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Synthesis and study of antibiotic peptides
Síntesi i estudi de pèptids antibiòtics

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"Science never solves a problem without creating ten more"

George Bernard Shaw

Aquest treball ha estat possible gràcies al suport que m'han donat les persones que han estat al meu costat durant aquests quatre anys de la carrera.

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REPORT

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1. SUMMARY

In the recent years, the effectiveness of some antibiotics is progressively decreasing due to the emerge of multidrug resistance bacteria while the development of new antibiotics is slow and complex procedure. Thus, it is one of the biggest public health problems because they can cause some disease which are difficult, and sometimes impossible, to treat. In general, this resistance is caused by irreversible mutations in bacterial genes and can be prevented by minimising the misuse of antibiotics.

KR-12 is the smallest antimicrobial peptide of human cationic LL-37 peptide. Thus, shorter peptide possessed the same antimicrobial and anti-inflammatory activities than the biggest one but without the associated mammalian cell toxicity. The production cost of KR-12 is much lower than LL-37 and it is also faster to obtain. It plays an essential role in protecting humans against infectious diseases and it has wound healing, immune modulating and anticancer effects.

The aim of this work is to synthesize this peptide by solid phase peptide synthesis using Fmoc/^{t}Bu protection strategy. Secondly, it has been designed and synthetized a KR-12 analog in order to improve its activity by accoupling a fatty acid group. Finally, these peptides have been characterized by using RP-HPLC and ESI mass spectroscopy. They have been obtained in moderated yield and high purity.

Furthermore, the microbiological activity of both peptides has been evaluated by determining their minimum inhibitory concentration (MIC) and, also, has been studied their toxicity by hemolytic assay.

Keywords: antibiotic peptides, KR-12 peptide, solid phase, microbiological activity, hemolysis.

2. RESUM

En els últims anys, l'efectivitat d'alguns antibiòtics està disminuint progressivament a causa de l'aparició de bacteris multi-resistents, mentre que, el desenvolupament de nous antibiòtics és un procés lent i complex. Això fa que sigui un dels majors problemes de l'actual salut pública, ja que, poden causar malalties difícil de ser tractades o inclús impossible. En general, aquesta resistència és causada per mutacions irreversibles en els gens bacterians i es pot prevenir minimitzant el mal ús dels antibiòtics.

El KR-12 és el pèptid antimicrobià més petit del pèptid humà catiònic LL-37. El més curt posseeix les mateixes activitats antimicrobianes i antiinflamatòries que el més llarg però sense la toxicitat associada a les cèl·lules dels mamífers. El cost de producció de KR-12 és molt inferior al del LL-37 i també és més ràpid d'obtenir. Aquest pèptid té un paper essencial en la protecció dels humans contra les malalties infeccioses i a part, té poder de cicatrització de ferides, efectes immuno-moduladors i contra el càncer.

L'objectiu d'aquest treball és sintetitzar aquest pèptid mitjançant síntesi de pèptids en fase sòlida utilitzant l'estratègia de protecció de Fmoc/tBu. En segon lloc, s'ha dissenyat i sintetitzat un anàleg del KR-12 per millorar la seva activitat acoblant un àcid gras. Finalment, aquests pèptids s'han caracteritzat mitjançant l'ús de RP-HPLC i ESI-MS. S'han obtingut amb rendiment moderat i un elevada puresa.

A més, l'activitat microbiològica d'ambdós pèptids s'ha avaluat determinant la seva concentració mínima inhibidora (MIC) i, també, s'ha estudiat la seva toxicitat mitjançant l'hemòlisis.

Paraules clau: pèptids antibiòtics, pèptid KR-12, fase sòlida, activitat microbiològica, hemòlisi.

3. INTRODUCTION

Antibiotics are chemical substances used as powerful medicines that fight bacterial infections, not caused by virus. They have the capacity in dilute solutions to inhibit the growth of bacteria or to destroy it.

If they are used properly, antibiotics can save lives. But with the advent of the antibiotic era, the overuse and inappropriate consumption have driven the rapid emergence of multidrug resistance pathogens. Antimicrobial resistance increases the morbidity, mortality length of hospitalization and healthcare costs. Antibiotic resistance is considered a major threat for human health. [1]

The growing drug resistance problem of pathogenic bacteria with traditional antibiotics calls for an urgent search for a new generation of antimicrobial agents. Antimicrobial peptides are ancient and potent weapons of the innate immunity of all life forms. The recently updated Antimicrobial Peptide Data base collects 3072 antimicrobial peptides. [2,3]

3.1. ANTIMICROBIAL PEPTIDES (AMPs)

Antimicrobial peptides are small molecular weight proteins with 10-50 amino acids in general. They are a new class of antibiotics. Unlike traditional antibiotics, they have broad-spectrum antimicrobial ability with lower cytotoxicity and accordingly, they have the potential to replace the use of traditional antibiotics against bacteria, viruses and fungi. So, they are an alternative to classical AMPs due to their non-cytotoxic activity and high efficacy. [4]

AMPs are characterized by an amphiphilic configuration, derived from the presence of cationic residues on one side of the peptide and hydrophobic residues on the opposite side. The amphipathic nature of AMPs allows them to interact with negatively charged bacterial membranes and perturb the membrane bilayer.

Human express a variety of AMPs, grouped into three major classes: cathelicidin, defensins, and histatins, which differ in structure and residue composition. [5]

3.2. SOLID PHASE PEPTIDE SYNTHESIS

Bruce Merrifield described a revolutionary method in 1969, the solid phase peptide synthesis (SPPS). This method allowed a much shorter and faster way to produce peptides with much higher yield than the classical synthesis. Nowadays, there are optimized techniques that allow to synthesize a peptide with 100 amino acids in just four days with reasonable yields. [6]

The concept of SPPS is based on attaching the first amino acid, the C-terminal residue, to a resin. To prevent the polymerization of the amino acid, the alpha amino group and the reactive side chains are protected with a temporary protecting group. The protective group is then removed and each protected amino acid N-terminal is added, one by one, with other necessary reagents, so that the peptide is synthesized from C-terminal to the N-terminal end. The cycle is repeated until the peptide sequence is complete. The peptide is washed after each step of the procedure, in order to remove by-products and excess reagents with vacuum filtration.

Ideally, the N alpha protecting group and the side chain protecting groups should be removable under completely different conditions, such as basic conditions to remove the N alpha protection and acidic conditions to remove the side chain protection. This selective protection is called "orthogonal" protection.

Finally, all the protecting groups are removed, and the peptide is cleaved from the resin by an acid treatment. [6,7]
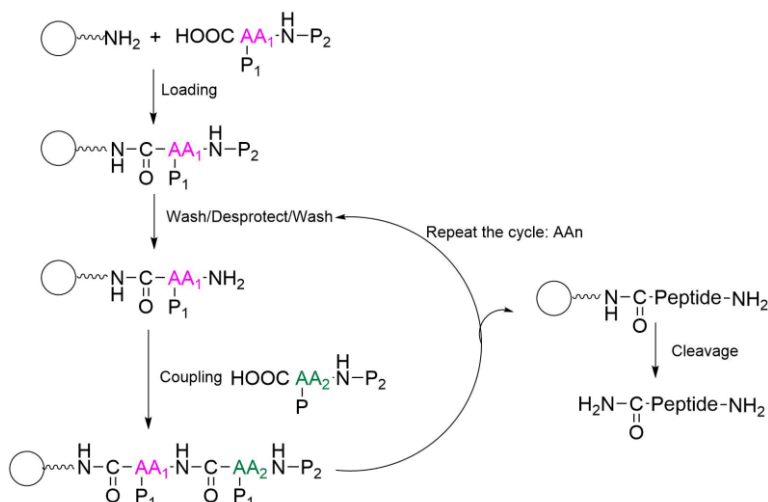

Figure 1. SPPS cycle (AA= amino acid, P_1= basic labil protecting group, P_1= acid labil protecting group).

