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Synthesis and study of antibiotic cyclopeptides
Síntesi i estudi de ciclopèptids antibiòtics

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If we knew what it was we were doing, it would not be called research, would it?

Albert Einstein

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REPORT

CONTENTS

1. SUMMARY

There is currently an important clinical worldwide challenge around the infections produced by multidrug-resistant bacteria. The discovery of effective antibiotics has diminished a lot in recent years, evidencing a great threat and an urgent need of new and safer antibiotics with broader spectrum to fight against these bacteria.

A new field of study consists in reconsidering polymyxin, a cyclopeptide that presents excellent antimicrobial activity against Gram-negative bacteria. However, its use has been reduced over the years due to their neuro and nephrotoxicity, so that they are currently used only as a last resort antibiotic. As a consequence, one of the research areas consists in the synthesis of polymyxin analogs with the aim of improving its biological activity and reducing its toxicity.

Regarding this work, three polymyxin analogs are firstly synthesized by solid phase peptide synthesis using Fmoc/^{t}Bu orthogonal protection strategy. Secondly, cyclization is carried out by the oxidation between two cysteine residues and finally, the analogs obtained are characterized by using RP-HPLC and ESI mass spectrometry. Moderately good yields and high purity are obtained for the analogs synthetized.

Subsequently, the microbiological activity of the analogs is evaluated by determining their minimum inhibitory concentration (MIC) and, later, their hemolytic activity is studied as a toxicity test.

Keywords: resistant bacteria, polymyxin analogs, solid phase, microbiological activity, toxicity.

2. RESUM

Actualment hi ha un repte clínic important a nivell mundial al voltant de les infeccions produïdes per bacteris resistents a múltiples fàrmacs. El descobriment d'antibiòtics eficaços ha disminuït molt en els últims anys, posant en evidència una gran amenaça i una necessitat urgent d'antibiòtics nous, de més ampli espectre i més segurs per tal de combatre aquests bacteris.

Un nou camp d'estudi es basa en la reconsideració de la polimixina, un ciclopèptid que presenta una excel·lent activitat antimicrobiana contra bacteris Gram-negatius. No obstant això, el seu ús ha disminuït al llarg dels anys a causa de la seva neuro i nefrotoxicitat, de manera que actualment s'utilitzen com antibiòtics d'últim recurs. Com a conseqüència, una de les àrees de treball consisteix en la síntesis d'anàlegs de polimixina amb l'objectiu de millorar la seva activitat biològica i reduir-ne la toxicitat.

Pel que fa aquest treball, primerament es sintetitzen tres anàlegs de polimixina mitjançant la síntesis de pèptids en fase sòlida utilitzant l'estratègia de protecció ortogonal Fmoc/^{t}Bu. En segon lloc, la ciclació es dur a terme mitjançant l'oxidació de dos residus de cisteïna i, finalment, els anàlegs obtinguts es caracteritzen utilitzant RP-HPLC i espectrometria de masses ESI. Els anàlegs sintetitzats s'obtenen amb rendiments moderadament alts i alta puresa.

Posteriorment, s'avalua l'activitat microbiològica dels anàlegs determinant la seva concentració mínima inhibitòria (CMI) i, després, s'estudia la seva activitat hemolítica com a prova de toxicitat.

Paraules clau: Bacteri resistent, anàlegs de polimixina, fase sòlida, activitat microbiològica, toxicitat.

3. INTRODUCTION

One of the major threats in the field of health worldwide is due to the increase in multidrug-resistant bacteria to existing drugs. This resistance is mostly caused by irreversible mutations in bacterial genes, resulting in bacteria acquiring multiple resistance mechanisms, thus becoming resistant to several antimicrobial agents. As a result, this may seriously limit the available treatments for current and future infections, increasing the healthcare cost. [1]

Between 1940 and 1970 the pharmaceutical industry released a steady flow of new antibiotics, including several with new mechanisms of action against bacterial resistance. However, the number of discoveries has drastically declined in the last few years. [2]

The growing gap between the increasing frequency of infections caused by multidrug-resistant bacteria and the decline in research is leading a crisis that must be addressed as a matter of urgency. The main goal is to design new antibiotics with a higher spectrum of action and security in order to combat bacteria that are prone to acquiring resistance to the antibiotics currently on the market. [3]

3.1. PEPTIDES AS ANTIBIOTICS

Historically, peptides have not been considered as successful therapeutic candidates due to their low oral bioavailability, since they are degraded and eliminated from the bloodstream quickly. Its expensive synthesis has also been one of the disadvantages. Despite this, its interest has increased in recent years by placing them as therapeutic candidates reconsidering their advantages; high efficiency, selectivity, specificity, low toxicity and the possibility of creating analogs. [4]

Antimicrobial peptides (AMPs) are a very diverse group of molecules, which are divided based on their composition and structure of amino acids. Against the great diversity, most of these peptides are composed of positively charges and amphipathic residues. [5] Their mechanism of action is based on the ionic and the hydrophobic interactions between the positive residues of AMPs and the anionic phospholipid components exposed to the membrane of the bacteria. [6]

3.2. SOLID PHASE PEPTIDE SYNTHESIS

Bruce Merrifield described a revolutionary method in 1969, the solid phase peptide synthesis (SPPS). Until that moment, the synthesis processes were very long, slow and with low yields. So this new method allowed the cited aspects to be improved. [7]

Solid phase peptide synthesis is a process which consists in attaching the C-terminal residue of the first amino acid with the protected alpha amino group, to a resin. The protective group is then removed and the N-protected and C-activated amino acids are incorporated stepwise. The cycle is repeated until the peptide sequence is completed.

For efficacious synthesis, the side chains must remain protected even with the repetitive N-alpha deprotections. Normally used an orthogonal protection, which consists in the completely different conditions that N-alpha protective group and the side chain protecting groups need to be eliminated.

The resin is washed after each step, so the excess of reagents and the impurities, which are not attached to the solid support, are removed by vacuum filtration. Finally, once the sequence has been synthesized the peptide is cleaved from the resin by and acid treatment. [7,8]

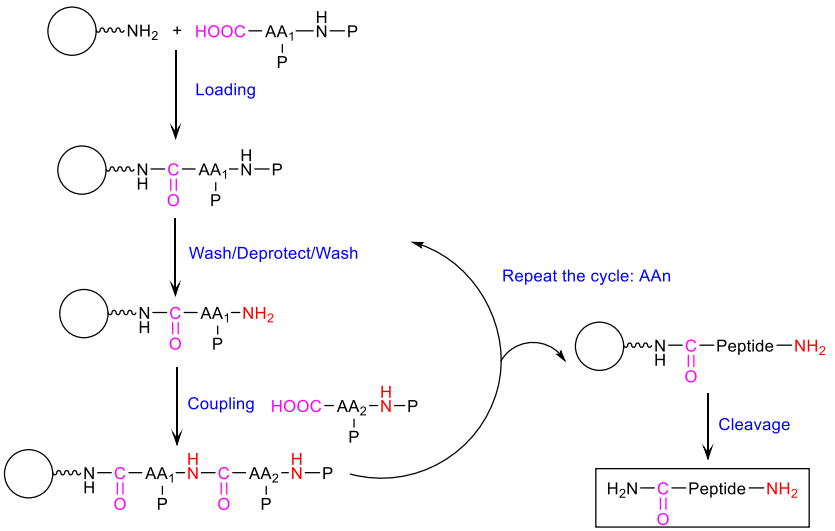

Figure 1. General structure of SPPS (P: protecting group).

