



IN VITRO ANTIMICROBIAL ACTIVITY OF CEREULIDE AND VALINOMYCIN COMPARE WITH CYCLIC D,L- α -PEPTIDES AND THEIR ENHANCED EFFECT

ARTHIT MAKARASEN¹, NANTHAWAN REUK-NGAM¹, PIYACHAT CHUYSINUAN¹, CHAIYAWAT AONSRI², NITIRAT CHIMNOI¹, MINORU ISOBE³ AND SUPANNA TECHASAKUL^{1,2,*}

¹Chulabhorn Research Institute, Vipavadee-Rangsit Highway, Bangkok 10210, Thailand

²The Center of Innovation in Chemistry (PERCH-CIC), Department of Chemistry, Kasetsart University, 50 Phaholyothin Road, Chatuchak, Bangkok 10900, Thailand

³National Tsing Hua University, Department of Chemistry Hsinchu, 30013, Taiwan

ABSTRACT

Cereulide and valinomycin are both cyclic depsipeptides with 12 stereogenic centers that have a very similar sequence in the structure. Both compounds are hexagonal cylinder-like framework and all the side chains stick outside of the framework. The framework of cyclic D,L- α -peptides were structured different from the cereulide and valinomycin which are performed by cyclic DDLL-peptides. The cyclic D,L- α -peptides consisted of an even number of alternating D- and L- α -amino acid which can adopt flat and stack to form hollow, β -sheet like tubular structures. The amino acid side chains are presented on the outside surface of the framework and the interior surface of such a tube is hydrophilic. In our studies, we found that the original cereulide and valinomycin demonstrated higher antimicrobial activity than the cyclic D,L- α -peptides which are synthesized by using cereulide and valinomycin as a model structure. Furthermore, the combinations of cereulide or valinomycin with clinically used drugs against fungi were found to enhance the antimicrobial efficacy by decreasing the MIC values between 2-128 times.

KEYWORDS Cereulide, Valinomycin, Cyclic peptide, D,L- α -peptides

*Corresponding author



SUPANNA TECHASAKUL
Chulabhorn Research Institute, Vipavadee-Rangsit Highway, Bangkok 10210, Thailand

INTRODUCTION

Peptides, a group of compounds consisting of two or more amino acids linked by peptide bond, are abundantly presented in living organisms. Thousands of peptides have been isolated from animals, plants and microorganisms. Based on their chemical structures, peptides can be divided into linear and cyclic peptides. Most of peptides isolated from plants and bacteria are cyclic peptides. Compared with linear peptides, cyclic peptides exhibit more potent biological activities, possibly due to the stable configuration provided by their cyclic structures. Pharmacological studies have proved that many peptides, including those isolated from plants and bacteria have a number of advantages over other chemical agents including their low molecular weight, relatively simple structure, lower antigenicity and fewer adverse actions, easy absorption, and a variety of route administration¹. In nature, cyclic peptides display high antimicrobial activity, which are also highly hemolytic and thus lack the selectivity required for a human antibiotic². Antibiotics produced by bacteria can be specific, effective only against closely related species, or broad spectrum, depending on their mode of action. Cereulide and valinomycin are both 36-membered cyclic depsipeptides with 12 stereogenic centers that have a very similar sequence of cyclo [-D-O-Leu-D-Ala-L-O-Val-L-Val-]₃ and cyclo [-D-O-Val-D-Val-L-O-Ala-L-Val-]₃, respectively. Cereulide produced by *Bacillus cereus* is known as an emetic toxin³⁻⁴. Valinomycin produced by *Streptomyces fulvissimus* is an antibiotic with a similar cyclic depsipeptide structure⁵. In three-dimensional structures of cereulide and valinomycin, the frameworks of these cyclic depsipeptides are very similar, these are composed of six peptides bonds and six ester bonds one after another with a stereochemistry of -(D-D-L-L)₃- with a three-fold symmetry axis. In both structures, three amide carbonyl groups in these compounds are arranged along the cylindrical side-wall planes, which can form β -turn hydrogen bonds with three NH protons. Each stereogenic alpha carbon is located at a corner in the top and bottom faces. The alpha protons of oxy acid turned inwards towards the cavity