3.3. KR-12

LL-37 is the only human cationic peptide. It plays an essential role in protecting humans against infectious diseases and it is produced by epithelial cells. This peptide is composed of 37 amino acids starting with a pair of leucines. It is known to have broad-spectrum of antibacterial activity against bacteria, viruses, fungi and parasites and can enter the cell to play a role in immune regulation. This peptide plays a decisive part in eliminating bacteria under physiological conditions. It is thought that LL-37 kills bacteria directly through regulating the body's defence system. When bacterial infection occurs, LL-37 is produced by epithelial cells and released from the neutrophil granules. Thus, patients who don't have this molecule are more susceptible to infections. In addition, it has wound healing, immune modulating and anticancer effects. [4,8]

Human LL-37 is relatively long and can be costly to synthesize chemically. Thus, it is usually to cut LL-37 by removing unwanted regions. KR-12 is the shortest antibacterial peptide of human LL-37, corresponding to residues 18-29. The amino acid sequence is KRIVQRIKDFLR. This sequence included five positively charged amine groups (3 Rs and 2 Ks), that could recruit negatively charged bacteria to the KR-12. [1,9]

Use of KR-12 peptide eliminates the toxicity problems associated with LL-37. Thus, shorter peptide possessed the same antimicrobial and anti-inflammatory activities than the biggest one but without the associated mammalian cell toxicity.

The use of AMP antimicrobial coatings in the Ti implants has been widely investigated, due to their lower cytotoxicity and minimal development of pathogen resistance compared to conventional antibiotics. Infection and poor bone-implant integration are two main reasons for titanium (Ti) implant failure. The KR-12 peptide can be covalently immobilized on the Ti surface by the covalent bond between the amine groups on the Ti surface and the carboxyl groups of the peptide. [10]

4. OBJECTIVES

The main objectives in this work are the following:

- Synthesis of KR-12 using solid phase peptide synthesis.
- Design and synthesis of KR-12 analog in order to improve its activity.
- Purify the obtained peptides by semi-preparative HPLC and characterize them by analytical HPLC and ESI mass spectrometry.
- Evaluate the antimicrobial activity of the synthesized peptides by using MIC test.
- Evaluate the toxicity of the synthesized peptides by hemolysis assay.

5. EXPERIMENTAL SECTION

5.1. MATERIALS

5.1.1. Solvents and reagents

5.1.1.1. Solvents

Solvent	Quality	Brand
Acetone	Synthesis	Scharlau
ACN	HPLC	Carlo Ebra
DCM[a]	Synthesis	VWR
DMF	Synthesis	Carlo Erba
Et_2O[b]	Synthesis	Scharlau
H_2O[c]	Milli-Q	-
Isopropanol	HPLC	Fisher Scientific
MeOH	HPLC	Fisher Scientific

(a) DCM is filtered through a basic alumina column.
(b) The dried Et_2O is kept over sodium.
(c) The deionized water is filtered with a Milli-Q (Millipore) system.

Table 1. Solvents and their specifications.

Reagents	Quality	Brand
Resin, RL and amino acids	-	Bachem
		Iris Biotech
		NeMPS
		Fisher Scientific
DIC	Pure, 99%	Sigma-Aldrich
DIEA	Reagent Plus®, 99%	Sigma-Aldrich
HOBt	Pure, 99%	Fluka
MHB	-	Oxoid
Na$_2$CO$_3$	-	Jescuder
Ninhydrin	Pure	Iris Biotech
Octanoic acid	Pure, ≥ 98%	Sigma-Aldrich
Piperidine	Reagent Plus®, 99%	Sigma-Aldrich
TFA	Synthesis	Fluorochem
TIS	Pure, 99%	Acros organics
TRIS	-	Sigma-Aldrich

Table 2. Reagents for peptide synthesis and products for biological assays and their specifications.

5.1.2. Instrumentation

Instrument	Brand and model
Autoclave	AUTESTER-E
Centrifuge	-Hettich ROTOFIX 32 A
	-Sigma 201M
HPLC	<u>-Analytitcal:</u> Shimadzu Serie 20 Prominence, with two pumps LC-20AD, automatic SIL-20A injector, controller CBM-20A and detector SPD-M20A
	<u>-Semipreparative:</u> Waters Delta Prep 3000, with a Waters 600E pump and a manual sample injector Eaters 712, Water 484 detector and a data recorder Pharmacia Biotech RED 101
Lyophilizer	Christ Alpha 2-4 LDplus
Mass spectrometer	Zq-Micromass (Waters)
Microplate reader	BioTek Synergy HT
Spectrophometer	Shimadzu UV-1700 PharmaSpec

Table 3. Instrumentation and their specifications.

5.2. METHODS

5.2.1. Ninhydrin test

Ninhydrin test or Kaiser test is a very sensitive assay used in SPPS to determine if the amino acid coupling has been done at all. The reaction of ninhydrin with primary amino groups of the N-terminal side form a dark blue or intensive purple colour. This means that the coupling reaction is not complete thus it's necessary to recouple. For a better yield, ninhydrin test should be performed after each coupling. [11]

To perform the test, three solutions are required:

Reagent A: ninhydrin (2,5g) are dissolved in EtOH (50mL). The solution prepared must be kept protected from light.

Reagent B: phenol (50mg) is diluted in EtOH (100mL). Then the solution is filtered.

Reagent C: KCN (65mg) is added in H_2O (100mL), 2mL of the resultant solution are added in 100mL of distilled pyridine. Then the solution is filtered.

To carry out the assay, a little sample of peptide-resin (washed with DMF, then with DCM and finally dried with a vacuum pump) is introduced in a small test tube. Two drops of each reagent (named above as A, B and C) are added and the mixture is heated in a sand bath at 110ºC for 3´.

After that, if the solution turns blue/purple (positive assay) free amino groups are present and a recoupling step is necessary. In contrast, if the solution remains yellow (negative assay) means that the coupling is successful at least 99,5% because no free primary amines are present.

As shows **Figure 2**, ninhydrin (1, which is a yellow compound) reacts with a primary amine of amino acid (2) forming and imine (3), in a nucleophilic addition-elimination reaction. Then, there is a decarboxylation reaction, and after that the imine (5) reacts with H_2O in a hydrolysis reaction to form an aldehyde and a free amine again (6). Compound 6 reacts with another equivalent of ninhydrin forming 7 and finally 8, which is the purple compound. [6]
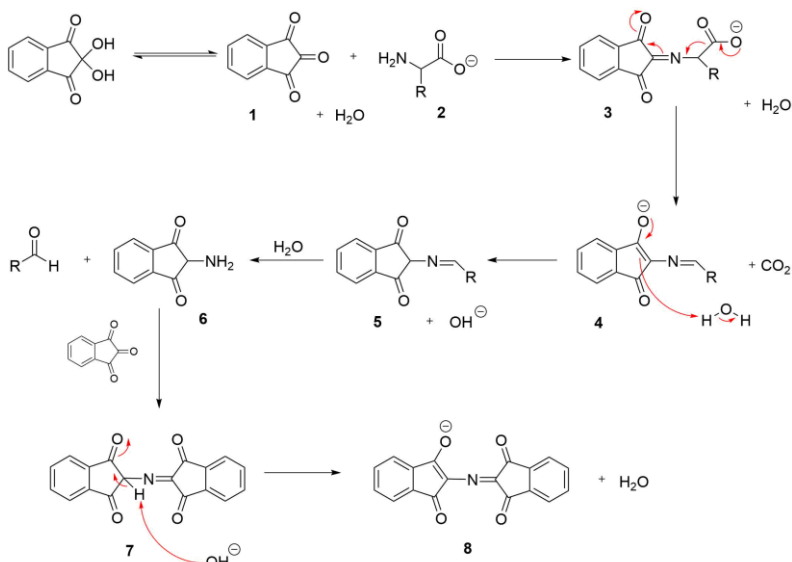
Figure 2. Reaction mechanism of ninhydrin assay.

