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Synthesis of antibiotic cyclopeptides
Síntesi de ciclopèptids antibiòtics

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REPORT

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1. SUMMARY

For the time being bacterial resistance has become one of the most important problems for public health. A large number of microorganisms have been developed, by natural selection, random mutations that have caused many of the antibiotics used previously are no longer effective. Therefore there is an urgent need for new drugs which can control these resistant bacteria.

Some cyclopeptides as polymyxin B and E (colistin) show good antibacterial activity against gram-negative resistant microorganisms. Even so, its administration is limited due to they can cause neuro and nephrotoxicity.

In the present work the synthesis of three polymyxin analogs has been carried out, with the aim of improving the pharmacological activity of this cyclopeptide in gram-positive and negative bacteria, and additionally to reduce its toxicity when being administered.

The analogs synthesis has been carried out by the solid phase peptide synthesis and the Fmoc/^{t}Bu strategy as amino acids protecting groups, and its cyclization has made by oxidation between two cysteine residues, forming a disulfide bond. The peptides obtained with high purity have been characterized by HPLC and ESI mass spectrometry.

Moreover, the antibiotic activity of the synthesized analogs has been determined through its minimal inhibitory concentration (MIC). We obtained good results and good selectivity against gram-negative bacteria (0.25 – 2 μg/ml) and better activity than polymyxin against gram-positive bacteria.

Keywords: peptide antibiotics, chemical synthesis, solid phase, microbiological activity

2. RESUM

Actualment la resistència bacteriana s'ha convertit en un dels problemes més importants per a la salut pública. Un gran nombre de microorganismes ha desenvolupat, per selecció natural, mutacions a l'atzar que han provocat que molts dels antibiòtics utilitzats anteriorment ja no siguin efectius. Per aquest motiu hi ha una gran necessitat de desenvolupament de nous fàrmacs que puguin combatre aquests bacteris resistents.

Els ciclopèptids com la polimixina B i E (colistina) presenten una bona activitat antibacteriana contra microorganismes resistents gramnegatius. Tot i això, la seva administració es veu limitada per la neuro i nefrotoxicitat que provoquen.

En aquest treball s'ha dut a terme la síntesi de tres anàlegs de la polimixina amb l'objectiu de millorar l'efectivitat farmacològica d'aquest ciclopèptid, tant en bacteris grampositius com gramnegatius, i de reduir la toxicitat que presenta en ser administrat.

La síntesi dels anàlegs s'ha dut a terme mitjançant la síntesi de pèptids en fase sòlida i l'estratègia de Fmoc/^{t}Bu com a grups protectors dels aminoàcids, i la seva ciclació s'ha realitzat per oxidació entre dos residus de cisteïna, formant un pont disulfur. Els pèptids obtinguts amb alta puresa s'han caracteritzat per HPLC i espectrometria de masses (ESI).

A més, s'ha determinat l'activitat antibiòtica dels anàlegs sintetitzats mitjançant la seva concentració mínima inhibitòria (CMI). S'ha obtingut bons resultats, amb bones selectivitats enfront bacteris gramnegatius (0.25 – 2 μg/ml) i millor eficàcia que la polimixina contra bacteris grampositius.

Paraules clau: pèptids antibiòtics, síntesi química, fase sòlida, activitat microbiològica

3. INTRODUCTION

In recent years, the effectiveness of some antibiotics is gradually decreasing due to the emergence of multidrug resistance bacteria. Many of the antibiotic currently used act by mechanisms capable of developing resistance. This means that bacteria which were sensitive to the antibiotic, experience mostly irreversible changes and become resistant. Levels of resistance had been increasing at an alarming rate, so this has become a serious problem for public health, therefore there is an urgent need for new antibiotics to overcome this problem [1,2].

3.1. PEPTIDES AS ANTIBIOTICS

Peptides are usually considered poor therapeutic candidates due to many factors, including their low oral bioavailability, the fact that they are easily removed from the circulatory system and its low ability to cross the physiological membranes. However, in last years pharmaceutical companies have increased their interest in peptides as potential therapeutic candidates because of their high effectiveness, which compensates the mentioned drawbacks [3,4].

3.1.1. Laboratory synthesis (SPPS)

A peptide is a chain of amino acids linked together by amide bonds. Peptides are synthesized rapidly within living cells, but until recently could only be artificially synthesized in very long, slow processes that had poor yields and gave impure products [5,6]. In 1969 Bruce Merrifield described a new method for peptide synthesis, known as solid phase peptide synthesis (SPPS). This technique results in high yields of pure products and works more quickly than classical synthesis [7], and is based on attaching the first AA to a water-insoluble polymer and on protecting the reactive groups of the other AA following an orthogonal scheme, in order to ensure the only formation of the desired peptide.

The general process for synthesizing peptides on a resin starts by attaching the first amino acid, the C-terminal residue, to the 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. Then the resin is filtered and washed to remove byproducts and excess reagents. Next, the N-alpha protecting group is removed in a deprotection process and the resin is again washed to remove byproducts and excess reagents. Then the next amino acid is coupled to the attached amino acid. This is followed by another washing procedure, which leaves the resin-peptide ready for the next coupling cycle. The cycle is repeated until the peptide sequence is complete. Then typically, all the protecting groups are removed, the peptide resin is washed, and the peptide is cleaved from the resin [8].

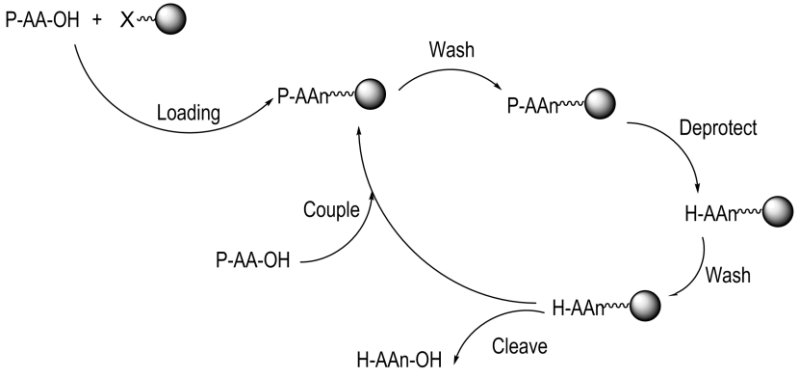


Figure 1. General SPPS cycle.

The side chains of many AA are reactive and may form side products if left unprotected. For successful peptide synthesis, these side chains must remain protected despite repeated exposure to N-alpha deprotection conditions. 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 scheme is called “orthogonal” protection [8].

3.1.2. Uses of synthetic peptides

Synthetic peptides have two main uses: as peptide drugs and as peptides for diagnostic purposes [5,9]:

- Peptide drugs are either naturally-occurring peptides or altered natural peptides. Many natural-occurring peptides are biologically active, such as polymyxins or some ADEPs. But in addition, the amino acids of an active peptide can be altered to make analogs of the original peptide. If the analog is more biochemically active

than the original peptide it is known as an *agonist*, and if it has the reverse effect is known as an *antagonist*.

- Peptides can be designed that change color under certain conditions, and these can be used for diagnostic purposes.

Antimicrobial peptides (AMPs) such as polymyxins provide a possible solution to the problem of bacterial resistance to conventional antibiotics. AMPs are a class of antibiotics that rarely spur the development of resistant microorganisms, because their main target is the lipid bilayer and their mechanism of action relies on ionic and hydrophobic interactions between this membrane (rich in anionic phospholipids) and the cationic character of most AMPs [10].

3.2. POLYMYXINS

As explained earlier, there is an urgent need for new antibiotics to overcome the problem of the resistance, particularly those active against gram-negative bacteria, such as *P. aeruginosa*. Therefore, polymyxins are increasingly being used as last-time therapy to treat infections caused by this kind of bacteria [11].

3.2.1. General aspects

Polymyxins are antimicrobial lipopeptides that were discovered more than 50 years ago. Polymyxin B (PxB) is a secondary metabolite produced by the bacterium *Bacillus polymyxa* (which is a gram-positive bacterium), with selectivity against gram-negative bacteria. These cationic peptides are mostly being used as last-resort antibiotics for otherwise untreatable serious infections, due to they can cause effects of neurotoxicity and nephrotoxicity. But even so, as explained above, PxB has an amphipathic nature and a cationic character (with five positive charges due to the amino acid Dab) that rarely cause the development of resistant bacteria [11,12].

So in view of the generally good antimicrobial activity of PxB and the low prevalence of resistance that exists, it is not surprising that there has been interest to design new polymyxin analogs which improve the microbiological, pharmacological and toxicological profiles of PxB [11].

3.2.2. Structure

The general structure of polymyxin B comprises a cyclic heptapeptide (with three positive charges due to the amino acid Dab) bound to a linear tripeptide, with two positive charges due to the same AA. As shows **figure 2**, the γ-amino group of 4-Dab is linked by an amide bond to the C-terminus of 10-Thr, while its α-amino group is connected to 3-Dab of the linar tripeptide. The α-amino group of 1-Dab is acylated with a hydrophobic chain (different for PxB1 and PxB2) [13].

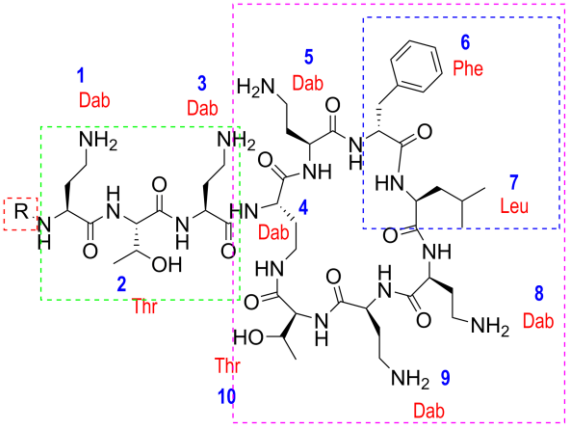


Figure 2. Chemical structure of PxB, where the functional segments are colored as follows: fatty acid chain (red) which is formed by (S)-6-methyloctanoic acid in polymyxin B1 and by 6-methylheptanoic acid in polymyxin B2; linear tripeptide segment (green); heptapeptide (pink); hydrophobic motif in the ring (blue).

The presence of positively charged residues, as well as the amphiphilic nature of PxB are crucial for its activity against gram-negative bacteria. These properties make polymyxins good candidates as antibiotics for therapeutic purposes. However, the severe toxicity to humans has limited their clinical use. This hurdle might be cleared by the development of non-toxic analogues [13].

4. OBJECTIVES

The main objectives of this present work are:

- Protection of the amino acid H-(D)-Cys-OH with the groups *p*-Npys and Boc in order to find a good synthesis strategy for obtaining the compound Boc-(D)-Cys(*p*-Npys)-OH.
- Synthesis of three polymyxin analogs to obtain new antibacterial agents. These bioactive peptides have to improve the activity of polymyxin. The Fmoc/tBu solid phase peptide synthesis (SPPS) will be used for this purpose.
- Purification and characterization of the peptides obtained by HPLC and ESI mass spectrometry.
- Evaluation of the antimicrobial activity of the synthesized analogs with MIC test, in gram-positive and gram-negative bacteria.