whereas the alpha protons of amino acid turned outwards⁶. Both compounds synthesized through an unusual non-ribosomal peptide synthesis (NRPS). There are specific functions as a potassium-ion selective ionophore to affect the function of mitochondria in living cells via the K⁺-selective binding property causes vacuole formation. Cereulide and valinomycin induced mitochondrial swelling in the presence of K⁺ ion, which causes a potassium-dependent drop in the transmembrane inner membrane potential owing to uptake of a K⁺ ion as positively charged ionophore complex⁶⁻⁸. Recently, Isobe and co-workers reported that cereulide showed K⁺-ion-selective ionophore properties at concentrations two times lower than valinomycin⁹. Additionally, both compounds showed the antimicrobial activity against selected gram-positive bacteria and fungi¹⁰⁻¹¹. The synthetic cyclic D,L- α -peptides possess unique structural features not found in the natural class of peptides antibiotics and their derivatives. Cyclic peptides with an even number of alternating D- and L- α -amino acid can adopt flat, ring-shaped conformation, of which the backbone amide functionalities are oriented perpendicular to the side chains and the plane of the ring structure. This cyclic peptide can stack to form hollow, β -sheet like tubular structures that are open-ended, presenting the amino acid side chains on the outside surface of the framework and the interior surface of such a tube is hydrophilic, while the exterior surface is hydrophobic¹². Cyclic peptide nanotubes (CPNs) are a class of artificial channels formed by closed peptide rings which consist of an even number of alternating D- and L- amino acid residues¹³⁻¹⁵. The internal diameter and external surface properties of nanotube depend on the number and kinds of amino acid residues in the peptide ring. The hydrophobic side chains of the cyclic D,L- α -peptide are self-assembly, insert into lipid bilayers, and transport ions or guest molecules across the membrane. It is mean that the cyclic peptide nanotubes could act as highly selective and efficient transmembrane channels for ions and small molecules which is acted as similar as the cereulide and valinomycin¹⁶⁻¹⁸. The cyclic

peptide nanotubes have been shown to assemble on the bacterial membrane by using the hydrophobic side chains inserted into the lipidic components of the membrane and the

hydrophilic residues remaining exposed to the hydrophilic components of the cell membrane¹⁹⁻²⁰.

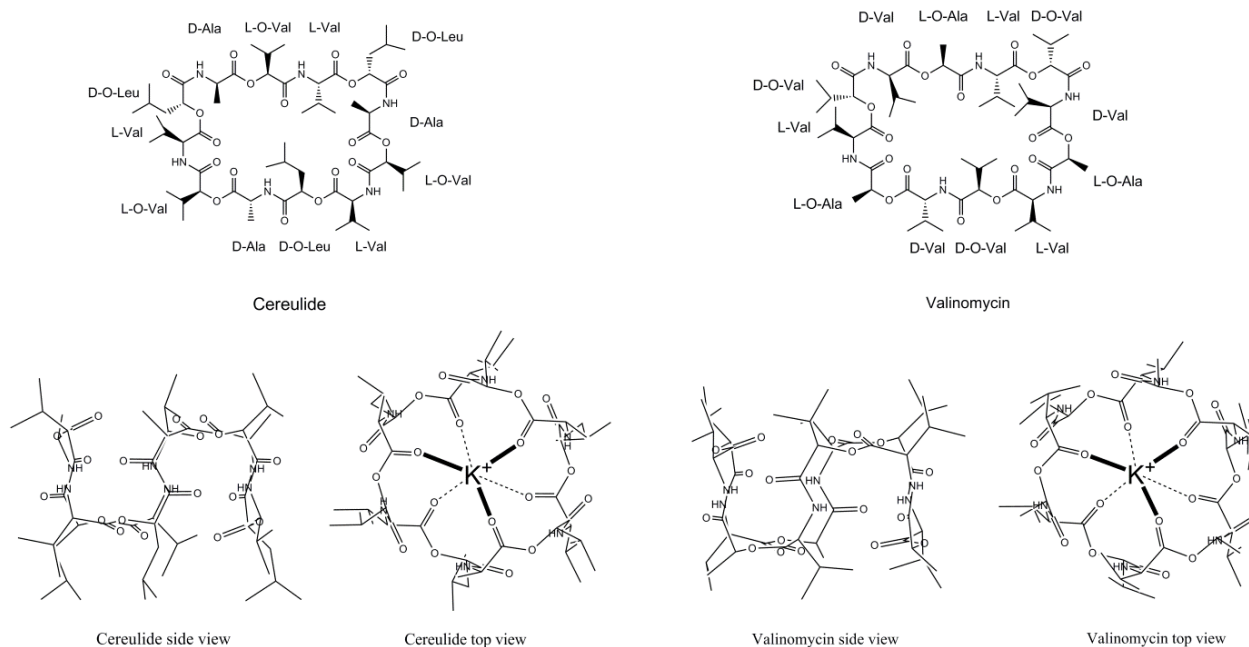


Figure 1
Framework of cereulide and valinomycin

Cereulide or valinomycin and cyclic D,L- α -peptides are both antimicrobial cyclic peptides that have a very similar structure as tubular frameworks when the cyclic D,L- α -peptides has formed like tubular structures (Figure 1). The comparison of the antimicrobial activity between cereulide or valinomycin and cyclic D,L- α -peptides has not been reported. Herein, we describe our efforts to compare the antimicrobial activity of cyclic D,L- α -peptides by using cereulide and valinomycin as a model structure with the original cereulide or valinomycin. The mimic cereulide and valinomycin which consisted of alternating D,L- amino acid with fully amide residue together with 12 stereogenic centers and the same kinds of amino acid residues as in the original cereulide or valinomycin were synthesized. Moreover, the alternating DD,LL- amino acids with fully amide residue were also synthesized to compare the antimicrobial activity with the original cereulide and valinomycin (Figure 2). All the cyclic peptide derivatives including the original structure as cereulide and valinomycin acted preferentially on gram-negative, gram-positive bacterial