5.2.2. Purification

High performance liquid chromatography is an analytical technique used to separate, identify and quantify each component in a mixture. It is based in the different molecular weight and polarity of the analytes. It has a column through which pass pressurized liquid solvent with the sample mixture at high pressure (it achieves 400 atmospheres) that makes it much faster.

Each component in the sample interacts differently with the adsorbent of the column, causing diverse retention times and leading to the separation of the components as they flow out of the column. The elution of bound components occurs by increasing the concentration of organic solution (ACN). This technique has a non-polar stationary phase and an aqueous mobile phase which is moderately polar. So, retention time is longer for molecules which are less polar while polar molecules elute faster.

5.2.2.1. Semi-preparative RP-HPLC

The peptides are purified by semi-preparative RP-HPLC. As they have different retention time produced by the different hydrophobicity of the molecule, this technique is used to purify compounds from a mixture. Finally, you will get the single compound at a certain purity level.

The column used is Phenomenex C18 of reverse phase (1 x 25 cm, 5 µm diameter particle). The samples are eluted at 2mL/min flow rate with a lineal gradient of 0,1% TFA/H_2O (A) and 0,1% TFA/ACN (B) and UV detection at 220 nm.

5.2.3. Characterization

Two different techniques have been used to carry out the characterization of the peptides.

5.2.3.1. Analytical RP-HPLC

The objective of analytical RP-HPLC is to identify and quantify the components of the mixture. The difference with the previous RP-HPLC is the amount of sample applied. In semi-preparative the quantity is higher than in analytical.

The column used is a Phenomenex C18 of reverse phase (0,46 x 25 cm) packed with octadecylsiloxane of 5 µm diameter particle. The samples are eluted at a flow rate of 1mL/min using the lineal gradients of 0,045% TFA/H_2O (A) and 0,036% TFA/ACN (B) and UV detection at 220 nm.

5.2.3.2. ESI mass spectroscopy

Mass spectroscopy is an analytical technique that separate the components of a sample by their mass and electrical charge. It produces a mass spectrum that plots the intensity of the mass-to-charge (m/z) ratio of ions in a mixture.

The electrospray ionization (ESI) is a soft ionization technique. It is a desorption ionization method and it is produced by applying a strong electric field to a liquid passing through a capillary tube. The transfer of ions from solution into the gaseous phase can thus be analysed by spectrometric analysis with increased sensibility. The different charged ions travel through the mass analyser and arrive at different parts of the detector according to their m/z ratio. The spectrum shows the m/z ratio on the x-axis and the abundance (%) on the y-axis.

The samples analyzed are peptide solutions in H_2O/ACN (1:1, v/v). A positive mass spectrum is obtained using the spectrometer ZQ-Micromass (Waters). The signals are caused by ionization with proton or another cation such as Na^+ or K^+. Moreover, multiply charged ions are also observed.

5.3. SOLID PHASE PEPTIDE SYNTHESIS

5.3.1. Synthesis (Fmoc/^{t}Bu strategy)

The peptide is synthesized manually by SPPS following the Fmoc/^{t}Bu strategy. It is carried out using polypropylene syringes from Terumo with a porous polyethylene filter from Teknokroma. After each coupling, by-products and reagents can be removed by vacuum filtration. The reagent mixture is stirred with a Teflon wand.

The concept of SPPS is based on attaching the first AA to a resin, then proceeding with peptide chain elongation. A resin is composed of a polymeric solid support linked permanently to a linker that facilitates temporary anchoring of the first AA to the polymeric solid support.

5.3.1.1. Loading the resin

The BHA commercial resin used (f=0,69) needs a pre-treatment because it is stored in a compact form. Thus, impurities will be eliminated. The protocol used is described in **Table 4.**

Step	Reagent	Function	Time	Repetitions
1	DCM	Wash	30''	5
2	TFA/DCM 40%	Wash and solvate	1'	1
3	TFA/DCM 40%	Wash and solvate	10'	2
4	DCM	Wash	30''	5
5	DIEA/CH$_2$Cl$_2$ 5%	Neutralization	2'	3
6	DCM	Wash	30''	5
7	DMF	Wash	30''	5

Table 4. Protocol used to load BHA resin.

5.3.1.2. Reference amino acid coupling

L-Fmoc-(L)-Val-OH used as reference amino acid in the synthesis of the two peptides is added to the BHA resin with DIC and HOBt with the minimum quantity of DMF for 1 hour. The coupling is confirmed by ninhydrin test and the coupling protocol used is shown in **Table 6.**

5.3.1.3. Rink Linker incorporation

To coupling the Rink Linker is necessary to remove Fmoc from amino acid reference in order to deprotect it, as shown in **Table 5**.

Step	Reagent	Function	Time	Repetitions
1	DMF	Wash	30''	5
2	20% piperidine/DMF	Deprotection	1'	1
3	20% piperidine/DMF	Deprotection	10'	2
4	DMF	Wash	30''	5

Table 5. Fmoc deprotection protocol.

After this, the Rink Linker incorporation is carried out by following the protocol shown in **Table 6** and by replacing step 2 for the addition of 1,8 eq. of RL, HOBt and DIC overnight to the BHA with a minimum quantity of DMF. After overnight reaction the coupling is confirmed by ninhydrin test.

5.3.1.4. Elongation of the peptide

To perform the peptide chain elongation, it is necessary to have the terminal amino group deprotected. After this step, the first amino acid can be coupled using an excess of amino acid, HOBt and DIC (3 eq.) with the minimum quantity of DMF for 1 hour as shown in **Table 6**. The protocol includes three general groups: the amino acid coupling, the ninhydrin test and the removal of Fmoc group. When the synthesis is finished, the peptide-resin is washed with DMF and DCM and dried in vacuum.

Step	Reagent	Function	Time	Repetitions
1	DMF	Solvate	30''	5
2	3 eq. Fmoc-AA-OH [a] 3 eq. HOBt 3 eq. DIC	Coupling	60'	1
3	DMF	Wash	30''	5
4	DCM	Wash	30''	5
5	Ninhydrin test [b]	Coupling control	3'	1
6	DMF	Solvate	30''	5
7	20% piperidine/DMF	Deprotection	1'	1
8	20% piperidine/DMF	Deprotection	10'	2
9	DMF	Solvate	30''	5

(a) The reagents are added to the resin with a minimum quantity of DMF
(b) If the ninhydrin test became positive, 1.5 eq of each reagent in DMF are added in the mixture for 30 extra minutes

Table 6. Elongation peptide chain protocol by Fmoc/^{t}Bu strategy.

At the end of the peptide sequence, the resin is divided in two equal parts: one of them will be finished (KR-12 peptide) and the other one will be coupled two glycines and a fatty acid group (KR-12 peptide analog).

5.3.1.5. Fatty acid coupling

After coupling the two Glycines to KR-12 peptide, the next step is to incorporate the fatty acid group following the procedure described in **Table 7.**

Step	Reagent	Function	Time	Repetitions
1	DMF	Solvate	30''	5
2	5 eq. Fatty acid (octanoic acid) 5 eq. HOBt 5 eq. DIC	Coupling	60'	1
3	DMF	Wash	30''	5
4	DCM	Wash	30''	5
5	Ninhydrin test	Coupling control	3'	1
6	DMF	Solvate	30''	5
7	DCM	Wash	30''	5

Table 7. Fatty acid coupling protocol.

