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Review Article

Cyclic Peptides based on Analogs of Dermaseptin S4 Fragments

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Abstract

A cationic peptide isolated from skins of American tropical frog Phyllomedusa Savage may offer a basis for design and synthesis of antimicrobial therapeutics. The presence of the positively charged amino acid lysine in the sequence of the natural product supplies the moieties essential for the biocide activity by the disintegration of the outer cell wall. Cyclic analogs based on the basic structural features of the natural cationic peptides did not improve the biocide properties as compared with their linear analogs.

In this work, we tried to explore a ring effect on the activity of small antimicrobial peptides.

The synthesis and hemolysis, as well as antibacterial activity as broad spectrum bactericides, is reported.

Keywords: Cationic Peptide; Antimicrobial Peptides; Membrane

Introduction and Background

It has been reported that small cyclic antimicrobial peptides disrupt membrane function by integrating into the lipid bilayer [1-3]. Clinical studies recommend cyclic antimicrobial peptide rhesus θ-defensin-1 (RTD-1) is a promising potential therapeutic agent for cystic fibrosis airway disease. RTD-1 is a recently discovered cyclic peptide that, like other well-studied antimicrobial peptides, appears to bind to the lipid matrix of the cell membrane in the initial stage of activity. It is far from certain that the molecular mechanism of ō-defensins will follow the same pattern as magainin and protegrin. The richness and versatility of peptide-membrane interactions prompted us to study this novel cyclic peptide RTD-1 [4].

The ability of microbes to develop resistance towards antimicrobial agent demands the search, design, and preparation of medicinal treatment to combat the severe life-threatening problem. Recently, The World Health Organization published a report focusing on antimicrobial resistance and global surveillance with the request to develop novel drugs and treatment for the healthcare of the world's population [5]. In the last century, the antibiotics revolution contributed by eradicating many bacterial infectious diseases (Cholera, Syphilis, Pneumonia, and Tuberculosis for example), to the increase of human life longevity in a dramatic manner. It also contributed at war time to treat the wounded of WWII from deadly infections. However, the built up of microorganism resistance was always a determining cause to look for better and more effective agents for the eradication of microbes.

Natural products, in particular, those that protect the living organisms from the invasion of microbes and parasites were one of the main guides for the drug developing people, as is the immune systems found in all kingdoms of living creatures. In these innate immune systems are materials that contain the means for the antimicrobial combat and defense [6,7].

In particular tropical frogs from the Phyllomedusa [8] family contain in their antimicrobial skin peptides, Mor [9] and collaborators thoroughly investigated Dernaseptin S4 (NH2-Ala-Leu-Trp-Met-Thr-Leu-Leu-Lys-Lys-Val-Leu-Lys-Ala-Ala-Ala-Lys-Ala-Ala-Leu-Asn-Ala-Val-Leu-Val-Gly-Ala-Asn-Ala-COOH).

(ALWKTLLKKVLKAAAKAALKAVLVGANA), as a representative of this biologically active set of cationic peptides. On the other hand, Juan R. Granja [10] concluded in his publication that " Cyclic peptides may be useful since they may disintegrate the living microbial cell." The ability of cyclic D, L-α-peptides, and the related β-amino-acid cyclic peptides to form a wide array of structurally analogous is remarkable. These are biologically active structures, with surface properties that are tailored individually and coupled with the ease of the peptide synthesis. They are expected to open

new opportunities in the treatment of existing and emerging infectious diseases through both rational design and selection from combinatorial cyclic peptide libraries. Cyclic cationic adopt unique conformation and interaction of the antimicrobial peptides in lipid bilayer [11].

Today, some of the most effective antimicrobial drugs are based on cyclic peptides; Examples are Polymyxin, Daptomycin and Bacitracin [12], Gramicidin S [13] and Bactenecin [14].

