รายงานวิจัยฉบับสมบูรณ์

โครงการ

Antimicrobial and cytotoxic agents based on the cyclic cereulide and valinomycin architecture

การสังเคราะห์และศึกษาการออกฤทธิ์ทางชีวภาพของสารประกอบไซคลิกเป็ปไทด์โดยใช้ลักษณะทางโครงสร้างของ cereulide และ valinomycin

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June 2014

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รายงานวิจัยฉบับสมบูรณ์

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ภาษาอังกฤษ : **Antimicrobial and cytotoxic agents based on the cyclic cereulide and valinomycin architecture**

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและสถาบันวิจัยจุฬาภรณ์

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Arthit Makarasen

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Abstract

Project Code: TRG5580009

Project Title: Antimicrobial and cytotoxic agents based on the cyclic cereulide and valinomycin architecture

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Project period: July 2012-2014

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Cyclic peptides with even number of alternating D- and L- amino acids can adopt flat, ring-shaped conformations in which the back bone amide functionalities are oriented perpendicular to the side chains and the plane of the ring structure. Under conditions that favor hydrogen bonding, such as adsorption onto lipid membranes, the cyclic peptides can stack to form hollow, β-sheet-like tubular structures that are open-ended, presenting the amino acid chains on the outside surface of the ensemble. Therefore, cyclic D,L-α-peptides may be able to selectively target and self-assemble in bacterial membrane and exert antibacterial activity by increasing the membrane permeability. Cereulide and valinomycin are both 36-membered cyclic depsipeptides with 12 stereogenic centers that have a very similar sequence in the structures. Both compounds are known as K^+-ion selective ionophores and cause a potassium-dependent drop in the transmembrane potential of mitochondria arising from the uptake of a K^+-ion charged ionophores complex. Cereulide and valinomycin may use the same functionalities as the cyclic D,L-α-peptides to form hollow and increasing the membrane permeability in the bacterial membrane. In the present of our project, we studies in the synthesis and cyclization of cyclic peptides with fully amide residue together with even number of alternating D- and L-amino acids and/or alternating DD- and LL- amino acid based on the cyclic cereulide and valinomycin architecture. All the desired products including with cereulide and valinomycin will study for the biological activities such as cytotoxic and antimicrobial actions. The activities for all derivative compounds may possess interesting biological properties.

Keywords: Cereulide, Valinomycin, antimicrobial, cyclic D,L-α-peptides, antibacterial activity, antifungal activity

บทคัดย่อ

รหัสโครงการ: TRG5580009

ชื่อโครงการ: การสังเคราะห์และศึกษาการออกฤทธิ์ทางชีวภาพของสารประกอบไซคลิกเปปเทด์ โดยใช้ลักษณะทางโครงสร้างของ cereulide และ valinomycin

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ไซคลิกเป็ปไทด์ซึ่งประกอบด้วยกรดอะมิโนชนิด D และ L เรียงสลับกันเป็นจำนวนคู่นั้น สามารถวางตัวอยู่ในแนวระนาบและเกิดการเชื่อมต่อระหว่างโมเลกุล โดยการใช้พันธะไฮโดรเจนของเอไมด์ฟังก์ชั่นระหว่างโมเลกุล ทำให้สามารถที่จะซึมผ่านเข้าสู่ผนังเซลล์ที่ประกอบด้วยชั้นไขมันได้ ดังนั้นสารประกอบไซคลิกเป็ปไทดชนิดนี้ จึงสามารถนำมาประยุกต์ใช้ในการยับยั้งการเจริญเติบโตของเซลล์แบคทีเรียได้ สำหรับสารประกอบ cereulide และ valinomycine ซึ่งประกอบด้วยกรดอะมิโน 2 ชนิด และมีลักษณะโครงสร้างที่เป็นวงเหมือนกัน แต่มีความแตกต่างกันในชนิดของกรดอะมิโนในโมเลกุล โดยสารประกอบทั้ง 2 ชนิดนี้ สามารถเกิดสารประกอบเชิงซ้อนกับ K^+ ไอออนได้ ซึ่งมีผลทำให้เกิดการซึมผ่านของไอออนออกจากเซลล์ได้ ดังนั้นลักษณะทางโครงสร้างของสารประกอบทั้ง 2 ชนิด คือ ไซคลิกเป็ปไทด์และ สารประกอบ cereulide และ valinomycin จึงเป็นที่น่าสนใจในการศึกษา โดยจะทำการสังเคราะห์สารประกอบไซคลิกเป็ปไทด์ที่ประกอบด้วยกรดอะมิโนชนิด D และ L เรียงสลับกันเป็นจำนวนคู่ให้มีลำดับของกรดอะมิโน เหมือนกันกับสารประกอบ cereulide และ valinomycin รวมทั้ง สังเคราะห์สารประกอบ cereulide และ valinomycin ชนิดที่ประกอบด้วยพันธะ amide ทั้งหมด แล้วนำสารประกอบเหล่านี้ ไปทำการศึกษาลักษณะการออกฤทธิ์ทางชีวภาพ เพื่อประเมินถึงผลต่อโครงสร้างที่มีต่อการออกฤทธิ์ทางชีวภาพต่อไป

คำหลัก: Cereulide, Valinomycin, antimicrobial, cyclic D,L-α-peptides, antibacterial activity, antifungal activity

Introduction

Peptides, a group of compounds consisting of two or more amino acids linked by peptide bond, and are abundantly present in living organisms. Thousands of peptides have been isolated from animals, plants and microorganisms. Based on their chemical structure, peptides can be divided into linear and cyclic peptides. The most 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 structure. 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 administration.

Cereulide (**1**) was first isolated from *Bacillus cereus* in 1994 by Agata et al.[1,2] and was chemically elucidated to have the structure as shown for compound **1** in 1995 by Isobe et al.[3] The 12 stereogenic centers of cereulide were established by chemical degradation and the higher structure was assigned from combination of NMR spectroscopy and molecular mechanics calculations. Chemical synthesis of **1** was achieved by Isobe et al. in 1995 and the lysine linked cereulide analogues were synthesized in 2009.[4,5] Cereulide (**1**), produced through an unusual non-ribosomal peptide synthesis (NRPS), is known as an emetic toxin.[6] Valinomycin (**6**), on the other hand, produced by *Streptomyces fulvissimus* is an antibiotic with similar cyclic depsipeptides structure.[7] Valinomycin, however, has very different activities from cereulide.[8,9] Cereulide (**1**) and valinomycin (**6**) are both 36-membered cyclic depsipeptides with 12 stereogenic centers having the 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 (Figure 1).

