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In vitro determination of the short-chain synthetic peptide RP13 antimicrobial activity

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ABSTRACT

Background. The proliferation of antibiotic-resistant microorganisms, along with the lack of new drugs against them, has elicited the interest of the scientific community on the study and development of endogenous synthetic compounds with bacteriostatic or bactericidal activity. In recent years, several short-chain, low molecular weight peptides isolated from natural sources such as plants and animals have demonstrated an array of antimicrobial activities. Despite having structural characteristics similar to microbicidal peptides isolated from human platelets, peptide RP11 does not exhibit antimicrobial activity. **Objective.** *In vitro* determination of the antimicrobial activity of the synthetic peptide RP13. **Material and methods.** Peptide RP13 was prepared modifying the original amino acids sequence of peptide RP11, reversing the position of the amino acids lysine and tyrosine in order to modify the conformation of the original peptide. These amino acids are localized close to the N-terminus of the peptidic chain. Peptide RP13 was prepared in solution using conventional methods for peptide synthesis. The antimicrobial activity of RP13 was assessed against the microorganisms *S. aureus, E. faecalis* and *E. coli* in a test solution and later evaluated by cultivation of plates during the first 2 h after inoculation of bacteria. RP13 activity antimicrobial was compared against tetracycline, a broad-spectrum antibiotic. **Results.** The new peptide RP13, resulting form the structural modification of the amino acid sequence of peptide RP11, displayed antimicrobial activity. RP13 demonstrated to be more efficient inhibiting the growth of gram-positive than gram-negative bacteria. **Conclusions.** The structural modification of peptide RP11, obtained from human platelets, resulted in a new peptide with improved antimicrobial activity. These results clearly demonstrate that peptides of natural origin, as well as their synthetic analogs, represent an attractive alternative against pathogenic agents.

Determinación* in vitro *de la actividad antimicrobiana del péptido sintético de cadena corta RP13

RESUMEN

Antecedentes. *La proliferación de microorganismos resistentes a los antibióticos, junto con la falta de nuevos fármacos para combatir a estos microorganismos, ha centrado el interés de la comunidad científica en el estudio y desarrollo de productos sintéticos endógenos con actividad bacteriostática o bactericida. En años recientes, algunos péptidos de cadena corta y bajo peso molecular aislados de fuentes naturales como plantas y animales han mostrado tener actividad antimicrobiana. A pesar de tener características estructurales semejantes a las proteínas microbicidas de plaquetas humanas, el péptido sintético RP11, no posee actividad antimicrobiana.* ***Objetivo.*** *Determinación* in vitro *de la actividad antimicrobiana del péptido sintético RP13.* ***Material y métodos.*** *El péptido RP13 se preparó modificando la secuencia original de aminoácidos presente en el péptido RP11, invirtiendo la posición de los aminoácidos lisina y tirosina, con la finalidad de modificar la conformación estructural del péptido original. Estos aminoácidos están localizados cerca del extremo N-terminal de la cadena peptídica. El péptido RP13 se preparó mediante síntesis en solución, usando técnicas convencionales de acoplamiento de aminoácidos. La actividad antimicrobiana de RP13 se evaluó usando los microorganismos* S. aureus, E. faecalis *y* E. coli *por el método de dilución y evaluada por cultivo de placas durante las primeras 2 h después de la inoculación de las bacterias. La actividad antimicrobiana de RP13 se comparó con la del antibiótico de amplio espectro tetraciclina.* ***Resultados.*** *El nuevo péptido RP13, obtenido de la modificación estructural de la secuencia original de aminoácidos del péptido RP11, mostró actividad antimicrobiana. RP13 inhibe el crecimiento de bacterias gram-positivas más eficientemente en comparación con las gram-negativas.* ***Conclusiones.*** *La modificación estructural del péptido RP11 resultó en un nuevo compuesto con actividad antimicrobia-*

na más eficiente Los resultados demuestran que péptidos de origen natural, así como sus análogos sintéticos, representan una posible alternativa en contra de agentes patógenos.

Key words. Antimicrobial peptides. Antimicrobial activity. Gram-negative. Gram-positive. Bacteria. Synthetic peptides.