Cyclic Antimicrobial cationic peptides [15,16]

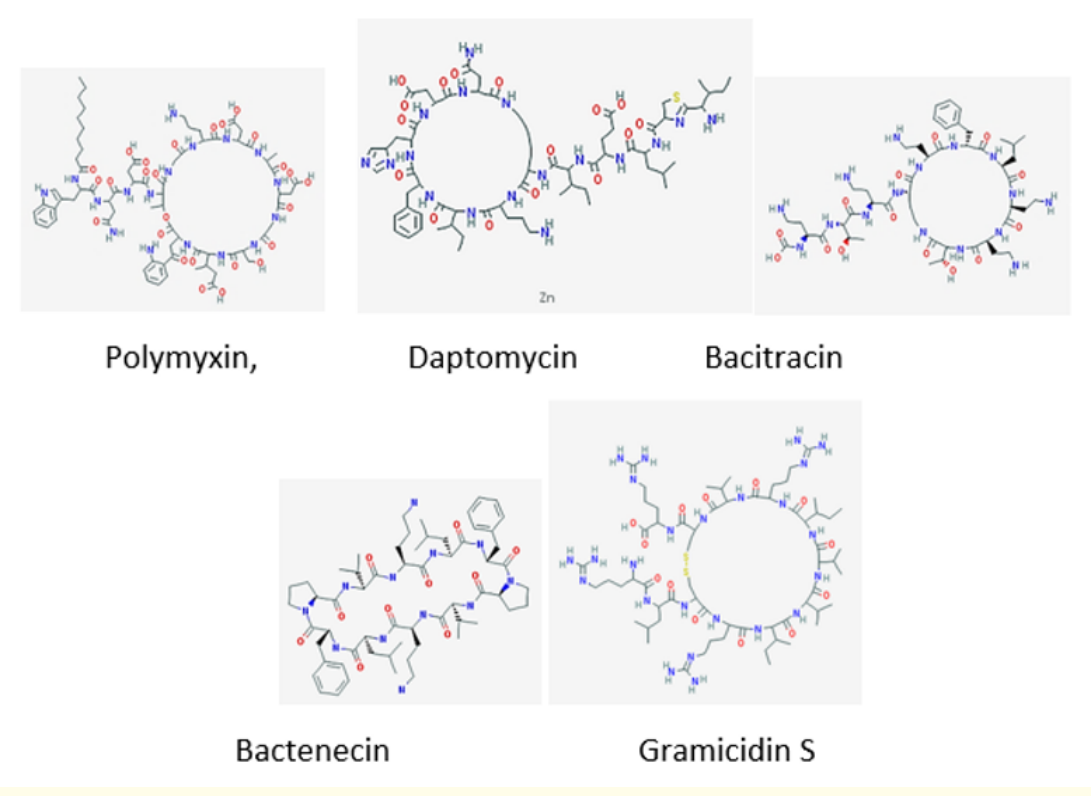


Figure 1

Rationale and Aim

Wu and Hancock and collaborators [11,17] observed in their work on open-chain and cyclic Bacitracin that the behavior of the cyclic form (S-S bond exists to form the macrocyclic ring system) and the open chain form (S-S bond is reduced) that there is a remarkable difference in antimicrobial effectiveness. The medium leads the researchers to differentiate between two mechanisms of action: The outer cell membrane is corrupted by the positively charged ends of the cationic amino acids in both cases. However, the cyclic form can form channels in the cell membrane [18] and thereby enter to the inner cytoplasm. Their internal cell processes may take place such as corruption of the genetic materials of the microorganism. The cyclic form is, therefore, more potent than the open chain in Bacitracin.

The discovery of a new family that consists of Short Cyclic Antimicrobial Peptides, the Ranacyclins [19] gives hope for selective eradication of bacteria lines. A possible mechanism for their biological activity was suggested in which a possible mechanism of pore formation by ranacyclins and pLR. Peptides bind in the first step predominantly by hydrophobic interactions and align parallel (stacking) to the outer membrane surface. Increasing peptide concentration results in the insertion of the peptides into the hydrophobic core of the membrane to form transmembrane pores. The authors suggested that these cyclic antimicrobial peptides may bear the fundamentals of selectivity in targeting the bacteria lines to be eradicated at later step of the biological activity.