5. EXPERIMENTAL SECTION

5.1. MATERIALS AND METHODS

5.1.1. Solvents, reagents and products

5.1.1.1. Solvents

Acetic acid	Pure quality, 99%	Sigma-Aldrich
AcOEt	Synthesis quality	Scharlau
Acetone	Synthesis quality	Scharlau
ACN	HPLC quality	Fischer
$CHCl_3$	Synthesis quality	Scharlau
DCM	Synthesis quality	Scharlau
DMF*	Synthesis quality	SDS
EtOH	99.5% quality	Panreac
Et_2O*	Synthesis quality	Scharlau
H_2O*	Milli-Q	
MeOH	HPLC quality	VWR

* The anhydrous DMF is prepared over molecular sieve and passed nitrogen stream before its use.

* The dried Et_2O is kept over Na.

* The deionised water is filtered with a Milli-Q Plus (Millipore) system.

5.1.1.2. Reagents

Resin, RL and amino acids		Bachem Iris Biotech Novabiochem PolyPeptide Fischer Scientific
DIC	Pure quality, 99%	Sigma-Aldrich
DIEA	Quality Reagent Plus ®, 99%	Sigma-Aldrich
DMSO	Synthesis quality	Acros Organics
DTNP	Synthesis quality	Merck
HCl	Aqueous 37%	Scharlau
Hexanoic acid	Quality 99.0%	Aldrich
HOBt	Pure quality, 99.0%	Fluka
$MgSO_4$ anhydrous		Jescuder
Na_2CO_3		Jescuder
Ninhydrin	Pure quality	Koch-Light
Octanoic acid	Quality ≥ 98.0%	Aldrich
Piperidine	Quality Reagent Plus ®, 99%	Sigma-Aldrich
TFA	Quality Reagent Plus ®, 99%	Sigma-Aldrich
TIS		Sigma-Aldrich

5.1.1.3. Products for biological assays

Mueller Hinton-Broth (MHB)	Oxoid
PxB	Sigma-Aldrich

5.1.2. General instrumentation

Autoclave	AUTESTER-E
Centrifuge	Hettich ROTOFIX 32 A
Liquid chromatography	Specified in **section 5.4.1.**
Lyophiliser	Virtis sentry 2.0. Freezemobile 25EL
Mass spectrometry	Specified in **section 5.4.2.**
pH measurement	Strips with colour scale, Panreac
Polyethylene filter	Applied Separations
Quartz cuvette	Helma Analytics
Rotary evaporator	Heidoph Laborota 4000
Sonicator	Selecta MEDI-II
Spectrophotometer	Shimadzu UV-2401 PC
Syringes	Terumo
Thin layer chromatography	Merck

5.1.3. Analytic methods

5.1.3.1. Ninhydrin test (Kaiser test)

It is a very sensitive test for primary amines, usually used in solid phase peptide synthesis to determinate if coupling reactions are complete. Ninhydrin reacts with free or deprotected amines producing an intense blue color (positive essay).

As shows **figure 3**, ninhydrin (1, which is a yellow compound) reacts with a primary amine of an amino acid (2) forming an imine (3), in a nucleophilic addition-elimination and reversible reaction. This is followed by a descarboxylation 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 blue compound [7].

To perform the test two solutions are required:

- Reagent A: a solution with phenol (40 g) in absolute EtOH (10 ml) is prepared in hot. Following, 100 ml of destilled pyridine are added to 2 ml of a solution with KCN (65 mg) in H2O (100 ml). These two solutions are stirred separately with 4 g of Amberlite MB-3 resin for 45 minutes. Then, are filtered and the filtrates are mixed.
- Reagent B: a solution of ninhydrin (2.5 g) in absolute EtOH (50 ml) is prepared. This solution must be protected from light.

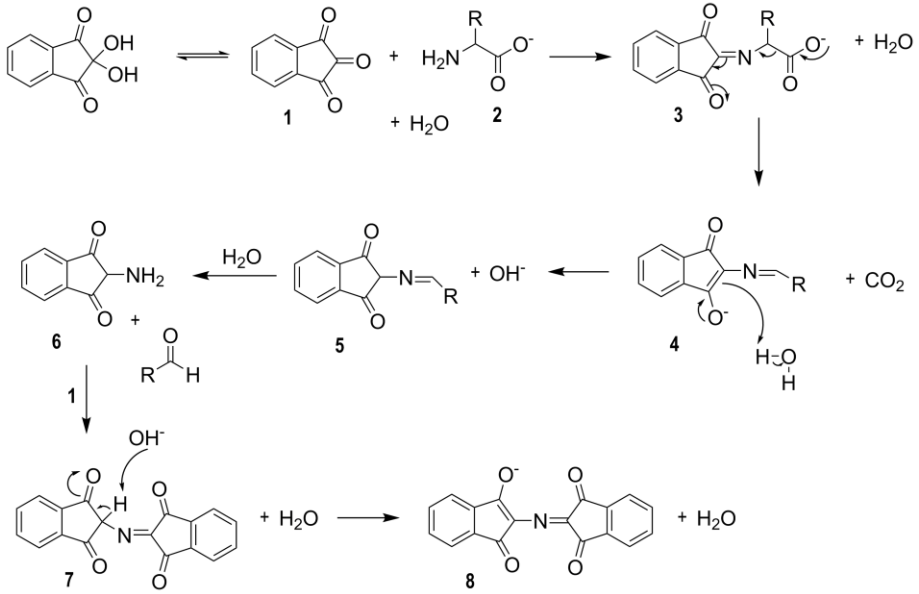


Figure 3. Reaction mechanism of ninhydrin test.

To carry out the essay, a little sample of peptide-resin (washed with DCM and dried with a vacuum pump) is introduced in a small test tube. Six drops of reagent A and two drops of reagent B are added. The tube is heated at 110ºC for 3 minutes.

After that, the solution remains yellow (negative assay) if no free primary amines are present. This means the coupling reaction is completed at least 99.5%. Contrary, the solution turns dark blue when primary amine is present (positive assay), so a recoupling step will be necessary.

5.1.3.2. Thin layer chromatography

Thin layer chromatography is performed on aluminium sheets which are coated with a thin layer of silica gel. Two mixtures are used as eluents: $CHCl_3$/MeOH/AcOH in proportion 89:10:1 (non-polar solvent) and the same components in proportion 59:40:1 (polar solvent). To visualize the TLC results, UV light at 254 nm and a ninhydrin solution are used.

- Ninhydrin stain solution: a solution of ninhydrin (3.33 g) in absolute EtOH (100 ml). The mixture should be kept protected from light. To visualize the TLC, this solution is spraying and after heat up a colour change is viewed.

The separation of amino acids by TLC is based on their polarity. When the solvent ascends the stationary phase by capillary action, it drags amino acids. Depending on their polarity, they have different affinities for the stationary and mobile phases, ascending the silica gel at different rates. This is measured by the R_f value. More polar AA are adsorbed more strongly to the sheet (which is relatively polar), and less polar AA ascend the sheet faster because they have more affinity for the mobile phase. Therefore, when the sheet is revealed with ninhydrin, the stain which is closer to the origin corresponds to the most polar AA [7].

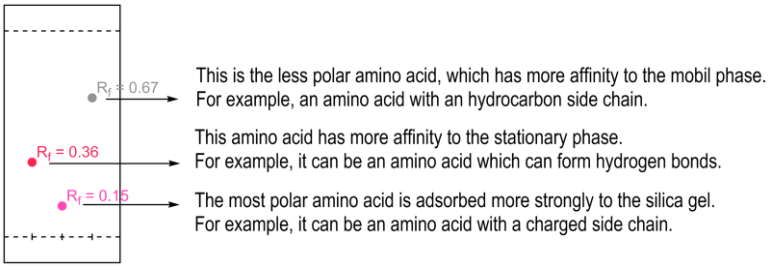
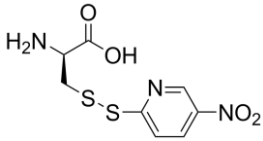


Figure 4. TLC separation of amino acids.

5.2. (H)-(D)-CYS-OH PROTECTION WITH *P*-NPYS AND BOC

1.058 g of DTNP (3.4 mmol, 3 eq.) are dissolved in 14 ml of AcOH/DMF (5:95, v/v) at 45 ºC for 4 h. When it is completely dissolved, 200 mg (1.1 mmol, 1 eq.) of H-(D)-Cys-OH·HCl·H_2O are added very slowly (for 6 h) at RT, with stirring and in an inert atmosphere. The mixture is stirred overnight at the same conditions, and the end of the reaction is checked by TLC. The solution is concentrated *in vacuo* and to the residue anhydrous Et_2O is added, in order to precipitate the product and to remove the excess of DTNP and other reaction byproducts. The

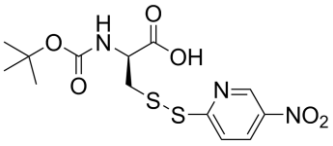
residue is washed, sonicated and centrifuged with Et_2O. Finally Et_2O is decanted to obtain the product H-(D)-Cys(*p*-Npys)-OH.



Brown solid. Yield: 406 mg, 129% (as evidence by TLC, it has not been possible to eliminate all DTNP and the byproduct 2-mercapto-5-nitropyridine). R_F = 0.46 ($CHCl_3$/MeOH/AcOH, 59:40:1, v/v), R_F = 0.08 ($CHCl_3$/MeOH/AcOH, 89:10:1, v/v).

The crude product obtained in the above reaction is dissolved in 5 ml of ACN, and 392 µl of DIEA are added to ensure the pH is 8. The mixture is stirred under N_2 atmosphere in order to avoid the oxidation of cysteine into cystine. Then a solution of 248 mg of Boc_2O in 2.2 ml of ACN is added. The mixture is stirred for 4 h at 45 ºC, and overnight at RT. After removal of most of the ACN *in vacuo*, the mixture is poured into 10 ml of H_2O. Na_2CO_3 is added until pH is 8. Then the mixture is washed with Et_2O and acidified to pH 2 with 37% HCl. After that it is extracted with AcOEt, and the extracts are washed with water, dried with $MgSO_4$, filtered and evaporated again to give the desired crude product, Boc-(D)-Cys(*p*-Npys)-OH.

The obtained product was not characterized correctly since, as explained in **section 7.2.3**., it was not pure (as evidence by TLC and HPLC). For that reason it was only characterized by ESI mass spectrometry and HPLC. The product was not purified because the final yield would be really low.



Yellow oil. Yield: 187 mg, 44%. TLC: R_F = 0.92 ($CHCl_3$/MeOH/AcOH, 59:40:1, v/v), R_F = 0.65 ($CHCl_3$/MeOH/AcOH, 89:10:1, v/v). HPLC: t_R = 28.03 min (lineal gradient from 5% B to 95% B for 30 min, at a flow of 1 ml/min; UV detection at 220 nm and 330 nm). Purity: 69%. HRMS (ESI^+): *m/z* calc. for $C_{13}H_{17}N_3O_6S_2^+$; 375.7 (41% $[M+H]^+$), 772.9 (8% $[2M+Na]^+$). HRMS (ESI^-): *m/z* calc. for $C_{13}H_{17}N_3O_6S_2^-$; 373.6 (58% $[M-H]^-$), 748.8 (99% $[2M-H]^-$).