Palabras clave. *Péptidos antimicrobianos. Actividad antimicrobiana. Bacterias Gram-negativa. Bacterias Gram-positiva. Péptidos sintéticos.*

INTRODUCTION

The increase in the number of infections caused by pathogens and the continuous appearance of antibiotic-resistant strains, have focused the attention of the scientific community on the need to discover new products of endogenous origin with different mechanisms of action than those of common use.[1,2] During the last decade, some short chain and low molecular weight peptides have shown a great efficiency in inhibiting bacterial growth and, at the same time, are effective molecules for the host mechanism of defense against invading pathogens. These compounds, called antimicrobial peptides, have been isolated from a variety of sources, such as plants and animals.[3,4]

Antimicrobial peptides have been classified on the basis of their biochemical properties or their structural characteristics such as anionic peptides, aromatic dipeptides and cationic peptides, which are the most abundant. Notable examples are cecropins, defensins, thionins, cathelicidins and thrombocidins,[5,6] which have been isolated from different parts of human body.[7,8] The main characteristic of these peptides is their electrostatic interaction with the lipidic components of the cell membrane of prokaryote organisms, deploying their bactericidal action by altering the permeability of the cell membrane causing the cell death.[9,10]

The antimicrobial activity of platelets has been described as a consequence of the release of a series of cationic peptides into the bloodstream in the presence of pathogens or when they are stimulated with thrombin. These compounds have been referred to as platelet microbicidal proteins (PMPs) or thrombin-stimulated microbicidal proteins (tPMPs). The result of this action is a rapid and highly efficient elimination of a great proportion of opportunistic microorganisms without having to activate the whole immune system and therefore this microbicidal peptides can be considered part of the first barrier of the host against pathogenic agents.[11-13]

Recent reports have shown that antimicrobial peptides can be prepared using biotechnology techniques or by chemical synthesis which can provide pure products on a large scale and, at the same time, generate new compounds to study their biological activity.[14,15]

The isolation, purification and structural characterization of a short-chain endogenous peptide isolated from factor IV of human platelets called C18G, which displays *in vitro* antimicrobial activity has been reported in the literature.[16] In a similar manner, structural analogs of these peptides have been synthesized with the goal of determining their structure-activity relationship. RP1 and RP11 peptides, consisting of 18 and 13 amino acid residues respectively, have been synthesized, and display characteristics similar to antimicrobial peptides isolated from platelets. For instance, RP1 showed antimicrobial activity against *E. coli*.[17]

The structural modification of a bioactive compound may have a significant impact on its physicochemical properties and changes in its activity, which can go from potentiation to total disappearance of the activity, and in some cases the appearance of different biological properties can occur. With this background in mind, peptide RP13 (H2N-ALKYRLFKKLKKFCO2H) was prepared as a structural analog of RP11 (H2N-ALYKRLFKKLKKFCO2H), which is inactive against *E. coli*. The structural modification consisted in reversing the position of the amino acids Lys and Tyr in the peptidic chain. The purpose of this change was to modify steric hindrance, between the side chains of Lys and Arg. Placing a Tyr residue between two positively charged residues must change the conformation of the peptide and therefore the activity of the peptide against gram-positive and -negative bacteria.[18]

MATERIAL AND METHODS

Peptide synthesis

RP13 peptide comprised of 13 amino acids (H_2N-ALKYRLFKKLKKF-CO_2H) was synthesized in solution following a convergent approach. Two main fragments were prepared: the first consisting of 6 and the second of 7 amino acid residues, which were bonded by conventional coupling methods using

dicyclohexylcarbodimide (DCC, Aldrich, St. Louis, USA), pentafluorophenol (PFF, Fluka, St. Louis, USA) or (benzotriazol-1-yloxy) tris (dimethylamino) phosphonium hexafluorophosphate (BOP, Aldrich, St. Louis, USA).

The amino acids were used orthogonally protected to avoid side reactions in each of the consecutive stages of coupling and deprotection.[19] RP13 peptide was finally obtained after removing the protective groups under mild basic conditions with lithium hydroxide (LiOH, Aldrich), followed by catalytic hydrogenation with palladium supported on carbon (Pd/C, Aldrich) for the deprotection of the amino groups.[20,21] The peptide was purified by recrystallization by pair of solvents (acetone/hexane) and obtained with an overall yield of 39%. A sample of the peptide was dissolved in 0.1% acetic acid (pH 5.5) and its purity was determined by reverse-phase HPLC analysis using a C18 column with H_2O/acetonitrile gradient elution system and 1% trifluoroacetic acid (TFA).[22]

Structural characterization of the final product and all intermediates was performed by proton (1H NMR, Varian, 700 MHz) and carbon Nuclear Magnetic Resonance (13C NMR, 700 MHz, Varian).