5.3.2. Cleavage

The aim of this step is to separate the peptide from the solid support and from the side-chain protecting groups. To achieve it, it requires a strong acid such as TFA. The procedure is carried out by acidolysis with 5 mL TFA/TIS/H_2O (95:3:2, v/v). This mixture is added to the resin in the syringe and it is left to react for 90 min. Triisopropylsilane (reductant) and water (nucleophile) act as cation scavengers.

TFA is removed by evaporation with N_2 stream and the crude product obtained is treated with Et_2O (20 mL) to precipitate the peptide. The solid peptide is isolated by centrifugation at 6000 r.p.m. for 10 min and the supernatant is poured off. The peptide is dissolved in H_2O/ACN, lyophilized and analysed by analytical HPLC and by ESI mass spectroscopy.

In addition, to check the evolution of the sequence, it is possible to carry out cleavage tests during the chain elongation with a little sample of the resin in 1mL of the cleavage solution. [11]

5.3.3. Purification and characterization

The peptides are purified by semi-preparative HPLC. Then they are lyophilized with high purities (≤99%) as determined by analytical RP-HPLC and followed by UV detection at 220 nm, as amine bonds absorb light at this wavelength. The peptides are obtained in TFA salt form and the yields achieved are shown in **Table 8**. The yields are moderately good, because the synthesis have a lot of steps. Moreover, various ninhydrin tests and mini-cleavages are carried out which causes product losses.

The purified peptides are characterized by analytical RP-HPLC and ESI mass spectroscopy. Chromatograms are shown in **Appendix 2** and ESI spectrums are in **Appendix 3**.

Peptide	Synthesis yield [%]	Purity [%]	RP-HPLC (t_R) [min] [a]	HRMS ESI+
KR-12	10,8	99,0	20,310	**m/z** 1571,81 (<1%, $[M+H]^+$), 786,23 (49%, $[(M+2H)/2]^{2+}$), 524,45 (100%, $[(M+3H)/3]^{3+}$), 389,34 (30%, $[(M+4H)/4]^{4+}$)
Analog	12,3	99,7	13,349	**m/z** 906,7 (39%, $[(M+2H)/2]^{2+}$), 604,8 (100%, $[(M+3H)/3]^{3+}$), 453,8 (53%, $[(M+4H)/4]^{4+}$)

(a) RP-HPLC: lineal gradient from 10% to 25% of B for 30 min, at flow 1 mL/min in KR-12 and 30% of B to 45% in analog.

Table 8. Purification yields and final characterization of synthesized peptides.

5.4 EVALUATION OF ANTIMICROBIAL ACTIVITY

One of the main objectives of this work is to characterize antibacterial activity of peptides. It has been used four bacterial strains, one of them is Gram-positive (*Staphylococcus aureus*) and the other ones are Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*).

5.4.1. Preparation of material and medium

First of all, the material and growth medium of bacteria must be autoclaved in order to eliminate any form of life. The medium used is Muller Hinton-Broth (MHB) and two different culture mediums need to be prepared: 1 x concentrated MHB (21 g in 1 L of Milli-Q Water) and 2 x concentrated MHB (42 g in 1 L of Milli-Q Water).

So, pipette tips, bowl for Multi-Channel Pipette, glass bottles, flask and the medium need to be autoclaved before its use.

5.4.2. Growth and inoculation of bacteria

The microorganisms used are:

- Gram-negative bacteria: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Acinetobacter baumannii* ATCC 19609.
- Gram-positive bacteria: *Staphylococcus aureus ATCC 25293.*

The day before the MIC test, in a test tubes are prepared one colony of each bacterium with 1xMHB solution and leave at 37ºC overnight.

The next day, the absorbance is measured and adjusted to 0,3-0,35 at 550nm. Then, after 4 hours at 37ºC is measured again and is adjusted to the same value. At that point, it is diluted 1:100 or 1:10 (depends on the bacteria) in a cultive bath and these solutions are ready to start the MICs.

5.4.3. Preparation of peptide solutions

For each peptide a solution of 512 µg/mL is prepared with Milli-Q water. This table shows the quantity of each one.

Peptide	Weight [mg]	Real weight [mg] [a]	Volume of Milli-Q Water [µL]
KR-12	0,86	0,63	1232
Analog	0,98	0,78	1529

(a) Peptides are positively charged so they contain the counterion CF_3COO^- from TFA. Thus, the real weight corresponds to the peptide without the counterion.

Table 9. Peptides solution for the MIC test

5.4.4. Determination of MIC

The determination is carried out in 96-well plate (8 rows: from A to H; 12 columns: from 1 to 12), one for each bacterium. In order to add the different volumes of solutions is used a multichannel pipette. The procedure followed is:

1) 50 µL of 2xMHB are added to column 1, 50 µL of 1xMHB to column 2 to 11 and 100 µL of 1xMHB to column 12.

2) 50 µL of peptide solution are added to column 1. With the help of the multichannel pipette, the mixture in column 1 is stirred and 50 µL of this column is added to column 2. This step is repeated until column 10 where 50 µL are picked up and thrown away. The concentration of peptide in each column decreases by a half to 128 µg/mL (column 1) to 0,25 µg/mL (column 10).

3) 50 µL of bacterial solution are added to column 1 to 11. So, the final concentration of bacteria in each well will be 10^5-10^6 CFU/mL. It is a standard value in order to be able to compare the results with others.

4)The last step is to incubate the plates at 37ºC for 20-22h.

Thus, column 11 is the positive control (it contains bacteria and MHB, so bacteria will grow without the peptide) and column 12 is the negative control (it just contains MHB).

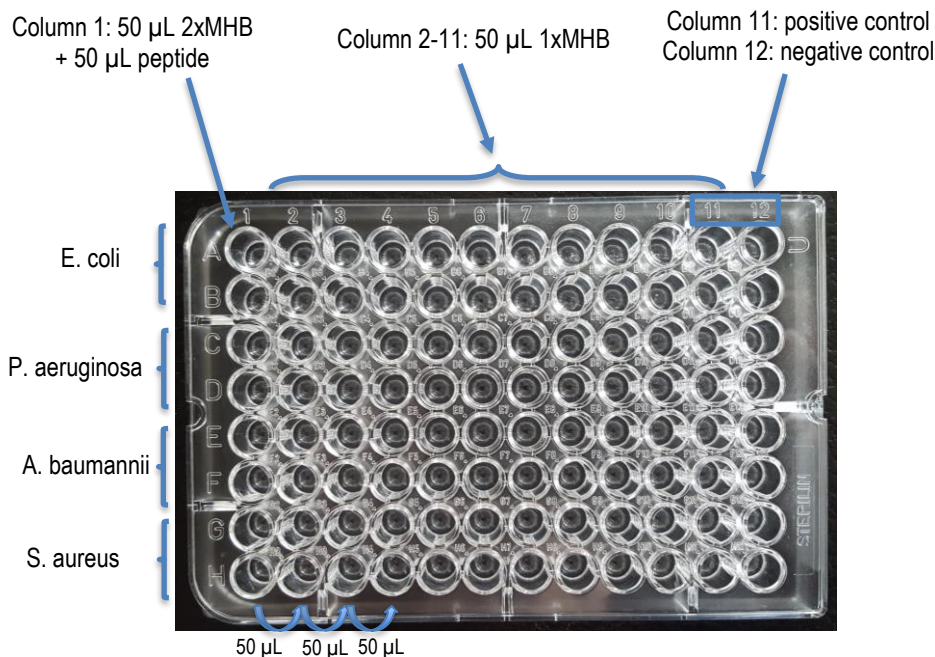The following day, it is evaluated the growth of bacteria by their turbidity with each peptide.

