

Synthesis of Peptaibolin, an Antimicrobial Peptide †

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† Presented at the 1st International Electronic Conference on Pharmaceutics, 1–15 December 2020; Available online: <https://iecp2020.sciforum.net/>.

Received: date; Accepted: date; Published: date

Abstract: To tackle one of the biggest global health problems, the resistance of microorganisms to antibiotics, a collective effort in the search for more effective agents against bacteria was required. Peptides with antimicrobial activity have been raising much attention as a promising alternative for antibiotics. Peptaibols, for instance, are a family of antimicrobial peptides (AMPs) with great biomedical potential, in which the Peptaibolin can be highlighted. This peptide has gained relevance due to its small amino acids content, only four, and its acetyl group and a phenylalaninol residue (Phol) at the *N*-terminal and *C*-terminal, respectively. Here, we report the synthesis of Peptaibolin through Solid Phase Peptide Synthesis assisted by Microwave heating (MW-SPPS), in a pre-loaded Phe-Wang resin. Starting from a loading of 0.51 mmol/g, two syntheses were made, using two different combinations of coupling reagents. The best option was DIC/Oxima achieving a yield of 50.0%. Proton Nuclear Magnetic Resonance (¹H-NMR) studies confirmed the peptide structure, while High Performance Liquid Chromatography (HPLC) verified the peptide purity. The peptide solubility was examined against several combinations of solvents. Peptaibolin was not soluble in water, only in organic solvents or in the combination of both. Antimicrobial testing was conducted using *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli* and *Pseudomonas aeruginosa*. Minimum inhibitory concentration studies demonstrated the resistance of bacteria to the peptide action and the peptide instability in bacterial growth conditions.

Keywords: antimicrobial peptides; peptide synthesis; solid-phase approach; bacteria resistance

1. Introduction

The discovery of antibiotics is considered one of the greatest and best achievements in the scientific world and for the pharmaceutical industry. For years, they have been the most effective treatment against one of the biggest causes of death worldwide, systemic infections, the result of the action of a wide spectrum of pathogenic microorganisms. However, nowadays, these are no longer an 100% effective solution in the fight against bacterial infections due to the development of resistance from both Gram-positive and Gram-negative bacteria, including: *Staphylococcus aureus*, *Enterococcus* spp., *Pseudomonas aeruginosa* and *Escherichia coli* [1–3]. To battle this obstacle, some functional strategies were developed to replace the action of conventional antibiotics with biomolecules with similar pharmacological characteristics, like peptides with antimicrobial activity (AMPs).

The effectiveness of AMPs is reflected in their mechanisms of action, affecting cell membranes in different ways, changing their permeability, or causing the membrane to rupture entirely. Such mechanisms are not common to all types of peptides and depend on their molecular properties and the type of bacterial and/or cellular target, including DNA, RNA, regulatory enzymes, and other

proteins. Generally, the damage caused to bacteria by AMPs is difficult to correct and the possibility that they develop resistance to these peptides is believed to be remote [4–6].

Peptaibols are a family of AMPs isolated from the fungal strain *Trichoderma* and have antibacterial and antifungal properties. The main characteristic of this type of AMP is the presence of non-proteinogenic amino acids which are not encoded by DNA, such as α -aminobutyric acid (Aib) and isovaline (Iva). This class of amino acids is often used in peptides of pharmacological interest since they are not recognized by hydrolytic enzymes, improving their biodegradability and increasing their bioavailability [7,8]. One of the most unique AMPs in this family is Peptaibolin because it is the smallest in the peptaibol family but at the same time, it has a three-dimensional structure comparable to the larger peptaibols. It contains an *N*-terminal acetyl group and a *C*-terminal phenylalaninol (Phol) residue, together with only four amino acids (Ac-Leu-Aib-Leu-Aib-Phol) (Figure 1). Crisma et al. studied Peptaibolin and concluded that this small peptide had antimicrobial activity, albeit moderate, in Gram-positive bacteria. Its mode of action is based on the permeabilization of the cell membrane causing its rupture. Through conformational studies by X-ray crystallography, it was detected that Peptaibolin has an extremely folded secondary α -helix structure and its antimicrobial activity is directly related to this fact, since this conformation allows for aggregation, unusual feature in peptides with few amino acid residues [9].