Antimicrobial test

Strains of *S. aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212 and *Escherichia coli* ATCC 32218 were used in this study to carry out the microbiological tests. As a result of the preliminary assays, it was confirmed that the microorganisms were susceptible to tetracycline, determining the minimum inhibitory concentration (MIC) values for an *in vitro* test in solution with conventional culture media.

S. aureus, E. faecalis and *E. coli* bacteria developed in Muller-Hinton broth (Fluka Biochemika, Steinheim Germany) for 18-20 h at 37 °C with constant agitation up to the logarithmic growth phase; the microorganisms were collected by centrifugation at 6000 rpm (Zentrifuge, Hettich, Tuttlingen Germany) and washed with saline solution (pH 7.2). *S. aureus* and *E. faecalis* were adjusted to an optical density of 0.261 and *E. coli* was adjusted to 0.104 by means of a spectrophotometer (Biomate 5, Thermospectrum, Loughborought, Manchester England) at λ = 540 nm wavelength. The cell density for each microorganism determined spectroscopically was confirmed by colony-forming units (CFU) through quantitative cultures in Muller-Hinton agar plates (Fluka Biochemika, Steinheim Germany).[23]

Antimicrobial activity of the peptide was evaluated during 3 h in a conventional Mueller-Hinton culture medium (Fluka, Biochemika Steinheim Germany) at pH 7.2, utilizing serial dilutions of the microorganism to be tested to a final cell density of 1 x 10^3 CFU/mL.

The RP13 peptide was used at concentrations of 50, 25, 12.5, 4, 2, 1 and 0.5 mg/mL for *S. aureus* and *E. faecali*. In the case of *E. coli*, the concentrations used were 100, 80, 60, 50, 25 and 12.5 mg/mL. To determine the inhibitory capacity of RP13, samples of 100 μL at 0, 30, 60, 90, 120 and 150 min of exposure of the peptide to the microorganism were plated in Mueller-Hinton agar plates and incubated at 37 °C for 18 h. In parallel tests, the MIC value of tetracycline for the microorganisms used in the assay was determined as a broad spectrum antibiotic reference.[23]

RESULTS

RP13 peptide showed a highly significant effect on the growth inhibition of gram-positive bacteria and was less efficient for gram-negative bacteria. These results show that susceptibility is directly related to the time exposure between the peptide and the microorganisms (Figures 1-3). The growth inhibition of the strains tested showed a depending relation on the concentration of RP13 peptide. A MIC value of 1 and 2 μg/mL was obtained respectively against *S. aureus* and *E. faecalis*, and a MIC value of 60 μg/mL against *E. coli* (Figure 4). The MIC values obtained for RP13 were higher than the value for tetracycline (Table 1).

DISCUSSION

Antimicrobial peptides have demonstrated a primarily inhibitory effect on bacterial growth, and their activity has also been evaluated in fungi, yeasts and viruses, obtaining good results in terms of their inhibitory activity.[24,25]

In a previous study, RP11 peptide was designed with the structural characteristics similar to the peptides derived from PMPs as well as microbicidal proteins stimulated by tPMPs. However, this peptide did not show good activity against *E. coli*, the only bacterium used to evaluate its antimicrobial activity.[17]

By studying the sequence of amino acids in RP11, it was speculated that there may be a strong steric hindrance among amino acids Arg-Lys-Tyr and/or polar interactions through hydrogen bonds among the side chains which restricts the free bond rotation