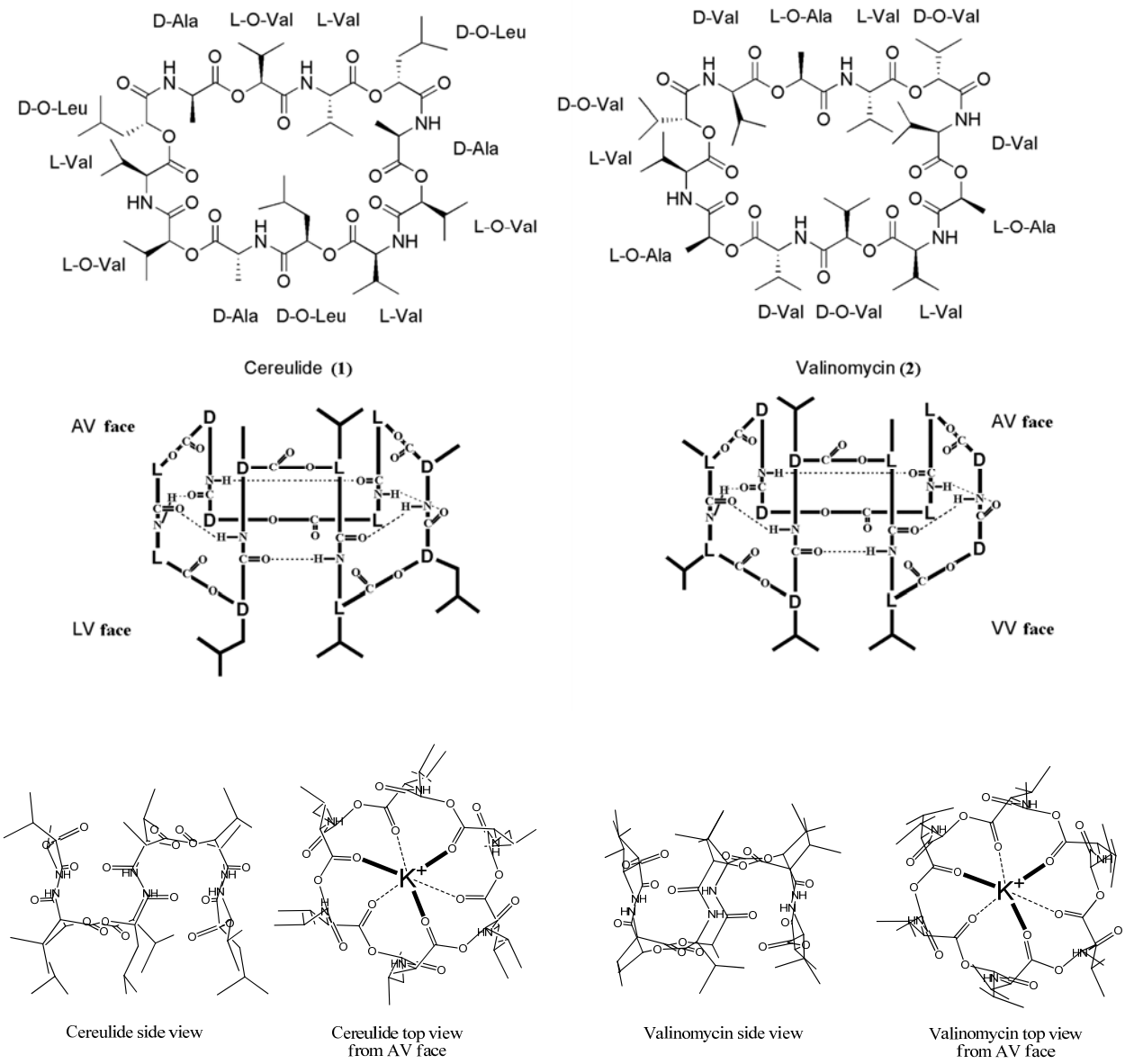


Figure 1. Structure of cereulide (**1**) and valinomycin (**2**) 2D and 3D.

In the three dimensional structures of cereulide and valinomycin, the frameworks of these cyclic depsipeptides are very similar and mirror image each other having difference only a side chain on the frameworks. Both compounds are composed of six peptide bonds and six ester bonds in one after another having stereochemistry as $(D\text{-}D\text{-}L\text{-}L)_3$ with 3-time symmetry axis, such that they are constructed by three times repeating a tetrapeptide.[3] Moreover, 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. All these facts imply that cereulide and valinomycin have enantiomeric framework. The metal-ionophoric properties of cereulide and valinomycin were reported with alkali metal ions such as Li^+, Na^+, K^+, Rb^+, Cs^+ and H^+. Complexation with inorganic and organic ammonium ions were assigned to show the similar higher structures judging from the NMR and ESI-MS spectroscopy.[3,10] Cereulide and valinomycin show high selectivity for K^+ ion by competing experiments in the complexation among those alkali metal ions. Both compounds specifically function as a potassium ion-ionophore to affect the function of mitochondria in living cells; thus, the K^+-selective binding property of cereulide largely causes vacuole formation. This activity can be recognized as the swelling of mitochondria in a HEp-2 cell bioassay.[1,11] The suppressive effect of cereulide on cytotoxicity was fast at toxic concentrations as low as the range of 20-30 μg/L.[12] Mikkola et al. found that cereulide and valinomycin induced mitochondria swelling in the presence of K^+ ion, which caused a potassium-dependent drop in the trans-membrane inner membrane potential due to uptake of K^+ ion as positively charged ionophore complex.[13] In 2009, Isobe et al. was also reported the competitive complexation between cereulide and valinomycin with different K^+ ion concentration which found that cereulide exhibited K^+ ion-selective ionophore property at lower K^+ ion concentration than valinomycin. Moreover, cereulide was performed complexes with Eu^{3+}, Fe^{3+}, Li^+, Rb^+ or Cs^+ ions at lower concentration than valinomycin.[14] Furthermore, Cereulide inhibits mouse leukemia cell (P388) with IC_{50} of 1.4×10^{-6} μg/ml, whereas adriamycin has activity at 1.9×10^{-3} μg/ml, which has widely been used as chemotherapy. Cereulide is also more active for inhibiting mouse colon cancer cells (Colon 26) with IC_{50} of 3.5×10^{-5} μg/ml than adriamycin with 1.7×10^{-2} μg/ml.[15]

The continuous use of antibiotics has resulted in multi-resistant bacteria strains all over the world and as expected, hospitals have become breeding grounds for human-associated microorganisms.[16] The rapid emergence of bacterial infections that are resistant to many drugs underscores need for new therapeutic agents. Cyclic D,L-α-peptides possess unique structural features not found in the natural class of peptide antibiotics and/or their derivatives.[17] Cyclic peptides with even number of alternating D- and L- amino acids can adopt flat, ring-shaped conformations in which the back bone amide functionalities are oriented perpendicular to the side chains and the plane of the ring structure. Under conditions that favor hydrogen bonding,

such as adsorption onto lipid membranes, the cyclic peptides can stack to form hollow, β-sheet-like tubular structures that are open-ended, presenting the amino acid chains on the outside surface of the ensemble.[18] Therefore, cyclic D,L-α-peptides may be able to selectively target and self-assemble in bacterial membrane and exert antibacterial activity by increasing the membrane permeability.

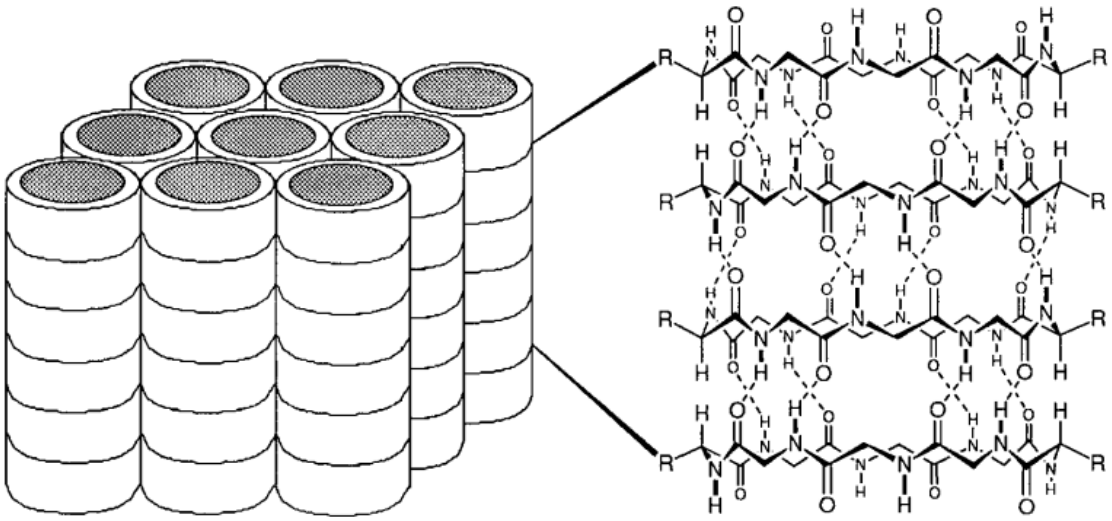
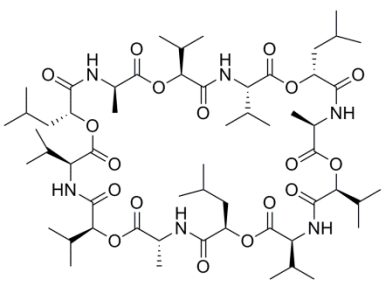


Figure 2. Cyclic peptide structures with alternating D- and L-amino acids adopting flat ring-shaped conformations and, depending on the peptide sequence and the conditions employed, assembling into ordered parallel arrays of solid-state nanotubes. The illustration emphasizes the antiparallel ring stacking and the presence of extensive intersubunit hydrogen-bonding interactions (for clarity most side chains are omitted).