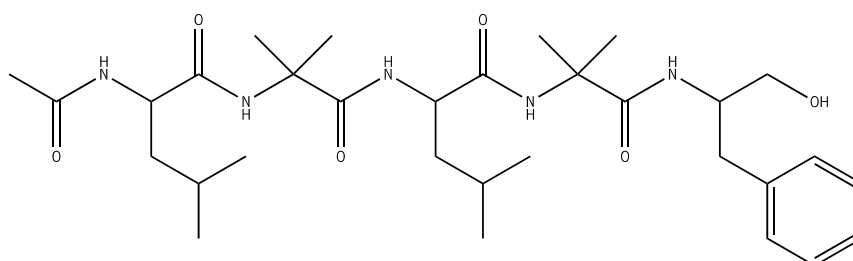


Figure 1. Structure of the AMP Peptaibolin containing an *N*-terminal acetyl group and a *C*-terminal phenylalaninol (Phol) residue, encompassing only four amino acid residues (Ac-Leu-Aib-Leu-Aib-Phol).

The synthesis of Peptaibolin and all peptaibols in general is significantly impaired by stereochemical impediment in residues such as Aib, where the reactivity during the coupling reaction is decreased compared to other amino acid residues. To overcome this difficulty, it is necessary to use a selection of suitable coupling reagents that allow the best possible activation of the carboxyl terminal of the amino acid Aib [10]. In this work, the combination of two groups of coupling reagents was studied: *N,N'*-Diisopropylcarbodiimide/1-Hydroxybenzotriazole (DIC/HOBt) and *N,N'*-Diisopropylcarbodiimide/Ethyl 2-cyano-2-(hydroxyimino)acetate (DIC/Oxima) to compare their reaction yields and purity using Solid Phase Peptide Synthesis assisted by Microwave heating (MW-SPPS) method, in a pre-loaded Phe-Wang resin. In addition, solubility and antimicrobial tests were performed.

2. Experiments

2.1. Materials

Microwave synthesis was performed on the manual peptide synthesizer Discover SPPS (CEM Corporation). The solid supports used were Fmoc-Phe-Wang resin with a functionalization degree of 0.51 mmol/g acquired from AAPptec. The ^1H nuclear magnetic resonance spectra were obtained on the Bruker Avance III 400 device at a frequency of 400 MHz, using the solvent peak as an internal reference, at 25 °C. The deuterated solvent used was deuterated chloroform (CDCl_3d_6) with a deuteration degree greater than 99.8% (Acros Organics). Coupling constants (J) were obtained in Hertz (Hz) and chemical shifts (δ) in parts per million (ppm). Analytical HPLC analysis was performed with a Licospher 100 RP18 column (5 μm) in an HPLC system composed of a Jasco PU-980 pump, coupled to a Shimadzu SPD-GAV UV/vis detector and a Shimadzu C-RGA Chromatopac

recorder. The dry reagents and solvents were used as purchased commercially (AAPPTec, Louisville, KY, USA; Acros Organic, Geel, Belgium; Sigma Aldrich, St. Louis, MO, USA; Merck, Kenilworth, NJ, USA). Growth media Mueller Hinton broth (MHB) was obtained from CondaLab. Gram-negative *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 25853) and Gram-positive *S. aureus* (ATCC 6538) and *S. epidermidis* (ATCC 35984) bacteria were supplied from American Type Culture Collection (ATCC).