3.3. POLYMYXINS

Polymyxins are a group of antimicrobial cyclic lipopeptides discovered in 1947 almost simultaneously by three different teams. They are produced by the fermentation of *Paenibacillus polymyxa* and they have a narrow antimicrobial spectrum since they are only active against Gram-negative bacteria. [9]

Polymyxins consist of a heterogeneous mixture composed of up to 30 tightly related lipopeptides, from which there are two highly well-known for their clinical use: Polymyxin B and colistin (Polymyxin E). They share the main structure, with five positive charges due to the amino acid Dab, and the only difference being at amino acid 6th.

Its use has diminished over the years until it has become last-resort antibiotic since some reports have shown its toxicity. However, nowadays they have regained a significant interest as a result of their high antimicrobial activity and low resistance, as a way to face the challenge of multidrug resistant bacteria. [9,10]

3.3.1. General structure

The general structure of polymyxins consists of a heptapeptide cycle unit to a linear tripeptide acylated at the N-terminus with a fatty acid tail.

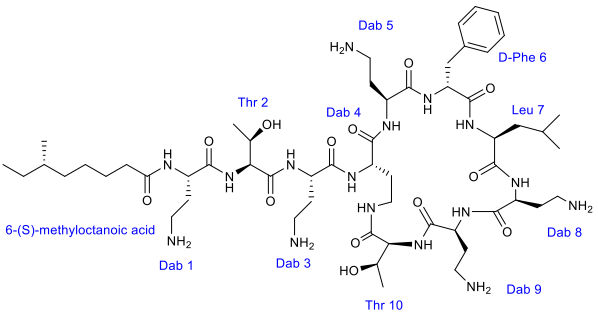

Figure 2. Polymyxin B1 structure. Amino acid positions are numbered from 1 to 10. The fatty acyl moiety and amino acids in the 6th and 7th position, define the hydrophobic features of the molecule. The rest of the amino acid residues are polar (Thr) and amino-containing basic residues (Dab).

As shown in **Figure 2**, an amide bond between the γ-NH2 of Dab-4 and the α-COOH of the Thr-10 amino acid closes the cycle, at the same time it binds to the tripeptide through the α-NH2 group from Dab-4. Both lipopeptides differ in position 6, in PxB it is occupied by (D)-Phe and (D)-

Leu in colistin. [9] This presence of lipophilic and hydrophilic groups converts polymyxins into amphipathic molecules, and essential property for antibacterial activity. [11]

3.3.2. Antibacterial mechanism

The mechanism of action of polymyxins is based on the interaction with the LPS, the main component of the OM of Gram-negative bacteria. This explains the reason why they are ineffective in front of Gram-positive bacteria.

LPS is a structural component composed of three domains: O-antigen, a core of polysaccharide and lipid A. Lipid A is the domain responsible for the packing of OM, and is the main target for polymyxins. LPS has several anionic charges due to the phosphate groups of the lipid A, so an electrostatic interaction with polycationic polymyxins occurred. In this way, a displacement of the divalent cations, Ca^{2+} and Mg^{2+}, which normally confer high rigidity and low permeability to OM, is generated. It causes the destabilization of LPS layer and allow the insertion of the acyl chain of polymyxin. [9,12]

The set of electrostatic and hydrophobic interactions causes the disruption of the OM and the entrance of polymyxin into the periplasmic space takes places. According to a suggested model, once in the periplasmic space, polymyxin will form contacts between the two phospholipid interfaces and promote an exchange of anionic phospholipids. As a consequence, the membrane lipid composition changes and trigged an osmotic imbalance that leads to cell death. [9]

However, the exact mode of action has not been described completely because other mechanisms involving intracellular targets can also play a role.

3.3.3. Toxicity

Toxicity of polymyxins is dose-dependent and reversible one the treatment is discontinued. The main adverse side effects of polymyxins are nephro and neurotoxicity.

The molecular mechanisms of toxicity are not well-understood but it seems to indicate that toxicity is related to the amphipathic nature of the molecule, due to the presence of hydrophobic residues and specially to the presence of charge Dab side chains at physiological pH. [9,13]

3.3.4. Polymyxin analogs

One of the research areas consists in the design of new polymyxin analogs with improved activity and reduced adverse side toxic effects. The main goal is to understand the relationship between activity and toxicity with the chemical structure of polymyxins.

First, it is known that amphipathicity in polymyxins is crucial for their activity. Thus, modifications in the 6 and 7 residues of the macrocycle and in the size of the fatty acid are applied. Secondly, in order to facilitate the synthesis, the fatty acid can be simplified by a linear one, without sterocenter. Finally, the amide bond can be substituted by a disulfide bond which could facilitate peptide proteolysis and potentially lower renal toxicity. This change implies modifying residues 4 and 10. [9]

4. OBJECTIVES

The main objectives in this work are the following:

- Synthesize three polymyxin analogs to obtain new antibacterial agents by using the solid phase peptide synthesis for this end.
- Purify the peptides obtained by semi preparative HPLC and characterize them by the use of analytical HPLC and ESI mass spectrometry.
- Determine the antimicrobial activity of the analogs in Gram-positive and Gram-negative bacteria by using MIC test.
- Evaluate the toxicity of the analogs by *in vitro* hemolysis assays.

5. EXPERIMENTAL SECTION

5.1. MATERIALS

5.1.1. Solvents, reagents and products

Solvent	Quality	Brand
ACN	HPLC	Fisher
DCM	Synthesis	VWR
DMF	Synthesis	Carlo Erba
Et_2O	Synthesis	Scharlau

H_2O	Mili-Q	-
MeOH	HPLC	Fisher

(a)DMC is filtered through a column of silica.
(b)The dried Et_2O is kept over sodium.
(c)The deionized water is filtered with a Milli-Q Plus (Millipore) system.

Table 1. Solvents and their specifications.

Reagent or product	Quality	Brand
BHA resin, RL and AA		Bachem Iris Biotech Novabiochem Polypeptide Fisher Scientific
Fatty acids	Reagent Plus®, 99%	Sigma-Aldrich
DIC	Pure, 99%	Sigma-Aldrich
DIEA	Reagent Plus®, 99%	Sigma-Aldrich
DMSO	Synthesis	Acros Organics
HOBt	Pure, 99%	Fluka
MHB		Oxoid
Ninhydrin	Pure	Koch-Light
Piperidine	Reagent Plus®, 99%	Sigma-Aldrich
PxE and PxB	Reagent Plus®, 99%	Sigma-Aldrich
TFA	Reagent Plus®, 99%	Sigma-Aldrich
TIS	Pure, 98%	Acros Organics
TRIS		Sigma-Aldrich

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

5.1.2. Instrumentation

Instrument	Brand and model
Autoclave	AUTESTER-E
Centrifuge	Hettich ROTOFIX 32 A and Beckman Coulter Allegra 25R Refrigerated Centrifuge
HPLC	-Analytical: Simadzu Serie 20 Prominence, with two pumps LC-20AD, automàtic SIL-20A injector, controller CBM-20A and detector SPD-M20A -Semipreparative: Waters Delta Prep 3000, with a Waters 600E pump and a manual sample injector Eaters 712, Water 484 detector, and a data recordar Pharmacia Biotech RED 101.

Lyophilizer	Christ Alpha 1-2 LDplus
Mass spectrometer	Zq-Micromass (wàters)
Microplate reader	Synergy HT
Sonicator	Selecta MEDI-II
Spectrophotometer	Shimadzu UV-1700 PC

Table 3. Instrumentation and its specifications.