Recently, Ghadiri, M. R. et al. has reported the design and construction of open-ended hollow tubular objects based on the self-assembly of flat, ring-shaped cyclic peptide subunits (Figure 2).[19] These artificial tubular constructs constitute a new class of synthetically readily accessible peptide-based biomaterials having unique structural and functional properties. Peptide nanotubes are constructed by highly convergent noncovalent processes by which cyclic peptides rapidly self-assemble and organize into ultra large well-ordered three-dimensional structures, upon an appropriate chemical- or medium-induced triggering. The properties of the outer surface and the internal diameter of peptide nanotubes can be adjusted simply by the choice of the amino acid side chain functionalities and the ring size of the peptide subunit employed.[19-21] This design flexibility, which is unique to this class of tubular structures, has

already enabled application of peptide nanotubes to the design of biologically-relevant trans-membrane ion channels and pore structures.[20]

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 as shown in Figure 3. 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.

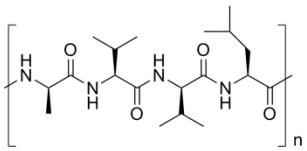


Cereulide (1)

HRMS (ESI-TOP): m/z [M+K$^+$]

Calcd for $C_{57}H_{96}N_6O_{18}K$: 1191.6418;

found 1191.6385

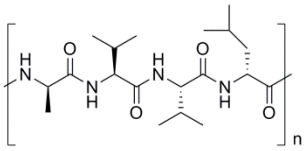


-(DLDL)$_2$-mimic cereulide (2)

HRMS (ESI-TOP): m/z [M+Na$^+$]

Calcd for $C_{38}H_{68}N_8O_8Na$: 787.5058;

found 787.5052



-(DDLL)$_2$-mimic cereulide (4)

HRMS (ESI-TOP): m/z [M+Na$^+$]

Calcd for $C_{38}H_{68}N_8O_8Na$: 787.5058;

found 787.5047

-(DLDL)$_3$-mimic cereulide (3)

HRMS (ESI-TOP): m/z [M+Na$^+$]

Calcd for $C_{57}H_{102}N_{12}O_{12}Na$: 1169.7638;

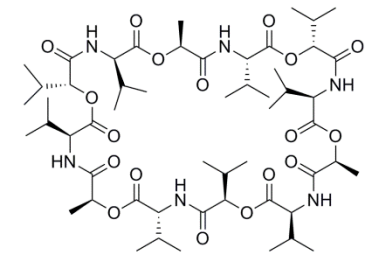
found 1169.7593

-(DDLL)$_3$-mimic cereulide (5)

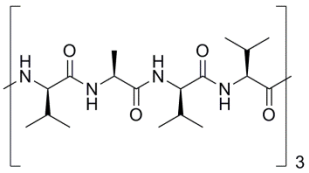
HRMS (ESI-TOP): m/z [M+Na$^+$]

Calcd for $C_{57}H_{102}N_{12}O_{12}Na$: 1169.7638;

found 1169.7567



Valinomycin (6)

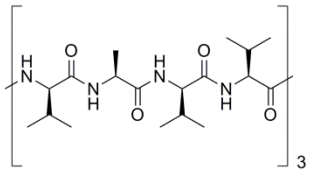


-(DLDL)$_3$-mimic valinomycin (7)

HRMS (ESI-TOP): m/z [M+Na$^+$]

Calcd for $C_{54}H_{96}N_{12}O_{12}Na$: 1127.7163;

found 1127.7127



-(DDLL)$_3$-mimic valinomycin (8)

HRMS (ESI-TOP): m/z [M+Na$^+$]

Calcd for $C_{54}H_{96}N_{12}O_{12}Na$: 1127.7163;

found 1127.7204

Figure 3. 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

1 Chemicals

Cereulide (**1**) was synthesized as described by Isobe et al.[4,5] 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 cereulide.[5] All products were purified with medium-pressure liquid chromatography and analyzed to confirm from nuclear magnetic resonance (NMR), mass spectrometry (MS). Proton and carbon NMR specta were obtained using a Bruker AvanceIII-300 spectometer 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).

2 Susceptibility testing and antifungal agents

2.1 Organisms and media

Gram-negative bacteria: Escherichia coli (*E. coli*) TISTR No.780, *Pseudomonas aeruginosa* (*P. aeruginosa*) TISTR No.781, *Salmonella typhimunium* (*S. typhimunium*) TISTR No.292, and *Shigella flexneri* (*S. flexneri*) ATCC 9199

Gram-positive bacteria: Staphylococcus aureus (*S. aureus*) TISTR No.1466, *Staphylococcus epidermidis* (*S. epidermidis*) TISTR No.518, *Enterococcus faecalis* (*E. faecalis*) TISTR No.379, and *Bacillus cereus* (*B. cereus*) TISTR No.687

Fungi: Aspergillus niger (*A. niger*) TISTR No.3254, *Aspergillus flavus* (*A. flavus*) TISTR No.3366, *Aspergillus sp.* TISIR No.3105, *Acremonium sp.* TISTR No.3487, and *Penicillium sp.* TISTR No.3118

Yeast: Candida albicans (*C. albicans*) TISTR No.5779 and *Cryptococcus albidus* (*C. albidus*) TISTR No.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^{o}C and fungi were maintained on potato dextrose agar (PDA) at 28^{o}C.

2.2 Preparation of inoculum.

The tested bacteria were cultured in nutrient broth (NB) and incubated for 18-24 h at 37^{o}C. The tested conidium-forming filamentous fungi, *C. albicans* and *C. albidus*, were made by grown on PDA or Sabouraud Dextrose agar (SDA) more than three days at 28^{o}C. The colonies were harvested, suspended in sterile saline, and their concentrations were adjusted to a 0.5 McFarland standard, the equivalence of 1-2 × 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} spore/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.

2.3 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 guideline.[22-25] 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 duplicate.

