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Synthesis of Some Analogs of Polymyxin E1 Antibiotic Using Automated Solid Phase Peptide Synthesis Assisted With Microwave Irradiation

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FIVE analogues of Polymyxin E1 antibiotic were synthesized via Fmoc- solid phase peptide synthesis (SPPS) strategy using Biotage® Initiator+Alstra™ microwave peptide synthesizer. The aim of this work is to study the importance of the cyclization conditions of the lactam ring on the purity and yield of the final compounds. For this purpose, replacing L-Thr10 with L-Asp allowed the application of different cyclization methodologies. In all analogues, the Dab5,8,9 residues were replaced with Arg, while the Dab4 residue involved in lactam cyclization was replaced with Lys. Modification of the linear tripeptide tail was applied to two of the analogues. The obtained compounds were characterized with different spectroscopic techniques and will be tested for their antimicrobial activity.

Keywords:
Amino acids, Polymyxin E1, Automated Solid Phase Peptide Synthesis, Microwave Irradiation, Fmoc- strategy

Introduction

The growing threat of antibiotic resistance by Gram-negative bacteria has become one of the most serious public health issues. There is a well-documented increase in the number of pathogens resistant to several commonly used antimicrobial drugs, and this great public concern has been further aggravated by the dramatic slowdown in the antimicrobial drug development pipeline [1]. The use of polymyxins represents an alternative line of therapy to treat infections caused by Gram-negative bacteria that are resistant to other currently available antibiotics. Introduction of modified versions of the clinically available polymyxins unfortunately abounded due to toxicity issues has been proposed recently [2]. Polymyxins act specifically on Gram-negative bacteria by disrupting their critical permeability barrier, the outer membrane (OM), through interaction with the anionic lipopolysaccharides (LPS) molecules. This interaction is followed by destruction of the physical integrity of the inner membrane, and finally cell death [3]. Among the different classes of polymyxins, Polymyxin E (Colistin) produced by the growth of *B. polymyxa* subsp. *colistinus* contains two major components, Polymyxin E_1 (Colistin A) and Polymyxin E_2 (colistin B), which are N-terminally acylated by 6-methyloctanoic acid (6-MOC) and 6-methylheptanoic acid, respectively [4-6]. Polymyxin E_1 decapeptide is a group of cyclic polycationic lipopeptides consisting of a cyclic heptapeptide (L-Dab^4-L-Dab^5- D-Leu^6-L-Leu^7-L-Dab^8- L-Dab^9-L-Thr^{10}), a linear tripeptide tail (L-Dab^1- L-Thr^2-L-Dab^3), and 6-MOC linked to the N-terminus of the tail. The heptapeptide ring is composed of seven amino acids, all in L- configuration, except D-Leu at the sixth position. The intramolecular lactam ring is formed between the amino group of side chain on the Dab^4 residue and the C-terminal carboxyl group of L-Thr^{10} residue as shown in Fig (1) [7].

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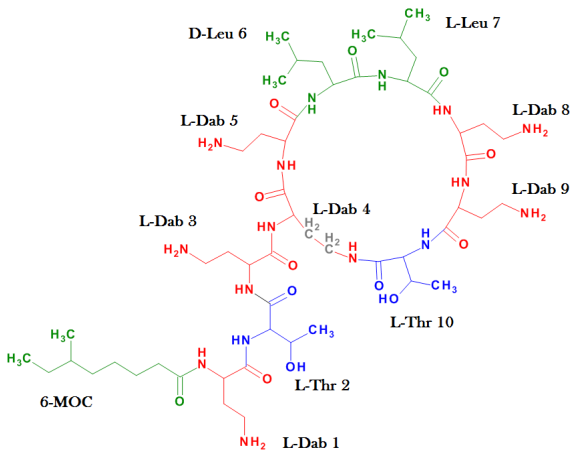


Fig.1. Structure of Polymyxin E_1

Dab: Diaminobutyric acid, Leu: Leucine, Thr: Threonine, 6-MOC: 6- methyloctanoic acid

Several works on different polymyxin analogues have been reported. For instance, the replacement of the 6-methylheptanoic fatty acid in Polymyxin B with aromatic Fmoc molecule resulted in analogues with high antibacterial activity and significant reduced toxicity [8]. Another study confirmed the importance of the presence of two Arg residues in favoring electrostatic interactions of polymyxin [9, 10]. An interesting work showed that adding the sequence formyl-Met-Leu-Phe to the linear tripeptide tail (L-Dab^1-L-Thr^2-L-Dab^3) conferred chemotactic and opsonic activities upon the conjugates, which were 8 to 10 times less toxic than the parent polymyxin antibiotic [11]. Finally, introduction of acyl derivatives to γ-Dab^1 and Dab^9 of polymyxin was investigated, where L-Arg-Dab^9 showed a ten folded improvement in the PD_{50} against *Pseudomonas* infection, while Arg-Dab^1 and Nitro-L-Arg-Dab^9 analogues were active but less than the parent compound [12]. More specifically, several works on polymyxin from our lab have been published over the years. One of the studies described the synthesis of the heptapeptide cyclic ring of Polymyxin B where all Dab residues were replaced with Lys [13]. In another study, the synthesis of Lys analogues of the cyclopeptide moiety of Polymyxin antibiotics M, D, E and B was performed and the obtained products were found to be biologically active [14].