5.3. Solid Phase Peptide Synthesis

5.3.1. General aspects

The solid phase peptide synthesis is carried out manually, using polypropylene syringes from Terumo (variable volume depending on the quantity of resin used in each case) with a porous polyethylene filter from Applied Separations. The reagent mixture is manually stirred

using a Teflon wand. After each coupling, the excess of reagents, solvents and other byproducts are eliminated by vacuum filtration.

5.3.2. Loading the resin

Peptide synthesis is performed using the resin BHA (f = 0.69 mmol/g). The analogs are prepared starting from 153.9 mg of resin (0.106 mmol, 1 eq.). Initially is necessary to do a pre-treatment, because it is stored in a compact form. The objective of this treatment is solvating the resin and washing the impurities. The protocol used for the resin BHA is described in **table 1**:

Step	Reagent	Operation	Time [min]
1	DCM*	Wash	5 x 0.5
2	40% TFA/DCM	Wash and solvate	1 x 1.0
3	40% TFA/DCM	Wash and solvate	2 x 10
4	DCM	Wash	5 x 0.5
5	5% DIEA/DCM	Neutralization	3 x 2.0
6	DCM	Wash	5 x 0.5
7	DMF	Wash	5 x 0.5

* DCM used is filtered through a column of silica.

Table 1. BHA resin loading protocol.

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

5.3.3.1. Reference amino acid incorporation

105 mg of Fmoc-Ala-OH as a reference amino acid (0.318 mmol, 3 eq.), 43 mg of HOBt (0.318 mmol, 3 eq.) and 49 µl of DIC (0.318 mmol, 3 eq.) are added to the BHA resin for 60 min, using anhydrous DMF as solvent. The reference amino acid is useful to do the amino acid analysis and to facilitate the solid phase peptide synthesis. The coupling is confirmed by the ninhydrin test. If it is incomplete (positive assay, blue beads) a recoupling step is necessary (1.5 eq. of reagents for 30 min).

5.3.3.2. Rink linker incorporation

To incorporate the Rink linker is necessary to deprotect the reference amino acid removing Fmoc, as shows **table 2**:

Step	Reagent	Operation	Time [min]
1	DMF	Wash	5 x 0.5
2	20% piperidine/DMF	Deprotection	1 x 1.0
3	20% piperidine/DMF	Deprotection	2 x 10
4	DMF	Wash	5 x 0.5

Table 2. Fmoc deprotection protocol.

To the BHA resin with reference amino acid, 172 mg of Fmoc-Rink linker (0.318 mmol, 3 eq.), 43 mg of HOBt (0.318 mmol, 3 eq.) and 49 µl of DIC (0.318 mmol, 3 eq.) are added, using the minimum quantity of anhydrous DMF as solvent. After 60 min, the coupling is tested by the ninhydrin assay. If it is positive, a recoupling is necessary with 1.5 eq. of each reagent during 30 min.

5.3.3.3. First amino acid incorporation

First the Rink linker must be treated with a solution of 20% piperidine/DMF (1 x 1 min, 2 x 10 min), washing the resin with DFM before and after the reaction. Removing of the Fmoc is tested by positive result in ninhydrin assay. Then the first amino acid is incorporated, using an excess of amino acid: 187 mg (0.318 mmol, 3 eq.) of Fmoc-(D)-Cys(Trt)-OH, 43 mg (0.318 mmol, 3 eq.) of HOBt and 49 µl (0.318 mmol, 3 eq.) of DIC in the minimum amount of anhydrous DMF during 60 min.

5.3.3.4. Elongation of the peptide chain

Peptide synthesis is performed manually following standard Fmoc procedures in the minimum amount of anhydrous DMF, with DIC as activate agent of the carboxylic acid and HOBt as an additive to minimize the racemization of amino acids and to improve the efficiency of peptide synthesis. As shown in **table 3**, every coupling is verified by ninhydrin assay. If the test is positive (blue colour), the coupling is repeated from the stage 4, adding 1.5 eq. of the reagents for 30 min. When the synthesis is finished, the peptide-resin is washed with DMF and DCM and dried in vacuum.

Step	Reagent	Operation	Time [min]
1	DMF	Wash	5 x 0.5
2	20% piperidine/DMF	Deprotection	1 x 1.0
3	20% piperidine/DMF	Deprotection	2 x 10
4	DMF	Wash	5 x 0.5
5	3 eq. Fmoc-AA-OH* 3 eq. DIC 3 eq. HOBt	Coupling	60
6	DMF	Wash	5 x 0.5
7	DCM	Wash	5 x 0.5
8	Ninhydrin assay	Test coupling	3

* It refers to: Fmoc-Dab(Boc)-OH, Fmoc-Xxx-OH, Fmoc-(D)-Yyy-OH, Fmoc-Thr(^{t}Bu)-OH.

Table 3. Elongation protocol of peptide chain in Fmoc/^{t}Bu synthesis strategy.

Once the last amino acid is incorporated in the sequence, the Fmoc protecting group is removed by treatment with a solution of 20% piperidine/DMF (1 x 1 min, 2 x 10 min). The resin is washed with DMF and DCM and divided into three parts for coupling the last amino acid (only in analogs 1A and 1B) and the corresponding fatty acid, as shows **table 4**.

Analog	Wet peptidyl-resin [mg]	Equivalent of dry resin [mg]	Last amino acid	Fatty acid
1A	134.5	45.55	Fmoc-Dab(Boc)-OH	C_6
1B	156.0	52.83	Fmoc-Dab(Boc)-OH	C_8
1C	163.9	55.51	Fmoc-Thr(^{t}Bu)—OH	C_x

Table 4. Last couplings of the three synthesized peptides.

5.3.3.5. Last amino acid and fatty acid coupling

In analogs 1A and 1B the last amino acid coupling is carried out following the same procedure of the peptide chain elongation (**table 3**, step 4 – 9).

When the peptide sequence is finished, the corresponding fatty acid is coupled in the three analogs following the protocol described in **table 5**.

Step	Reagent	Operation	Time [min]
1	DMF	Wash	5 x 0.5
2	20% piperidine/DMF	Deprotection	1 x 1.0
3	20% piperidine/DMF	Deprotection	2 x 10
4	DMF	Wash	5 x 0.5
5	5 eq. fatty acid 5 eq. DIC 5 eq. HOBt	Coupling	60
6	2.5 eq. fatty acid 2.5 eq. DIC 2.5 eq. HOBt	Coupling	30
7	DMF	Wash	5 x 0.5
8	DCM	Wash	5 x 0.5
9	Ninhydrin assay	Test coupling	3

Table 5. Fatty acid coupling protocol.

5.3.4. Cleavage from the resin

When the synthesis is finished, lateral chains of amino acids are deprotected and the peptide is cleaved from the resin. This procedure is carried out by acidolysis with 5 ml TFA/TIS/H_2O (95:3:2, v/v) for 90 min. Then the peptidyl-resin is washed with 95% TFA/H_2O. TFA is removed by evaporation with N_2 stream, and the oily residue obtained is treated with 25 ml of dry Et_2O to obtain the peptide precipitate. The solid peptide is isolated by centrifugation at 60 r.p.m. for 10 min and the supernatant is poured off. The peptide crude is dissolved in H_2O/ACN (1:1, v/v), lyophilized and analyzed by analytical HPLC and by ESI mass spectrometry.

- Cleavage yield: analog 1A: 49% (18.07 mg of crude peptide), analog 1B: 62% (27.30 mg of crude peptide), analog 1C: 43% (18.51 mg of crude peptide).

5.3.5. Cyclization

The linear peptide is added to a solution of 40 ml of 5% DMSO/H_2O (the concentration is 0.5 mg/ml). The peptide cyclization is carried out in solution to form a disulfide bond between the two Cys residues, by an oxidation reaction. It is left to react for 8 h, and controlled by HPLC. When the oxidation reaction is finished, the solution is lyophilized.

- Cyclization yield: analog 1A: 94% (17.00 mg of crude oxidized peptide), analog 1B: 100% (27.30 mg of crude oxidized peptide), analog 1C: 95% (17.60 mg of crude oxidized peptide).

5.3.6. Purification and characterization

The crude oxidized peptides are purified by Semipreparative High Performance Liquid Chromatography using the following conditions of purification: lineal gradient from 20% of B to 50% of B for 30 min, at flow of 2 ml/min; UV detection at 220 nm.

- Purification yield: analog 1A: 38% (6.40 mg of purified peptide), analog 1B: 25% (6.80 mg of purified peptide), analog 1C: 19% (3.40 mg of purified peptide).

The pure fractions are put together and lyophilized, and then the characterization of the products is carried out by RP-HPLC and HRMS ESI^+.

Analog	Synthesis yield	Purity	RP-HPLC* (t_R)	HRMS ESI^{+*}
1A	18%	>99%	27.8 min	*m/z* 1176.6 (4% $[M+H]^+$), 588.7 (46% $[(M+2H)/2]^{2+}$), 392.7 (100% $[(M+3H)/3]^{3+}$), 294.6 (100% $[(M+4H)/4]^{4+}$)
1B	16%	>99%	33.6 min	*m/z* 1204.6 (10% $[M+H]^+$), 602.8 (98% $[(M+2H)/2]^{2+}$), 402.1 (100% $[(M+3H)/3]^{3+}$)
1C	8%	>99%	33.5 min	*m/z* 1119.5 (12% $[M+H]^+$), 560.3 (100% $[(M+2H)/2]^{2+}$), 373.7 (78% $[(M+3H)/3]^{3+}$)

* RP-HPLC: lineal gradient from 20% of B to 50% of B for 30 min, at flow 1 ml/min; UV detection at 220 nm. The chromatograms are shown in **appendix 5**. HRMS ESI+: spectra are shown in **appendix 6**.

Table 6. Final yield and characterization of the synthesized products.

5.4. CHARACTERIZATION AND PURIFICATION METHODS

5.4.1. High Performance Liquid Chromatography

5.4.1.1. Analytical scale

This technique is used to separate, identify and quantify the components in a mixture. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out the column.

The purity of peptides is assessed using a Nucleosil C_{18} reverse-phase column (0.4 x 25 cm) packed with octadecylsiloxane of 5 μm particle diameter and 120 Å pore size, and the elution is carried out at 1 ml/min flow with mixtures of 0.045% TFA/H_2O (A) and 0.036% TFA/ACN (B). Due to that reverse phase has a non-polar stationary phase and an aqueous (polar) mobile phase, retention time is longer for molecules which are less polar, while polar molecules elute more readily. The UV detection is carried out at 220 nm, because the wavelength of absorbance for a peptide bond is 190-230 nm.

