for both cecropins B1 and B2, respectively. However, there were some differences in the MIC values of cecropins B1 and B2 when *S. aureus* was used. The full MIC values of synthetic cecropins B1 and B2 and other antimicrobial agents are listed in Table 2.

3.1 Minimal Inhibitory and Bacteriocidal Concentration (MIC & MBC)

Synthetic cecropins B1 and B2 showed strong inhibitory activity toward all gram negative and gram positive bacterial strains used in this study. It was observed that cecropin B2 showed stronger activity towards *S. aureus* (MIC = 1.656 µg/ml) than B1 (MIC = 3 µg/ml). Both cecropins B1 and B2 showed approximately similar MIC values against *P. aeruginosa* (MIC = 0.75 µg/ml and 0.828 µg/ml respectively. Full MIC values of synthetic cecropins B1 and B2 and other antimicrobial agents are listed in Table 2.

However, cecropin B2 showed a strong activity against *E.coli* with MIC value of just 0.207 µg that reflects the role of the tryptophan residue in increasing the effectiveness of the peptide. *Corynebacterium spp* is known for its high sensitivity to antimicrobial peptides as shown by the results in this study for both cecropins B1 and B2. *S. aureus* showed more sensitivity to cecropin B2 than cecropin B1. *B. cereus* was inhibited at the same MIC values with both cecropins B1 and B2. Nisin is known to be effective on gram positive microorganisms as shown in this study. However, it did not inhibit gram negative bacteria *P. aeruginosa* and *E. coli* in this study, reflecting the difference in mechanism of action of the cationic antimicrobial peptides. A previous comparative study between the Hancock method used in this study and CLSI standard method showed that the former exhibited lower MIC values for cationic peptides cecropin P1 and nisin.[15] A similar comparative study in our laboratory proved this fact (data not shown).

3.2 Fluorescence quenching

Interaction of the cecropins B1 and B2 peptides with the *Pseudomonas aeruginosa* (PA) membrane was investigated by following the fluorescence change of tryptophan in energy and

time domains. One factor affecting the tryptophan fluorescence is the polarity of its surrounding environment. We have shown recently that the sensitivity of the fluorescence peak position of tryptophan is clearly dependent on the solvent polarity.[16] In a solvent such as 1,4-dioxane (an apolar solvent),[17] fluorescence is peaked at 334 nm, whereas this peak is red-shifted as the solvent polarity increases. The maximum red shift was observed in aqueous solution (peak at 355 nm). The detected unstructured fluorescence in all the solvents is due to the ^1L_a state of tryptophan. This state is solvent-sensitive because the ^1L_a transition more directly involves the polar nitrogen atom of the indole ring in the tryptophan molecule.[17]

Figure 1 shows the steady-state fluorescence spectra of cecropin B1 with and without the PA membrane. The spectra show a blue shift (peak at 350 nm) and an increase in the fluorescence intensity when the peptide cecropin B1 is added to the membrane (compared with the peptide in buffer). This is a typical pattern when tryptophan is transferred from aqueous solution with free motion (peak at 355 nm) to a more hydrophobic environment with decreased flexibility. The results indicate some interaction between the sole tryptophan residue in the peptide with the membrane. In order to clarify the mode of binding between the cecropin B1 peptide and the membrane, we tested the modified peptide cecropin B2 which carries an additional terminal tryptophan as shown in its sequence above. The fluorescence spectra for the new peptide show a slight blue shift when mixed with the membrane, but no increase in intensity, compared to the peptide in buffer (Figure 1). It has been reported that peptides with multi-tryptophan residues produce fluorescence spectra that are difficult to interpret.[18] Nevertheless, comparing the spectral change when both peptides are mixed with the membrane shows that the mode of binding between the peptide and the membrane is vertical (penetration) and not parallel. If the latter is the case, an increase in the fluorescence peak intensity should be observed for cecropin B2-PA because both tryptophan molecules are expected to be in direct contact with the membrane.

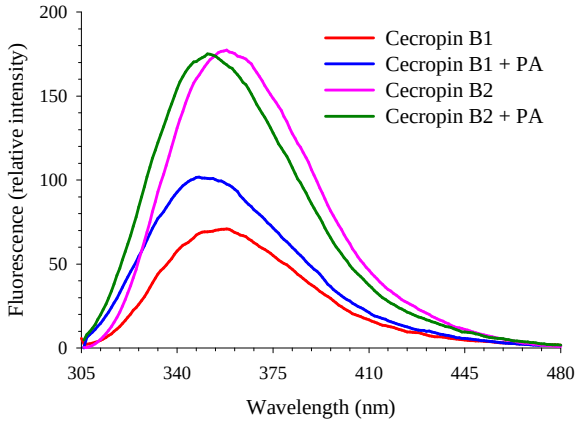

Fig. 1 Fluorescence spectra of the synthetic peptides cecropins B1 and B2 in the absence and present of the PA membrane (1:1 molar ratio). The solvent was 10 mM HEPES buffer of pH 7.4 λ_{ex} = 295 nm. The concentration of all species was 0.05 mM.