Figure 3. Preparation of the microtiter plates for the MIC test.

5.5. EVALUATION OF TOXICITY

The next assay is to determine the toxicity of the KR-12 peptide and its analog and compare them.

5.5.1. Hemolytic assay

The hemolytic activity is investigated using rabbit's blood. The procedure followed was:

1.   First of all, blood needs to be washed with TBS (TRIS 10mM and NaCl 150mM). The mixture is centrifuged (10', 4000 r.p.m., 4ºC), removing the supernatant (TBS) in order to separate the precipitate (red blood cells). Isolated red blood cells are washed thrice with buffer/TBS.

2.   When concentrated red cells are obtained, they are adjusted to a certain value of absorbance at 540nm by adding drop of blood to a X quantity of TBS.

3.   For each peptide, 1mL of a solution of 1024µg/mL is prepared dissolving the correspondent amount of the peptide in TBS buffer. Next step is to prepare 10 Eppendorf with the same quantity of blood but with different concentration of peptide. The first Eppendorf are mixed 200 µL of TBS with 200 µL of peptide solution. The mixture in this Eppendorf is stirred and 200 µL of it is added to Eppendorf 2 which also contains 200 µL of TBS. This step is repeated until Eppendorf 10 where 200 µL are picked up and thrown away. Then, 200 µL of blood is added. The concentration of peptide in each one decreases by a half to 256 µg/mL (Eppendorf 1) to 0,5 µg/mL (Eppendorf 10). Then, it is needed to confirm that the absorbance is 1.

4.   In addition, a 0% and 100% hemolysis controls are needed. Both are prepared with the same quantity of blood as before but the medium in 0% is TBS while in 100% of hemolysis it must be water or Triton X-100 (surfactant).

5.   All these solutions are incubated at 37ºC for 1h.

6.   After that, they are centrifugated (5', 4000 r.p.m., 4ºC)

7.   It is added 200µL from the supernatant of each Eppendorf to each well of a microtiter plate.

8.   The absorbance of the mixture is read at 540nm.

6. KR-12 ANALOGS

The main objective in this work is to obtain KR-12 peptide using solid phase peptide synthesis. In order to improve its activity a new antimicrobial peptide derived from this one is synthesized.

Many studies demonstrated that the attachment of aliphatic fatty acid to N-terminus of positively charged peptides can compensate the hydrophobicity of the peptide chain. This generate new artificial lipopeptides more hydrophobic with potent antibacterial activities. Thus, the peptide chain causes more hydrophobic interactions with bacteria and may facilitate the penetration of the antimicrobial peptide. Also, this incorporation can destabilize the membrane. [12,13]

6.1. DESIGN

The principal changes between both peptides are that analog has a fatty acid group coupled:

- In order to obtain the analog, first of all it is necessary to have KR-12 peptide synthesized at all. After, two glycines are coupled as spacer. Finally, the fatty acid group (octanoic acid) is added. Theoretically, this change will affect the hydrophobicity of the molecule.

The sequence of the synthesized peptides is shown in **Table 10** and the structure is shown in **Figure 4.**

Peptide	Sequence
KR-12	Lys-Arg-Ile-Val-Gln-Arg-Ile-Lys-Asp-Phe-Leu-Arg-NH$_2$
Analog	**C8-Gly-Gly-**Lys-Arg-Ile-Val-Gln-Arg-Ile-Lys-Asp-Phe-Leu-Arg-NH$_2$

Table 10. Sequence of peptides synthesized.
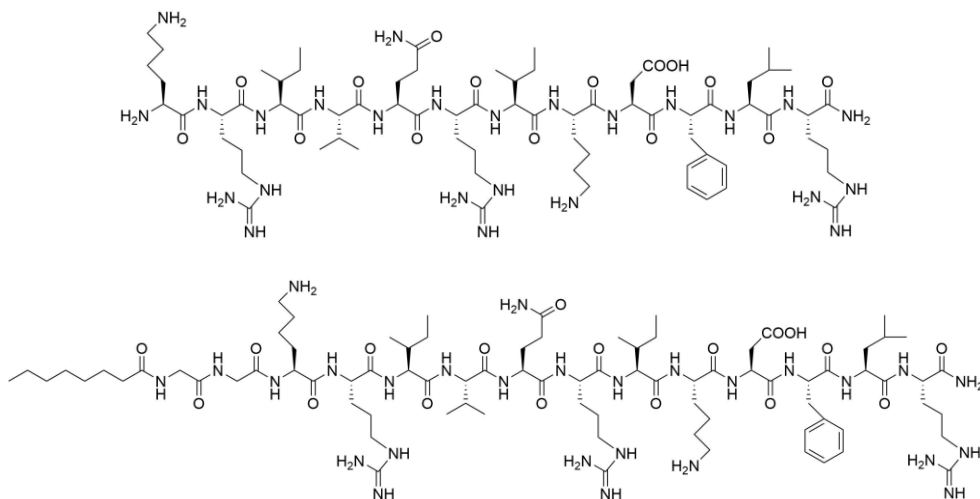
Figure 4. Structure of KR-12 (above) and structure of analog (below)

6.2. SYNTHESIS

6.2.1. Synthesis strategy

These peptides are synthesized manually by SPPS following the Fmoc/^{t}Bu protection strategy. To prevent the polymerization of the amino acid, the alpha amino group and the reactive chains are protected with a temporary protecting group. In this protection, the alpha nitrogen of

the amino acids is protected with the base labile Fmoc group while the side chains are protected with acid labile groups. This strategy is called orthogonal protection, which it means that the side chain protecting groups can be removed without affecting the N-terminal protection and vice versa. It is an advantage because Fmoc is removed in each coupling (in basic conditions); while the protecting group of the lateral chain remains unaffected until de cleavage (in acidic conditions).

First, Fmoc-Val-OH is coupled to the resin BHA as a reference amino acid. This solid support is a benzhydrylamine linker bound to a polystyrene matrix, which need a pre-treated as explained in **Section 5.3.1.1.** This couple facilitates the SPPS because it could be used for amino acids analysis. The reaction is carried out with three equivalents of each reagent in DMF for 1 hour, shown in **Section 5.3.1.4.**

Once the Fmoc is removed, the Rink Linker is coupled following the same steps as is explained in **Section 5.3.1.3.** Linkers are chemical entities to "link" the peptide sequence to the resin during SPPS. The Linker used is a benzhydrylamine functionalized with two methoxy groups at *ortho* and *para* positions. This coupling enables the cleavage of the RL-peptide bond with a solution of TFA in H_2O and obtain the peptide with the C-terminal end in the carboxamide form.
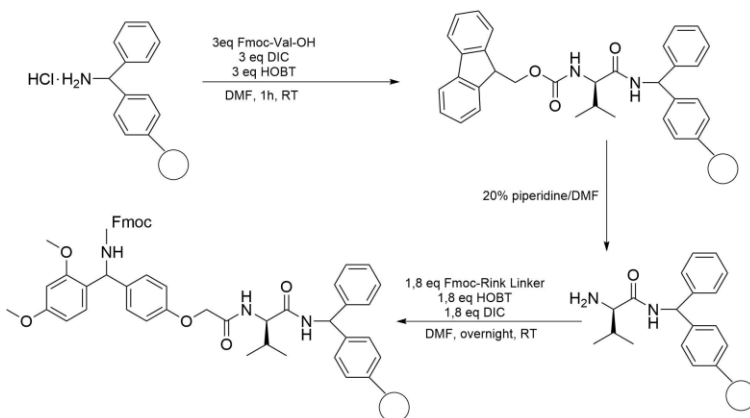
Figure 5. Reference AA and RL coupling to BHA resin.