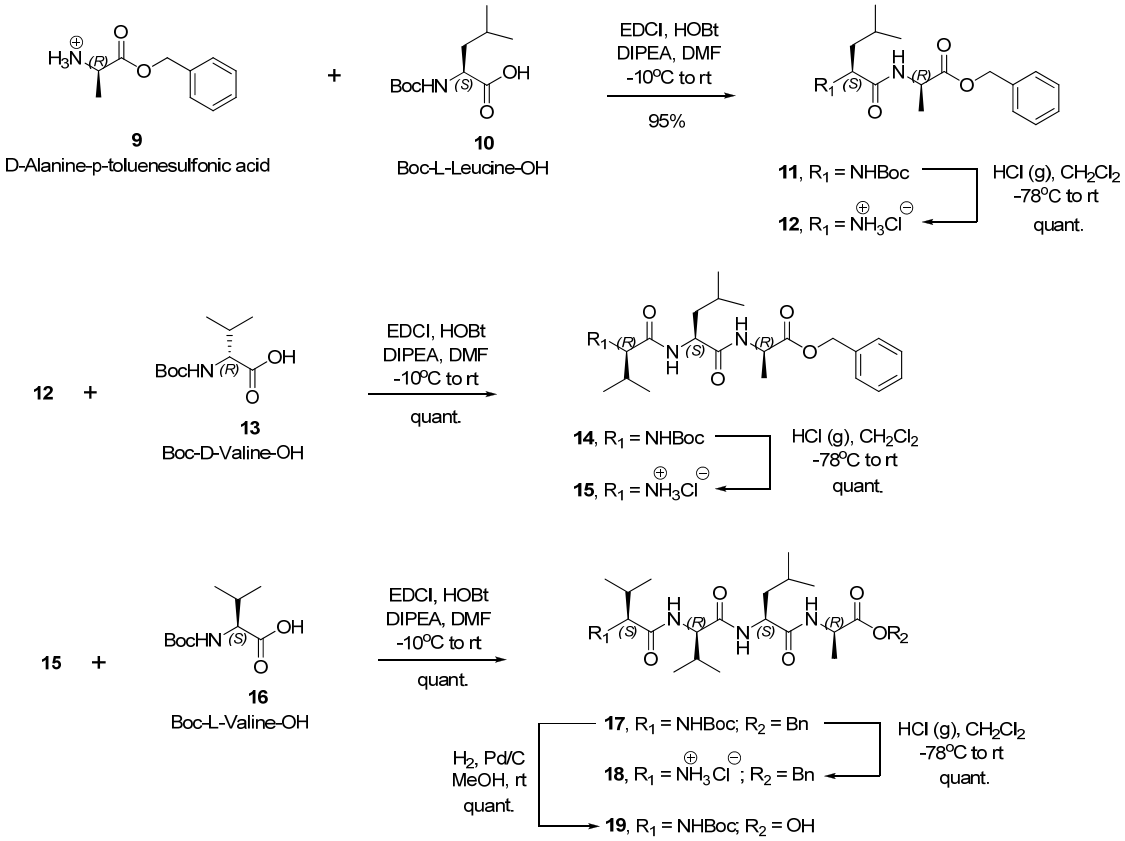
Results and discussion

Cereulide was obtained by using the synthesis method as described in the literature.[5] 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. The linear peptides and the cyclic peptides product were synthesized as described as scheme 1-10. All desired cyclic peptides were successfully carried out in moderate yield. The cyclization products were purified on short column chromatography and confirmed by ^{1}H NMR and mass spectral data. The characteristic NMR spectra all the intermediate compounds were analyzed. ^{1}H 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.

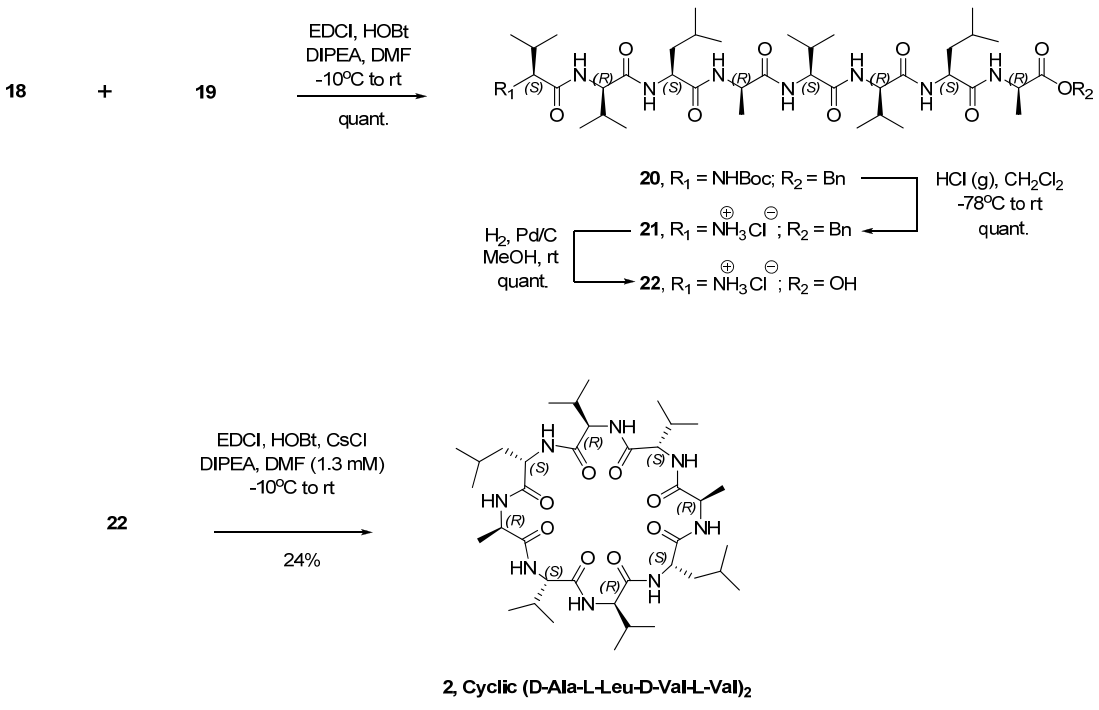
The original cereulide, valinomycin and desired cyclic peptides, cereulide (**1**), -$(DLDL)_3$-mimic cereulide (**3**), -$(DDLL)_3$-mimic cereulide (**5**), valinomycin (**6**), -$(DLDL)_3$-mimic valinomycin (**7**), -$(DDLL)_3$-mimic valinomycin (**8**), were tested for screening antimicrobial activity. The antibacterial and antifungal activities were carried out against the eight bacteria (*E. coli*, *Ps. aeruginosa*, *S. typhimunium*, *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, -$(DLDL)_3$-mimic cereulide (**3**), -$(DLDL)_3$-mimic valinomycin (**7**) and -$(DDLL)_3$-mimic valinomycin (**8**), were inactive for all bacterial and fungal strains except for -$(DDLL)_3$-mimic cereulide (**5**) was moderately active with some bacterial strains. The octa-cyclic peptide -$(DLDL)_2$-mimic cereulide (**2**) and -$(DDLL)_2$-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, -$(DLDL)_2$-mimic cereulide (**2**), -$(DLDL)_3$-mimic cereulide (**3**) and -$(DLDL)_3$-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, -$(DDLL)_2$-mimic cereulide (**4**), -$(DDLL)_3$-mimic cereulide (**5**) and -$(DDLL)_3$-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.

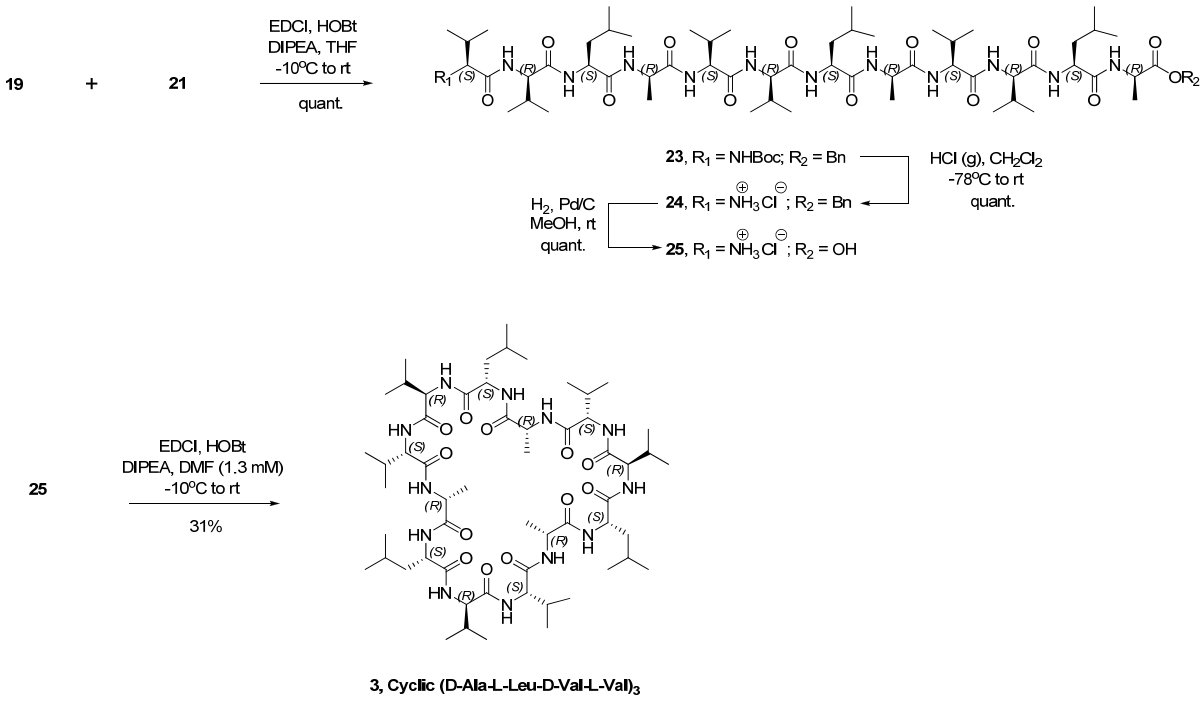
Due to the cereulide and valinomycin are strongly active against some bacteria and fungi, structural modification could be performed to improve the activity which is potentially useful in the developing new antimicrobial therapeutic agents using the cereulide or valinomycin as the core structure.