2.2. Synthesis of Peptaibolin by MW-SPPS on Wang Resin

2.2.1. DIC/HOBt as Coupling Reagents

Starting from 1.1 g of Fmoc-Phe-Wang resin (0.51 mmol/g), Fmoc group was removed by adding 5 mL of a 20% piperidine solution in dimethylformamide (DMF) to the reaction vessel containing the resin. The vessel was placed with the optical fiber for temperature measurement in the microwave and the SPPS deprotection program was chosen at 75 °C and 20 watts (W) for 30 s. After this initial deprotection, the 20% piperidine solution in DMF was filtered and 7 mL of this solution was added again, and the same program was selected for 3 min. The solution was filtered and washes were performed: DMF (3x) and methanol (MeOH) (3x), repeating this washing cycle 4 times. Then, all amino acids were sequentially coupled by introducing Fmoc-Aib-OH, Fmoc-Leu-OH, Fmoc-Aib-OH and Fmoc-Leu-OH sequentially. The *N*-protected amino acid (0.0028 mol, 5 equiv.), HOBt (0.0028 mol, 0.4756 g, 5 equiv.) and DIC (0.0028 mol, 0.35 mL, 5 equiv) were dissolved and the reaction mixture containing the amino acid Aib required a stirring period of 2 h and the reaction mixture containing the amino acid leucine (Leu) remained stirring for 5 min. After two cycles of coupling with the conditions of 75 °C and 20 W for 5 min, the following washes were performed: DMF (3x) and MeOH (3x), 3 wash cycles.

Prior to the separation of the peptide from the resin, acetylation was carried out, in which the resin was suspended in 7 mL of dry DMF, 1 mL of *N,N*-Diisopropylethylamine (DIPEA) (14 equiv.) and then 0.4 mL of acetic anhydride (7 equiv.) were added. The mixture was left under stirring for 1.5 h. After that period, the resin was washed with DMF (2x), MeOH (2x), dichloromethane (DCM) (3x) and ethyl ether (3x). The resin was dried in the desiccator at room temperature.

After obtaining the final sequence and acetylation, the peptide Ac-Leu-Aib-Leu-Aib-Phol, was separated from the Wang resin. In a 3-port flask, sodium borohydride (NaBH₄) (5 equiv.) was suspended in dry tetrahydrofuran (THF) (16 mL/g resin) under nitrogen atmosphere, dry ethanol (EtOH) (4 mL/g resin) and lithium bromide (LiBr) (5 equiv.). The mixture was vigorously stirred for 15 min in an ice bath and a further 15 min at room temperature. The resin was added to the previous mixture and left to stir at room temperature for a period of 24 h.

The separation of the resin from the reaction mixture was done through filtration into a porous plate funnel and washed with THF (2x), EtOH (2x) and ethyl acetate (EtOAc) (2x), directly into a 100 mL round bottom flask. Acetic acid was added dropwise in order to eliminate all the borohydride present in the mixture. The solvent was evaporated without heating and proceeded to the extraction, using a combination of water (H₂O):EtOAc:saturated sodium chloride (NaCl) solution (3x). The organic phase was collected and dried using anhydrous magnesium sulfate (MgSO₄). After filtration, the solvent was evaporated to dryness without heating. Ethyl ether was added and placed in the cold. The solution was centrifuged until a white solid was obtained.

2.2.2. DIC/Oxima as Coupling Reagents

Starting from 1.1 g of Fmoc-Phe-Wang resin (0.51 mmol/g), the protective group was removed by adding 5 mL of a 20% piperidine solution in DMF to the reaction vessel containing the resin, the vessel was placed reaction with the optical fiber for temperature measurement in the microwave and the SPPS deprotection program was chosen at 75 °C and 20 W for 30 s. After this initial deprotection, the 20% piperidine solution in DMF was filtered and 7 mL of this solution was added again, and the same program was selected again for 3 min. The solution was filtered and washes were performed: DMF (3x) and MeOH (3x), repeating this washing cycle 4 times. Then, all amino acids were sequentially coupled by introducing Fmoc-Aib-OH, Fmoc-Leu-OH, Fmoc-Aib-OH and Fmoc-Leu-