The apparatus used is *Shimadzu Serie 20 Prominence* which consists of two pumps LC-20AD model, autosampler SIL-20A, photo-diode Array detector SPD-M20A and System Controller CBM-20A.

5.4.1.2. Semipreparative scale

This is a technique to isolate and purify compounds from a mixture. The goal is to get the single compounds at a certain purity level. It is different from analytical HPLC because of the amount of sample applied to the column. In analytical HPLC the applied sample amount is very small compared to the amount of stationary phase in the column. Therefore very good separations can be achieved. To purify higher amounts of sample in a single run the loadability of a column has to be increased [14].

Peptides are purified by preparative HPLC using a Phenomenex® C_{18} column (25 x 1 cm, 5 μm diameter). Elution is carried out at 2 ml/min flow with 0.1% TFA/H_2O (A) and 0.1% TFA/ACN (B), and UV detection at 220 nm. The apparatus used is a Waters Delta Prep 3000 (Preparative Chromatography System) which consists of a controller and a pump *Waters 600 E* model, a manual sample injector *Waters 712*, a detector of variable wavelength *Waters 484* and a chart recorder *Pharmacia Biotech REC 101*.

5.4.2. Electrospray Ionization Mass Spectrometry

ESI is a technique used in mass spectrometry to produce ions using an electrospray in which a high voltage is applied to a liquid to create an aerosol. It is a soft ionization technique typically used to determine the molecular weights of proteins, peptides and other biological macromolecules. It is useful because its process does not fragment the macromolecules into smaller charged particles; rather it turns the macromolecule being ionized into small droplets. These droplets will then be further desolvated into even smaller droplets, which creates molecules with attached protons. These protonated and desolvated molecular ions will then be passed through the mass analyzer to the detector, and the mass of the sample can be determined.

This quantitative analysis is done by considering the mass to charge ratios of the various peaks in the spectrum. This spectrum shows the *m/z* ratio on the x-axis and the relative intensity (%) on the y-axis. In positive mode, the ions observed are created by the addition of a hydrogen cation (denoted $[M+H]^+$), or of another cation such as sodium ($[M+Na]^+$) or potassium ($[M+K]^+$). In addition, multiply charged ions such as $[M+nH]^{n+}$ are often observed [15,16].

The samples analyzed are solutions of peptides in H_2O/ACN (1:1 v/v), and mass spectra are obtained in positive mode using the spectrometer *ZQ-Micromass (Waters)*.

5.5. EVALUATION OF ANTIMICROBIAL ACTIVITY

5.5.1. Preparation of material and medium

Material and growth medium of bacteria must be sterilized to ensure the elimination of any form of life. Pipette tips, glass bottles, flasks and MilliQ-water are autoclaved (subjecting them to high pressure saturated steam at 121ºC for 15-20 min).

Different culture mediums are prepared and also autoclaved: 1 x concentrated MHB (9.6 g in 400 ml MilliQ-water), 2 x concentrated MHB (19.4 g in 400 ml MilliQ-water) and 1 x concentrated MHB with Agar (6g) for plates.

5.5.2. Growth and inoculation of bacteria

The microorganisms used are:

- Gram-negative bacteria: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027.
- Gram-positive bacteria: *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213.

Four bacteria suspensions are prepared following the protocol of Amsterdam, D. [17,18]. 50 µL of each suspension in MHB adjusted according to McFarland standard is added to the microtiter wells, so the final concentration of each bacterium in every well is 10^6 UFC/ml.

5.5.3. Preparation of peptides solutions

About 1 mg of each peptide is needed to prepare the solutions in MilliQ-water. The starting concentration of microplates for MIC testing is 128 µg/ml, so the wished concentration of peptide solutions is 512 µg/ml (128x4).

Peptide	**Weight** [mg]	**Real weight*** [mg]	**Volume of MilliQ-water** [µl]
1A	1.1	0.77	1504
1B	0.9	0.63	1230
1C	1.0	0.70	1367
PxB	0.9	0.76	1494

*Real weight of the synthesized peptides is 70% of their total weight, because they contain the counterion CF_3COO^- from TFA. Real weight of PxB is 86% of its total weight, because commercial product contains $2 \cdot H_2SO_4$.

Table 7. Peptides solutions for the MIC test.

5.5.4. Determination of MIC

To carry on MIC test four microtitres of 96 wells are needed (8 rows x 12 columns, **figure 5**). In each microplate one peptide will be analyzed (PxB and the three synthesized analogs).

- 50 µl of 2xMHB are added to column 1.
- 50 µl of 1xMHB are added to columns 2-11.
- 100 µl of 1xMHB are added to column 12.
- 50 µl of peptide solution are added to column 1. With a multichannel pipette, the mixture in column 1 is stirred and 50 µl are added to column 2. The mixture in 2 is also stirred and 50 µl are added to column 3. Thus successively through column 10, so we get peptide concentration diluted by half. So that the final concentrations of the peptides ranging from 28 µg/ml (column 1) to 0.25 µg/ml (column 10).
- Finally, the four bacterial suspensions are added to microtitres. Each bacterium only takes two rows. Microplates are incubated at 37 ºC for 20 – 22 h.
- Column 11 is the positive control. It only contains 50 µl of bacteria and 50 µl of medium, so we can ensure the growth of bacteria without peptide solution.
- Column 12 is the negative control. It only contains MHB growth medium, so we can ensure there is no bacteria growth because the medium is sterilized.

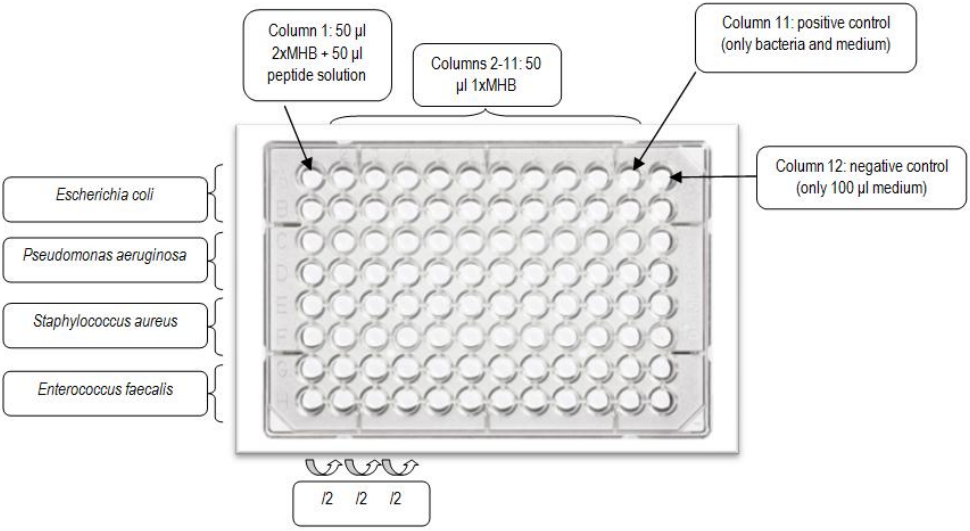


Figure 5. Preparation of the microplates for the MIC test.

6. (D)-CYS PROTECTION

One of the objectives of this present work was to find an effective method to synthesize the compound Boc-(D)-Cys(*p*-Npys)-OH, since, as explained later, the compound *para*-nitro-2-pyridinesulfenyl (*p*-Npys) has certain advantages over other protecting groups.

6.1. *P*-NPYS AS AN ACTIVATING AND PROTECTING GROUP

To form an asymmetric disulfide bond between two lateral chains of cysteine, the activation of the thiol function of one of the AA is required, followed by the addition of the second AA in the free thiol form. One of the most successful and widely used method employs aromatic sulfenyl protecting/activating groups, such as Nps, PyrS and Npys. The formation of the disulfide bond is driven by the low pKa of the aromatic thiol, and hence the reaction can be performed under acidic conditions. However, the Npys and other activated Cys derivatives are not compatible with the Fmoc/^{t}Bu strategy, as they are not stable to the strongly basic conditions required for the removal of the Fmoc group. Consequently, the activation of Cys in peptides assembled by Fmoc/^{t}Bu strategy is often performed with Npys-Cl, but sulfenyl chlorides have limited stability and can react with other groups.

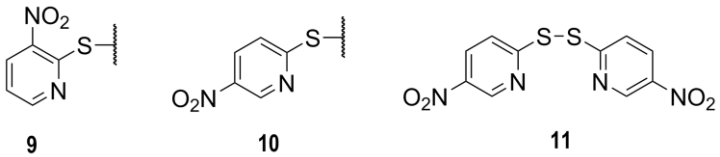


Figure 6. Protecting groups Npys and *p*-Npys, and reagent DTNP.

The Npys group (9) not only acts protecting the thiol function of Cys, but also the Npys-modified cysteine can react selectively with the free thiol group of another cysteine molecule to afford a new disulfide bond. This group is easily removed under neutral conditions using tertiary phosphine and water, by a reduction reaction, but it is sufficiently resistant to acids such as TFA, HCl and HF.

The use of 2,2'-dithiobis(5-nitropyridine) or DTNP (11) as an activating reagent for the thiol function of cysteine generates the protecting/activating group *p*-Npys (10), which is an isomer of Npys. This compound facilitates the synthesis and increases the acidity of the thiol because of the *para* nitro group, making it a better leaving group than Npys [19,20].

6.2. BOC PROTECTING GROUP

Boc protecting groups are used to temporarily protect the N-alpha nitrogen groups of the amino acids. This group is usually used in a Boc/Bzl synthesis strategy, which is not a true orthogonal protection scheme, because both groups are acid labile [8].

Boc group is orthogonal to *p*-Npys group, because they are removable under completely different conditions. While Boc is removed under moderate acid conditions (50% TFA/DCM), *p*-Npys requires reduction conditions (DTT, tertiary phosphine). In addition, Boc group can be introduced with the reagent Boc_2O to a *p*-Npys-protected AA, because this reaction requires medium basic conditions (pH 8) as shows **figure 7**.

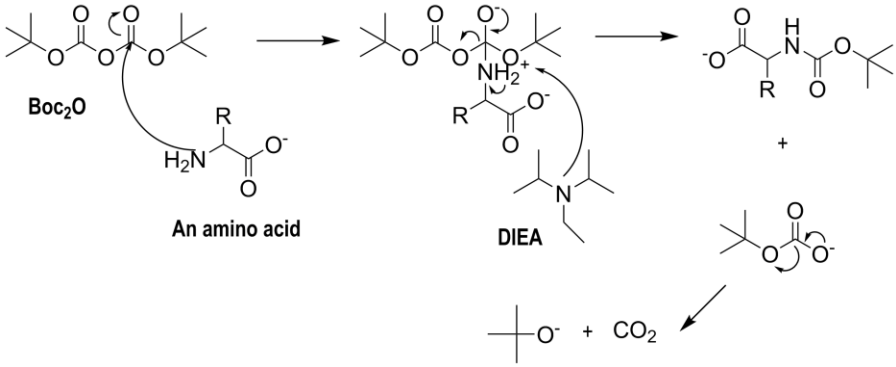


Figure 7. Reaction mechanism of Boc protection, in a nucleophilic substitution of an acyl group where a carbamate (Boc protected amino acid) is formed.