For the elongation of the peptide chain, the coupling of amino acid to the peptide resin is the crucial part of the SPPS method for a good rate and yield. Each amino acid coupling is carried out with 3 eq. of Fmoc-AA-OH, 3 eq. of DIC and 3 eq. of HOBt in DMF for 1h. The efficiency of the coupling needs to be controlled by ninhydrin test.

The peptide synthesis is based on the formation of an amide bond between a carboxylic acid and an amino group of and amino acid. First of all, it is necessary to activate the acid carboxylic, because if not, an acid-basic reaction will take place. In order to avoid this, DIC is used as an activate agent of the carboxylic acid of the amino acid by introducing a good leaving group on the carbonyl carbon. An O-acylisourea is formed and can react with the amine to yield the amide.
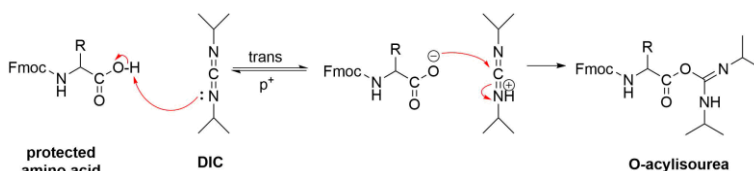

Figure 6. Mechanism of carboxylic acid activation

The first priority is to find the optimal combination of high coupling rate and minimal racemization. The product, O-acylisourea, increases the tendency to racemize. The mechanism involves the abstraction of the α-hydrogen from the α-carbon of the activated amino acid by direct formation of an enolic intermediate. Racemization also occurs by an alternative mechanism forming a 5-membered oxazolone ring. (**Figure 7**) [11]
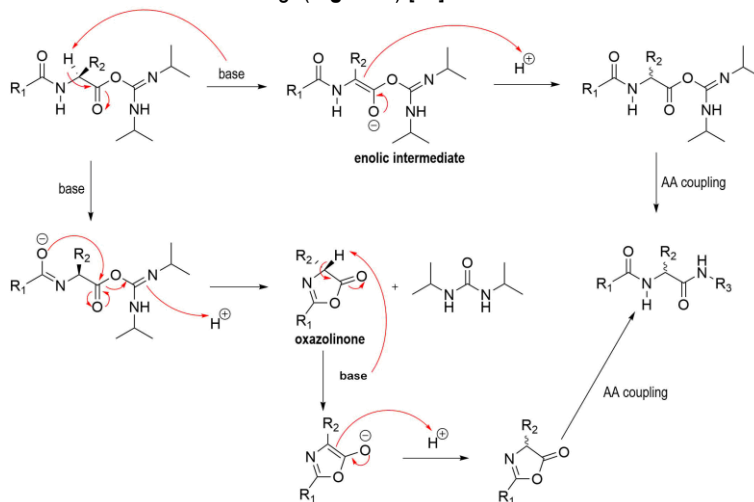
Figure 7. Mechanism of base-catalysed racemization during activation.

In order to improve the efficiency of the SPPS technique by minimizing the racemization, a nucleophile such as HOBt is added. It forms active esters that will react with amino groups with little racemization, shown in **Figure 8.**
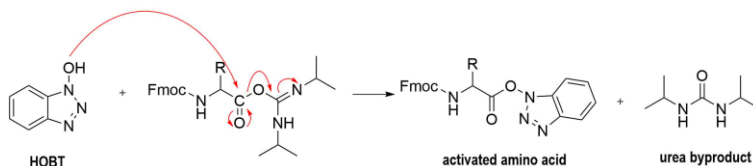
Figure 8. Mechanism of HOBt coupling.

Finally, the activated carboxylic acid can be attached by an unprotected amino group of a second amino acid via nucleophilic addition to form a tetrahedral intermediate, which is easily broken because the benzotriazolyl ester is a good leaving group, see in **Figure 9**. The urea by-product and excess reagents are eliminated by vacuum filtration.
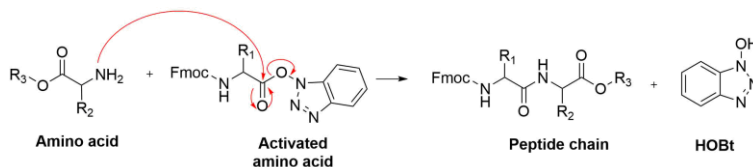
Figure 9. Mechanism of chain elongation.

Before the next amino acid coupling the Fmoc group must be removed. Fmoc is a basic labile protector group, thus it will be treated with 20% piperidine/DMF solution as it is explained in **Section 5.3.1.4**. The Fmoc group is removed when a base abstract the relatively acidic proton from the fluorenyl ring system, leading to a β-elimination and the formation of dibenzofulvene and carbon dioxide. The mechanism for deprotection is shown in **Figure 10.**
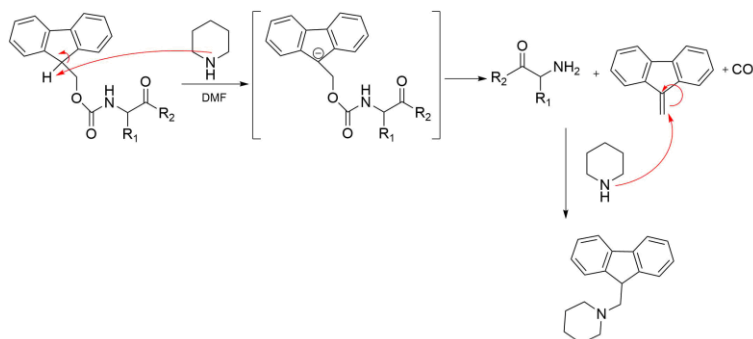
Figure 10. Mechanism of Fmoc deprotection.

6.2.2. Purification and characterization

The peptides synthesized are obtained with moderate yield because in SPPS there are many steps where it is easy to loss product. These steps could be ninhydrin test, mini-cleavage in order to verify the correctly elongation of the chain and also side reactions that leads to the not desired product. The results are obtained are shown in **Table 11.**

Peptide	η cleavage [%]	η purification [%]	η synthesis [%]	Purity [%]
KR-12	91	18	10,8	99
Analog	94	20	12,3	>99

Table 11. Synthesis yields and purity of the synthesized peptides.

7. DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION

7.1. GRAM-NEGATIVE AND GRAM-POSITIVE

The difference between Gram-negative and Gram-positive it is based on their cell wall composition. Gram-positive bacteria have cell walls composed of thick layers of peptidoglycan (20-30 nm) and Gram-negative have thin layer (8-12 nm).

In the positive one, these bacteria have a single layered, are straight, less elastic and more rigid. While in the negative one, these bacteria have bilayer, are wavy, uneven, more elastic and less rigid. Moreover, they have outer membrane (external to the peptidoglycan cell wall) with lipopolysaccharide component not found in Gram-positive bacteria.

In Gram stain test the Gram-positive retains crystal violet dye and stain blue/purple, meanwhile, Gram-negative can be decolorized and stain pink/red.
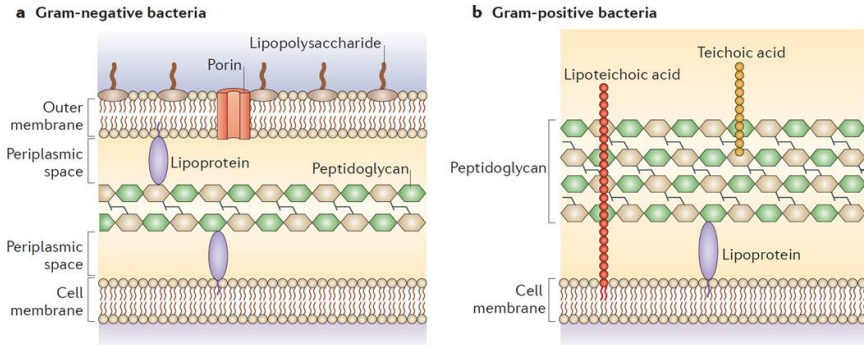
Figure 11. Difference between Gram-positive and Gram-negative cell wall. [14]

7.2. BACTERIA

A brief description of each bacteria:

-*Escherichia coli* (ATCC 25922): is a Gram-negative and rod-shaped bacterium that can be found in the intestines of some animals. Most types of *E. coli* are harmless and can help digestion processes, food breakdown and absorption and vitamin K production. But some strains can cause bloody diarrhea, several anemia or kidney failure when someone gets infected by contaminated water or food. Nowadays, the resistance of this bacteria to antibiotics has increased.