5.2. METHODS

5.2.1. Ninhydrin test

The ninhydrin test, also known as Kaiser test, is an assay used in SPPS to determine the completeness of the amino acid coupling. Ninhydrin reacts with primary amino groups resulting in an intense blue color (positive assay).

To perform the test, three solutions are required:

-Reagent A: ninhydrin (2.5g) are dissolved in EtOH (50mL). The obtained dissolution 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 test, a little part of the peptide-resin cleaned and dried is introduced in a small test tube after each coupling. Two drops of each reagent (stated above as A, B and C) are added and the tube is heated at 110ºC for 3' in a sand bath.

If the dissolution remains yellow (negative assay) means a successful coupling because of the absence of free amines, while if the solution turns blue (positive assay) free primary amino groups are present and a recoupling step is necessary.

As shows **Figure 3**, ninhydrin (1) reacts with a primary amine of an amino acid (2) to form the imine (3) by a nucleophilic addition-elimination reaction. After that, a decarboxylation reaction produces the imine (5) which reacts with H_2O to form an aldehyde and a free amine again (6). This latter free amine can react with another equivalent of ninhydrin forming 8, which is the responsible compound for blue color. [7]

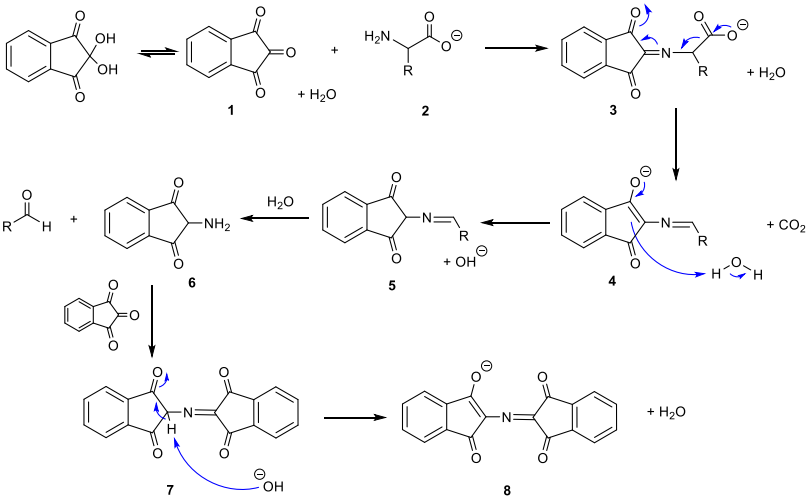

Figure 3. Reaction mechanism of ninhydrin assay.

5.2.2. Purification

The peptides purification is carried out by using a semi-preparative RP-HPLC. In this case, the retention time is bigger for non-polar molecules. Moreover, the retention time in presence of polar solvents will increase and decrease with hydrophobic solvents.

The column used is Phenomenex C18 of reverse phase (1x25 cm, 5µm diameter particle). The elution is carried out 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 techniques have been used to carry out the characterization of the peptides.

5.2.3.1. Analytical RP-HPLC

This technique is used to identify and quantify the components of a mixture. The amount of sample applied in this case is very small compared to the amount of stationary phase in the column.

The column used is a Nucleosil C18 of reverse phase (0.4x25 cm, 5µm diameter particle). The elution is carried out at 1mL/min flow rate with a lineal gradient of 0.045% TFA/H_2O (A) and 0.036% TFA/ACN (B) and UV detection at 220 nm.

5.2.3.2. Electrospray ionization mass spectrometry

Mass spectrometry is an analytical technique that converts molecular analytes into ions and provides qualitative and quantitative information. The ESI is a soft ionization technique extensively used for producing gas phase ions (without fragmentation) of thermally labile large supramolecules.

The samples analyzed are peptide solutions in H_2O/ACN (1: 1, v / v). A positive mass spectrum is obtained where the signals are caused by ionization with a cation hydrogen, sodium or potassium.

5.3. SOLID PHASE PEPTIDE SYNTHESIS

5.3.1. Synthesis strategy

The peptide sequences are manually synthesized by using polypropylene syringes equipped with a porous polyethylene filter. After all reactions of coupling, the excess of reagents, solvents and other byproducts are removed by vacuum filtration. In this work, the SPPS is carried out by Fmoc/^{t}Bu strategy.

5.3.1.1. Loading the resin

First of all, the BHA commercial resin used (f=0.69 mmol/g) needs a pre-treatment because it is stored in a compact form. As a result, an impurity-free resin is obtained. The analogs A and B are prepared starting from 252.72 mg of dry resin (0.174 mmol) and C from 122.7mg (0.085 mmol). The protocol used is described in **Table 4**.

The DMC is used to wash and dry the resin, TFA to wash it from impurities and DIEA to neutralize.

Step	Reagent	Time [min]
1	DCM	5x0.5
2	40% TFA/DCM	1x1
3	40% TFA/DCM	2x10
4	DCM	5x0.5
5	5% DIEA/DCM	3x2
6	DCM	5x0.5

Table 4. Protocol used to load BHA resin.

5.3.1.2. Reference amino acid coupling

The amino acid Fmoc-Val-OH is used as reference amino acid in the synthesis of the three analogs. The coupling protocol used is shown in **Table 5**.

5.3.1.3. Rink Linker incorporation

After the Fmoc removal, the RL incorporation is carried out by following the protocol shown in **Table 5** and by substituting Step 2 for the addition of 1.8 eq. of HOBt, DIC and RL to the BHA resin overnight.

5.3.1.4. Elongation of the peptide chain

It is necessary to have the terminal amino group deprotected to perform the peptide chain elongation. Then, the first amino acid of the chain and the following are coupled in the same way as shown in **Table 5**. The protocol includes the amino acid coupling, the Kaiser test to check the coupling and the removal of the Fmoc group. The sequences of the peptides chain of the three analogs are specified in **Section 6.1**.

Step	Reagent	Time [min]
1	DMF	5x0.5
2	3 eq Fmoc-AA-OH (a) 3 eq HOBt 3 eq DIC	1x60
3	DMF	5x0.5
4	DCM	5x0.5
5	Ninhydrin test (b)	1x3
6	DMF	5x0.5
7	20% piperidine/DMF	1x0.5
8	20% piperidine/DMF	2x10
9	DMF	5x0.5

(a) The amount of each reactive used depends on the weight of dry resin. The reagents are added to the resin with a minimum quantity of DMF.

(b) If the coupling is not completed, 1.5 eq. of the reagents are added for 30 extra minutes in DMF.

Table 5. Amino acids coupling protocol by Fmoc/^{t}Bu strategy.

5.3.1.5. Fatty acid coupling

Once the last amino acid is coupled and the Fmoc group is removed, the amount of resin in the first syringe (252.72mg of dry resin) is divided in two equal portions in order to perform the

analogs A and B. The analog C is synthetized directly in the whole amount of the resin (122.7mg of dry resin) in the second syringe.

The corresponding fatty acid is coupled on the peptidyl-resin of the three analogs. The protocol for this coupling is shown in **Table 5**, by substituting Step 2 for the addition of 5 eq. of DIC and 5 eq. of fatty acid to the peptidyl-resin for an hour and skipping Steps 6-9.

5.3.2. Cleavage from the resin

Once the analogs are synthesized, the side chains of the amino acids remain protected. The objective of this step is to separate the peptide from the solid support while removing the acid-labile protecting groups of the sidechains. This procedure is carried out by acidolysis with 5mL of TFA/TIS/H_2O (95: 3: 2, v / v) for 90' to the peptidyl-resin.