6.3. SYNTHESIS AND CHARACTERIZATION OF BOC-(D)-CYS(*P*-NPYS)-OH

As explained in **section 5.2.2.**, the lateral chain of H-(D)-Cys-OH (12) was initially protected with the group *p*-Npys. The amino acid was added very slowly to a solution of DTNP to ensure the no-formation of a dimer of cysteine (cystine) in an oxidation reaction. For the same reason the reaction took place in an inert atmosphere, without oxygen. The reaction was followed by TLC, using a relatively polar mobile phase formed by $CHCl_3$/MeOH/AcOH in proportion 59:40:1, v/v. The final product obtained was a brown solid formed by the synthesized *p*-Npys-AA (13) and for a small part of the non-reacted AA, the excess of DTNP and the byproduct 2-mercapto-5-nitropyridine (14), which could not be removed, as evidence by TLC (**figure 8, 9**).

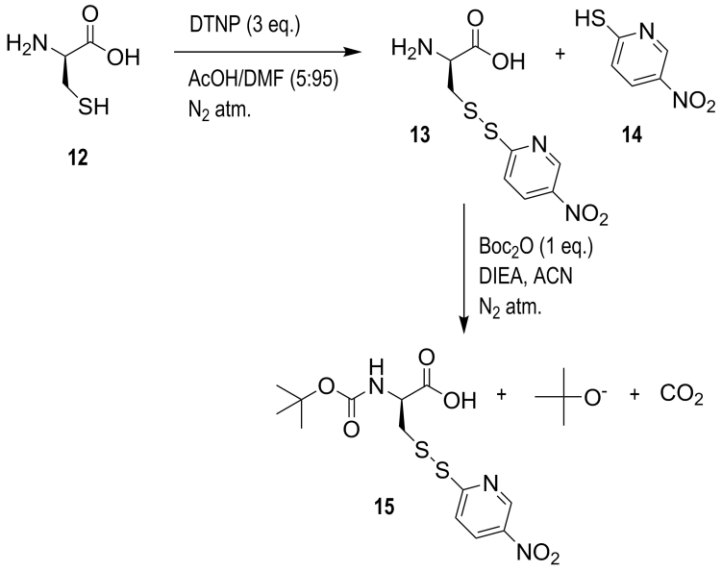


Figure 8. Synthesis of Boc-(D)-Cys(*p*-Npys)-OH.

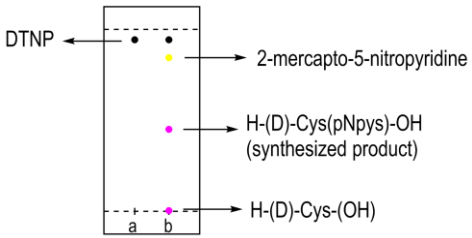


Figure 9. TLC sheet where (a) corresponds to DTNP and (b) to the synthesized product H-(D)-Cys(pNpys)-OH. All stains were revealed in UV light at 254 nm. The AA and the synthesized product were revealed with ninhydrin and without heating, since they contain a primary amine. The mercaptonitropyridine was recognized by its yellow color, and commercial DTNP was used as a standard.

In a second part of the synthesis, the N alpha group of H-(D)-Cys(*p*-Npys)-OH (13, **figure 8**) was protected with the Boc group, with the reagent Boc_2O in a nucleophilic substitution reaction, using DIEA as a base. The reaction took place in an inert atmosphere and was followed by TLC, using a relatively non-polar mobile phase formed by $CHCl_3$/MeOH/AcOH in proportion 89:10:1, v/v**. Figure 10** shows the TLC sheet when the reaction was completed and the product Boc-(D)-Cys(*p*-Npys)-OH (15, **figure 8**) was obtained.

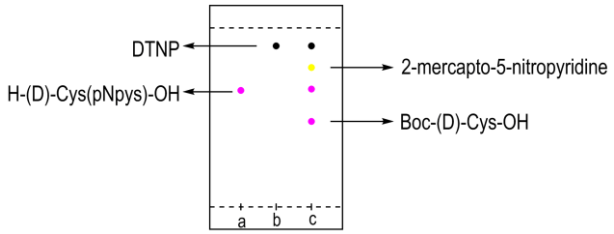


Figure 10. TLC sheet where (a) corresponds to the commercial product Boc-(D)-Cys(*p*Npys)-OH, (b) to DTNP and (c) to the synthesized product Boc-(D)-Cys(*p*Npys)-OH. All stains were revealed in UV light at 254 nm, excepting the Boc-(D)-Cys-OH stain. The synthesized product was revealed with ninhydrin, HCl vapors and heating. Nitropyridine was recognized by its yellow color, and commercial DTNP was used as a standard.

Boc group is not visible with UV light at 254 nm, because it does not absorb at this λ. Moreover, N-alpha protected amino acids cannot be revealed with ninhydrin because their amine groups are not free. Therefore, to reveal the stains of compounds which have Boc group it's necessary to apply HCl vapors and to heat, in order to deprotect them. The mechanism of this reaction is showed in **figure 11**.

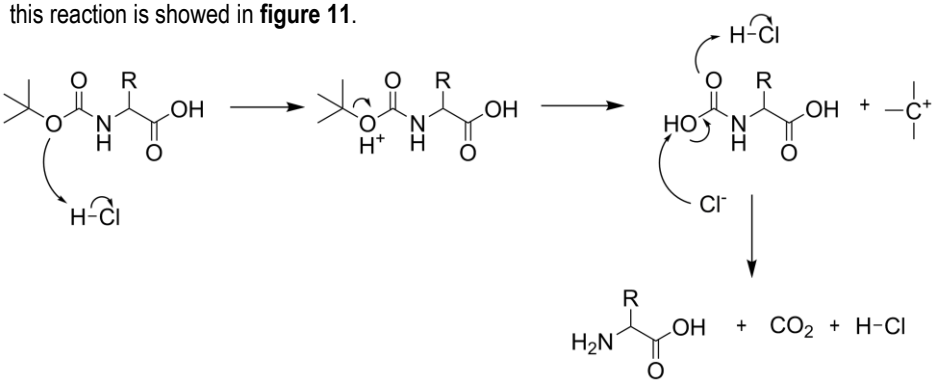


Figure 11. Boc deprotection mechanism in an acidic hydrolysis with HCl.

Finally, the work up of the product was made by extractions. Initially water was added and the product was washed with diethyl ether. Thus the initial reagents DTNP and Boc_2O and the byproduct 2-mercapto-5-nitropyridine stayed in the organic phase, as evidence by TLC. The aqueous phase was acidified with HCl and extracted with AcOEt. The free AA and the Boc-protected AA stayed in the aqueous phase, while the synthesized product moved to the organic phase. AcOEt was evaporated, dried and filtered to obtain the product Boc-(D)-Cys(*p*-Npys)-OH.

As shows TLC sheet ($CHCl_3$/MeOH/AcOH in proportion 89:10:1, v/v**, figure 12**) the obtained product was not pure, and that is why a good characterization was not made. The product synthesized with a yield of 44% was characterized by ESI mass spectrometry and HPLC, and it was not purified because the yield would be very low.

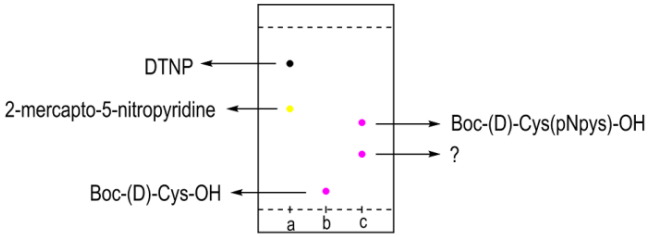


Figure 12. TLC sheet where (a) corresponds to the diethyl ether phase, (b) to the aqueous phase and (c) to the AcOEt phase. The byproduct in phase (c) could not be determined; therefore the desired product was not purified.

7. SYNTHESIS OF POLYMYXIN ANALOGS

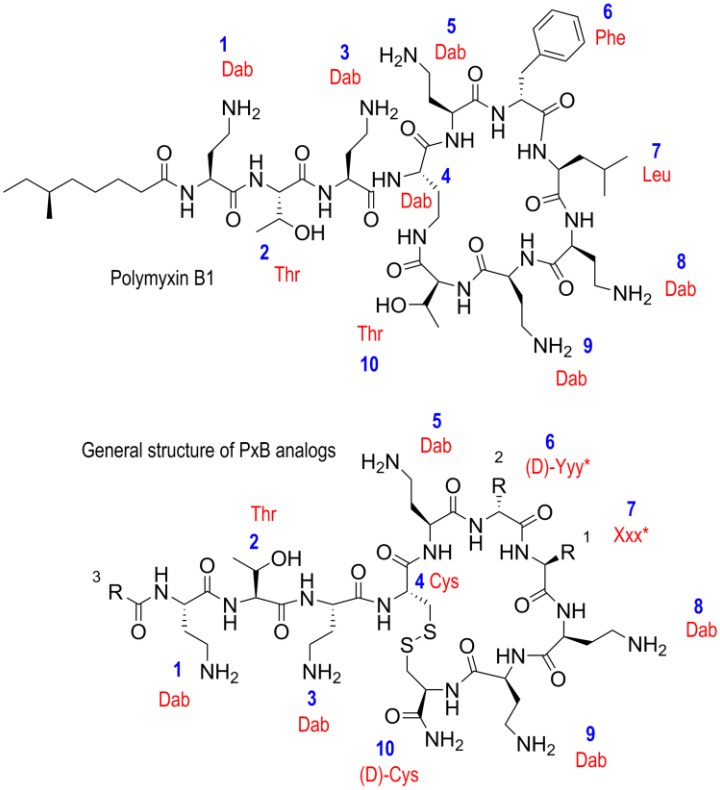
7.1. DESIGN

The main objective of this present work was to obtain analogs of PxB which conserve or improve its efficiency against gram-negative microorganisms, or that expand its range of action, being active against gram-positive microorganisms and, if possible, reducing the toxicity of PxB.

As shows **figure 13**, the differences between PxB and the synthesized analogs are:

- Substitution of the amide bond between residues Dab-4 and Thr-10, which forms the cyclic heptapeptide, for a disulfure bridge between the residues that are in the same position, Cys-4 and (D)-Cys-10. This modification keeps the size of the cycle and simplifies the synthetic process.
- Substitution of the residues 6 and 7, which represent the hydrophobic part of the cycle, for the amino acids Xxx and (D)-Yyy. These residues cannot be public for reasons of confidentiality.

- Variation of the acid (S)-6-methyloctanoic for different fatty acids in analogs 1A and 1B, and substitution of Dab-1 for the fatty acid Cx in analog 1C. These modifications, along with substitution of residues 6 and 7, vary the hydrophobicity of the molecule.



* In analog 1C the AA 1-Dab is substituted for the fatty acid Cx.

Figure 13. Comparison between PxB and the synthesized analogs.