OH sequentially. The *N*-protected amino acid (0.0028 mol, 5 equiv.), Oxima (0.0028 mol, 0.3986 g, 5 equiv.) and DIC (0.0028 mol, 0.35 mL, 5 equiv) were dissolved and the reaction mixture containing the amino acid Aib required a stirring period of 2 h and the reaction mixture containing the amino acid Leu remained stirring for 5 min. After two cycles of coupling with the conditions of 75 °C and 20 W for 5 min, the following washes were performed: DMF (3x) and MeOH (3x), 3 wash cycles. Prior to the separation of the peptide from the resin, acetylation was carried out, in which the resin was suspended in 7 mL of dry DMF, 1 mL of DIPEA (14 equiv.) and then 0.4 mL of acetic anhydride (7 equiv.) were added. The mixture was left under stirring for 1.5 h. After that period, the resin was washed with DMF (2x), MeOH (2x), DCM (3x) and ethyl ether (3x). The resin was dried in the desiccator at room temperature.

After obtaining the final sequence and acetylation, the peptide Ac-Leu-Aib-Leu-Aib-Phol, was separated from the Wang resin. In a 3-port flask, NaBH₄ (5 equiv.) was suspended in dry THF (16 mL/g resin) under nitrogen atmosphere, dry ethanol (4 mL/g resin) and LiBr (5 equiv.). The mixture was vigorously stirred for 15 min in an ice bath and a further 15 min at room temperature. The resin was added to the previous mixture and left to stir at room temperature for a period of 24 h.

The separation of the resin from the reaction mixture was done through filtration into a porous plate funnel and washed with THF (2x), EtOH (2x) and EtOAc (2x), directly into a 100 mL round bottom flask. Acetic acid was added dropwise in order to eliminate all the borohydride present in the mixture. The solvent was evaporated without heating and proceeded to the extraction, using a combination of H₂O:EtOAc:saturated NaCl solution (3x). The organic phase was collected and dried using anhydrous MgSO₄. After filtration, the solvent was evaporated to dryness without heating. Ethyl ether was added and placed in the cold. The solution was centrifuged until a white solid was obtained.

2.3. Peptaibolin Characterization: Proton Nuclear Magnetic Resonance (¹H NMR)

For structural confirmation, 5 mg of peptide was dissolved in 500 µL of CDCl₃d₆ and transferred to an NMR tube and NMR spectrum was recorder at 400 MHz.

2.4. Peptaibolin Characterization: High Performance Liquid Chromatography (HPLC)

To confirm the purity, three eluent solutions were prepared. Filtering all solvents, 300 mL of the eluent solution acetonitrile/water (ACN/H₂O) (1:1), ACN/H₂O (2:1) and ACN/H₂O (1:2) was prepared adding 0.1% trifluoroacetic acid (TFA), and all solutions were placed in an ultrasonic bath for 20 min.

2.5. Solubility Tests Preparation

Several solvents have been tested so that the dissolution of Peptaibolin takes place. For each solvent tested, a specific peptide mass was weighed and tried to dissolve in 1 mL of solution. For the combination of 10% dimethyl sulfoxide (DMSO) in H₂O (2 mg); for 10% hydrochloric acid (HCL)/H₂O (1.9 mg); for 100% HCL (1.8 mg); for phosphate-buffered saline (PBS) (2.4 mg); for NaCl/H₂O (2 mg); for Acetic acid/H₂O (1.9 mg); for 10% CDCl₃ (2 mg) and for ACN/H₂O (2.1 mg).

2.6. Minimum Inhibitory Concentration (MIC)

Working solutions at 8 mg/mL were prepared using the most suitable solvent. Aliquots were added to the first column of 96-well plates in a volume of 100 µL. Serial dilutions (1:2) were made with MHB in the consecutive wells, to a final volume of 50 µL. Then, to each of these wells, 50 µL of the bacteria suspensions prepared at 2 × 10⁷ CFUs/mL in MHB were added (inoculated from one colony and grown overnight at 37 °C and 100 rpm); meaning the starting peptide concentration in contact with the bacteria was at 4 mg/mL. Agent-free bacteria suspensions (positive) and culture media (negative) were used as controls. Absorbance readings at a wavelength of 600 nm (EZ READ 2000 Microplate Reader, Biochrom, UK) were made before and after plate incubation for 24 h at 37 °C and 100 rpm. The MIC value for each peptide/bacteria combination was established as the

concentration at which bacteria did not show any growth, determined visually, and confirmed by the differences in absorbance readings.