The results indicate that the cecropin B1 peptide penetrates the membrane from the Lys side. In this case, the terminal tryptophan in cecropin B2 will not be in direct contact with the membrane. This is in agreement with the inhibition results shown above (Tables 2 and 3) in which both cecropin B1 and cecropin B2 show approximately similar effect with PA. We confirmed the mode of binding by carrying out a quenching experiment using the water-soluble fluorescence quencher acrylamide. Acrylamide is known to quench the fluorescence of tryptophan (and indole) without penetrating into the membranes or the lipid bilayers[19,20] As shown in Figure 2, quenching of tryptophan fluorescence is less effective when the peptide is mixed with the membrane (compared to free peptide in buffer). However, quenching is still detected for the peptide/membrane mixture, indicating that the tryptophan molecule is not fully sequestered in the hydrophobic interior of the membrane. We can then conclude that the tryptophan residue penetrates into the membrane, but resides close to the interface region.

The Stern-Volmer constants (K_{sv}) were calculated from the relationship $F_0/F = 1 + K_{sv}[Q]$, where F_0 is the fluorescence intensity of the peptide in the absence of acrylamide (Q), F is the fluorescence intensity of the peptide in the presence of increasing concentrations of acrylamide, and [Q] is the acrylamide concentration. From the slopes of the graphs in Figure 2, K_{sv} was estimated to be 1735 ± 80 M^{-1} for cecropin B1 free in buffer, and 589 ± 50 M^{-1} for cecropin B1 bound to the membrane. The values reflect the reduced quenching effect on the tryptophan fluorescence when the cecropin B1 peptide is bound to the PA membrane. It should be noted here that if there is any free peptide in solution, the K_{sv} value will be altered. To avoid this, an excess membrane concentration should be used compared to that of the peptide. However, excess membrane concentration may enhance aggregation and the results may not be reliable. Also, high concentration of the peptide may lead to self-association which was reported for other peptides to take place at concentrations ≥ 0.1 mM (using circular dichroism measurements).[18] To avoid aggregation, we performed all the fluorescence experiments at low concentrations of both the membrane and the peptide (0.05 mM; 1:1 molar ratio).

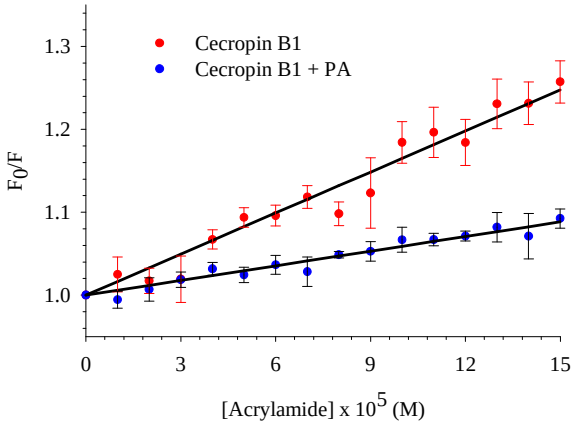

Fig. 2 Stern-Volmar plots for the fluorescence quenching of tryptophan in cecropin B1 by acrylamide in the absence and presence of the PA membrane. The concentration of PA and cecropin B1 was 0.05 mM (1:1 molar ratio). λ_{ex} = 295 nm.

Time-resolved fluorescence decay transients are shown in Figure 3 for cecropin B1 in the absence and presence of the PA membrane. As shown in the figure, the overall fluorescence decay is slightly slower in the presence of the

membrane, indicating less exposure of the tryptophan residue to buffer.