cells and fungi which allowed for the determination of the impact on antimicrobial activity and their potential applications in the medical treatment. In addition, the wide use of antibiotics in the treatment of bacterial infections has led to the emergence and spread of resistant strains in human and animals thus, the antibiotic combinations have long been used to provide antimicrobial activity against multiple potential pathogens for initial empirical treatment of critically ill patients²¹. In the present, clinically important bacteria and fungi are characterized not only by single drug resistance, but also by multiple antibiotic resistances. Due to the emergence of multidrug-resistant pathogens, the combination treatment, using two or more antibiotic drugs, has become common. The aim of this study was compared the antimicrobial activity of all cyclic peptides and investigated their enhancement of biologically active cyclic peptides with clinically used antibiotic drugs in order to access their potential applications in the medical treatment toward the development of analogs that possess greater selectivity for bacterial cells.

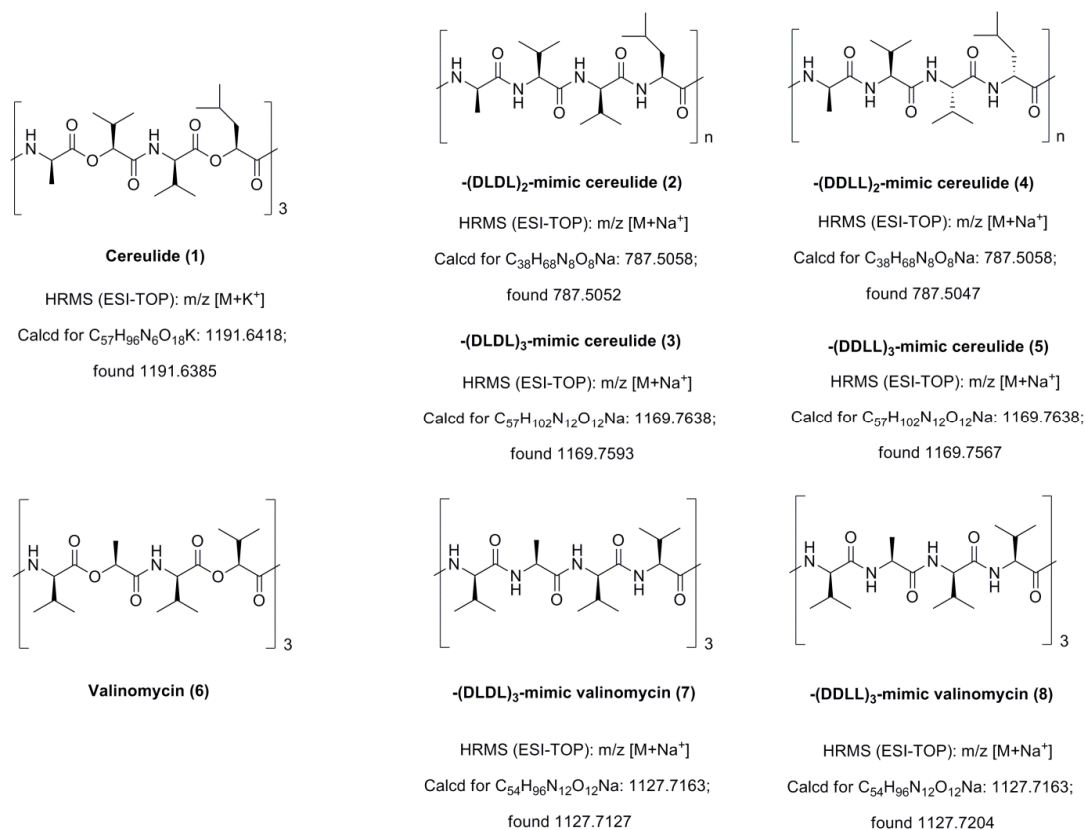


Figure 2

Structure and HRMS analysis of cyclic peptide; cereulide (1), -(DLDL)_n- mimic cereulide (2,3), -(DDLL)_n-mimic cereulide (4,5); valinomycin (6), -(DLDL)₃- mimic valinomycin (7), -(DDLL)₃-mimic valinomycin (8)

MATERIALS AND METHODS

(i) Chemicals

Cereulide 1 was synthesized as described by Isobe et al.²²⁻²³. Valinomycin 6 was purchased from Sigma Aldrich Co. Ltd. The mimic cereulide and valinomycin with fully amide residue 2-5, 7, 8 were obtained by using linear peptide synthesis in solution phase using amide formation in the methods for total synthesis of cereulide²³. All products were purified with medium-pressure liquid chromatography and analyzed to confirm from nuclear magnetic resonance (NMR) and mass spectrometry (MS). Proton and carbon NMR spectra were obtained using a Bruker Avance III-300 spectrometer at 300 MHz and 75 MHz, respectively. High-resolution (HR) mass spectra were measured with ESI-TOF, MicroTOF mass spectrometer (Bruker Daltonics, Germany). Medium-pressure liquid chromatography was performed on a Young Lin Acme 9000 HPLC system composed of

semi-prep gradient pump and semi-prep UV/Vis Detector. The apparatus was equipped with a YMC Diol-HG S-20 μm column (15 × 460 mm).