Solid Phase Peptide Synthesis (SPPS), pioneered by Robert Bruce Merrifield in 1960s, involves the construction of a peptide chain on an insoluble solid support, allowing simple separation and purification of intermediates from soluble reagents and solvent by filtration and washing. Excess reagents are employed to help in driving reactions to completion and minimizing product losses as the peptide remains attached to the support throughout the synthesis [15]. The application of microwave heating to SPPS, introduced extensively in the early 90s, is particularly advantageous, resulting in shorter cycle times, higher repetitive yields, and ultimately purer peptides [16]. Automated SPPS offers a suitable technology to produce chemically engineered peptides. Following the first liquid-handling apparatus in 1965, many simplified and advanced peptide synthesizers have been developed, that differ mainly in the mixing technique, the type of solution transfer, the synthesis scale and the automated monitoring [17]. The introduction of Fmoc-based SPPS allowed for simpler designs of automated peptide synthesizers. These simplified and robust synthesizers have resulted in high-quality peptides with good reproducibility and short time periods [18]. Biotage® Initiator+ Alstra™ system is a fully automated microwave assisted peptide synthesizer, ideal for both small and large scale synthesis. The provided tools allow an extensive overview for the scheduling and visualization of operations in order to simplify the programming of complex peptide modifications [19]. One of the published applications of this automatic system included the on-resin synthesis reactions of cyclic peptide mupain-1 analogues which were achieved with a high degree of purity [20].

In this work, we performed the synthesis of several analogues of Polymyxin E_1, using fully automated Biotage® Initiator+Alstra™ microwave peptide synthesizer. Different cyclization techniques were applied to the SPPS methodology, where five Dab residues were replaced with the proteinogenic amino acid Arg, in addition to some modifications to the tripeptide tail.

Experimental

Protected amino acids all are in L-configuration except Leu^6 in D-configuration. TentaGel™ S-NH_2 polymer as Polystyrene-Polyethylene glycol graft co-polymer (PS-PEG-NH_2), with loading capacity 0.26 mmol/g and mesh size 90 µm on a 0.026 mmol scale, 4-Hydroxymethylbenzoic Acid (HMBA) linker and coupling agent Disopropylcarbodiimide (DIC) were obtained from commercial supplier Sigma-Aldrich. The α-amino groups of amino acids were protected by 9-fluorenylmethoxycarbonyl (Fmoc), and the side chains of Asp, Arg, Lys and Thr were protected by (OtBu), (Pbf), (Boc), and (tBu) respectively. N-Hydroxybenzotriazole (HOBt) was provided from Fluka Chemika. Benzotriazol-l-yl-oxy-tri(dimethylamino)phosphonium hexafluorophosphate (BOP) was provided from Sisco Research Laboratories Pvt. Ltd and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC.HCl) was provided from Roth Carl company. Dichloromethane (DCM) HPLC grade, Methanol (MeOH) HPLC grade were provided from Fisher Scientific and N, N-dimethylformamide (DMF) from s d fine chem-limited. Colistin Sulfate was obtained from Amon Chemical Company.

Kaiser reagent is a mixture of the following solutions in equivalent ratio: 500 mg Ninhydrin in 10 ml absolute ethanol, 80 mg Phenol in 20 ml absolute ethanol, 2 ml (0.001M) aqueous Solution of KCN in 100 ml Pyridine.

In a Kaiser tube, one drop of each of the three solutions was added to about 5 beads of the solid polymer. The mixture was heated for 5 min at 110-120°C then the color is observed. In case of coupling process, colorless beads were obtained, while in case of deprotection process, blue colored beads were noticed.

Thin layer chromatography (TLC) was performed on Silicagel 60 F_{254}-coated plates (Merck) to check for the purity of compounds. The TLC developing system used for detection was S1: Butanol/water/acetic acid/pyridine system (60:24:6:20). Reversed phase HPLC with Column water symmetry C18 (3.5µm *4.6*150 mm) was used for purity characterization. It was equipped with Agilent 1200 series pump G1311A, auto-sampler G1329 A and Agilent multi wave length detector GG1315B. The mobile phase consisted of a mixture of A: 0.1% Formic acid in H_2O and B: 0.1% Formic in acetonitrile. The mixture used was A: B (50%: 50%) The column flow-rate was maintained at 1.0 ml/min.