The sequence of the synthesized peptides is shown in **table 8**:

Peptide	Sequence
1A	Hexanoyl-Dab-Thr-Dab-*cyclo*-[Cys-Dab-(D)-Yyy*-Xxx*-Dab-Dab-(D)-Cys]
1B	Octanoyl-Dab-Thr-Dab-*cyclo*-[Cys-Dab-(D)-Yyy*-Xxx*-Dab-Dab-(D)-Cys]
1C	Cx*-Thr-Dab-*cyclo*-[Cys-Dab-(D)-Yyy*-Xxx*-Dab-Dab-(D)-Cys]

*Xxx = $NH_2CH(R_1)COOH$; Yyy = $NH_2CH(R_2)COOH$; Cx = $(R_3)COOH$. Amino acids and fatty acid structure can't be disclosed for reasons of confidentiality

Table 8. Sequences of the synthesized PxB analogs.

7.2. SYNTHESIS

7.2.1. Synthesis strategy

The analogs of polymyxin were synthesized following the Fmoc/^{t}Bu protection strategy. In this protection scheme, 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 based either on the tert-butyl protecting group or the trityl (triphenylmethyl) group. This is an orthogonal protection system, since the side chain protecting groups can be removed without displacing de N-terminal protection and visa versa. It is advantageous when side chains need to be selectively modified, as when the peptide is selectively labeled or cyclized through the side chain [8].

The solid phase peptide synthesis was carried manually and performed using the resin BHA, which had to be pre-treated as explains **section 5.3.2**.

Initially, Fmoc-Ala-OH was coupled to the resin BHA as a reference amino acid, facilitating the SPPS because it avoids steric and electronic problems. The reaction was carried out using DIC and HOBt, for 1 hour in anhydrous DMF.

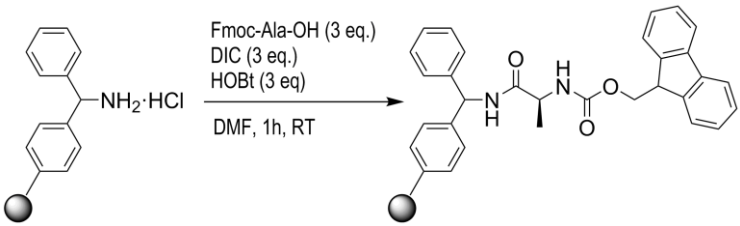


Figure 14. Coupling of the first amino acid to the resin BHA.

Carbodiimides are commonly used to prepare amides, esters and acid anhydrides from carboxylic acids, so diisopropylcarbodiimide (DIC) was used as an activate agent of the carboxylic acid of the amino acid. This reagent and its urea byproduct are more water soluble, so the byproduct and any excess reagent are removed by aqueous extraction. Carbodiimide activation of AA derivatives often causes a partial racemization of the AA, so in peptide

synthesis, adding an equivalent of 1-hydroxybenzotriazole (HOBt) minimizes this problem, improving the efficiency of the SPPS [8]. The mechanism of this coupling is shown in **figure 15**.

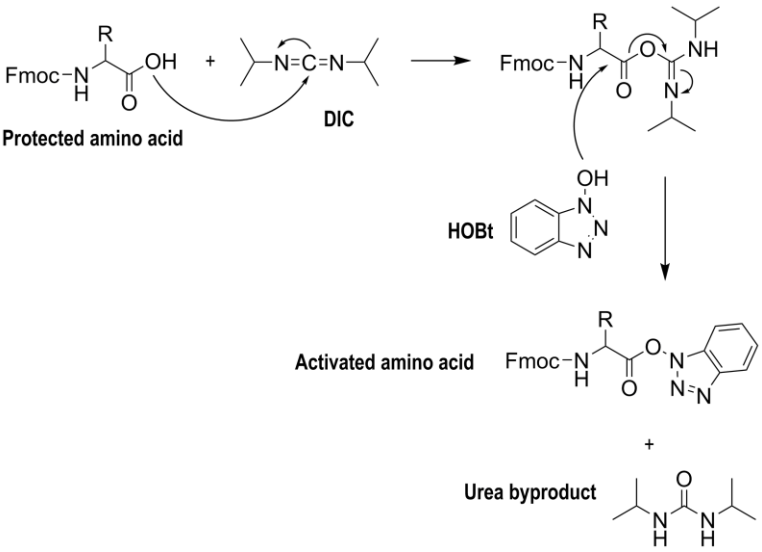


Figure 15. Activation of the AA. The attack of HOBt is based on a reaction of addition-elimination.

After this coupling, the Fmoc group of the reference amino acid was removed with a solution of 20% of piperidine in DMF, as is explained in **section 5.3.3.2.** (**table 2**). The Fmoc group is removed when a base abstracts the relatively acidic proton from the fluorenyl ring system, leading to a β-elimination and the formation of dibenzofulvene and carbon dioxide, as shows **figure 16**. Dibenzofulvene is a reactive electrophile and would readily attach irreversibly to the deprotected amine unless it was scavenged. Secondary amines such as piperidine add to dibenzofulvene and prevent deleterious side reactions. Hence piperidine is typically used to remove the Fmoc group and also scavenge the dibenzofulvene by-product.

After Fmoc removal, Fmoc-Rink linker was coupled to the reference amino acid, following the procedure shown in **section 5.3.3.2**. Linkers are chemical entities used to "link" a compound to a resin bead during SPPS. The nature of the linker determines the kind of chemistry that can be performed, and the conditions under which products can be cleaved from the resin [8]. The RL used was a benzhydrylamine functionalized with two methoxy groups at *ortho* and *para* positions. This enables the cleavage of the peptide-RL bond with a solution of TFA in H_2O, and obtaining the peptide with the C-terminal end in carboxamide form.

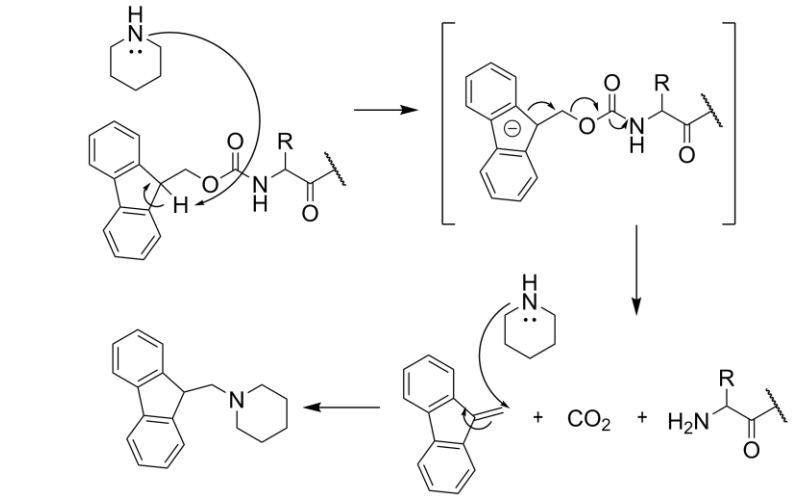


Figure 16. Fmoc deprotection mechanism.

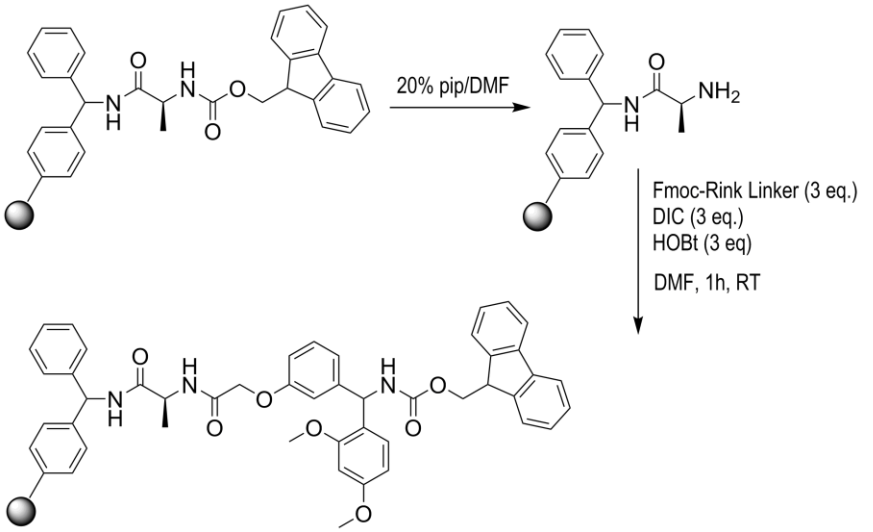


Figure 17. Rink linker incorporation to the peptidyl-resin.

For the elongation of the peptide chain, each AA coupling was carried out with 3 eq. of Fmoc-AA-OH, 3 eq. of DIC and 3 eq. of HOBt in anhydrous DMF for 1 h. The efficiency of the reactions was controlled by ninhydrin test (**section 5.1.3.1.**).

As explained above, lateral chains of AA were protected with acid labile groups. The protecting group of the lateral chain of Cys residues was Trt, in order to obtain a fully deprotected linear precursor after the cleavage from the resin, and allowing the disulfide bond

formation in aqueous solution by oxidation. Regarding other trifunctional amino acids, Dab was protected with Boc group and Thr was protected with ^{t}Bu group.

Once the last amino acid was incorporated in the sequence, fatty acids were coupled in the three analogs following the protocol described in **section 5.3.3.5**.

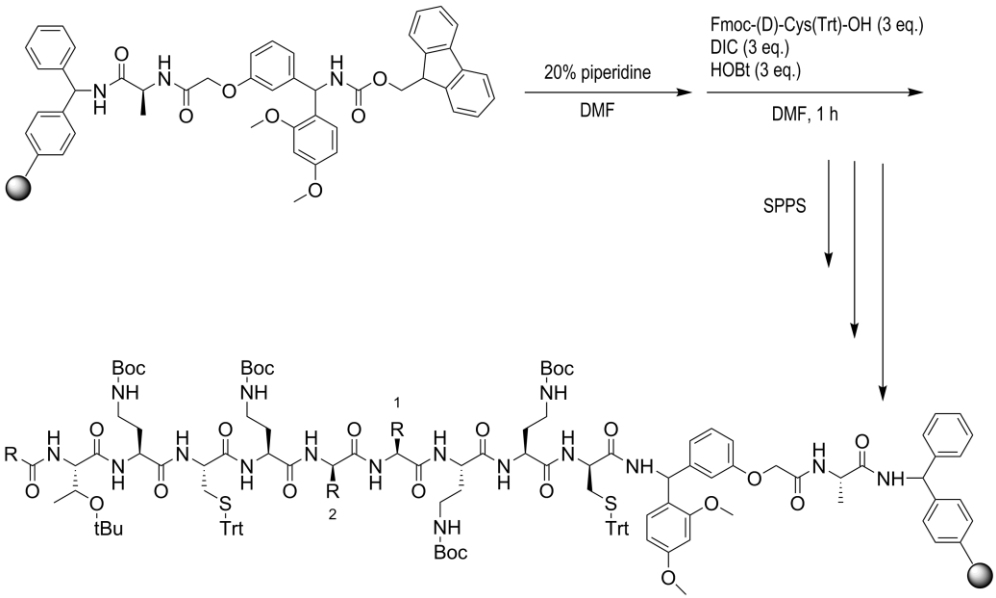


Figure 18. Formation of the linear peptidyl-resin with Fmoc/^{t}Bu SPPS.