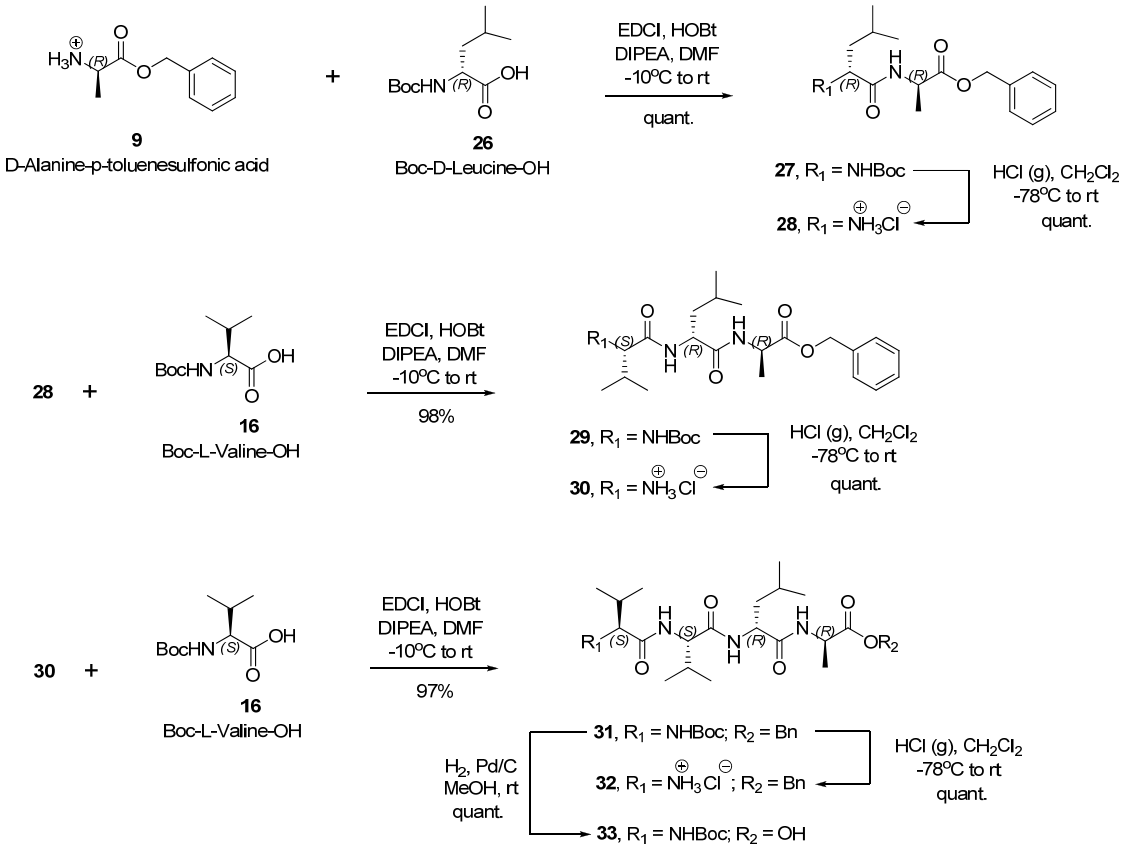
Scheme 1. Synthesis of L-Val-D-Val-L-Leu-D-Ala



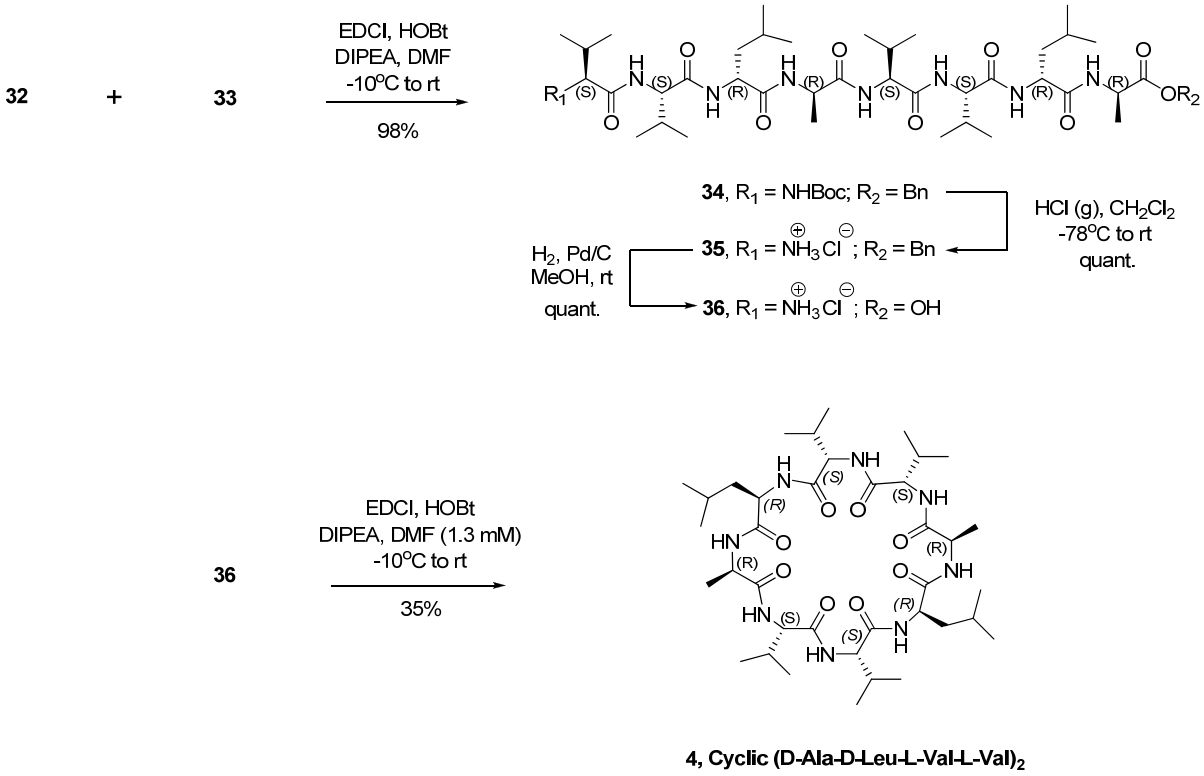
Scheme 2. Synthesis of –(DLDL)$_2$- mimic cereulide (2)