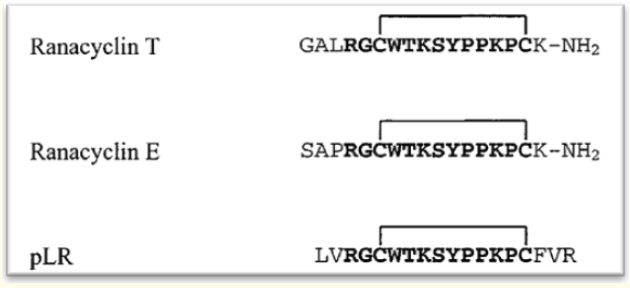


Figure 2

Ranacyclins

Bearing this in mind, we were interested to explore the possibility to obtain from the linear peptides Dermaseptin S4 and its shorter derivative cyclic peptides and to learn in preliminary experiments about the ability to the linear cationic peptides in the cyclic version to eradicate both Gram + as well as Gram – Bacteria. We also were interested to know if the cyclic compounds will affect human red blood cell hemolysis.

The purpose of the study and its importance

The general research objective is to develop cyclic peptides based on Dermaseptin S4 with Potential anti – bacterial properties.

a. Synthesis of peptides aged 9-11 amino acids, linear and cyclic
b. Checking activity on bacterial Gram + and Gram – and reaction towards human red blood cells hemolysis.

Results

Although it is accepted in literature that It is microbicide for bacteria and fungi at low micro molar concentrations. Antibacterial activity of the cyclic peptide was threefold greater than that of an open-chain analogs [20,21]. Cyclization can also constrain a bioactive peptide in its active conformation, thus lowering the entropic cost of binding to its biological target [18]. Similarly, Stelsed [22] and collaborators studied the mechanistic significance of the cyclization on the biological activity in eradication of both gram negative and gram positive bacteria. Thus, it was investigated by comparing the antibacterial activities of the cyclic peptide to that of the tri-disulfide-containing acyclic analog from which it was produced. The peptides were tested for activity against *S. aureus* and *E. coli* in an agar diffusion antimicrobial assay. It was found that the cyclized peptide was three times as active as the acyclic analog against both organisms, indicating that cyclization confers a substantial increase in antimicrobial potency.

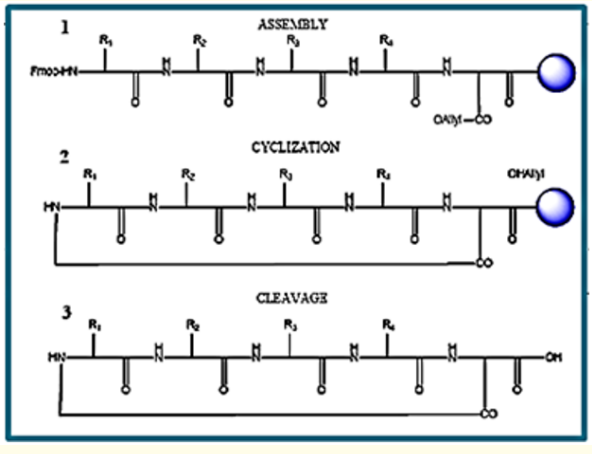
Mitsuzaki [23] and colleagues reported that the cyclic β-sheet antimicrobial peptide tachyplesin I (T-SS) was found to show 280-fold higher affinity for lipopolysaccharides (LPS) compared with acidic phospholipids, whereas the linear α-helical peptide F5W-magainin 2 (MG2) could not discriminate between LPS and acidic phospholipids.

The controversy about the properties of cyclic vs open chained bactericide peptide led us to inquire the activity issue.

We examined the antibacterial and report on the synthesis and efficiency of some 30 cyclic peptides and learn about the structural, including chirality, on their antimicrobial utility.