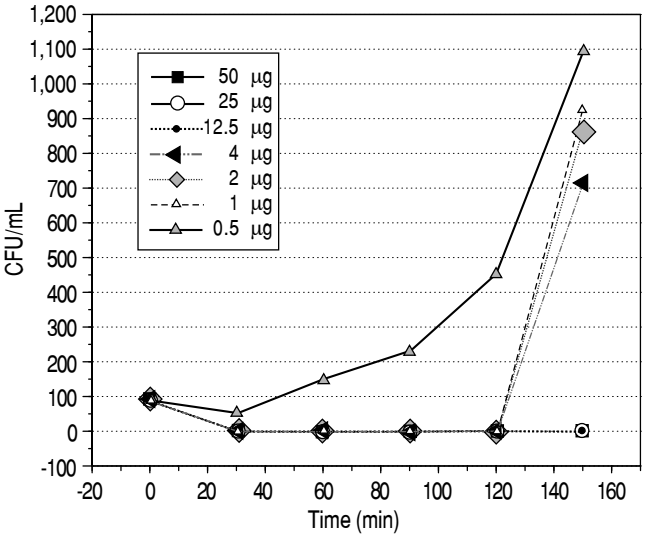

Figure 1. Susceptibility of S. aureus *in the presence of peptide RP13. Approximately 10^3 bacteria per milliliter were cultured at 37 °C in the presence of the peptide RP13. Colony forming units (CFU) were obtained by quantitative cultures taken every 15 min during 2 h.*

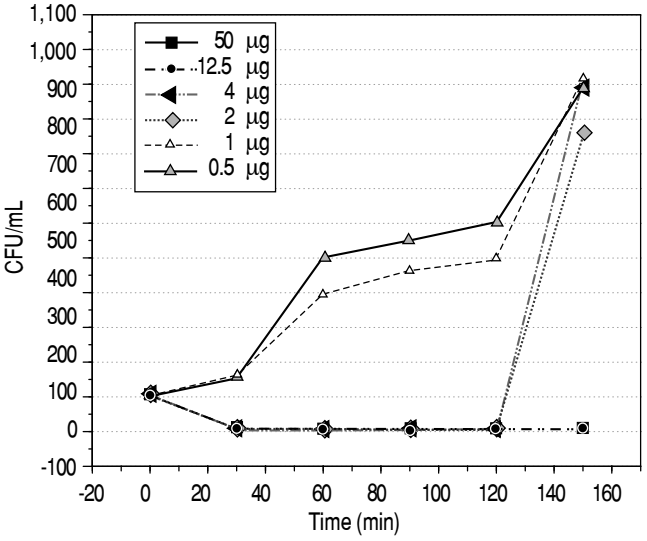

Figure 2. Susceptibility of E. faecalis in the presence of peptide RP13. Kinetics of antimicrobial activity of the peptide RP13 against E. faecalis *in a test of 2 h in culture medium Muller-Hilton to 37 °C by taking samples every 15 min tested for obtaining the colony-forming units (CFU).*

required for antimicrobial action on the membrane of *E. coli*. For this reason, we thought that reversing the position of the amino acids Lys-Tyr might reduce the problems and promote antimicrobial activity against gram-positive and -negative bacteria.

Fortunately, it was confirmed that antimicrobial activity of the peptides is closely related to the three

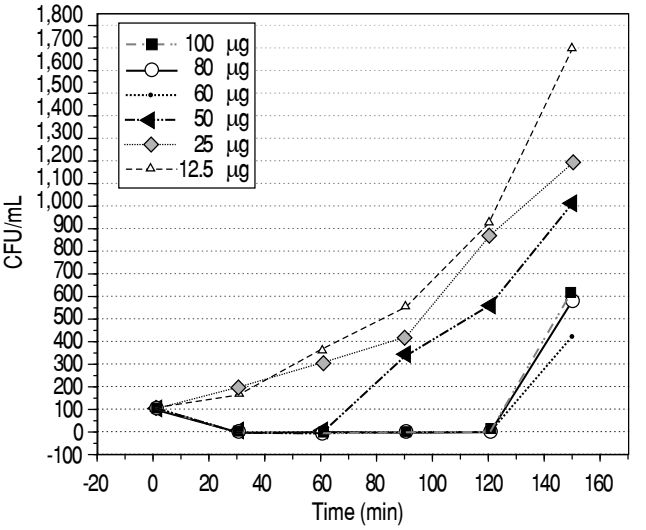

Figure 3. Susceptibility of E. coli *in the presence of peptide RP13. Effect of the peptide RP13 on the bacterial growth of* E. Coli. *The susceptibility was evaluated in a trial of 2 h by taking samples every 15 min to obtain the colony-forming units (CFU).*

Table 1. Results of antibacterial activity (MIC) from RP13 peptide, 2 h after incubation with microorganisms at 37 °C.