In this way, the resin is filtered and the combined filtrates are evaporated under a nitrogen flow to eliminate TFA. Then, the peptide is isolated by treating the crude product with Et_2O in order to induce precipitation. The product centrifuged and decanted is left to dry in the air and the dry peptide is obtained. This procedure is repeated twice.

- Cleavage yield: analog A 53% (79.8mg), analog B 59% (89.2mg) and analog C 79% (120.3mg)

5.3.3. Cyclization

The cyclization of the linear peptide is carried out by dissolving the peptide at a 1.5 mg/mL concentration is a solution of DMSO/H_2O at 3%. The mixture reactions at RT is monitored by analytical RP-HPLC. Finally, when the oxidation reaction is finished, the solution is lyophilized.

- The cyclized peptides are obtained as oils, for the presence of DMSO, and their yields are assumed to be 100%.

5.3.4. Purification and characterization

The peptides are purified by RP-HPLC at a semi-preparative scale and the UV detection at 220 nm. A lineal gradient using a flow range of 2mL/min is used with the following specifications in each analog: a lineal gradient from 15% to 30% of (B) for 30' in analog A and B and 20% to 35% of (B) for 30' in analog C. The pure fractions obtained are combined and lyophilized.

- Purification yield: analog A 52% (41.8mg), analog B 38% (33.9mg) and analog C 43% (51.3mg)

The characterization of purified peptides is performed by analytical RP-HPLC and ESI mass spectrometry. In RP-HPLC, a lineal gradient using a flow range of 1mL/min is used from 15% to 30% of (B) in analog A and B and 20% to 35% of (B) in analog C for 30' respectively. Chromatograms are shown in **Appendix 3** and ESI spectra at **4**.

Analog	Purity [%]	RP-HPLC t_R [min]	HRMS ESI$^+$
A	>99	14.530	**m/z** 1147.8 (7%, $[M+H]^+$), 574.5 (85%, $[(M+2H)/2]^{2+}$), 383.4 (100%, $[(M+3H)/3]^{3+}$)
B	>99	18.434	**m/z** 1161.8 (7% $[M+H]^+$), 581.8 (89% $[(M+2H)/2]^{2+}$), 388.0 (100%, $[(M+3H)/3]^{3+}$), 291.3 (25%, $[(M+4H)/4]^{4+}$)
C	>99	24.893	**m/z** 1237.8 (8%, $[M+H]^+$), 619.7 (100%, $[(M+2H)/2]^{2+}$)

Table 6. Final characterization of synthesized analogs.

5.4. EVALUATION OF ANTIMICROBIAL ACTIVITY

The MIC values for the analogs and commercial PxE are evaluated in order to compare the activity against Gram-negative and Gram-positive bacteria. [14,15]

5.4.1. Preparation of material and medium

Different culture mediums of MHB at different concentrations are prepared: 1xMHB (9.6g in 400mL MiliQ-water), 2xMHB (19.4g in 400mL MiliQ-water) and 1xMHB with Agar (23g MHB in 1L MiliQ-water with 6g of Agar). All material and liquid growth medium must be sterilized with Autoclave before its use.

5.4.2. Preparation of bacteria and peptides

The microorganisms used are *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Acinetobacter baumannii* ATCC 19606 and *Staphylococcus aureus* ATCC 25923.

These bacteria need to be cultured in MHB overnight followed by MHB with Agar overnight culture. The day before the test, bacteria are cultured in test tubes with MHB liquid medium overnight. Next day, the absorbance of the suspensions is adjusted to 0.2 at 550 nm.

For each analog and commercial PxE a solution of 512 µg/mL is prepared and then it is diluted in order to obtain a 128 µg/ml solution.

The stock solution is prepared with MiliQ-water taking into account the current proportion of the peptide in the product obtained with the TFA counterions. In the case of commercial PxE, the same procedure is performed taking into account that 1 mg of commercial PxE corresponds to 0.85 mg of free base polymyxin.

5.4.3. MIC determination

The determination is carried out in 96-well polypropylene microtiter plates, one for each analog and one for polymyxin. Its preparation is explained below and it is shown in **Figure 4**. To add the different volumes a multi-channel pipette is used:

1) 50µL of 2xMHB in column 1, 50µL 1xMHB in columns 2-11 and 100µL 1xMHB in column 12.

2) 50µL of peptide solution in column 1. The mixture in column 1 is stirred and 50 µl of it is added to column 2. This process is repeated until column 10. The concentration of peptide in each column decreases gradually from 32 µg/mL in column 1 to 0.0625 µg/mL in column 10.

3) 50µL of bacteria suspension adjusted to 10^6 CFU/mL in columns 1-11. Each bacteria is inoculated in two rows.

4) Incubation of microtiter plate for 20-22 h at 37ºC. The plates are examined for visible bacteria growth as evidenced by turbidity.

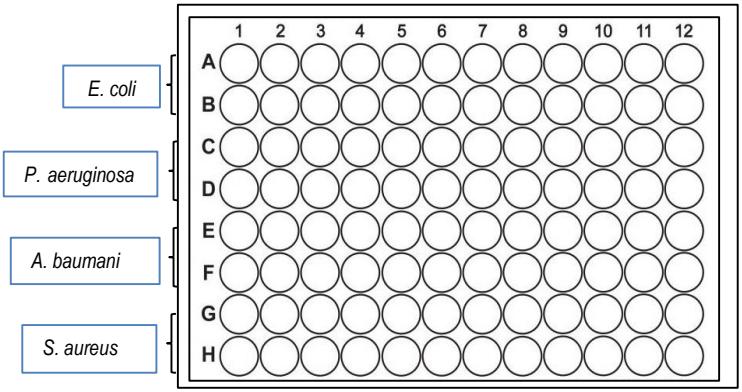

Figure 4. Microtiter plates design for MIC test.

Columns 11 and 12 correspond to positive (MHB and bacteria) and negative (only MHB) controls respectively.

5.5. EVALUATION OF TOXICITY

The next step is to measure the hemolytic activity of the analogs A and B and compare it with the PxE values. The hemolytic activity is examined in rabbit's blood. [14,15]

5.5.1. Blood treatment

It is necessary to wash blood with TBS (10mM TRIS; 150mM NaCl) by the centrifugation of blood (10', 4000 r.p.m., 4ºC), removing supernatant and adding buffer and repeat it three times. Finally, the concentrated red cells are obtained and they are adjusted to absorbance 1 at 540 nm by adding a variable amount of TBS and one drop of blood. A standard amount of erythrocytes is obtained to compare the results.

The positive control where there is total hemolysis is prepared by the centrifugation of 200 µL of red cells during 3' at 10000 r.p.m. The supernatan is then removed and the erythrocytes pellet is dissolved with 200µL of destilled water.

5.5.2. Preparation of peptide solutions

For each analog and commercial polymyxin, 1mL of a solution of 2mM is prepared by dissolving the correspondent amount of peptide in TRIS buffer. Then, a part of it is diluted in order to obtain 0.5mM solution.

5.5.3. Hemolytic assay

The assay is carried out in 96-well polyproylene microtiter plates, one for two peptides and one for PxE. Its preparation is explained below and it is shown in **Figure 5**. To add the different volumes a multi-channel pipette is used:

1) 200 µL of red cells adjusted solution to colums 1-9 and 11 in rows A-C and F-H. 200 µL of TBS to column 10 in rows A-C and F-H and 200 µL of total hemolysis solution to column 12 and rows A-D

2) Addition of the needed amount of each peptide solution in order to obtain the desired concentration in each well. The concentration of peptide in each column increases from 10 µM in column 1 to 367 µM in column 10.