(ii) Susceptibility testing and antifungal agents

(ii. i) Microorganism and culture media

Gram-negative bacteria *Escherichia coli* (*E. coli*) TISTR 780, *Pseudomonas aeruginosa* (*P. aeruginosa*) TISTR 781, *Salmonella typhimurium* (*S. typhimurium*) TISTR 292, and *Shigella flexneri* (*S. flexneri*) ATCC 9199

Gram-positive bacteria *Staphylococcus aureus* (*S. aureus*) TISTR 1466, *Staphylococcus epidermidis* (*S. epidermidis*) TISTR 518, *Enterococcus faecalis* (*E. faecalis*) TISTR 379, and *Bacillus cereus* (*B. cereus*) TISTR 687

Fungi *Aspergillus niger* (*A. niger*) TISTR 3254, *Aspergillus flavus* (*A. flavus*) TISTR

3366, *Aspergillus sp.* TISIR 3105, *Acremonium sp.* TISTR 3487, and *Penicillium sp.* TISTR 3118

Yeast *Candida albicans (C. albicans)* TISTR 5779 and *Cryptococcus albidus (C. albidus)* TISTR 5684 Microorganisms were obtained from the culture collection center, Institute of Scientific and Technological Research (TISTR), Thailand. Microorganisms were used for antimicrobial test organisms. The bacteria were maintained on nutrient agar (NA) at 37°C and fungi were maintained on potato dextrose agar (PDA) at 28°C.

(ii. ii) Preparation of inoculum.

The tested bacteria were cultured in nutrient broth (NB) and incubated for 18-24 h at 37°C. The tested filamentous fungi and yeasts were prepared by grown for more than three days at 28 °C on PDA and Sabouraud Dextrose agar (SDA), respectively. The colonies were harvested, suspended in sterile saline, and their concentrations were adjusted to a 0.5 McFarland standard, the equivalence of $1-2 \times 10^8$ cfu/ml. Then the samples were further diluted 1:10,000 in Muller Hinton broth (MHB) or Sabouraud Dextrose broth (SDB) to 1×10^4 cfu/ml. For filamentous fungi (*A. niger*, *A. flavus*, *Aspergillus sp.*, *Acremonium sp.* and *Penicillium sp.*) and spore suspension were adjusted to 0.4×10^4 to 5×10^4 spores/ml in sterile saline. Oxacillin, gentamicin, ciprofloxacin were used as standards against bacteria strains. Amphotericin B and nystatin powder were used as standards against fungal strains. All standards antibiotic drugs were obtained from Sigma Chemicals Co., St. Louis, Mo. Antimicrobial agents were prepared as stock solutions at concentration of 4 mg/ml in DMSO for susceptibility and enhanced effect tested.

(ii. iii) Minimal inhibitory concentration (MIC) of the cyclic peptides

The minimal inhibitory concentration (MIC) was determined using the two-fold broth microdilution method in accordance with NCCLS guideline²⁴⁻²⁶. Concentrations of the cyclic peptides ranging from 0.39-200 µg/ml were used. After incubating 24 h for bacteria or 48-72 h for fungi, the lowest concentration of compounds that inhibited the growth of organism was considered as MIC. The experiment was performed in triplicate.

(iii) Enhancement of the cyclic peptides and the clinically used antibiotic drugs

(iii. i) Agar wells assay.

Sterile Glass Pasteur pipettes were used for cutting and removing agar for preparation of well on MHA plate. The surface was spread with 0.1 ml of microorganism culture (about 10^8 cfu/ml). The antibiotic and cyclic peptides at 0.5 MIC were used to combination assay in each well whereas 0.5 MIC at 200 µg/ml of the cyclic peptides were applied for the cyclic peptides which performed the MIC value greater than, or equal to 200 µg/ml. The plates were incubated at 37°C, 24 hours for bacteria and 28°C, 48-72 hours for fungi. The results were recorded by measuring the diameters of the zones of growth inhibition surrounding the well²⁷. The experiment was performed in triplicate.

(iii. ii) Minimal inhibitory concentration (MIC) of selected cyclic peptides combined with the clinically used antibiotic drugs.

The enhanced effect of the combinations was performed by serial two-fold dilution technique. Standard powder forms of amphotericin B and nystatin were stored at 2 to 8°C until use. The serial two-fold dilutions of each drug to at least double the MIC were prepared in each well and the synergistic cyclic peptides at 0.5 MIC were used to the combination assay. Each well was inoculated with 0.1 ml of 10^5 cfu/ml culture of the test microorganism and then incubated for 24 h at 37°C (bacteria) or 48 h at 30°C (fungi). The results were interpreted in triplicate.