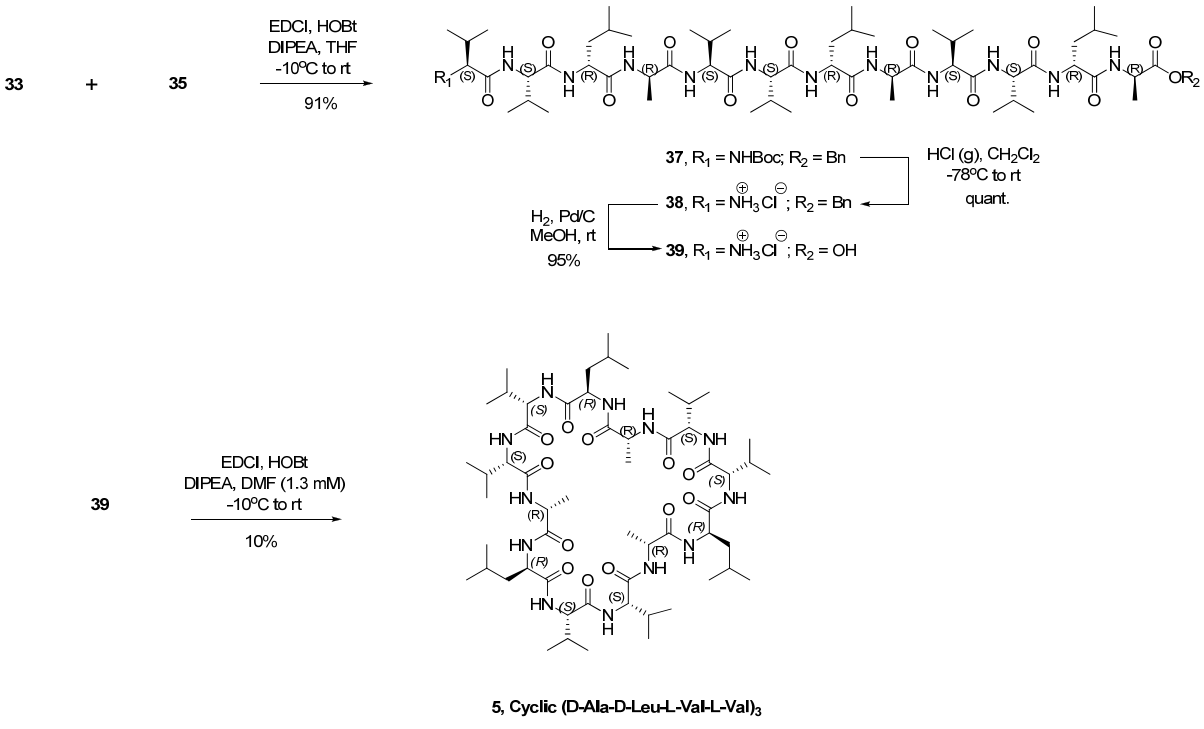
Scheme 3. Synthesis of –(DLDL)$_3$- mimic cereulide (3)



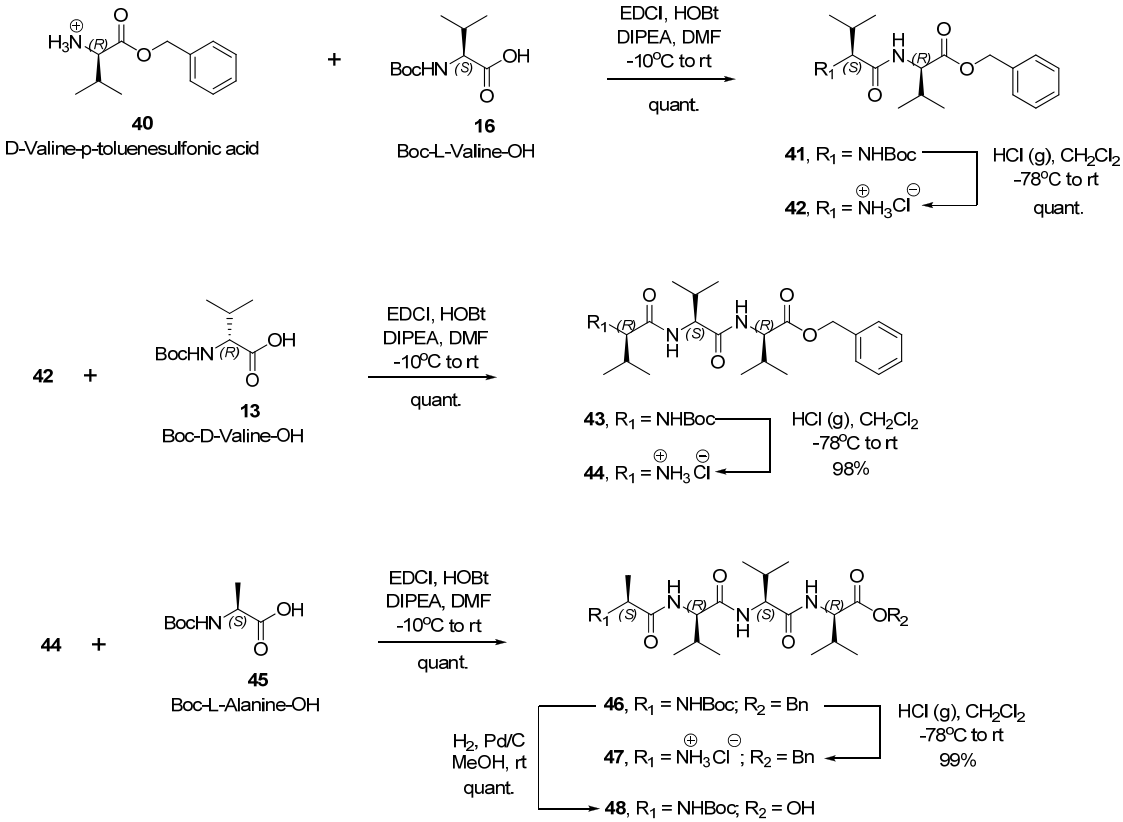
Scheme 4. Synthesis of L-Val-L-Val-D-Leu-D-Ala



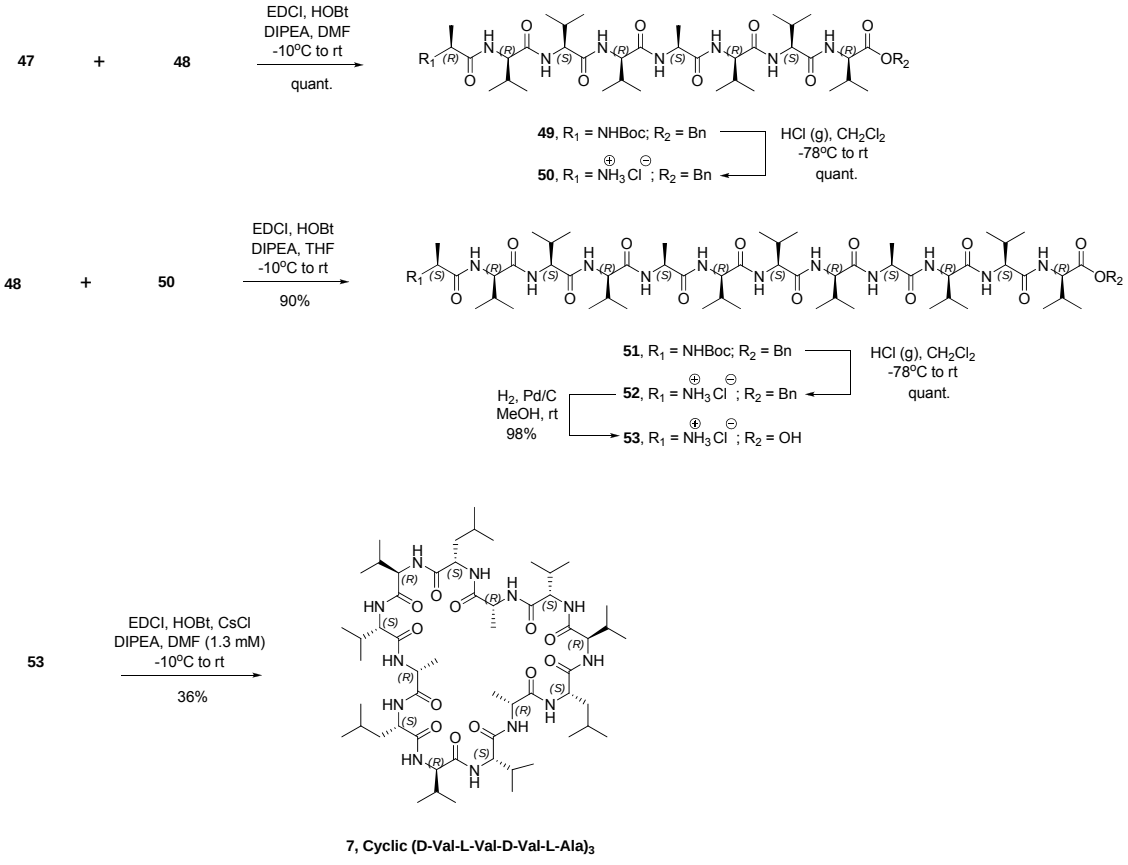
Scheme 5. Synthesis of –(DDLL)$_2$- mimic cereulide (4)