-*Pseudomonas aeruginosa* (ATCC 27853): is a Gram-negative and rod-shaped bacterium that can cause disease in plants and animals. It can cause urinary tract infections, respiratory system infections, gastrointestinal infections, blood infections and others. Usually it is found in soil, water and skin flora. It is a multidrug resistant pathogen and has a low susceptibility to antibiotics.

-*Acinetobacter baumannii* (ATCC 19609): is a Gram-negative short, round and rod-shaped bacterium. They are often found in soil samples and occasionally it can be found in environmental soil and water samples. This bacterium has a high antibiotic resistance that is one of the responsible for nosocomial infections.

-*Staphylococcus aureus* (ATCC 25293): is a Gram-positive and round-shaped bacterium that is usually found on the skin and hair as well as in the noses and throats of animals. This bacterium can cause many different typed of infections such as pneumonia, endocarditis, osteomyelitis. Like *A. baumannii*, is one on the main cause of nosocomial infections.

7.3. RESULTS AND DISCUSSION

The results of the MIC test are determined visually using an amplifier to observe the turbity and then it is possible to determine bacteria growth.

MIC is the lowest concentration of antibiotic which inhibit the visible growth of bacteria. Thus, the values in **Table 12** are referred to the peptide concentration in the first well where the bacteria did not grow.

Bacteria / Peptide	MIC [µg/mL]			
	E. coli	*P. aeruginosa*	*A. baumannii*	*S. aureus*
KR-12	32-64	64	>128	>128
Analog	16	32	32	32

Table 12. MIC results.

First of all, it is necessary to emphasize that there is no difference with the effectiveness against Gram-negative and Gram-positive bacteria. All the values are very high so both peptides are not very active against them. But when a fatty acid group is coupled (Analog) this substituent increases the hydrophobicity of the molecule, which improves the activity.

It has been demonstrated that KR-12 is more active against *E. coli*, then with *P. aeruginosa* and finally with *A. baumannii* and *S. aureus* equally. Analog is also more active with *E. coli* and then with the others equally.

So, the results of MIC test show that de KR-12 analog presents the best antimicrobial activity because with a lower concentration of it can inhibit the growth of bacteria.

8. HEMOLYSIS ASSAY

8.1. GENERAL ASPECTS

Hemolysis is the rupture of red blood cells (erythrocytes) with release of hemoglobin into the plasma. Erythrocytes have a lifespan of 110-120 days. After dying they break down and are removed from the circulation. With diseases or medicaments this breakdown of red blood cells is

increased. This requires that the bone narrow must produce more erythrocytes than normal. They can break down due to defects in the cell, infections or immunity reactions for example.

The hemolysis assay is strongly influenced by the tonicity of the medium in which red blood cells are placed. If erythrocytes are placed in a hypotonic solution such as distilled water, the water induces lyses of erythrocyte membrane because goes into red blood cells through an osmosis process. On the contrary, if it is used TBS (which is a buffer solution made by TRIS and NaCl), this solution avoids hemolysis caused from medium because it gives the isotonic salt concentration. Moreover, TBS is used to keep the physiological conditions (pH between 7,4 and 8,0) of most living microorganisms. So, distilled water is used to obtain the 100% hemolysis control and TBS is used as 0% of hemolysis control. A toxicity evaluation is obtained from the percentage of lysis cells according to peptide concentration.

8.2. RESULTS AND DISCUSSION

The percentage of hemolysis is calculated using the following formula:

$$\% \text{ hemolysis} = \left[\frac{A_{\text{sample}} - A_{C0}}{A_{C100} - A_{C0}}\right] \times 100$$

Figure 12. Percentage of hemolysis equation (A: absorbance)

The relationship between the percentage of hemolysis and the concentration of each peptide is shown in **Figure 13.**
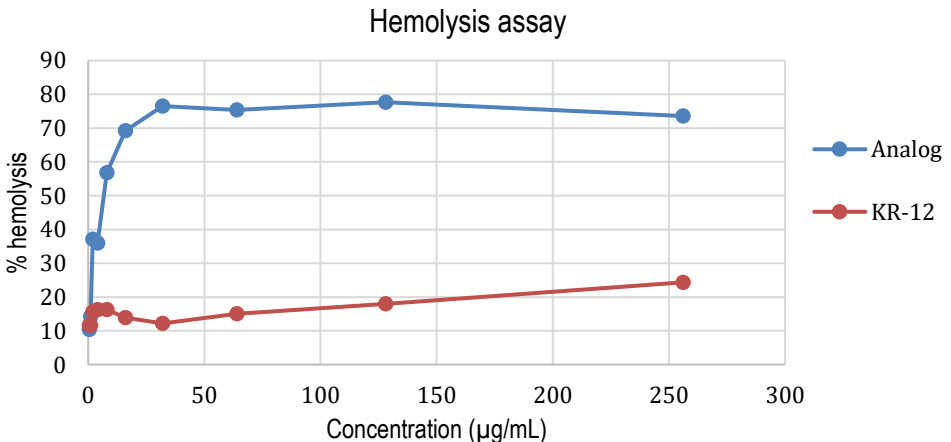
Figure 13. Plot of peptide concentration vs percentage of hemolysis.

For each peptide, the analysis was done twice. Such as some results of one KR-12 sequence were contaminated, after a few days the test was repeated. But the results were not coherent because it had passed 8 days since blood was extracted and it was not good. Thus, I have two replicates of Analog and just once of KR-12.

To evaluate percent hemolysis, the release of hemoglobin into the surrounding medium is measured via absorbance on a microtiter plate.

In the first place, with this assay has been demonstrated that when the concentration of peptide increases the % of hemolysis also gets bigger.

The main results of this investigation are the considerable difference between both peptides. The analog which has been coupled a fatty acid is more hydrophobic and is also more hemolytic.

A good antibacterial peptide should have low MIC values (more active) and low hemolytic activity (less toxic). KR-12 is less active but has a good hemolytic activity. Analog was created in order to improve KR-12 activity but as a consequence of this decrease in MIC values the peptide has become more toxic.

10. CONCLUSIONS

The conclusions reached in this work are:

- Solid phase peptide synthesis by Fmoc/tBu strategy is very effective and useful to obtain the desired peptides with high purities (≥99%).
- It is possible to purify the obtained peptides by semi-preparative HPLC. However, the yields are moderate because this synthesis have multiple steps which causes product losses. Also, analytical HPLC and ESI mass spectroscopy are good techniques to characterize them.
- The determination of minimum inhibitory concentration allows to know and compare the antimicrobial activity of the synthesized peptides.
- The hemolysis assays allows to know and compare the toxicity of the synthesized peptides.
- It has been possible to obtain a KR-12 analog more active. But as consequence of the hydrophobicity of the fatty acid group added the peptide is also more toxic.