RESULTS AND DISCUSSION

Cereulide was obtained by using the synthesis method as described in the literature²²⁻²³. The mimic cereulide and valinomycin with fully amide residue and the same kinds of amino acids with the cereulide or valinomycin can be synthesized by using the building block amino acids together with EDCI/HOBt as peptide coupling agents which are commercially available to synthesize dipeptide, tripeptide, tetrapeptide, respectively. The octapeptide and dodecapeptide were performed by coupling the tetrapeptide unit in two and three-times, respectively. However, column chromatography was necessary to obtain linear peptides in good purity. The linear

peptides were converted to the cyclic peptides using EDCI/HOBt under low concentration at 1.5 mM to provide desired cyclic peptides. All desired cyclic peptides were successfully carried out in moderate yield. The cyclization products were purified on short column chromatography and confirmed by ^1H NMR and mass spectral data. The characteristic NMR spectra of all the intermediate compounds were analyzed. ^1H NMR and ^{13}C NMR spectra of all cyclic products clearly indicate the presence of all respective amino acid moieties. The mass spectra data of final desired product are consistent with the molecular formula as shown in Figure 2. The original cereulide, valinomycin and desired cyclic peptides, cereulide 1, $-(\text{DLDL})_3$ -mimic cereulide 3, $-(\text{DDLL})_3$ -mimic cereulide 5, valinomycin 6, $-(\text{DLDL})_3$ -mimic valinomycin 7, $-(\text{DDLL})_3$ -mimic valinomycin 8, were tested for screening antimicrobial activity. The antibacterial and antifungal activities were carried out against the eight bacteria (*E. coli*, *P. aeruginosa*, *S. typhimurium*, *S. flexneri*, *S. aureus*, *S. epidermidis*, *E. faecalis* and *B. cereus*) and seven fungal strains (*A. niger*, *A. flavus*, *Aspergillus sp.*, *Acremonium sp.* and *Penicillium sp.*, *C. albicans*, *C. albidus*). The results (summarized in Table 1 and 2) indicated that cereulide 1 and valinomycin 6 was strongly active against only the bacterial strains *E. faecalis* and the fungal strains *C. albicans* and *C. albidus* which was found to be active as similar as to the standards. On the contrary, $-(\text{DLDL})_3$ -mimic cereulide 3, $-(\text{DLDL})_3$ -mimic valinomycin 7 and $-(\text{DDLL})_3$ -mimic valinomycin 8, were inactive for all bacterial and fungal strains except for $-(\text{DDLL})_3$ -mimic cereulide 5 was moderately active with some bacterial strains. The octa-cyclic peptide $-(\text{DLDL})_2$ -mimic cereulide 2 and $-(\text{DDLL})_2$ -mimic cereulide 4 were demonstrated similar to other mimic cereulide and mimic valinomycin. The screening antimicrobial activities were concluded that the cyclic D,L- α -peptides, $-(\text{DLDL})_2$ -mimic cereulide 2, $-(\text{DLDL})_3$ -mimic cereulide 3 and $-(\text{DLDL})_3$ -mimic valinomycin 7 were demonstrated less activity than the original cereulide and valinomycin although the sequence of amino acid residues inside the ring are the same. Moreover, $-(\text{DDLL})_2$ -mimic cereulide 4, $-(\text{DDLL})_3$ -mimic cereulide 5 and $-(\text{DDLL})_3$ -mimic valinomycin 8, consisted of the

same sequence and configuration of amino acid residue as in cereulide or valinomycin structure, demonstrated the different spectrum of antimicrobial activity from the original structure. Cereulide and valinomycin are structured in hexagonal cylinder-like framework by the hydrogen bonding along the side-wall plans and the complexation with the K^+ ion via the oxygen atom in ester bond whereas the cyclic D,L- α -peptides are adopted flat and formed β -sheet like tubular structures via hydrogen bonding for each of molecule and the amino acid side chains on the outside of the molecules. However, the cavity size of cereulide and valinomycin are similar in the size whereas the cyclic D,L- α -peptides is larger than that of cereulide and valinomycin. Accordingly, cereulide and valinomycin should have a different ability to inhibit bacterial and fungal strains. Moreover, the cyclic DD,LL-peptide demonstrated less activity than the cereulide and valinomycin even if the configuration and the sequence of amino acids are the same. The results mean that the structure of cereulide and valinomycin which are consisted of ester and amide bond have more stability and complex structure for biological activities than other cyclic peptide such as cyclic D,L- α -peptides. We assume that the structure of cereulide and valinomycin should become more significant and systematic when binding or penetrating though to the cell membranes.