The lifetime data from our measurements are summarized in Table 4. Most of the reported data for tryptophan decay in aqueous solution indicate two lifetimes of ~ 3.1 and ~ 0.5 ns.[17,21,22] As shown in Table 4, two lifetimes were measured when cecropin B1 is mixed with the PA membrane. The biexponential nature of the fluorescence decay establishes strong support for the heterogeneity in the ground state of tryptophan. This heterogeneity indicates that the membrane has a degree of flexibility that allows the tryptophan side chain to exist in two different rotamers. The effect of the membrane is observed in an increase of about 20% in the long lifetime component (compared to the same component in the absence of PA). The relative contribution from the short component is also slightly increased in the presence of PA. The overall average lifetime for cecropin B1 ($\tau_{average}$ in Table 4) is increased in the presence of the PA membrane by about 15%, compared to the average lifetime in buffer. This observation is in line with the mechanism of binding mentioned above in which the cecropin B1 peptide penetrates the membrane, yet stays close to the interface. The slight increase in the tryptophan average lifetime is a consequence of a small degree of isolation inside the membrane.

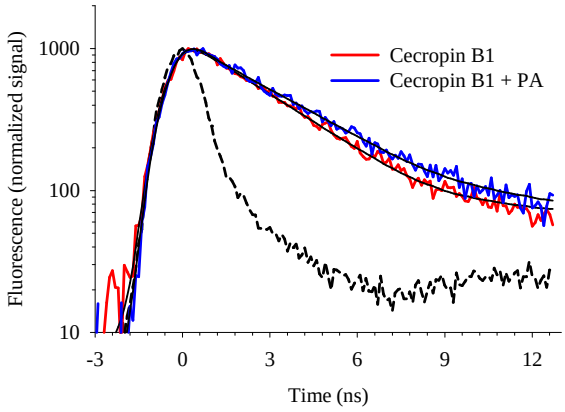

Fig. 3 Fluorescence decay transients of cecropin B1 in the absence and presence of the PA membrane. λ_{ex} = 295 nm. Fluorescence was detected using a Schott WG-320 nm cut-off filter. IRF is shown by a dashed line. The best fits are shown in black solid lines.

4 Discussion

Boosting microbicidal potency was achieved through end-tagging of antimicrobial peptides with hydrophobic oligopeptides.[23] A larger effect was reported for Trp and Phe stretches than for aliphatic ones. The enhancement of microbicidal effects was correlated to a higher degree of bacterial wall-rupture. Linking more than one Trp residues (up to five units) to the C-terminal end of some peptides shows substantial peptide adsorption, membrane lysis, and bacterial killing.[24] It was observed that increasing the peptide helicity correlates with increasing antimicrobial potency.[25] The effect of Trp substitutions was demonstrated to increase the peptide's helicity and amphiphilicity, thus resulting in higher peptide adsorption, increased peptide-induced liposome leakage and antimicrobial potency.[24,26]

The current results indicate that cecropin B1 shows a slight enhancement in MIC and MBC, as indicated in Tables 2 and 3, respectively. Adding a second Trp to the opposite end of the lipid did not show any induced enhancement in such activities. This is in line with our fluorescence results which indicate that the Trp residue in cecropin B1 is interacting with the *P. aeruginosa* membrane, whereas the second Trp residue in cecropin B2 is not in direct contact with the membrane. The observed similar blue shift in the fluorescence peak of both cecropin peptides points to a vertical penetration from the Lys side in which the terminal Trp in cecropin B2 is expected to stay far from the membrane.

It was shown in a previous study that elimination of the peptide positive net charge drastically reduced bactericidal effect on *P.* aeruginosa.[27] However, short analogues of cecropin B presented in this study with slightly reduced net charge (Table 1) retained the bactericidal activity against the proposed pathogen namely *P. aeruginosa.*

The most possible mechanism of membrane disruption is thus shown in Fig. 4 whereby unfolded cationic cecropins tend to create cracks through the negatively charged outer membrane of *P. aeruginosa* by neutralizing the charge over a patch of the outer membrane. The cecropins can directly attach to the cation binding sites on the lipopolysaccharides and depolarize the membrane. After transiting through the outer membrane, cecropins bind to the negatively charged phospholipids where this cationic peptides fold to their amphipathic structure. After insertion into the membrane interface, the cationic cecropins will either aggregate into a micelle-like complex and span across the membrane or flip-flop across the membrane driven by the transmembrane electrical potential gradient (approximately – 140 mV).[28] This causes a huge leakage of the ions and other water-soluble molecules, thereby compromising the cell integrity.