General strategy for the preparation of linear and cyclic peptides on solid support (SPPS) (carboxylic and amine inside chains form the cyclic structure by production of a new amide bond).

The strategy is based on a stepwise buildup of the linear peptide chain on solid support (Rink Amide), removal of the F-moc protection of the NH2 terminus, removal of the allyl ester and then cyclization on the solid support followed by cleavage of the cyclic-peptide from the solid support.



Scheme 1: Strategy for the synthesis of linear and cyclic cationic peptides.

Chemical Synthesis: Cyclic Peptide synthesis [24,25].

Linear Peptide synthesis: Linear Peptides were prepared in a reactor on a solid substrate (Fmoc Rink-Amide MRHB resist). Ay first, the preparation of the linear peptides was carried out. Then, the bulk was divided into two batches. The resis was downloaded in separate reactors one for the linear and the other for cyclization [26-28].

We have used F-moc, t-Butyl and allyl [29] as protection for residue protection.

For the Lys side chain amino group, we applied the carbonyl tert-butyloxy. (Boc) amino acid -Fmoc - Asp - OAL first Resin added to allow cyclization before removal from the solid phase. Removal of the F-moc protection group was carried out by a solution of 20% piperidine in DMF 2x10) for 10 minutes. Washing was done by using N-Methyl Pyrrolidone (NMP) for 3 times each 5 minutes and Dichloromethane (DCM) for 2 times each of duration of 5 minutes in an alternating manner. Upon completion of the connection the t-Bu ester protecting group was removed by treatment with Pd (PPh3) 40.1mmol in DCM-AcOH-MMP (37:2:1) under an atmosphere of nitrogen for two hours on the resin bearing the peptide.

The linear peptide was washed with DCM and NMP - then washed twice with 20% piperidine in DMF- and another rinse with NMP and DCM. NMP –resin slurry was separated into two reactors. Linear peptides were obtained by final Fmoc removal.

Application of the Head to side chain Cyclization strategy for the synthesis of cyclic peptides from the linear peptides [10,30]:

The cyclization was carried out by treatment with PyBOP, 1mmol HOBt 1mmol and DIEA 2mmol with 6 ml of NMP in the reactor. The changed reactor was connected to a shaker for one hour. We then rinsed with NMP) three washes for five minutes each (and DCM 2) drops to five minutes each (Off. Remove Resin peptides made using trifluoroacetic acid (TFA)-H2O-Triisopropylsilane (TIS) (95:2.5:2.5) for one hour Cocktail Shaker and excess liquids were removal by nitrogen stream followed by washing with diethyl ether and decantation gave the following products, they were kept frozen at -4ºC until further use. The following were prepared:

The following compounds were prepared and their biological activity as bactericides were examined using the MIC method [31] as follows (Figure 3)

Inspecting the data presented in Figure 3 leads us to the following conclusion

- The existence of Lysine (or Arginine) units in all peptides are essential to the bactericidal properties of all peptides. One can locate such sequences in the Ranacyclins T and E.
- The ratio between cationic amino acids to hydrophobic may vary from 10:90 to 90:10 cationic vs hydrophobic amino acids in the peptide. No remarkable effect was detected on the grounds of MIC results between cyclic and their linear analogs. However, the eradication of both Gram+ and Gram- bacteria is observed by all substances tested without much difference - linear or cyclic structure.
- Comparing the bactericidal effect of the cyclic peptides with that of short linear fragments (5 amino acids short peptides) of Dermaseptin S4 reveal that there is no remarkable change in the biological activity, It seems the presence of the Lysine side chain is essential for the bactericidal activity of all the compounds synthesized and tested [32].

It seems that the mechanism in which antimicrobial peptides eradicate bacteria depends mainly on secondary peptide sequences rather that spatial architecture as chirality or cyclic constructs. The positively charges amino acids (Lys, Arg) including the location of those in the framework of the bioactive peptide or surrogate determine the mode and efficiency of the eradication process.