Due to the cereulide and valinomycin are strongly active against some bacteria and fungi, both compounds were selected to combine with antibiotic agent as oxacillin, gentamicin and ciprofloxacin for antibacterial assay, amphotericin B and nystatin for antifungal assay. Our data confirm good enhance activity of cereulide or valinomycin and antifungal agents whereas cereulide and valinomycin did not display better inhibition of the bacterial growth compared with the reported synthetic antibiotics. Combined antibiotic therapy may produce synergistic effects in the treatment of microorganism infection. Multidrug resistance is widespread among gram-negative and gram-positive bacteria. For this reason, there has been interested in developing single drugs or combinations of drugs that have the antimicrobial activity against multiple microorganisms. However, the data presented

in this paper could represent a novel tool potentially valuable as an adjuvant for antimicrobial chemotherapy concerning their intrinsic antifungal activity and their enhanced interactions. There are no reports on the enhanced antimicrobial activity of cereulide and valinomycin with antibiotics in the literatures. In our experiment, the results for each cereulide or valinomycin-amphotericin B/nystatin combination initially demonstrated enhancement by decreasing the MIC value between 2-128 times of the MIC values of the antibiotic drugs as shown in Table 3, however, the mechanism of these positive interactions

appears to be complex. The major value of this study is demonstrated that the combination of cereulide or valinomycin with amphotericin B and nystatin primarily results in the enhancement effect and comparison of the structural activity between cereulide or valinomycin and cyclic D,L- α -peptides or cyclic DDLL-peptides. Further structural modification could be performed to improve the activity which is potentially useful in the developing new antimicrobial therapeutic agents using the cereulide or valinomycin as the core structure.

Table 1
***In vitro* antibacterial activity of DLDL, DDLL amino acid cyclic peptides, cereulide and valinomycin^a**

| Compounds | Minimun inhibitory concentration ($\mu\text{g/ml}$) | | | | | | | |
|-------------------------|---|----------------------|-----------------------|--------------------|------------------|-----------------------|--------------------|------------------|
| | <i>E. coli</i> | <i>P. aeruginosa</i> | <i>S. typhimurium</i> | <i>S. flexneri</i> | <i>S. aureus</i> | <i>S. epidermidis</i> | <i>E. faecalis</i> | <i>B. cereus</i> |
| Oxacillin | - | - | - | - | 0.78 | 0.39 | 12.5 | 25 |
| Gentamicin | 1.56 | 1.56 | 0.78 | 1.56 | 3.12 | 0.78 | 6.25 | 0.78 |
| Ciprofloxacin | 2.34 | 0.39 | 0.39 | 0.78 | 0.195 | 0.39 | 1.56 | 0.195 |
| Cereulide (1) | >200 | >200 | >200 | >200 | >200 | >200 | 6.25 | >200 |
| - (DLDL) ₂ - | | | | | | | | |
| Cereulide (2) | >200 | >200 | >200 | 100 | >200 | >200 | 200 | >200 |
| - (DLDL) ₃ - | | | | | | | | |
| Cereulide (3) | >200 | >200 | >200 | >200 | 200 | >200 | 100 | >200 |
| - (DDLL) ₂ - | | | | | | | | |
| Cereulide (4) | >200 | >200 | >200 | 150 | 200 | 200 | 200 | >200 |
| - (DDLL) ₃ - | | | | | | | | |
| Cereulide (5) | >200 | >200 | >200 | 75 | 100 | 200 | 75 | >200 |
| Valinomycin (6) | >200 | >200 | >200 | >200 | >200 | >200 | 0.78 | >200 |
| - (DLDL) ₃ - | | | | | | | | |
| Valinomycin (7) | >200 | >200 | >200 | >200 | >200 | >200 | >200 | >200 |
| - (DDLL) ₃ - | | | | | | | | |
| Valinomycin (8) | >200 | >200 | >200 | >200 | >200 | >200 | >200 | >200 |

^a*E. coli* = *Escherichia coli* (gram (-) bacteria)

S. typhimurium = *Salmonella typhimurium* (gram (-) bacteria)

S. aureus = *Staphylococcus aureus* (gram (+) bacteria)

E. faecalis = *Enterococcus faecalis* (gram (+) bacteria)

(-) means not used in experiment

Ps. aeruginosa = *Pseudomonas aeruginosa* (gram (-) bacteria)

S. flexneri = *Shigella flexneri* (gram (-) bacteria)

S. epidermidis = *Staphylococcus epidermidis* (gram (+) bacteria)

B. cereus = *Bacillus cereus* (gram (+) bacteria)

Table 2
***In vitro* antifungal activity of DLDL, DDLL amino acid cyclic peptides, cereulide and valinomycin**