As 0% and 100% hemolysis controls, buffer and erythrocytes in distilled water were employed in column 10 and 12 respectively.

3) Incubation of the plate 1h at 37ºC with agitation

4) Centrifugation of the plate (10 min, 4000 r.p.m., 4ºC)

5) Addition of 100µL of each well from the supernatant to a new 96-well plate and reading the absorbance at 540 nm.

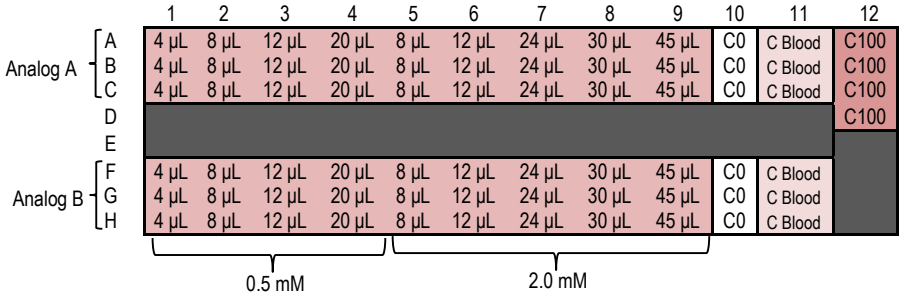

Figure 5. Microtiter plates design for hemolysis test.

6. POLYMYXIN ANALOGS

The principal objective in this work is to synthetize polymyxin analogs using SPPS in order to obtain new antimicrobial agents with the intention that its activity and toxicity are improved.

6.1. DESIGN

The principal changes between polymyxin and the analogs synthesized are explained in **section 3.3.4** and are based on modifications in the hydrophobic parts and the cycle closure.

The differences between polymyxin and the synthetized analogs in this work are the following:

- Substitution of the amide bond between residues Dab-4 and Thr-10 for a disulfide bond which requires Cys-4 and (D)-Cys-10 as new residues. This variation maintains the size of the molecule and facilitates the synthetic process.

- Substitution of residues 6 and 7 for new ones that increases the hydrophobic cycle motif.
- Substitution of the fatty acid chain by a linear one, without stereocenter, and increasing its length from analog A to C to show the hydrophobicity effect.

The sequences of the analogs and shown in **Table 7** and the structures in **Appendix 2**.

Peptide	Sequence
PxB	**(S)-6-methyl-octanoyl**-Dab-Thr-Dab-cyclo-[**Dab**-Dab-**(D)-Phe-Leu-**Dab-Dab-**Thr**]
PxE	**(S)-6-methyl-octanoyl**-Dab-Thr-Dab-cyclo-[**Dab**-Dab-**(D)-Leu-Leu-**Dab-Dab-**Thr**]
A	**Acyl1**- Dab-Thr-Dab-cyclo-[**Cys**-Dab-**R6-R7-**Dab-Dab-**(D)-Cys**]
B	**Acyl2**- Dab-Thr-Dab-cyclo-[**Cys**-Dab-**R6-R7-**Dab-Dab-**(D)-Cys**]
C	**Acyl3**- Dab-Thr-Dab-cyclo-[**Cys**-Dab-**R6-R7-**Dab-Dab-**(D)-Cys**]

Table 7. Sequence of PxB, PxE and the three analogs synthesized. The fatty acid, R6 and R7 for each analog cannot be disclosed for confidentiality reasons.

6.2. SOLID PHASE PEPTIDE SYNTHESIS

Solid phase peptide synthesis is a process to synthesize peptides that is based on the successive incorporation of protected amino acids to an amino acid anchored to a solid suport matrix by its C-terminus until the desired sequence is completed. Thus, the peptide is synthesized from C-terminus to the N-terminus end.

6.2.1. Synthesis strategy

The Fmoc/^{t}Bu protection strategy is used during the SPPS. This strategy is used to prevent the polymerization of the amino acid, so the base-labile Fmoc group protect temporally the alpha nitrogen of the amino acid while the acid-labile ^{t}Bu group is used in the side chains. It consists in an orthogonal protection system, since the side chain remain protected even with the N-alpha deprotections and vice versa. [7,8]

The peptide synthesis is based on the reaction between a carboxylic acid and an amino group of and amino acid to form an amide bond. However, activation is required in the acid group, since if the two groups react directly the product would not be the expected one, but a stable salt (acid-base reaction). In order to avoid this, DIC is used as an activate agent to form an O-acylisourea which can react with the amine to yield the amide. Nevertheless, this activation increases the tendency of racemization, so a nucleophile such as HOBt is added to improve the efficiency of the technique. The mechanism is shown in **Figure 6.**

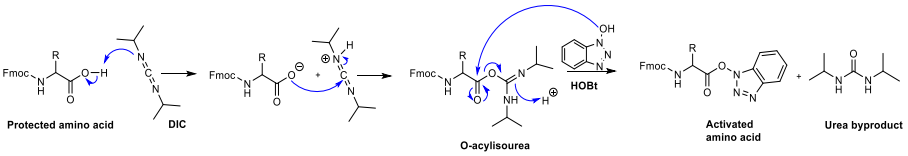

Figure 6. Mechanism of carboxylic acid activation and HOBt coupling.

Finally, the activated carboxylic acid can be attacked by an unprotected amino group of a second amino acid as we can see in **Figure 7.**

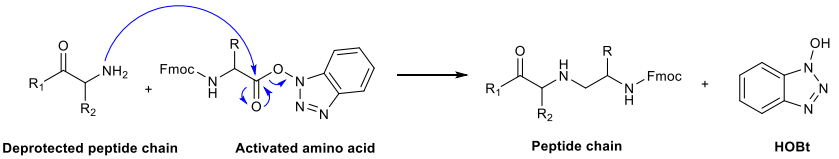

Figure 7. Mechanism of chain elongation.

Before the next amino acid coupling the Fmoc group must be removed, so a basic treatment such as 20% piperidine/DMF is used as it is explained in **Table 5**. The mechanism for deprotection is shown in **Figure 8**.

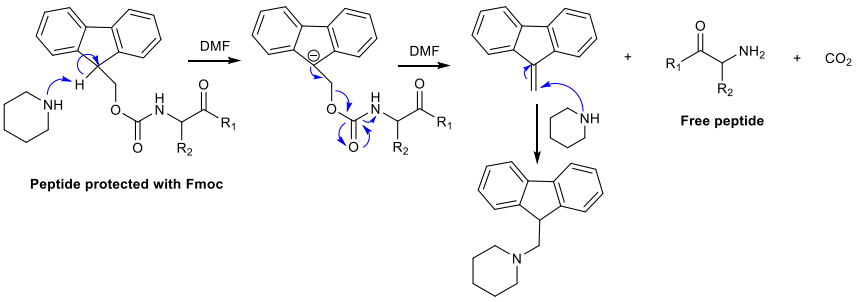

Figure 8. Mechanism of Fmoc deprotection.

Following this mechanisms, the synthesis of the analogs was carried out on BHA resin. This solid support consists of a benzhydrylamine linker bound to a polystyrene matrix, which need to be pre-treated as explained in **Section 5.3.1.1**. The bond between BHA resin and the peptide sequences is highly stable, and is for that reason that a linker was put between the peptide

sequence and the resin. Moreover, the addition of a reference amino acid facilitates the SPPS because it avoids steric and electronic problems.