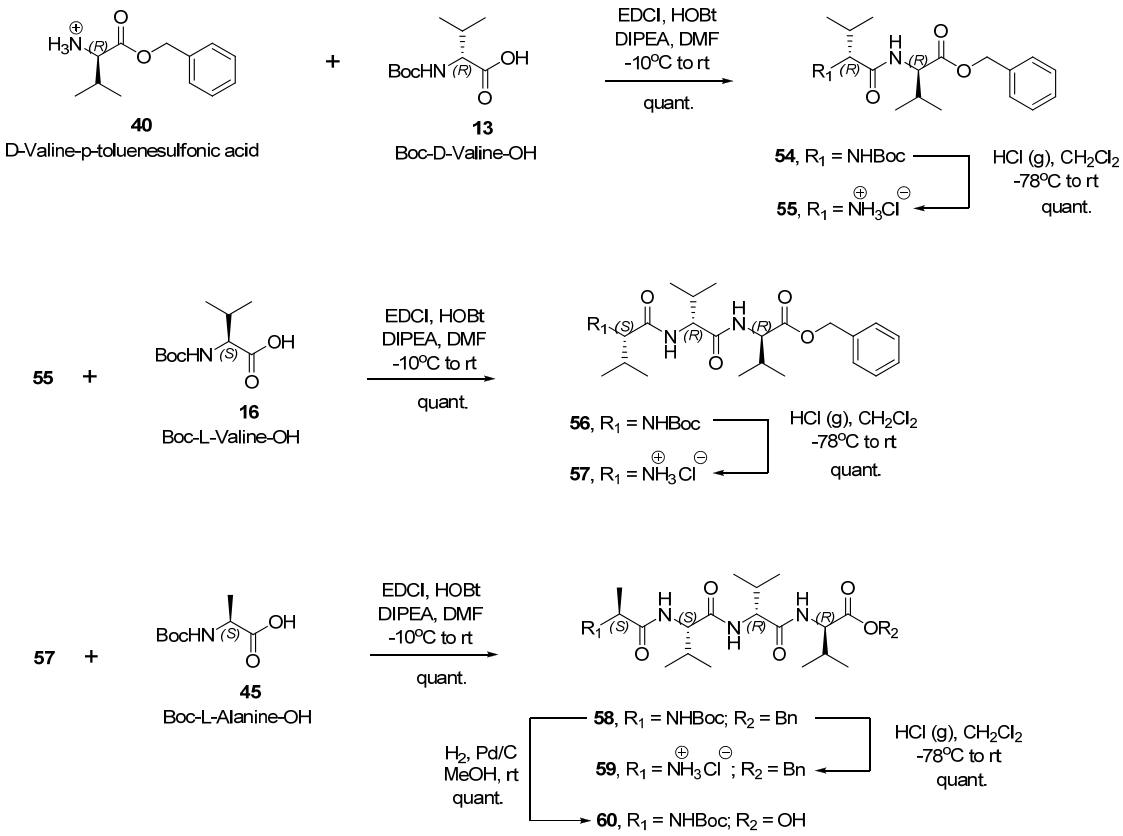
Scheme 6. Synthesis of –(DDLL)$_3$- mimic cereulide (5)



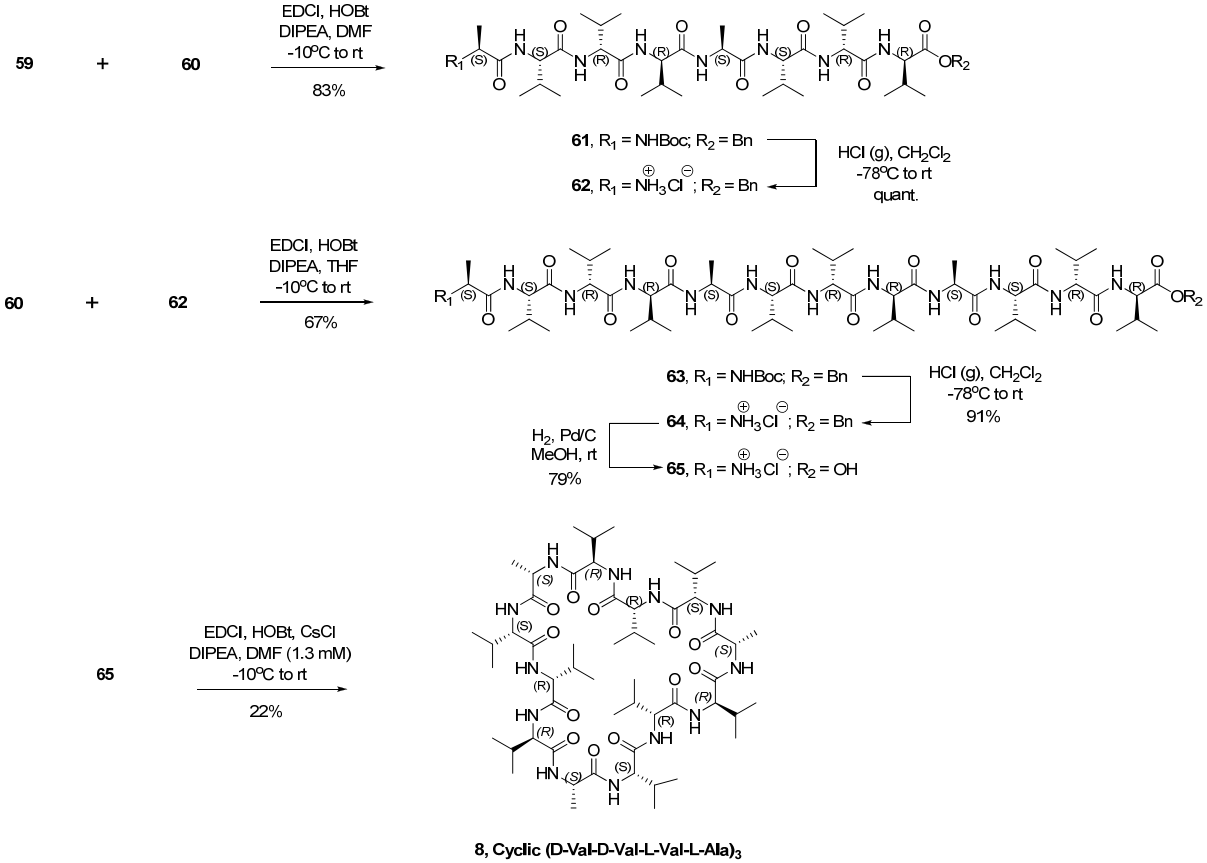
Scheme 7. Synthesis of L-Ala-D-Val-L-Val-D-Val



Scheme 8. Synthesis of –(DLDL)$_3$- mimic valinomycin (7)



Scheme 9. Synthesis of L-Ala-L-Val-D-Val-D-Val



Scheme 10. Synthesis of –$(DDLL)_3$- mimic valinomycin (8)

Table 1. In vitro antibacterial activity of DLDL, DDLL-α- amino acid cyclic peptides, cereulide and valinomycin

Compounds	Minimun inhibitory concentration (μg/ml)							
	E. coli	*Ps. aeruginosa*	*S. typhimunium*	*S. flexneri*	*S. aureus*	*S. epidermidis*	*E. faecalis*	*B. cereus*
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
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

E. coli = Escherichia coli (gram (-) bacteria); *Ps. aeruginosa* = Pseudomonas aeruginosa (gram (-) bacteria); *S. typhimunium* = Salmonella typhimunium (gram (-) bacteria); *S. flexneri* = Shigella flexneri (gram (-) bacteria); *S. aureus* = Staphylococcus aureus (gram (+) bacteria); *S. epidermidis* = Staphylococcus epidermidis (gram (+) bacteria); *E. faecalis* = Enterococcus faecalis (gram (+) bacteria); *B. cereus* = Bacillus cereus (gram (+) bacteria)

(-) means not used in experiment

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

Compounds	Minimun inhibitory concentration (μg/ml)						
	Aspergillus niger	*Aspergillus flavus*	*Aspergillus species*	*Acremonium species*	*Penicillium species*	*Candida albicans*	*Cryptococcus albidus*
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
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

References

1) N. Agata, M. Mori, M. Ohta, S. Suwan, I. Ohtani, M. Isobe, *FEMS Microbiol. Lett.* **1944**, *121*, 31-34.