| Compounds | Minimun inhibitory concentration ($\mu\text{g/ml}$) | | | | | | |
|-------------------------|---|---------------------------|----------------------------|---------------------------|----------------------------|-------------------------|-----------------------------|
| | <i>Aspergillus niger</i> | <i>Aspergillus flavus</i> | <i>Aspergillus species</i> | <i>Acremonium species</i> | <i>Penicillium species</i> | <i>Candida albicans</i> | <i>Cryptococcus albidus</i> |
| Amphotericin B | 1.56 | 3.12 | 3.12 | 6.25 | 6.25 | 0.78 | 0.78 |
| Nystatin | 0.78 | 1.56 | 0.78 | 6.25 | 12.5 | 6.25 | 3.12 |
| Cereulide (1) | >200 | 200 | 200 | 150 | 200 | 3.12 | 1.56 |
| - (DLDL) ₂ - | | | | | | | |
| Cereulide (2) | >200 | >200 | >200 | >200 | 200 | >200 | >200 |
| - (DLDL) ₃ - | | | | | | | |
| Cereulide (3) | >200 | >200 | >200 | >200 | >200 | >200 | >200 |
| - (DDLL) ₂ - | | | | | | | |
| Cereulide (4) | >200 | 200 | >200 | 200 | 150 | >200 | >200 |
| - (DDLL) ₃ - | | | | | | | |
| Cereulide (5) | >200 | >200 | 200 | 200 | 200 | 200 | >200 |
| Valinomycin (6) | 200 | 50 | >200 | 75 | 200 | 1.56 | 1.56 |
| - (DLDL) ₃ - | | | | | | | |
| Valinomycin (7) | >200 | 200 | >200 | >200 | 200 | >200 | >200 |
| - (DDLL) ₃ - | | | | | | | |
| Valinomycin (8) | 200 | 200 | 200 | >200 | 200 | >200 | >200 |

Table 3
Minimal inhibitory concentration (MIC) of selected compounds combined with other clinically used antibiotics^d

| Compounds | Minimun inhibitory concentration (µg/ml) | | | | | | |
|----------------------------------|--|---------------------------|----------------------------|---------------------------|----------------------------|-------------------------|-----------------------------|
| | <i>Aspergillus niger</i> | <i>Aspergillus flavus</i> | <i>Aspergillus species</i> | <i>Acremonium species</i> | <i>Penicillium species</i> | <i>Candida albicans</i> | <i>Cryptococcus albidus</i> |
| Amphotericin B | 1.56 | 3.12 | 3.12 | 6.25 | 6.25 | 0.78 | 0.78 |
| Nystatin | 0.78 | 1.56 | 0.78 | 6.25 | 12.5 | 6.25 | 3.12 |
| Cereulide (1) + A ^a | 1.56 | 0.195 | 1.56 | 0.195 | 1.56 | 0.195 | 0.012 |
| (gain ^c) | (1) | (16) | (2) | (32) | (4) | (4) | (64) |
| Valinomycin (6) + A ^a | 0.097 | 0.097 | 3.12 | 0.097 | 0.78 | 0.097 | 0.0061 |
| (gain ^c) | (16) | (32) | (1) | (64) | (8) | (8) | (128) |
| Cereulide (1) + N ^b | 0.78 | 0.195 | 0.39 | 0.78 | 6.25 | 1.56 | 0.39 |
| (gain ^c) | (1) | (8) | (2) | (8) | (2) | (4) | (8) |
| Valinomycin (6) + N ^b | 0.39 | 0.097 | 0.78 | 0.39 | 6.25 | 1.56 | 0.195 |
| (gain ^c) | (2) | (16) | (1) | (16) | (2) | (4) | (16) |

^aA = Amphotericin B

^bN = Nystatin

^cgain > 1 mean decrease concentration of antibiotic, 1 mean no effect

^dCereulide (1) and Valinomycin (6) did not display better inhibition of the bacterial growth compared with the reported synthetic antibiotics

CONCLUSION

The biological evaluation of all compounds compared with the original cereulide and valinomycin found that the antimicrobial activity of the original cereulide and valinomycin was higher than that of the cyclic D,L- α -peptides which were synthesized by using cereulide and valinomycin as a model structure. Furthermore, the combinations of the cereulide or valinomycin and the clinically used drugs against fungi were found enhancing the antimicrobial activity. The potentially useful of cereulide or valinomycin and theirs analogs are developed in the new antimicrobial therapeutic agents.

REFERENCES

- Shinde NV, Dhake AS, Haval KP, Biological activities of cyclic peptides: an overview. Res. J. Pharm. Biol. Chem. Sci., 4: 142-158, (2013).
- Joo SH, Cyclic peptides as therapeutic agents and biochemical tools. Biomol. Ther., 20: 19-26, (2012).
- Agata N, Mori M, Ohta M, Suwan S, Ohtani I, Isobe M, A Novel dodecadepsipeptide, cereulide, isolated from *Bacillus cereus* vacuole formation in HEp-2-cells. FEMS Microbiol. Lett., 121: 31-34, (1994).
- Agata N, Ohta M, Mori, M, Production of an emetic, cereulide, is associated with a specific class of *Bacillus cereus*. Curr. Microbiol., 33: 67-69, (1996).
- Perkins JB, Guterman SK, Howitt CL, Williams VE, Pero J, Streptomyces genes involved in biosynthesis of the peptide antibiotic valinomycin. J. bacterial., 172: 3108-3116, (1990).
- Suwan S, Isobe M, Ohtani I, Agata N, Mori M, Ohta M, Structure of cereulide, a cyclic dodecadepsipeptide toxin from *Bacillus cereus*, and studies on NMR characteristics of its alkali metal complexes including a conformational structure of the K⁺ complex. J. Chem. Soc., Perkin Trans. 1., 7: 765-775, (1995).
- Teplova V, Mikkola R, Tonshin A, Saris NL, Salkinoja-Salonen MS, The higher toxicity of cereulide relative to valinomycin is due to its higher affinity for potassium at