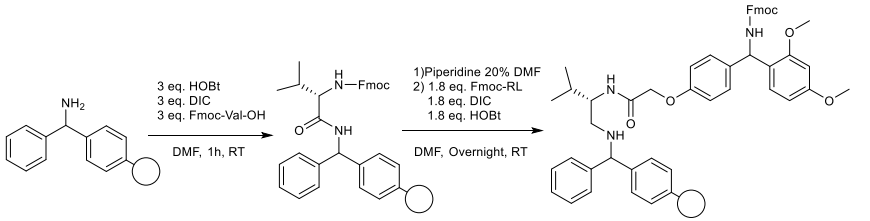

Figure 9. Reference AA and RL coupling to BHA resin.

For the elongation of the peptide chain, the procedure indicated in **Section 5.3.1.4** was carried out. The efficiency of the couplings was controlled by ninhydrin test.

Once the last amino acid was incorporated in the sequence, fatty acids were coupled in the three analogs following the protocol described in **Section 5.3.1.5** and the following linear peptide chain is obtained.

As **Figure 10** shows, lateral chains remained protected with acid-labile groups, so cleavage and full deprotection of the peptidyl-resin was carried out by acidolysis reaction specified in **Section 5.3.2**. During this process, highly reactive cationic species are generated from the protecting groups. Unless these species are trapped, side reactions take place. To prevent this, nucleophilic reagents (known as scavengers) are added to the TFA to quench these ions. In this work, TIS and H_2O are used for this end. So finally, total unprotected linear peptides were obtained.

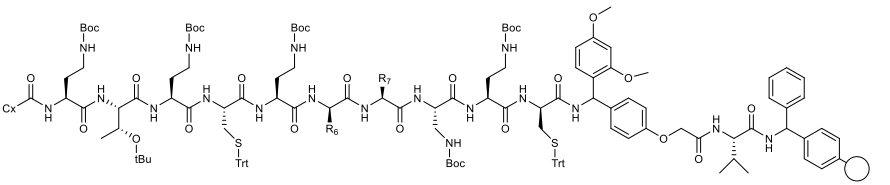

Figure 10. Lineal structure of an analog with the lateral chains protected.

After deprotection, the two residues of cysteine form a disulfide bond by oxidation at high dilution to prevent dimerization of the peptide.

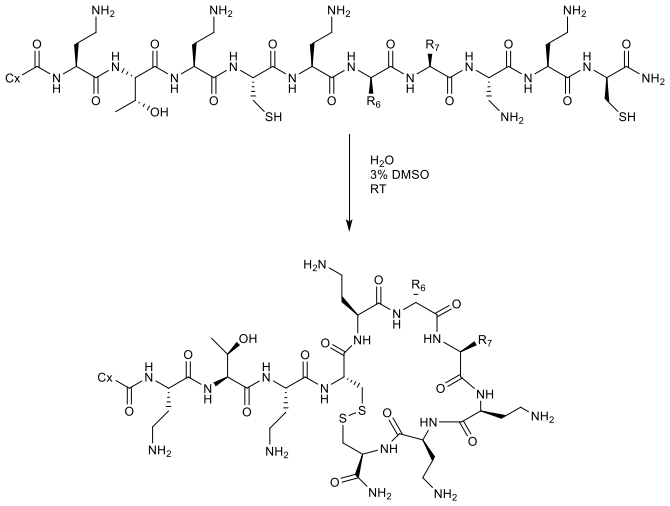

Figure 11. Cyclization of the linear analog by mild oxidizing agent

The reaction of cyclization was monitored by analytical RP-HPLC. As shown in **Figure 12**, the disappearance of the peak of the linear peptide and the subsequent emergence of a new peak corresponding to the cyclic peptide is observed. The peptides were cyclated after 90-120 h approximately.

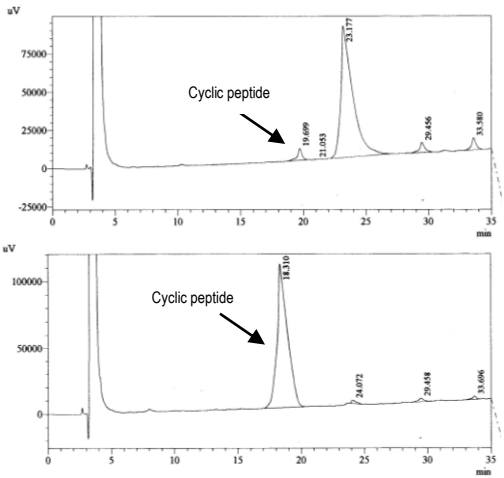

Figure 12. Chromatograms of cyclization of analog B. At t=0h the percentage of the cyclic peptide was 3% and after 120 h it increased to 98%.

Finally, the solutions were lyophilized to obtain the crude peptides.

6.2.2. Synthesis results

The cycled peptides are obtained as oils due to the presence of DMSO because it has not been eliminated in lyophilization. Then, they were purified and characterized obtaining good purities and moderately good yields. The characterization results are shown in **Appendices 3** and **4**.

The partial yields are the following:

Peptide	ɳ cleavage [%]	ɳ cyclization [%]	ɳ purification [%]	ɳ synthesis [%]	Purity [%]
A	53	100	52	28	>99
B	59	100	38	22	>99
C	79	100	43	34	>99

Table 8. Reaction yields and purity of analogs A, B and C

Synthetic yields are good, despite the many steps and tests that are needed in SPPS and therefore it causes the loss of the amount of the product. Also, due to side reactions that no leads to the desired product.

The step of purification is the step with lower yields and in this case indicates that the expected products are the main components of crude product, but there are also impurities of bad couplings.

7. MICROBIOLOGICAL ACTIVITY

7.1. GENERAL ASPECTS

To evaluate the antibiotic activity of the synthesized peptides MIC tests are performed. These tests aim to quantify the lower concentration of an antibiotic that inhibits the visible growth of the microbe, which corresponds to the minimal inhibitory concentration (MIC).

The method used consists of a series of dilutions of the peptide (32-0.0625 µg/mL) in a medium of microbial growth that is inoculated with a standardized number of organisms (10^6

UFC/ml) and its incubated during a time of 20-22 h at 37ºC. Finally, the plates are examined to obtain visible evidence of the growth bacteria by turbidity [16,17]

Accurate control of the concentration of organisms in the growth medium is required in order to obtain good and comparable results. In this test, the absorbance is adjusted to 0.2 at 550 nm In this way, this procedure helps us to relate the turbidity of the solution with the concentration of present bacteria ($1.5 \cdot 10^8$ CFU /ml)

Despite knowing that polymyxin acts on Gram-negative bacteria, three of these bacteria and a Gram-positive bacteria were chosen to make the analysis to confirm their actions.

7.2. RESULTS AND DISCUSSION

After the overnight incubation, the MIC values are determined visually using an amplifier to observe bacteria growth as evidence by turbidity. The results are shown in **Table 9**.

		MIC [µg/mL]			
		PxE	**A**	**B**	**C**
GRAM -	***E. coli***	1-0.5	32	32	2
	P. aeruginosa	1	>32	16-32	2
	A. baumannii	1	>32	>32	>32
GRAM +	***S. aureus***	>32	>32	>32	4

Table 9. MIC test results of PxE and three analog synthesized

The MIC results show that the analogs do not improve the effectiveness of PxE in Gram-negative bacteria. However, analog C is the only one which has improved the activity of PxE in Gram-positive bacteria.

A correlation is observed in the analogs between their fatty acid length and their activity. Analog C with the largest hydrocarbon chain presents better antimicrobial activity against Gram-negative bacteria than analog A and B but without exceeding PxE results.