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12. ACRONYMS

AA	Amino acid
ACN	Acetonitrile
AMP	Antimicrobial peptide
ATCC	American Type Culture Collection
BHA	Benzhydrylamine resin
Boc	*tert*-butyloxycarbonyl
tBu	*tert*-butyl
CFU	Colony-forming unit
DCM	Dichloromethane
DIEA	N,N'-Diisopropylethylamine
DIC	N,N'-Diisopropylcarbodiimide
DMF	N,N'-Dimethylformamide
Eq.	Equivalents
ESI	Electrospray ionization
f	Functionality
Fmoc	Fluorenylmethyloxycarbonyl
h	Hours
HOBT	1-Hydroxybenzotriazole
HPLC	High Performance Liquid Chromatography
m/z	Mass-to-charge ratio
MHB	Muller Hinton-Broth
MIC	Minimum inhibitory concentration
min	Minutes

mL	Milliliter
MS	Mass spectroscopy
nm	Nanometres
Pbf	2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl
RL	Rink Linker
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
r.p.m.	Revolutions per minute
SPPS	Solid Phase Peptide Synthesis
TBS	Tris-buffered saline
TFA	Trifluoroacetic acid
TIS	Triisopropylsilane
TRIS	Tris(hydroxymethyl)aminomethane
Trt	Triphenylmethyl (trityl)
UFC	Colony-forming unit
UV	Ultraviolet

APPENDICES

APPENDIX 1: AMINO ACIDS AND PROTECTING GROUPS
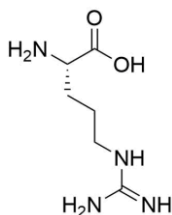
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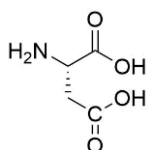
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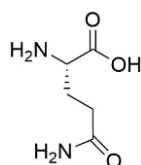
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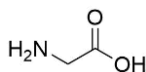
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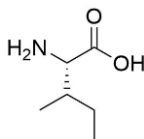
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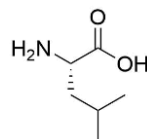
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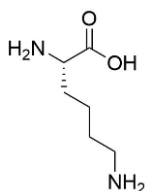
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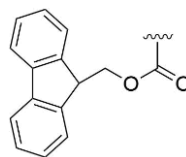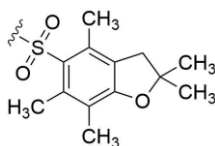L-Lys
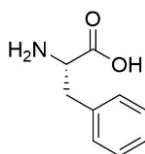
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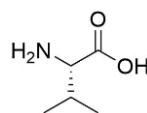
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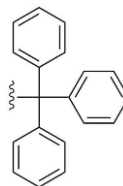L-Val
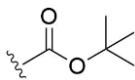
Tert-butyloxycarbonyl
(Boc)

Tert-butyl
(ᵗBu)

Fluorenylmethyloxycarbonyl
(Fmoc)

2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl
(Pbf)

Tryphenylmethyl
(Trt)

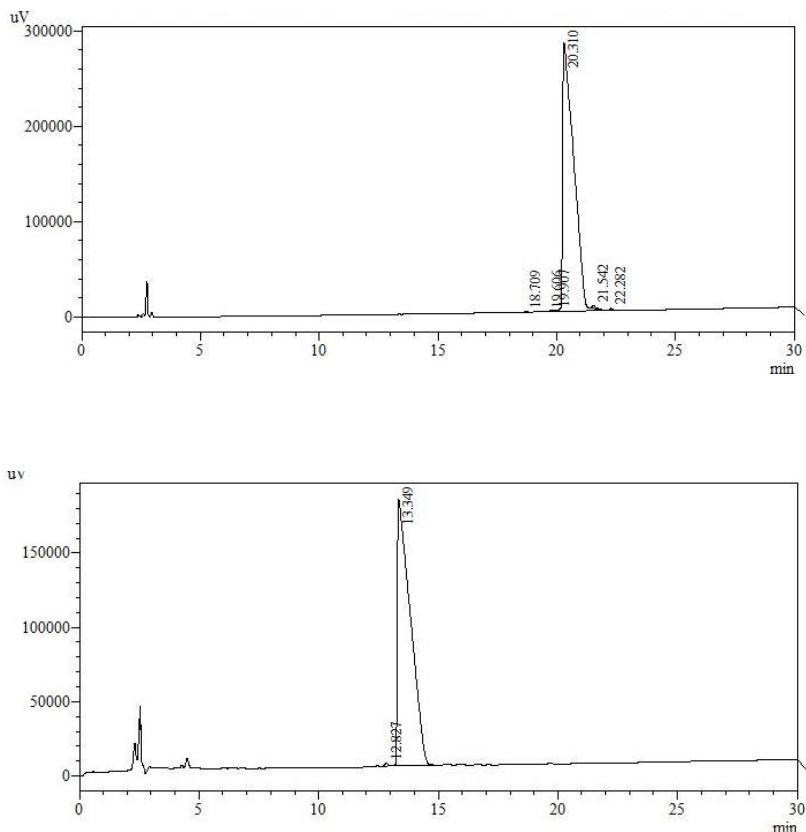# APPENDIX 2. CHROMATOGRAMS OF PURE PEPTIDES

Figure 14. HPLC chromatograms of KR-12 and analog with linear gradient from 10% to 25% and 30% to 45% of B for 30 minutes respectively; UV detection at 220nm.
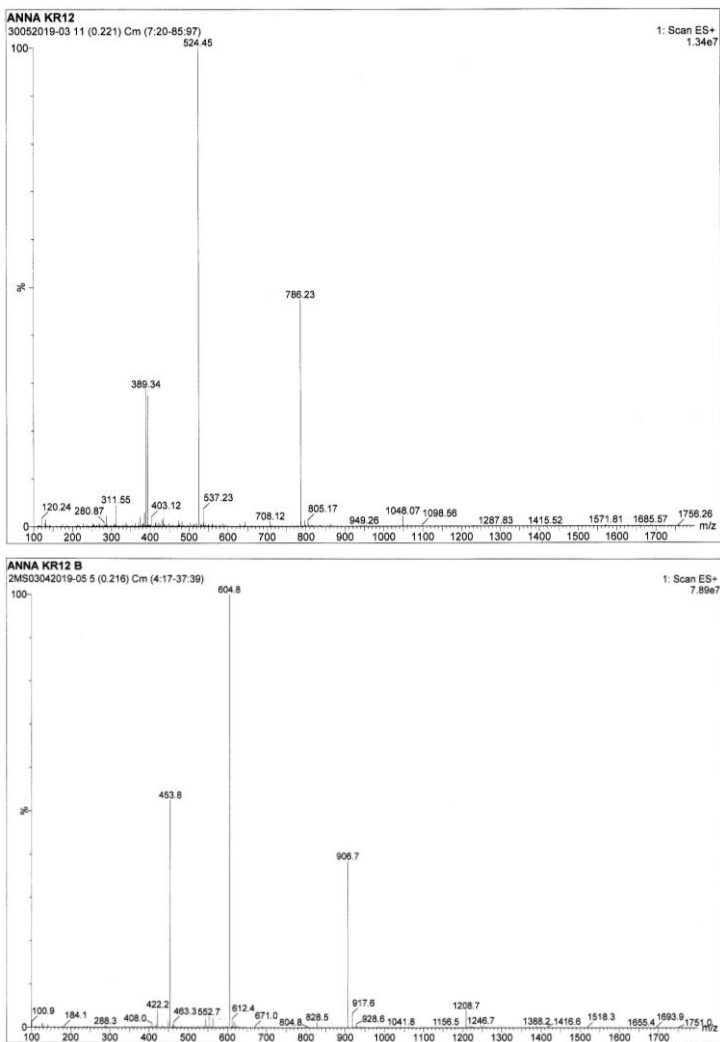
APPENDIX 3. ESI SPECTRA OF PURE PEPTIDES

Figure 14. ESI spectra of pure peptides: KR-12 above and analog below.