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- physiological plasma concentration. *Toxicol. Appl. Pharm.*, 210: 39-46, (2006).
8. Mikkola R, Saris NL, Grigoriev PA, Andersson MA, Salkinoja-Salonen, MS, Ionophoric properties and mitochondrial effects of cereulide: the emetic toxin of *B. cereus*. *Eur. J. Biochem.*, 263: 112-117, (1999).
 9. Makarasen A, Yoza K, Isobe M, Higher structure of cereulide, an emetic toxin from *Bacillus cereus*, and special comparison with valinomycin, an antibiotic from *Streptomyces fulvissimus*. *Chem. Asian. J.*, 4: 688-698, (2009).
 10. Tempelaars MH, Marcel H, Rodrigues S, Abee T, Comparative analysis of antimicrobial activities of valinomycin and cereulide, the *Bacillus cereus* emetic toxin. *Appl. Environ. Microbiol.*, 77: 2755-2762, (2011).
 11. Ladeuze S, Lentz N, Delbrassinne L, Hu X, Mahillon J, Antifungal activity displayed by cereulide, the emetic toxin produced by *Bacillus cereus*. *Appl. Environ. Microbiol.*, 77: 2555-2558, (2011).
 12. Buriak JM, Ghadiri MR, Self-assembly of peptide based nanotubes. *Mater. Sci. Eng.: C*, 4: 207-212, (1997).
 13. De SP, Morosetti S, Scipioni A, Peptides with regular enantiomeric sequences: a wide class of modular self-assembling architectures. *J. Nanosci. Nanotechnol.*, 7: 2230-2238, (2007).
 14. Datois V, Sanchez-Quesada J, Cabezas E, Chi E, Dubbelde C, Dunn C, Granja J, Gritzen C, Weinberger D, Ghadiri MR, Systemic antibacterial activity of novel synthetic cyclic peptides. *Antimicrob. Agents Ch.*, 49: 3302-3310, (2005).
 15. Ghadiri MR, Self-assembled nanoscale tubular ensembles. *Adv. Mater.*, 7: 675-677, (1995).
 16. Ghadiri MR, Granja JR, Buehler LK, Artificial Trans-membrane Ion Channels from Self-Assembling Peptide Nanotubes. *Nature*, 369: 301-304, (1994).
 17. Sanchez-Quesada J, Ghadiri MR, Bayley H, Braha O, Cyclic peptides as molecular adapters for a pore-forming protein. *J. Am. Chem. Soc.*, 122: 11757-11766, (2000).
 18. Ashkenasy G, Ghadiri MR, Boolean logic functions of a synthetic peptide network. *J. Am. Chem. Soc.*, 126: 11140-11141, (2004).
 19. Chapman R, Danial M, Koh ML, Jolliffe KA, Perrier S, Design and properties of functional nanotubes from the self-assembly of cyclic peptide templates. *Chem. Soc. Rev.*, 41: 6023-6041, (2012).
 20. Fernandez-Lopez S, Kim HS, Choi EC, Delgado M, Granja JR, Khasanov A, Kraehenbuehl, K, Long G, Weinberger DA, Wilcoxon KM, Ghadiri MR, Antibacterial agents based on the cyclic D,L-alpha peptide architecture. *Nature*, 412: 452-455, (2001).
 21. Mainous III AG and Pomeroy C, Management of Antimicrobials in Infectious Diseases, 2nd ed.; Humana Press: Springer, (2010).
 22. Isobe M, Ishikawa T, Suwan S, Agata N, Ohta M, Synthesis and activity of cereulide, a cyclic dodecadepsipeptide ionophore as emetic toxin from *Bacillus cereus*. *Bioorg. Med. Chem. Lett*, 5: 2855-2858, (1995).
 23. Makarasen A, Nishikawa T, Isobe M, Synthesis of Four Lysine-Linked Cereulide Analogues Showing Ionophoric Activity Towards Potassium Cations as Lead Compounds for Emetic toxin-Detection by Immunoassays. *Synthesis*, 13: 2184-2220, (2009).
 24. National Committee for Clinical Laboratory Standard. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Conidium-forming Filamentous Fungi Proposed standard M38-P, Wayne, PA, (1998).
 25. National Committee for Clinical Laboratory Standard. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi. Approved standard M38-A, Wayne, PA, (2002).
 26. National Committee for Clinical Laboratory Standard. Method for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically Approved standard, M7-A9, 9th ed, Wayne, PA, (2012).
 27. Bell SC, Grundy WE, Preparation of agar wells for antibiotic assay. *Appl. Microbiol.*, 16: 1611-1612, (1968).