Microorganism	MIC peptide RP13 (µg/mL)	MIC tetracycline (µg/mL)
S. aureus	1	0.5
E. faecalis	2	1
E. coli	60	1

dimensional structure that the peptide can adopt in solution, a property that depends on the amino acids sequence on the peptide chain.[26] There are reports where the peptides containing basic amino acids such as Lys, His and Arg provide a cationic character to the molecule, and the presence of hydrocarbon residues such as Leu or Ile, aromatic compounds such as Phe and Tyr favor the specific formation of hydrophobic or hydrophilic regions, which are directly correlated with the amphipathic structure of the peptides, a characteristic that increases its antimicrobial activity against bacteria.[27,28] For this purpose, RP13 peptide was prepared with its peptidic chain containing six basic amino acids, four aromatics, and three hydrocarbons in a sequence that allows clear differentiation of the hydrophilic and hydrophobic regions and a strong cationic character, in order to increase the electrostatic interactions between the amino acids arginine and tyrosine.

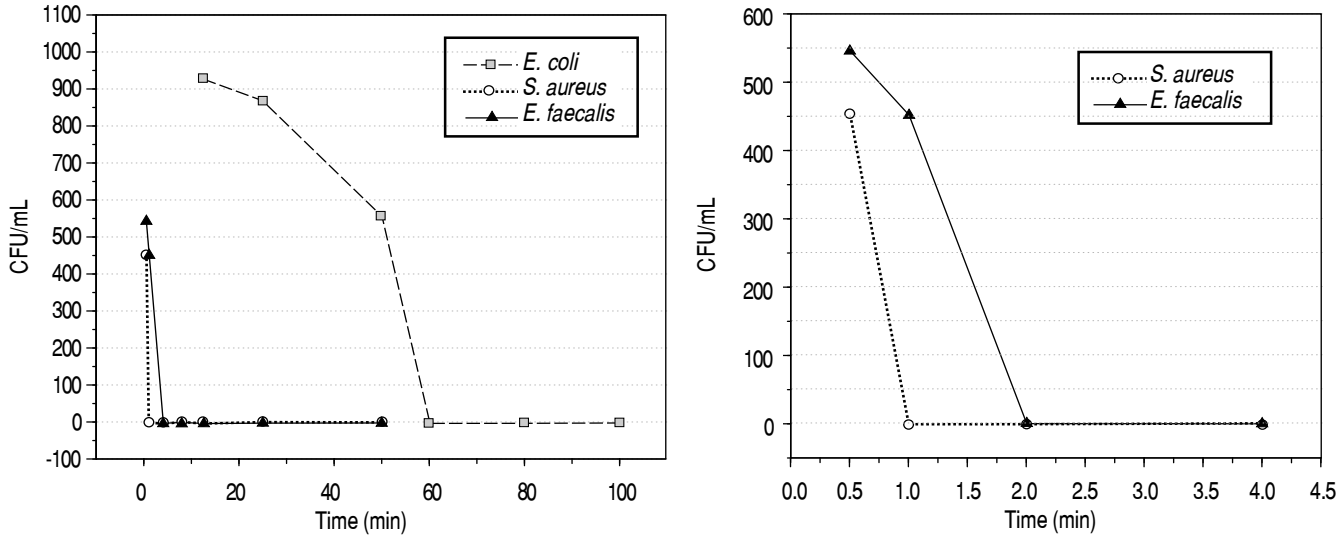

Figure 4. A. Antimicrobial activity of synthetic peptide RP13, 2 h after incubation with the indicated microorganisms. Minimum inhibitory concentration of RP13 peptide against the bacteria tested in vitro *assay dilution for a period of 2 h. Minimum inhibitory concentration values (MIC) of the RP13 peptide against the bacteria tested were obtained by tube dilution technique for a period of 2 h.* **B.** *Defining the zone of inhibition of the synthetic peptide RP13, 2 h after incubation with Gram-positive bacteria.*