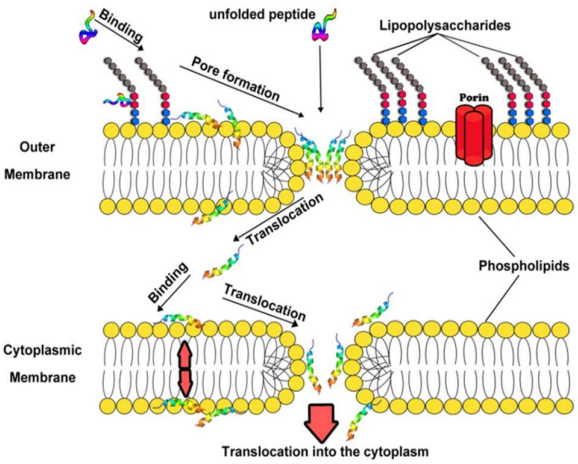

Fig. 4 Proposed mechanism of interaction of cecropin-like peptides with the cell envelope of *P. aeruginosa*.

5 Conclusions

In this work, the interaction of synthetic cecropin B with *P. aeruginosa* was studied using fluorescence measurements to detect the change in the fluorescence signal of the intrinsic Trp residue in the peptide chain. The original synthetic peptide (cecropin B1) was modified by attaching a second Trp residue to the Val terminal (cecropin B2) in order to elucidate the mode of attack onto the *P. aeruginosa* membrane. The activity of both peptides was detected against a wide range of bacteria, compared to natural peptides. A fluorescence enhancement

and a blue shift was observed when cecropin B1 was mixed with *P. aeruginosa*, compared to the fluorescence peak of cecropin B1 in buffer. Only a blue shift was observed when cecropin B2 was used. The results indicate perpendicular penetration of the peptides into the membrane from the Lys side. We observed a partial quenching of the Trp fluorescence of cecropin B1 when acrylamide was added. The quenching is much less when the peptide is mixed with *P. aeruginosa*, confirming the perpendicular mode of penetration. The estimated values of the Stern-Volmer constants (K_{sv}) indicate that the Trp molecule of cecropin resides close to the interface region. A degree of flexibility inside the membrane was observed in the form of two fluorescence decay components for the Trp residue in which the molecule can adapt two different rotamers.

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Table 1 Comparison between the cecropin B-like peptides used in this study with the natural cecropin B peptide

Peptide	Amino acid sequence	Total hydrophobic ratio	Net charge	Molecular weight	Reference
Cecropin B	KWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL	48 %	+7	3835.0	Ref. 7
Cecropin B1	KWKVFKKIEKMGRNIRNGIV	40%	+6	2444.6	Current study
Cecropin B2	KWKVFKKIEKMGRNIRNGIVW	42%	+6	2630.6	Current study

Table 2 MIC values of different antimicrobial peptides

Antimicrobial	MIC values (µg/ml)				
	P. aeruginosa	*E. coli*	*S. aureus*	*Corynebacterium spp*	*B. cereus*
Synthetic cecropin B1	0.75 µg	0.75 µg	3 µg	0.09375 µg	1.5 µg
Synthetic cecropin B2	0.828 µg	0.207 µg	1.656 µg	0.103 µg	1.656 µg
Ampicillin	1000 µg	≤ 15.62 µg	≤ 15.62 µg	≤ 15.62 µg	2000 µg
Penicillin G	250 µg	3.90 µg	≤ 0.9 µg	≤ 0.9 µg	125 µg
Nisin A	NI*	NI*	0.734 µg	≤ 0.367 µg	2.937 µg
Tetracyclin	≤ 1.56 µg	≤ 1.56 µg	≤ 1.56 µg	≤ 1.56 µg	≤ 1.56 µg

*No Inhibition.

Table 3 Minimal bactericidal concentration of synthetic cecropins B1 and B2

Antimicrobial	MBC values				
	P. aeruginosa	*E. coli*	*S. aureus*	*Corynebacterium spp*	*B. cereus*
Synthetic cecropin B1	1.5 µg	1.5 µg	6 µg	0.09375 µg	6 µg
Synthetic cecropin B2	1.656 µg	0.414 µg	3.321 µg	0.09375 µg	3.312 µg

Table 4 Fluorescence spectral position and lifetime data[a]

	λ_{em}^{max} (nm)	τ_1[b]	α_1	τ_2[c]	α_2	$\tau_{average}$	χ^2
Synthetic cecropin B1	355	0.37	0.06	2.60	0.94	2.47	1.01
Synthetic cecropin B1 + PA	350	0.38	0.11	3.14	0.89	2.84	1.07

[a] λ_{ex} = 295 nm. Fluorescence was detected using a Schott WG-320 nm filter. [b] Uncertainty in measurements is ± 0.05 ns. [c] Uncertainty in measurements is ± 0.10 ns. Relative contributions are shown by the α values.