Then the cleavage of the peptide from the resin and de removing of the lateral chains protecting groups were carried out by acidolysis, with a treatment of TFA/TIS/H_2O (95:3:2, v/v). Triisopropylsilane (TIS) function is to reduce carbocations.

So finally, totally unprotected linear precursors were obtained.

7.2.2. Cyclization

The cyclization of the synthesized peptides was performed by oxidation of the thiol groups of Cys residues to form a disulfide bond, as shows **figure 19**. Lineal peptides were added to a solution of 5% DMSO/H_2O, therefore its concentration was 0.5 mg/ml. Under conditions of high dilution formation of cyclic dimers and oligomers is avoided.

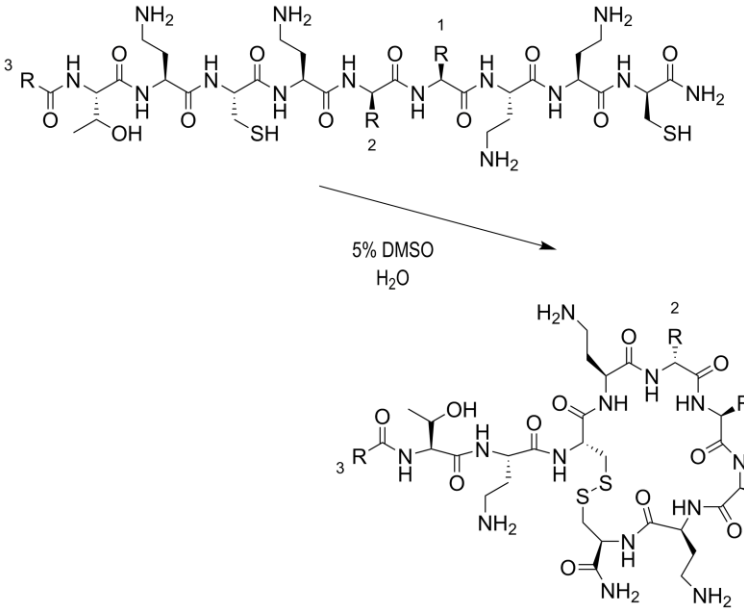
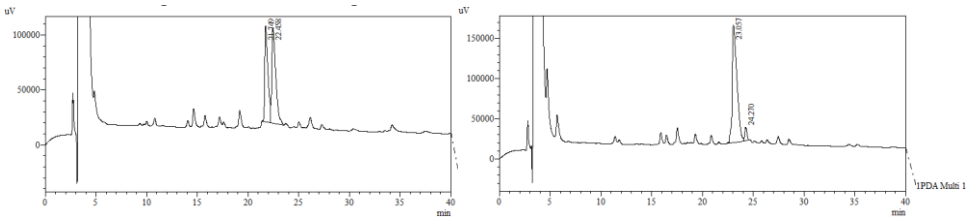


Figure 19. Cyclization by an oxidation reaction of the linear peptides to form the definitely analogs.

The oxidation reaction was followed by RP-HPLC. The peptides were cyclisized after 8 h.



t = 1 h t = 8 h

Figure 20. Oxidation process of the analog 1B. At t=1h the percentage of the cyclic peptide was 44%, and after 7 hours it increased to 95%. The samples were analyzed with a lineal gradient from 20% to 50% of B for 30 min. (Solution A: 0.045% TFA/H20, solution B: 0.036% TFA/ACN; UV detection at λ 220 nm).

In all cases a fast disappearance of the chromatographic peak of the linear peptide and the subsequent emergence of a new peak corresponding to the cyclic peptide is observed. Cyclization yields were 94 – 100%; therefore it is an optimal methodology for a complete peptide cyclization.

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

7.2.3. Purification and characterization

The crude peptides obtained after cyclization were purified by RP-HPLC in a semipreparative scale. Final peptides were obtained with good purities (>99%), as is shown in RP-HPLC and ESI spectra (**appendices 5, 6**). The yields are shown in **table 9**:

Peptide	η cleavage	η cyclization	η purification	η synthesis
1A	49%	94%	38%	18%
1B	62%	100%	25%	16%
1C	43%	95%	19%	8%

Table 9. Cleavage, cyclization, purification and final yields of the synthesized analogs.

The cleavage yield was calculated from the weight of crude peptide after TFA treatment, using as a theoretical value the calculated from the initial dry resin and its functionalization. The cyclization yield was calculated from the weight of peptide obtained after cyclization and the weight obtained after TFA treatment. The purification yield was obtained from the weight of the final pure peptide and the weight of the cyclic peptide. Finally, the total yield was expressed as a combination of the cleavage, cyclization and purification yields.

Synthesis yields are quite low, especially in analog 1C. Due to it was the first time these peptides were synthesized, it was necessary to make a large number of mini-cleavages in order to control if the chains were elongating correctly.

8. Determination of Minimum Inhibitory Concentration

8.1. General aspects

One of the main objectives of this present work was to determinate the antimicrobial activity of the synthesized peptides.

This activity is expressed with minimum inhibitory concentrations, which are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism

after overnight incubation. MICs are used by diagnostic laboratories mainly to confirm resistance, but most often as a research tool to determinate the *in vitro* activity of new antimicrobials [21].

The MIC of the synthesized PxB analogs was determined following the protocol described for Amsterdam, D. [17,18]. As explained in **section 5.5**, four bacteria suspension were prepared (with a concentration of 10^6 UFC/ml). A stock solution of each peptide in MilliQ-water was also prepared, from which we obtained a range of concentrations from 0.25 µg/ml to 128 µg/ml. Peptides and bacteria were incubated with the growth medium MHB at 37 ºC for 20 – 22 h.

8.2. BACTERIA

To carry on these assays the microorganisms used were:

- Gram-negative bacteria: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027.
- Gram-positive bacteria: *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213.

In the following section there is a brief description of these bacteria [22]:

8.2.1. Gram-negative bacteria

- *Escherichia coli* is the typical standard model of gram-negative bacteria. It is a rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms. Most *E. coli* strains are harmless. These strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K and preventing colonization of the intestine with pathogenic bacteria. But there are also harmful strains which can cause many excretory system infections, such as cystitis, urethritis, meningitis, mastitis, pneumonia… In recent years the resistance of this bacterium to antibiotics has increased.
- *Pseudomonas aeruginosa* is a model of gram-negative resistant bacteria. It is a pathogen microorganism which can cause chronic infections, and has a low susceptibility to antibiotics. It may cause pneumonia, excretory system infections…

Because it thrives on moist surfaces, this bacterium is also found on and in medical equipment, so can cause cross-infections in hospitals and clinics.

8.2.2. Gram-positive bacteria

- *Staphylococcus aureus* is at present the main cause of nosocomial infections. This is favored by the fact that it is found in the skin and mucous membranes of humans, allowing through surgical wounds can enter the patient's bloodstream. This bacterium can produce a wide range of diseases, ranging from skin infections and relatively benign, such as folliculitis, until disease life-threatening, such as osteomyelitis, meningitis, sepsis, endocarditis and pneumonia. Resistant strains of this bacterium are resistant to penicillin, and they are treated with aminoglycosides or oxacillin.
- *Enterococcus faecalis* is found in the intestine of humans and other warm-blooded organisms. It indicates fecal contamination, so its present in foods indicates poor hygiene or poor conservation, but it is also present on some foods like cheese, where acts as natural bacteria flora. It may be resistant to all antibiotics in use.

8.3. RESULTS AND DISCUSSION

After overnight incubation, MIC of the synthesized peptides was determined visually using an amplifier. Polymyxin B (PxB) was used as a standard.

The results of this qualitative test are shown in **table 10**.

Peptides / Bacteria		MIC [μg/ml]			
		PxB	**1A**	**1B**	**1C**
Gram -	***E. coli***	<0.25	2 – 4	2	2
	P. aeruginosa	<0.25	<0.25	<0.25	0.25
Gram +	***S. aureus***	32	32	4	4
	E. faecalis	>128	>128	16 – 32	64 – 128

Table 10. MIC results for PxB and the three synthesized analogs.

The results of MIC test show that the analog 1B presents the best antimicrobial activity. In addition of being effective against gram negative bacteria, has a good selectivity against gram positive bacteria, actually better than polymyxin. Therefore, comparing with the analog 1A (which does not improve the effectiveness of PxB), it can be concluded that the 8C fatty acid chain has provided more antibacterial activity than the 6C chain. The analog 1C also shows a good effectiveness against gram positive bacteria, especially into the bacterium *S. Aureus.*

9. CONCLUSIONS

The final conclusions of this present work are:

- The synthesis methodology using the solid phase peptide synthesis, the resin BHA and the Fmoc/^{t}Bu strategy allows obtaining the desired peptides with high purities (>99%). Although synthesis yields have not been very high, it is a simple and effective methodology.
- The determination of minimum inhibitory concentration (MIC) of the synthesized analogs has led to good results, with high selectivity against gram negative bacteria and acceptable activity against gram positive bacteria. MIC values are comparable and even better, in some cases, than polymyxin B. The best obtained results correspond to the analog 1B, which has a residue of Dab in position 10 and a fatty acid of eight carbons.
- The synthesis of the protected amino acid Boc-(D)-Cys(*p*-Npys)-OH has not been successful, due to the low purity of the crude compound obtained and the expected low yield after purification.