R.S. Hodges [9,33] and collaborators investigated the role interactions of cell membrane with cyclic antimicrobial cationic peptides and presented graphic description on the of cyclic Structure and amphipathic topology of the cyclic cationic antimicrobial peptides. The L-Lys and D-Lys residues are denoted by K and dK, respectively. From inspecting this presentation, the -CH2- NH2 groups present in our structures of the cyclic and linear antimicrobial peptides, is interacting with the outer cell membrane. This may be the cause in our investigation for the bactericide activity, The lysine NH2 groups are disintegrating the cell membranes in a similar manner in linear and cyclic peptides investigated in this paper.

Peptide synthesis and purification

All peptides were synthesized by solid phase peptide synthesis usingpre-coupled Boc-Pro-phenylacetamidomethyl resin (Novabiochem, San Diego, CA) on an Applied Biosystems model 430A peptide synthesizer (Foster City, CA) using standard t-butyloxycarbonyl chemistry (25) as reported previously (26). Side chain protecting groups were 2-bromobenzyloxycarbonyl for tyrosine and formyl for lysine and ornithine. Side chain formylation was carried out by the procedure of Kitagawa., *et al*. (27) using either Na-Boc-lysine or Na-Boc-ornithine. Peptides were cleaved from the resin using anhydrous hydrogen fluoride (20 ml/g resin) in the presence of 10% anisole for 1 h at 25°C. Peptides were extracted from the resin with glacial acetic acid and lyophilized. Crude linear peptides were purified by reversed-phase HPLC on a Synchropak RP-4 preparative C8 column (250 3 21.2 mm inner diameter, 6.5-mm particle size, 300-Å pore size) (Synchrom, Lafayette, IN) using a Beckman System Gold HPLC system (San Ramon, CA). The flow rate was 5 ml/min with a linear AB gradient of 0.25% B/min where solvent A was 0.05% trifluoroacetic acid/H2O and solvent B was 0.05% trifluoroacetic acid/acetonitrile. Purity of peptides was verified by analytical reversed-phase HPLC on a Zorbax SB-C8 column (250 3 4.6 mm inner diameter, 5-mm particle size, 300-Å pore size) (Rockland Technologies, Wilmington, DE) using a Hewlett-Packard 1090 chromatograph with a linear AB gradient of 2% B/min and a 1 ml/min flow rate. Identity of peptides was confirmed by mass Spectrometry on a Fisons VG Quattro triple quadrupole mass spectrometer (Manchester, United Kingdom) fitted with an electrospray ionization source operating in positive ion mode.