3. Results and Discussion

3.1. Synthesis of Peptaibolin by MW-SPPS on Wang Resin

Peptaibolin was synthesized according to the MW-SPPS method where each amino acid is sequentially coupled with the aid of a combination of coupling reagents in order to favor the activation of the carboxylic group and potentiate the formation of the peptide bond between two amino acid residues. Despite the fact that Peptaibolin is a widely studied peptide, which mechanisms of action and antimicrobial activity have already been proven and further studies have been already performed by replacing the amino acid Aib with its analogues to potentiate its activity, the search for different coupling reagent combinations has never been much addressed. Two combinations were tested: DIC/HOBt and DIC/Oxima. After obtaining the white solid by centrifugation and after a drying period in a desiccator, the mass obtained from each peptide was weighed and their yields were calculated. It was proved that the combination of DIC/Oxima coupling reagents had a higher yield as well as a higher purity, confirmed by the retention time obtained by the analytical HPLC technique using ACN/H₂O (1:1) + 0.1% TFA as eluent (Table 1).

Table 1. Comparison between the two coupling reagent combinations in terms of obtained mass, reaction yield and retention time purity.

Coupling Reagents	Mass (g)	Yield (%)	Retention Time (min)
DIC/HOBt	0.1222	40.6	6232
DIC/Oxima	0.1520	50.0	6942

Structural confirmation was made only by ¹H NMR for each peptide synthesized with different coupling reagents where it was proved that both peptides contained all the amino acids coupled in the right positions.

DIC/HOBt: RMN de ¹H (CDCl₃d₆, 400 MHz) δ_H = 0.90–1.00 (12H, m, 4xCH₃-Leu); 1.37 (3H, s, J = 6.8 Hz, CH₃-Aib); 1.38 (3H, s, J = 6.4 Hz, CH₃-Aib); 1.48 (3H, s, CH₃-Aib); 1.54 (3H, s, CH₃-Aib); 1.58–1.89 (6H, m, βCH₂-Leu + γCH-Leu); 2.14 (3H, s, CH₃CO); 2.77–2.83 (2H, m, βCH₂-Phe); 3.62–3.69 (2H, m, CH₂OH); 3.96 (1H, s, αCH-Leu); 4.02–4.08 (1H, m, αCH-Leu); 4.29 (1H, s, αCH-Leu); 7.17–7.30 (5H, m, CH-Phol); 7.85 (1H, s, NH-Leu); 8.02 (1H, s, NH); 8.11 (1H, s, NH).

DIC/Oxima: RMN de ¹H (CDCl₃d₆, 400 MHz) δ_H = 0.97–1.00 (12H, m, 4xCH₃-Leu); 1.38 (3H, s, CH₃-Aib); 1.39 (3H, s, CH₃-Aib); 1.48 (3H, s, CH₃-Aib); 1.54 (3H, s, CH₃-Aib); 1.58–1.89 (6H, m, βCH₂-Leu + γCH-Leu); 2.14 (3H, s, CH₃CO); 2.73–2.83 (5H, m, βCH₂-Phe); 3.61–3.72 (2H, m, CH₂OH); 3.96 (1H, s, α-CH); 4.02–4.08 (1H, m, αCH-Leu); 4.29 (1H, s, α-CH-Leu); 7.15–7.30 (5H, m, CH-Phol); 7.86 (1H, s, NH); 8.06 (1H, s, NH); 8.15 (1H, s, NH).

3.2. Evaluation of Solubility Properties of Peptaibolin

The solubility of an AMP is an important factor and its crucial that the peptide is soluble in suitable and compatible solvents for antimicrobial tests. The use of toxic solvents is not interesting for this type of tests since they cannot be applied in a biological context and therefore, cannot be biomedically applied. Solubility tests were performed only on the peptide synthesized with DIC/Oxima, mostly because of the highest amount of mass and yield. The most suitable solvent should be 100% H₂O due to its inert properties, being sure that it would not interfere with the antibacterial results. However, it was concluded that the peptide was not soluble in water, requiring the addition of other types of organic solvents or even the dissolution of the peptide in a total volume of organic solvents (Table 2).