The effectiveness against the bacteria *A. baumannii* could not be improved in any analog. A hypothesis of their repetitive ineffectiveness is due to the presence of disulfide bond in the analogs.

8. HEMOLYSIS ASSAYS

8.1. GENERAL ASPECTS

Hemolysis is the rupture of red blood cells and the release of their contents into the surrounding fluid. The hemolysis results evaluate hemoglobin release in the plasma, as an indicator of red blood cell lysis, after test agent exposure.

The hemolysis process is strongly influenced by the tonicity of the medium in which erythrocytes are placed. For example, if red blood cells are placed in a hypotonic solution such as distilled water, the water goes into red blood cells through an osmosis process and it causes cell rupture. This process is used to obtain the total hemolysis control in this work. Besides, TBS (Tris-buffered saline) which is a useful buffer solution formed by TRIS and NaCl (in order to give the isotonic salt concentration), avoids hemolysis caused from medium. Also, TBS is used to pretend physiological conditions of most living organisms such as the pH value which range from 7.4 to 8.0.

The protocol consists in a series of dilutions of the peptide which are mixed with pre-treated blood and incubated for 60 minutes at 37°C. The cells are centrifuged and the absorbance of the supernatant, which includes hemoglobin, is measured at 540 nm. A toxicity evaluation is obtained from the percentage of lysis cells according to peptide concentration.

A good antibacterial agent should have low MIC values maintain low hemolytic activity, a measure of toxicity.

8.2. RESULTS AND DISCUSSION

The results are represented by concentration of each peptide versus the hemolysis percentage which was calculated according to the equation in **Figure 13**.

$$\% \text{ hemolysis} = 100 \times \left[\frac{(A_{SAMPLE} - A_{C\ BLOOD})}{(A_{C100} - A_{C\ BLOOD})}\right]$$

Figure 13. Percentage of hemolysis equation. (A:absorbance)

The dependence between the percentage of hemolysis and the concentration of each peptide is shown in **Figure 14.**

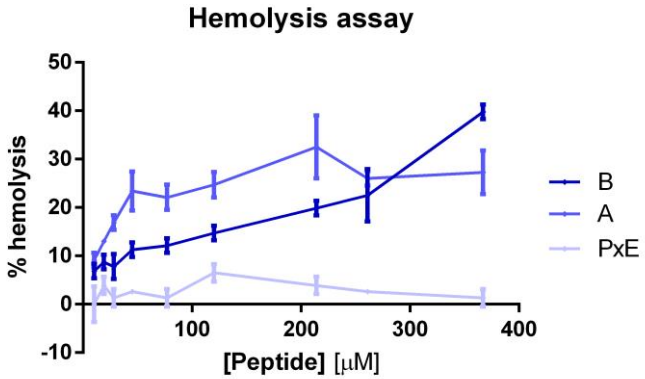

Figure 14. Plot of peptide concentration vs percentage of hemolysis. The results are plotted with the main values and its error.

As shown in **Figure 14**, the percentage of hemolysis increases with a non lineal tendency.

Comparing the analogs A and B with PxE, the plot does not shown the expected results. Both analogs present a smaller length of fatty acid chain than PxE, so analogs are expected to have less hemolytic activity. Analog C has not been tested in this work since it has been previously tested.

By comparing the analogs, the results obtained conclude that there is a correlation between the length of their fatty acid and their hemolytic values. The analog C, which is more hydrophobic, is also more hemolytic.

The amphipathicity is important in polymyxin mechanism of action. Polymyxins are cationic amphipathic molecules that cause cell membrane damage, suggesting a detergent-like mode of action.

The results are not conclusive since in our analogs three types of structural changes have been introduced with the hypothesis of reducing the hemolysis and finally, it is observed that this is not the case. It will be necessary to continue studying to which structural change belongs the increase in the hemolysis.

Although *in vitro* tests give valuable information of toxicity, *in vivo* studies have to be performed to assess the potential application of these compounds.

9. CONCLUSIONS

The conclusions reached in this work are the following:

- Three new antibacterial agents are obtained by using solid phase peptide synthesis. This technique is an effective and manageable methodology that allows to acquire the desired peptides with high purities (>99%) and good reaction yields.
- The MIC determination allows knowing and comparing the antimicrobial activity of the different antibiotics. The results meet the expectations since a larger fatty acid chain contributes to a better microbiological activity and lower MIC values. The analog C presents the best antimicrobial activity for its effectiveness against Gram-negative bacteria and its improved action against the Gram-positive bacteria compared with PxE.
- The hemolytic assays allow to know and compare the toxicity of different antibiotic agents. Analogs A and B have not been successful since they have higher hemolytic values than PxE and therefore, they present more toxicity.

Hence, results conclude that modifications made in polymyxin structure in the present work need further increase in microbiological activity and in toxicological properties of polymyxin.

10. REFERENCES AND NOTES

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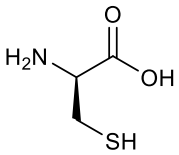
11. ACRONYMS

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
DMSO	Dymethyl sulfoxide
Eq.	Equivalents
ESI	Electrospray ionization
f	Functionality
Fmoc	Fluorenylmethyloxycarbonyl
h	Hours
HOBt	1-Hydroxybenzotriazole

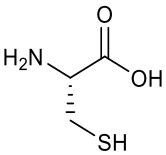
LPS	Lipopolysaccharides Structures
m/z	Mass-tot-charge ratio
MHB	Muller Hinton-Broth
MIC	Minimum inhibitory concentration
min	Minutes
mL	Milliliter
MS	Mass spectrometry
nm	Nanometres
OM	Outer-membrane
PxB	Polymyxin B
PxE	Polymyxin E
RL	Rink linker
RP-HPLC	Reverse Phase High Performance Liquid Cromatography
r.p.m.	Revolutions per minute
SPPS	Solid Phase Peptide Synhtesis
TBS	Tris-buffered saline
TFA	Trifloroacetic acid
TIS	Triisoprpylsilane
TRIS	Tris(hydroxymethyl)aminomethane
Trt	Triphenylmethyl (trityl)
UV	Ultraviolet

APPENDICES

APPENDIX 1: AMINO ACIDS AND PROTECTING GROUPS



(D)-Cysteine (DCys)



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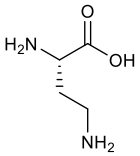
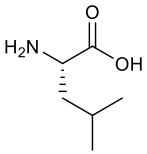
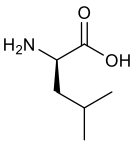


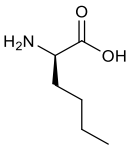
(L)-(2,4)-Diaminobutyric acid (Dab)



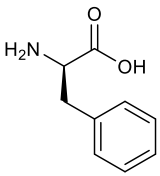
(L)-Leucine (Leu)



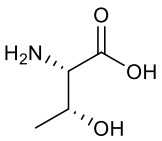
(D)-Leucine (DLeu)



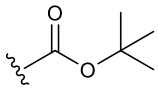
(L)-Norleucine (Nle)



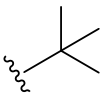
(D)-Phenylalanine (DPhe)



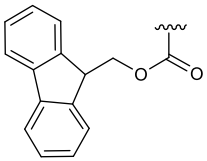
(L)-Threonine (Thr)



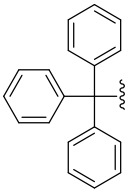
Tert-butyloxycarbonyl (Boc)



Tert-butyl (^{t}Bu)