Copd Numb	sequence	charge	Mol mass	MIC E.Coli linear	MIC E.Coli cyclic	MIC S. Aureus linear	MIC S. Aureus cyclic
RK1L	Gly-Lys-Gly-Lys-Gly-Lys-Gly-Lys-Gly-Asp	3+	931	50		50	
RK1C	Gly-Lys-Gly-Lys-Gly-Lys-Gly-Lys-Gly-Asp	3+	913		30		25
RK2L	Gly-Lys-Gly-Lys-Gly-Lys-Gly-Gly-Lys-Gly-Asp	3+	988	50		50	
RK2C	Gly-Lys-Gly-Lys-Gly-Lys-Gly-Gly-Lys-Gly-Asp	3+	970		40		25
RK3L	Lys-Lys-Gly-Lys-Gly-Lys-Gly-Lys-Asp	4+	945	40		50	
RK3C	Lys-Lys-Gly-Lys-Gly-Lys-Gly-Lys-Asp	4+	927		45		50
RK4L	Lys-Gly-Gly-Lys-Gly-Lys-Gly-Gly-Lys-Asp	3+	931	40		50	
RK4C	Lys-Gly-Gly-Lys-Gly-Lys-Gly-Gly-Lys-Asp	3+	913		30		50
RK5L	Gly-Lys-Gly-Val-Lys-Lys-Gly-Val-Gly-Lys-Asp	3+	1072	45		50	
RK5C	Gly-Lys-Gly-Val-Lys-Lys-Gly-Val-Gly-Lys-Asp	3+	1054		50		35
RK6L	Ala-Lys-Ala-Ala-Lys-Ala-Lys-Ala-Lys-Asp	3+	1001	45		50	
RK6C	Ala-Lys-Ala-Ala-Lys-Ala-Lys-Ala-Lys-Asp	3+	983		50		50
RK7L	Gaba-Gaba-Lys-Gaba-Lys-Gaba-Lys-Gaba-Asp	3+	943	50		50	
RK7C	Gaba-Gaba-Lys-Gaba-Lys-Gaba-Lys-Gaba-Asp	2+	925		50		50
RK8L	Gly-Val-Val-Lys-Lys-Gly-Lys-Lys-Val-Asp	3+	1057	25		50	
RK8C	Gly-Val-Val-Lys-Lys-Gly-Lys-Lys-Val-Asp	3+	1039		30		50
RK9L	Val-Val-Gly-Val-Lys-Gly-Lys-Gly-Lys-Gly-Asp	2+	1043	50		50	
RK9C	Val-Val-Gly-Val-Lys-Gly-Lys-Gly-Lys-Gly-Asp	2+	1025		50		50
RK10L	Lys-Ala-Leu-Val-Gly-Val-Lys-Gly-Lys-Lys-Asp	3+	1142	50		45	
RK10C	Lys-Ala-Leu-Val-Gly-Val-Lys-Gly-Lys-Lys-Asp	3+	1124		20		50
RK11L	Val-Gly-Val-Lys-Gly-Lys-Gly-Lys-Gly-Lys-Val-Asp	3+	1171	50		50	
RK11C	Val-Gly-Val-Lys-Gly-Lys-Gly-Lys-Gly-Lys-Val-Asp	3+	1153		30		50
RK12L	Val-Gly-Val-Val-Lys-Gly-Lys-Gly-Lys-Lys-Asp	3+	1114	45		50	
RK12C	Val-Gly-Val-Val-Lys-Gly-Lys-Gly-Lys-Lys-Asp	3+	1096		50		50
RK13L	Gly-Val-Gly-Val-Gly-Gly-Lys-Gly-Lys-Asp	1+	872	60		60	
RK13C	Gly-Val-Gly-Val-Gly-Gly-Lys-Gly-Lys-Asp	1+	854		60		60
RK14L	DLys-Gly-Gly-DLys-Gly-DLys-Gly-Gly-DLys-Asp	4+	945	40		50	
RK14C	DLys-Gly-Gly-DLys-Gly-DLys-Gly-Gly-DLys-Asp	4+	927		40		50
RK15L	DLys-Gly-Gly-DLys-Gly-DLys-Gly-Gly-DLys-Asp	3+	931	40		50	
RK15C	DLys-Gly-Gly-DLys-Gly-DLys-Gly-Gly-DLys-Asp	3+	913		40		50

table 1: linear peptides and their cyclic analogs MIC comporison E.Coli and S.Aureus

Figure 3

Cyclization of Peptides—Pure linear formylated peptides were cyclized at a concentration of 2 mg/ml in N,N-dimethylformamide using 3 eq of each of benzotriazolyl N-oxytri-dimethylamino-phosphonium hexafluorophosphate, 1-hydroxybenzotriazole, and diisopropylethylamine. The progress of the cyclization reaction was monitored by analytical reversed-phase HPLC and was typically complete after 12 h. Cyclic peptides were deformylated (10% HCl in methanol, 37°C for 24 h) and purified by preparative reversed-phase HPLC. Purified cyclic peptides were homogeneous by analytical reversed-phase HPLC and gave correct primary ion molecular weights by mass spectrometry as well as appropriate amino acid analysis ratios. Peptide concentration used for all assays was based.

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