Table 2. Combinations of solvents tested and the effects obtained on Peptaibolin in a total volume of 1 mL solution.

Solvents Tested ($V_T = 1$ mL)	Dissolution Effect
10% DMSO/H ₂ O	Soluble
ACN/H ₂ O	Dissolves only with large amounts of ACN
NaCl/H ₂ O	Does not dissolve
Acetic Acid/H ₂ O	Peptide precipitation
PBS	Does not dissolve
10% HCl/H ₂ O	Does not dissolve
100% HCL	Does not dissolve
100% H ₂ O	Does not dissolve
10% CDCl ₃	Partially soluble

3.3. Antibacterial Assessment

MIC evaluations were conducted using 10% DMSO solvent in water. This was found as the most suitable solvent for the peptide and as such was applied in contact with the bacteria. After 24 h of incubation, precipitates were detected in the most Peptaibolin concentrated wells, namely those containing 4 mg/mL of the peptide solution. Bacteria was also found to grow over the incubation period. Data reported no action against the selected microorganisms, meaning that if it was possible to find a MIC for this peptide it would be superior to 4 mg/mL. In terms of biomedical or pharmacological applications, this concentration is considered too elevated and, as such, not suitable for a cost-effective mass production.

4. Conclusions

Although the discovery of Peptaibolin as an AMP is not a recent topic in scientific research and there have been several studies focusing on its optimization, this peptide is most often synthesized according to standard procedures and without varying its combination of coupling reagents. Nowadays, there are a variety of coupling reagents commercially available from the most traditional carbodiimides, such as DIC, which is most frequently used in SPPS, to auxiliary nucleophiles such as HOBt. According to the results obtained, it can be concluded that the DIC/Oxima combination, in addition to maintaining the characteristics of Peptaibolin, provides better yields with the same level of purity. Furthermore, this alternative is even more positive because it replaces the traditional HOBt, which has explosive characteristics. Solubility tests were also performed to study the behavior of Peptaibolin in various solvents and to test its solubility. Being a small peptide and showing nonpolar characteristics, it was expected that its dissolution on H₂O would be an obstacle, which has been proven, since this peptide is only soluble in organic solvents or in organic solvents in combination with H₂O, making it unsuitable for biomedical applications as AMP. Its lack of antibacterial activity was also proven with a certain amount of Peptaibolin being dissolved in 10% DMSO/H₂O (the most promising solvent). Microbiological tests were carried out on Gram-positive bacteria and Gram-negative bacteria. However, MIC studies demonstrated the resistance of bacteria to the peptide action and the peptide instability in bacterial growth conditions.

Author Contributions: A.R.M.R. performed the experiments; A.R.M.R., H.P.F., S.P.G.C. and S.M.M.A.P.-L. discuss the results; H.P.F., S.P.G.C. and S.M.M.A.P.-L. acquired the funding; H.P.F. and S.M.M.A.P.-L. supervised the work. All authors have read and agreed to the published version of the manuscript.

Funding: The authors acknowledge Fundação para a Ciência e Tecnologia-FCT (Portugal) for funding through CQUM (UID/QUI/00686/2020) and project PTDC/QUI-COL/28052/2017. The NMR spectrometer Bruker Avance III 400 is part of the National NMR Network and was purchased within the framework of the National Program for Scientific Re-equipment, contract REDE/1517/RMN/2005 with funds from POCI 2010 (FEDER) and FCT. Authors also acknowledge FCT for funding the project PEPTEX with reference PTDC/CTM-TEX/28074/2017 (POCI-01-0145-FEDER-028074). They acknowledge project UID/CTM/00264/2020 of Centre for Textile Science and Technology (2C2T), funded by national funds through FCT/MCTES.

Conflicts of Interest: The authors declare no conflict of interest.

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