The results obtained showed that there was considerable homogeneity between *S. aureus* and *E. faecalis*, observing a kinetic behavior similar for the inhibitory growth activity. However, there were minimal differences, because *S. aureus* was more sensitive to the antimicrobial effect the RP13 peptide and had a MIC of 1 μg/mL. The antimicrobial activity was maintained for 2 h at low concentrations (0.5-4 μg/mL) and this behavior was maintained only at concentrations > 12 μg/mL (Figure 1). *E. faecalis* showed less sensitivity to RP13 peptide with a MIC value of 2 μg/mL. The tendency of the inhibitory effect had a similar behavior to that showed by *S. aureus* (Figure 2). With respect to *E. coli,* the results were different; RP13 peptide had a low antimicrobial activity compared to its activity against gram-positive bacteria. Thus, *E. coli* showed a MIC value of 60 μg/mL at 2 h of incubation, and the antimicrobial effect remained after 2.5 h only at concentrations > 100 μg/mL (Figure 3). The results of antimicrobial activity are similar to evaluations performed with endogenous antimicrobial peptides as well as with synthetic peptides, where the inhibitory growth activity was more prominent in gram-positive rather than gram-negative bacteria.[29,30]

Evaluation of the antimicrobial activity of the original reported RP11 peptide did not show a significant effect inhibiting the growth of *E. coli,* the only microorganism evaluated in this study.[17] In the case of RP13, a MIC value of 60 μg/mL was obtained, which is between the interval reported for other peptides with antimicrobial properties. Similarly, Azuma, *et al.*,[31] evaluated a series of 20 peptides whose linear main chain was composed of between 20 and 30 amino acids residues and observed that ca. 30% of the peptides had a MIC value of ≤ 60 μg/mL for gram-negative bacteria. Similar results with regard to MIC values were described by Jang, *et al.*,[32] in their study of inhibitory activity of halocidine derivatives, a peptide isolated from tunicates. Additionally, it has been reported that the isolated peptides from human platelets are less efficient against *E. coli* compared with the inhibitory power against gram-positive bacteria.[33,34] Linear peptides are not the only peptides that demonstrated this tendency, Janieszewska, *et al.*,[35] described a series of short-chain dendrimeric peptides with highly cationic character, 60% had MIC values ≤ 80 μg/mL. The most likely reason for this activity may be the different constitution of the cell membranes of gram-negative and gram-positive bacteria, the site where it has been postulated that these compounds act to exert their antimicrobial activity.[5,9,36]

Most of the reported evaluations have been carried out in conventional culture media, being Mueller-Hinton the most used. When this media was used, the peptide had a greater activity at short times. With incubation periods between 2 and 3 h or larger, the peptide lost its antimicrobial activity.

Determinations of the antimicrobial properties of peptides have been performed modifying the conditions of the culture, utilizing serum biometrix plasma and whole blood, simulating the physiological conditions of the human body by pre-incubating the peptide before the contact with the microorganism. However, the results were not significant because MIC values did not show an increase in the antimicrobial activity.[17]

The main disadvantage of this class of compounds of peptidic origin is the proteolytic degradation, leading to the loss of the activity activity which is reflected in a short period of inhibition. Moreover, the activity may also decrease due to the culture medium used. It has been reported that compounds of peptidic origin and others, such as dextrin, polysaccharides and sulfates interact strongly with the constituents of the culture medium, a factor to be considered for future evaluations of antimicrobial peptides.[37]

In conclusion, antimicrobial peptides are promising candidates against resistant pathogens. One of the greatest advantage of it use is the absence or occasional resistance that the microorganisms may generate against them. Currently, their clinical utility is limited because of problems of enzymatic degradation and availability from natural sources in sufficient quantities for clinical trials and consequently chemical synthesis is required for to the preparation of this type of compounds. Thus far, once a potentially active peptide is identified as an antimicrobial agent, the synthesis of derivatives by manipulation of its structure may allow the preparation of compounds with an improved biological activity.

The sequence of amino acids that form the peptidic chain of compound RP13 plays a pivotal role in its structure and consequently in its antimicrobial activity. The change in the position of the amino acids lysine and tyrosine resulted in the inhibition of cellular growth against gram-positive bacteria *(S. aureus* and *E. feacalis)* and even *E. coli*, a gram-negative bacterium, shows susceptibility to RP13 peptide. The results obtained in the evaluation trial of its inhibitory activity, represent a contribution to growing list of synthetic antimicrobial peptides and positions it as a strong candidate in the search for alternatives to combat infectious diseases.

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