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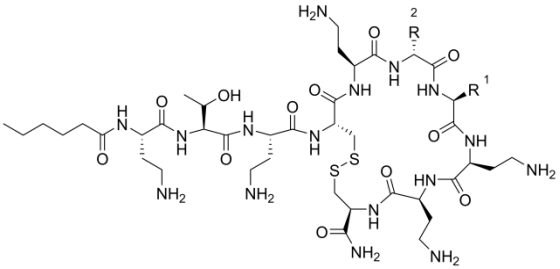
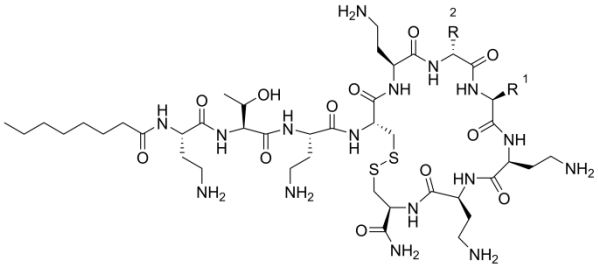
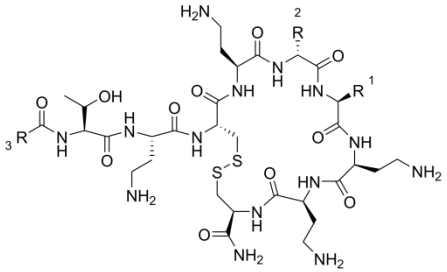
11. ACRONYMS

AA	Amino acid
ACN	Acetonitrile
ADEPs	Acyldepsipeptides
AMPs	Antimicrobial peptides
ATCC	American Type Culture Collection
BHA	Benzhydrylamine resin
Boc	*tert*-butyloxycarbonyl
^{t}Bu	*tert*-butyl
DCM	Dichloromethane
DIEA	N,N-Diisopropylethylamine
DIC	N,N'-Diisopropylcarbodiimide
DMF	N,N'-Dimethylformamide
DMSO	Dimethyl sulfoxide
DTNP	2,2'-Dithiobis(5-nitropyridine)
Eq.	Equivalents
f	Functionality
Fmoc	Fluorenylmethyloxycarbonyl
HOBt	1-Hydroxybenzotriazole
HPLC	High Pressure Liquid Chromatography
m/z	Mass-to-charge ratio
MHB	Mueller Hinton-Broth (culture media)
MIC	Minimum inhibitory concentration
Min	Minutes

Nps	3-nitrophenyl
Npys	3-nitro-2-pyridinesulfenyl
Npys-Cl	3-nitro-2-pyridinesulfenyl chloride
p-Npys	5-nitro-2-pyridinesulfenyl
PxB	Polymyxin B
PyrS	2-pyridinesulfenyl
RL	Rink linker
r.p.m.	Revolutions per minute
SPPS	Solid phase peptide synthesis
TFA	Trifluoroacetic acid
TIS	Triisopropylsilane
TLC	Thin layer chromatography
Trt	Triphenylmethyl (trityl)
UFC	Colony-forming unit
UV	Ultraviolet

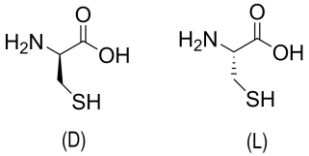
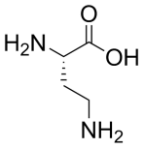
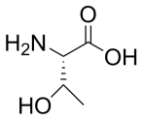
APPENDICES

APPENDIX 1: SYNTHESIZED ANALOGS STRUCTURE

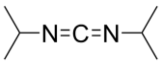
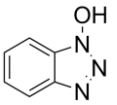
Name	Structure
1A	 Hexanoic-Dab-Thr-Dab-*ciclo*-[Cys-Dab-Yyy*-Xxx*-Dab-Dab-(D)-Cys]
1B	 Octanoic-Dab-Thr-Dab-*ciclo*-[Cys-Dab-Yyy*-Xxx*-Dab-Dab-(D)-Cys]
1C	 Cx*-Thr-Dab-*ciclo*-[Cys-Dab-Yyy*-Xxx*-Dab-Dab-(D)-Cys]

*Xxx = $NH_2CH(R_1)COOH$; Yyy = $NH_2CH(R_2)COOH$; Cx = R_3COOH. Amino acids and fatty acid structure can't be public for reasons of confidentiality

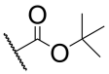
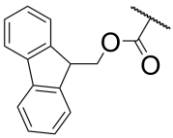
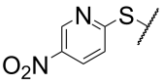
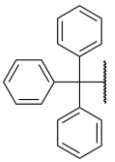
APPENDIX 2: AMINO ACIDS USED

Amino acid	Type	Structure
C Cysteine (Cys)	Proteinogenic	 (D) (L)
L-(2,4)-Diaminobutyric acid (Dab)	Non-proteinogenic	
T Threonine (Thr)	Proteinogenic	

APPENDIX 3: COUPLING AGENTS AND ADDITIVES

Abbreviation	Name	Structure
DIC	N,N'-Diisopropylcarbodiimide	
HOBt	1-Hydroxybenzotriazole	

APPENDIX 4: PROTECTING GROUPS

Abbreviation	Name	Structure	Lability
Boc	*Tert*-butyloxycarbonyl		TFA
^{t}Bu	*Tert*-butyl		TFA
Fmoc	Fluorenylmethyloxycarbonyl		Piperidine
p-Npys	*para*-5-nitro-2-pyridinesulfenyl		Tertiary phosphine, DTT
Trt	Triphenylmethyl (trityl)		TFA

APPENDIX 5: CHROMATOGRAMS OF PURE PEPTIDES

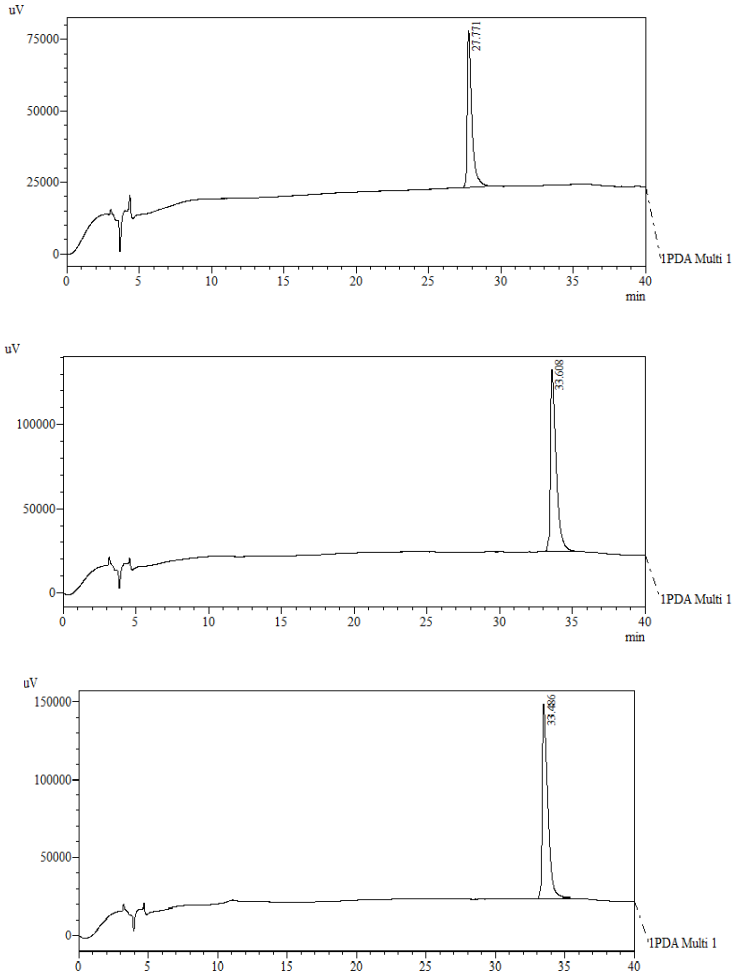
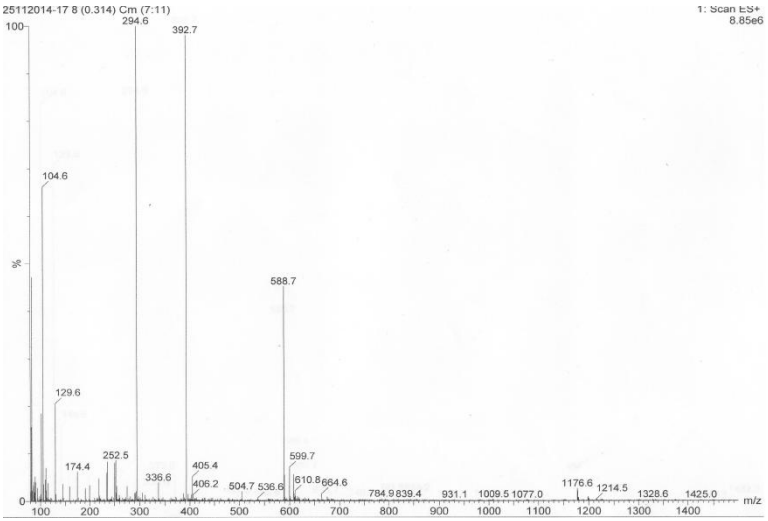
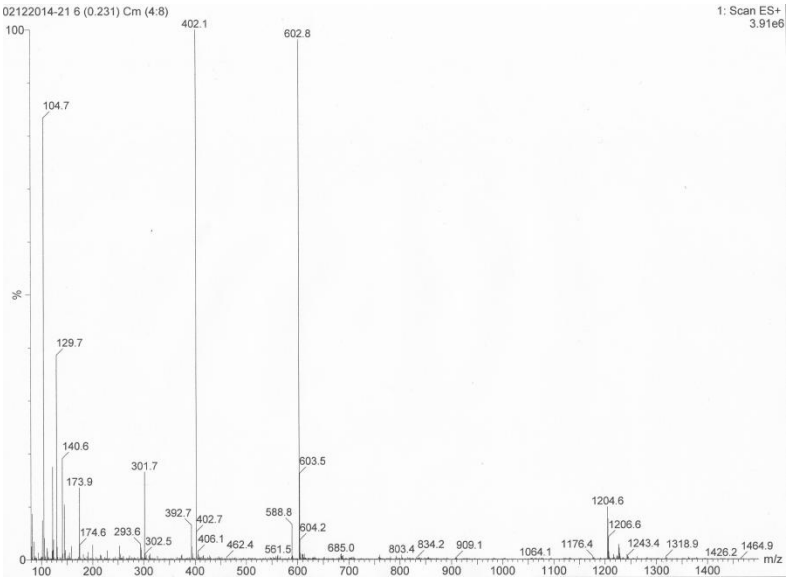


Figure 21. HPLC chromatograms of pure peptides: 1A, 1B and 1C. Lineal gradient from 20% to 50% for 30 min; UV detection at 220 nm.

APPENDIX 6: ESI SPECTRA OF PURE PEPTIDES





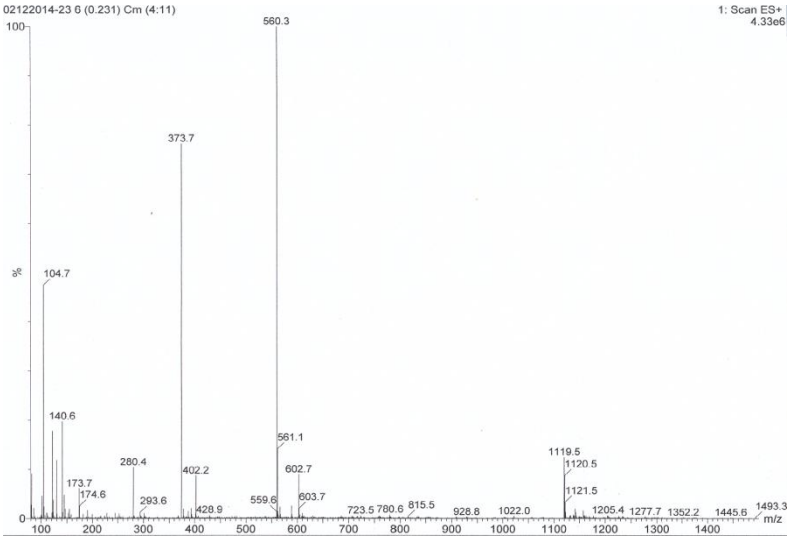


Figure 22. ESI-HRMS spectra of pure peptides: 1A, 1B and 1C.