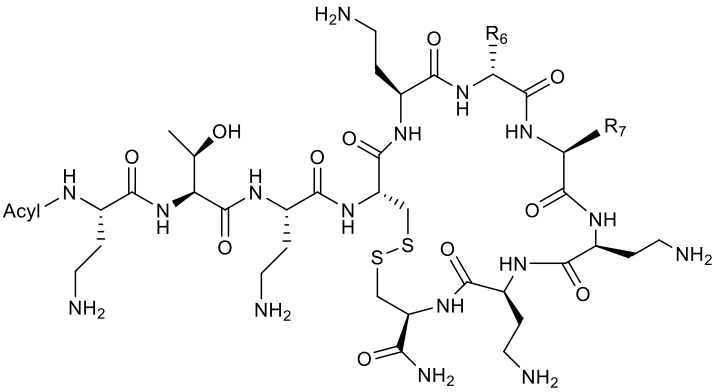


Fluorenylmethyloxycrbonyl (Fmoc)



Triphenylmethyl (trityl) (Trt)

APPENDIX 2: STRUCTURE OF POLYMYXIN ANALOGS



Acyl$_x$ - Dab-Thr-Dab-cyclo-[**Cys**-Dab-**R6-R7-**Dab-Dab-**(D)-Cys**]

Figure 15. General structure of polymyxin analogs.The amino acids R6, R7 and the acyl chain cannot be disclosed for confidentiality reasons.

APPENDIX 3: CHROMATOGRAMS OF PURE PEPTIDES

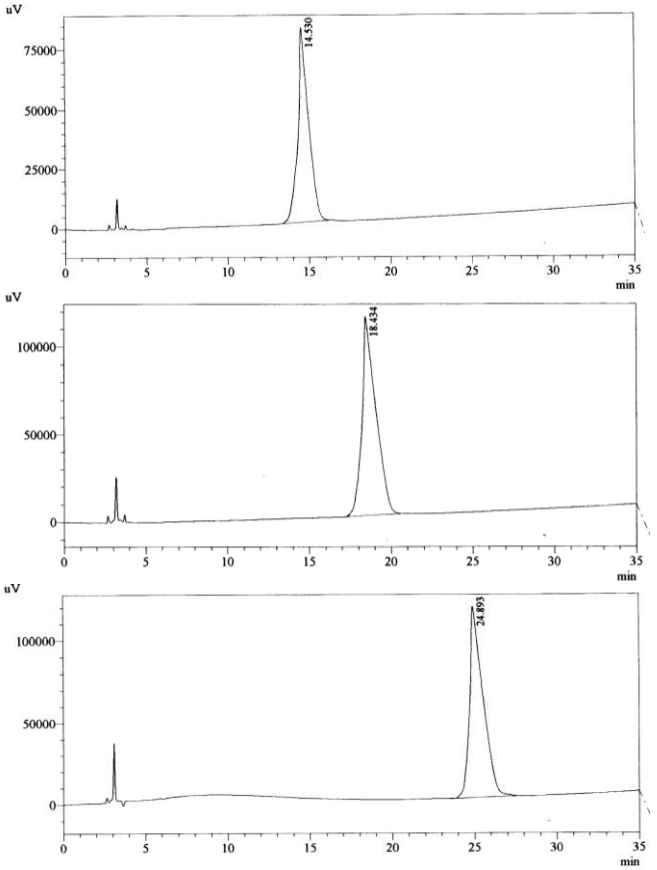
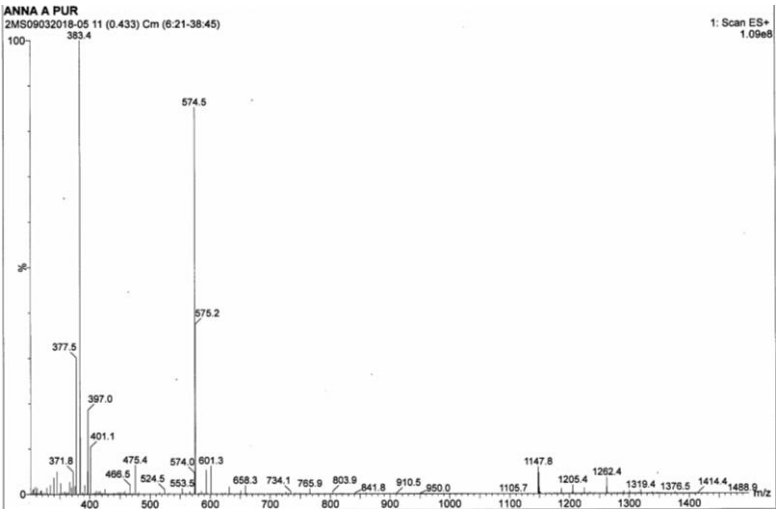
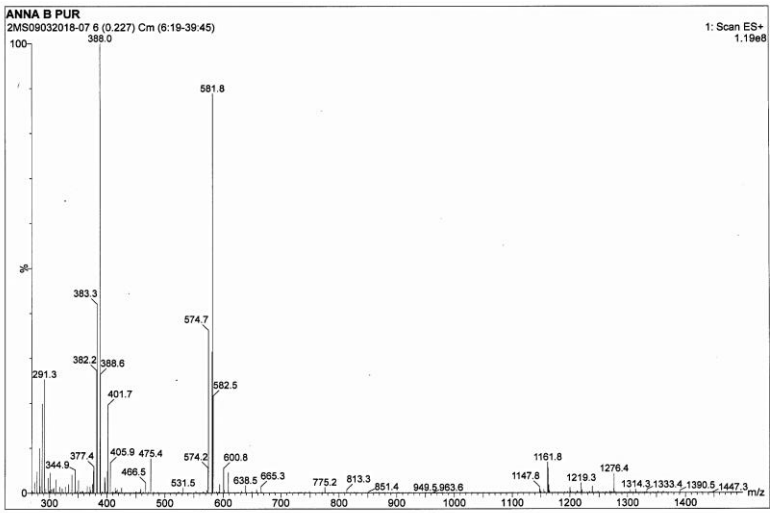


Figure 16. HPLC chromatograms of analogs A, B and C.

APPENDIX 4: ESI SPECTRA OF PURE PEPTIDES





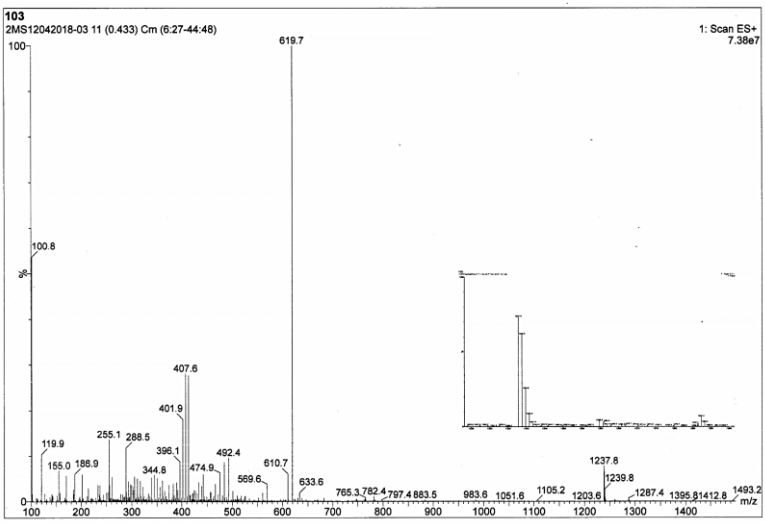


Figure 17. ESI spectra of analogs A, B and C.