2) N. Agata, M. Ohta, M. Mori, M. Isobe, *FEMS Microbiol. Lett.* **1995**, *129*, 17-20.

3) a) S. Suwan, M. Isobe, I. Ohtani, N. Agata, M. Mori, M. Ohta, *J. Chem. Soc. Perkin Trans. 1.* **1995**, 765-775; b) M. Isobe, M. Ohta. *JP 07242643*, **1995**. [*Chem. Abstr.* **1995**, *124*, 143741.]

4) M. Isobe, T. Ishikawa, S. Suwan, N. Agata, M. Ohta, *Bioorg. Med. Chem. Lett.* **1995**, *5*, 2855-2858.

5) A. Makarasen, T. Nishikawa, M. Isobe, *Synthesis.* **2009**, *13*, 2184-2204.

6) a) A. Rajkovic, M. Uyttendaele, J. Debevere, *Int. J. Food Microbiol.* **2007**, *114*, 92-99; b) P. E. Granum, Foodborne Pathogens. 2005, 409-417; c) J. L. Schoeni, A. C. Lee Wong, *J. Food Prot.* **2005**, *68*, 636-648; d) M. Toh, M. C. Moffitt, L. Henrichsen, M. Raftery, K. Barrow, J. M. Cox, C. P. Marquis, B. A. Neilan, *J. Appl. Microbiol.* **2004**, *97*, 992-1000; e) N. Agata, M. Ohta, K. Yokoyama, *Int. J. Food Microbiol.* **2002**, *73*, 23-27; f) P. E. Granum, T. Lund, *FEMS Microbiol. Lett.* **1997**, *157*, 223-228; g) N. Agata, M. Ohta, M. Mori, *Curr. Microbiol.* **1996**, *33*, 67-69; h) S. Hughes, B. Bartholomew, J. C. Hardy, J. M. Kramer, *FEMS Microbiol. Lett.* **1988**, *52*, 7-12; i) J. Melling, B. J. Capel, *FEMS Microbiol. Lett.* **1978**, *4*, 133-135.

7) N-L. Katarina, D. Max, *Helv. Chim. Acta* **1975**, *58*, 432-442.

8) a) A. Rajkovic, M. Uyteendaele, A. Vermeulen, M. Andjelkovic, I. Fitz-James, P. in't Veld, Q. Denon, R. Verhe, J. Debevere, *Lett. Appl. Microbiol.* **2008**, *46*, 536-541; b) K. Shinagawa, Y. Ueno, D. Hu, S. Ueda, S. Sugii, *J. Vet. Med. Sci. / the Japanese Society of Veterinary Science* **1996**, *58*, 1027-1029.

9) a) R. Mikkola, N.-E. L. Saris, P. A. Grigoriev, M. A. Anderson, M. S. Salkinoja-Salonen, *Eur. J. Biochem.* **1999**, *263*, 112; b) H. Ristow, J. Salnikow, H. Kleinkauf, *FEBS Lett.* **1974**, *42*, 127-130.

10) a) S. Pitchayawasin, M. Isobe, M. Kuse, T. Franz, N. Agata, M. Ohta, *Int. J. Mass. spectrom.* **2004**, *235*, 123-129; b) S. Pitchayawasin, M. Kuse, K. Koga, M. Isobe, N. Agata, M. Ohta, *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3507-3512.

11) a) H. Mahler, A. Pasi, J. M. Kramer, P. Schulte, A. C. Scoging, W. Bär, S. Krähenbühl, *N. Engl. J. Med.* **1997**, *336*, 1142-1148; b) N. Agata, M. Ohta, M. Mori, M. Isobe, *FEMS Microbiol. Lett.* **1995**, *129*, 17-20; c) K. Shinagawa, H. Konuma, H. Sekita, S. Sugii, *FEMS Microbiol. Lett.* **1995**, *130*, 87-90.

12) A. Paananen, R. Mikkola, T. Sareneva, S. Matikainen, M. Hess, M. Andersson, I. Julkunen, M. S Salkinoja-Salonen, T. Timonen, *Clin. Exp. Immunol.* **2002**, *129*, 420-428.

13) V. V. Teplova, R. Mikkola, A. A. Tonshin, N.-E. L. Saris, M. S. Salkinoja-Salonen, *Toxicol. Appl. Pharmacol.* **2006**, *210*, 39-46.

14) A. Makarasen, K. Yoza, M. Isobe, *Chem. Asian. J.* **2009**, *4*, 688-698.

15) a) Y. Q. Cheng, *Chem. Biochem.* **2006**, *7*, 471-477; b) M. Ehling-Schulz, M. Fricker, H. Grallert, P. Rieck, M. Wagner, S. Scherer, *BMC Microbiol.* **2006**, *6*, 20; c) M. Ehling-Schulz, N. Vukov, A. Schulz, R. Shaheen, M. Andersson, E. Märtlbauer, S. Scherer, *Appl. Environ. Microbiol.* **2005**, *71*, 105-113; d) F. M. Hoton, L. Andrup, I. Swiecicka, J. Mahillon, *Microbiology (Reading, U.K.)*, **2005**, *151*, 2121-2124; e) C. Apetroaie,; M. A. Andersson, C. Spröer, I. Tsitko, R. Shaheen, E. L. Jääskeläine, L. M. Wijnands, R. Heikkilä, M. S. Salkinoja-Salonen, *Arch. Microbiol.* **2005**, *184*, 141-151; f) D. Kamimura, O. Kyoo, K. Yamada, K. Yazawa, *JP 08277219*, **1996**. [*Chem. Abstr.* **1996**, *126*, 42678.]

16) A. MAINOUS III and C. POMEROY, Management of Antimicrobials in Infectious Diseases. Humana Press, **2001**. 349 p. ISBN 0-89603-821-1.

17) a) D. T. Bong, T. D. Clark, J. R. Granja, M. R. Ghadiri, *Angew. Chem. Int.* **2001**, *40*, 988-1011; b)M. R. Ghadiri, J. R.Granja, R. A. Milligan, D. E. McRee, N. Khazanovich, *Nature* **1993**, *366*, 324-327; c)H. G. Boman, *Annu. Rev. Immunol.* **1995**, *13*, 61-92; d) Z. Oren, Y. Shai,

Biopolymers **1998**, *47*, 451-463; e) D. Andreu, L. Rivas, *Biopolymers* **1998**, *47*, 415-433; f) Z. Oren, Y. Shai, *Biochemistry* **2000**, *39*, 6103-6114.

18) a) M. R. Ghadiri, J. R. Granja, L. Buehler, *Nature* **1994**, *369*, 301-304; b) J. R. Granja, M. R. Ghadiri, *J. Am. Chem. Soc.* **1994**, *116*, 10785-10786; c) H. S. Kim, J. D. Hartgerink, M. R. Ghadiri, *J. Am. Chem. Soc.* **1998**, *120*, 4417-4424.

19) (a) M. R. Ghadiri, J. R. Granja, R. A. Milligan, D. E. McRee, N. Khazanovich, *Nature* **1993**, *366*, 324-327. (b) N. Khazanovich, J. R. Granja, D. E. McRee, R. A. Milligan, M. R. Ghadiri, *J. Am. Chem. Soc.* **1994**, *116*, 6011-6012. (c) M. R. Ghadiri, *Adv. Mater.* **1995**, *7*, 675-677.

20) (a) M. R. Ghadiri, J. R. Granja, L. Buehler, *Nature* **1994**, *369*, 301-304. (b) J. R. Granja, M. R. Ghadiri, *J. Am. Chem. Soc.* **1994**, *116*, 10785-10786.

21) M. R. Ghadiri, K. Kobayashi, J. R. Granja, R. K. Chadha, D. E. McRee, *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 93-95.

22) 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.

23) National Committee for Clinical Laboratory Standard. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi. Approved standard M38-A.; Wayne, PA, 2002.

24) 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.

25) Bell, S.C.; Grundy, W.E. *Appl. Microbiol.* **1968**, *16*, 1611