Infra -red spectra were recorded on FTIR, FT-IR 1650 Perkin- Elmer (Germany) spectrometer. 5 mg of sample was mixed with 100 mg of Potassium bromide (KBr) disc and scanned from 4000 to 400 cm^{-1}

Molecular masses of compounds were determined by Water UPLC/MS/MS (Acquity UPLC) mass spectrometer, equipped with mass selective detector (Xevo -TQS) with mass range from 2 to 2048 Da, capillary volt 3.2, dessolvation temperature 450°C, dessolvation gas 800L/H and nebulizer gas 7 L/H.

Melting points were determined using Stuart SMP 30 apparatus.

The peptides were prepared by Fmoc-SPPS on a Biotage® Initiator+Alstra ™ microwave peptide synthesizer equipped with a10.4" touch screen. It is used for experimental planning, instrument control, and reaction monitoring. A flexible reagent setup with modular amino acid racks and reagent bottles makes the Biotage® Initiator+ Alstra™ ideal for the synthesis of our compounds. Digital syringe pumps guarantee accurate dispensing of all reagents. Oscillating mixing within the microwave cavity ensures homogeneous heat distribution and allows for larger scale capability up to 2 mmol.

Synthesis of the Partially Protected Peptide Analogues (I-III) with Cyclization in Solution

1-Attachment of Anchoring group 4-Hydroxymethylbenzoic Acid (HMBA) to TentaGel™ S-NH_2

A solution of (0.0174 gm, 0.11 mmol) HOBt, (0.0173 gm, 0.11 mmol) HMBA and (0.0143 gm, 0.11 mmol, 0.0176 mL) DIC in 3 mL DMF in a

10 mL vessel was stirred for activation 5 min at room temperature. Then the solution was added to 10 mL reactor vial containing pre-swelled TentaGel™ S-NH_2 (0.1gm, 0.026 mmol) and mixed by oscillation with microwave irradiation at 75°C for 10 min. The completeness of reaction was checked by Kaiser Test. The polymeric support was filtered off and washed several times with DMF, DCM, MeOH, and finally with ether.

2-Attachment of the First Amino Acid to the Polymeric support in all sequences (I-III)

A solution of (0.0174 gm, 0.11 mmol) HOBt, (0.0453 gm, 0.11 mmol) Fmoc-Thr(tBu)-OH and (0.0143 gm, 0.11 mmol, 0.0176 mL) DIC in 3 mL DMF in 10 mL vessel was stirred for activation 5 min at room temperature then was added to 10 mL reactor vial containing a polymeric support (0.1gm,0.026 mmol) and mixed by oscillation with microwave irradiation at 75°C for 10 min. The completeness of reaction was checked by Kaiser Test. The resin was filtered off and washed several times with DMF, DCM, MeOH and finally with ether.

Spectroscopic Determination of the Solid Support Coupling Capacity was done according to Meienhofer method [21] where: The optical density at 267nm [u.v. λ max 267nm (ε 17.500) or at 290 nm (ε 5.800) and 301nm (ε 7.800)] of a sample of (0.1 mL, diluted to l mL with CH_2C1_2) presents the Fmoc-acid acyl content in mmol per gram of resin according to the following equation:

[c (mmol/g) = (OD x 10^4)/17.500)].

3-Deprotection of Fmoc N^{α} -Amino Protecting Group

A solution of 20%Piperdine /DMF (4.5 mL) was added to 10 mL reactor vial containing a pre-swelled polymeric support attached to Fmoc-Thr(tBu)-OH (0.1 gm, 0.026 mmol) ~~of~~ and was mixed by oscillation at room temperature until Kaiser Test showed a positive result. Then the polymeric support was filtered off and washed several times with DMF, DCM, DMF, DCM, MeOH and finally with ether.

4-Coupling of Next Fmoc N^{α} Protected Amino Acid

A solution of (0.0174 gm, 0.114 mmol) HOBt, (0.0739 gm, 0.114 mmol) Fmoc-Arg(Pbf)-OH and (0.0143 gm,0.0176 mL ,0.114 mmol)DIC in 3 mL DMF in 10 mL vessel was stirred for activation 5 min at room temperature then was added to 10 mL reactor vial containing a pre-swelled polymeric support attached to the first amino acid (0.1 gm , 0.026 mmol) and was mixed by oscillation for10 min with microwave irradiation at 75°C then sample of polymer bound peptide was tested by Kaiser reagents to monitor coupling . The polymeric support bound peptide to was then filtered off and washed several times with DMF, DCM, DMF, DCM, MeOH and finally with ether.

5-Capping of Unreacted Amino Group

A mixture of 1mL of 5M AC_2O in DMF and 2.5 mL of 2M DIEA in DMF was added to 10 mL reactor vial containing a pre-swelled protected peptide resin (0.1gm, 0.026mmol) and was shacked at room temperature for 10 min. Then, the resin was filtered off and washed several times with DMF, DCM, DMF, DCM, MeOH and finally with ether.

6-Sequential Deprotection and Coupling of Next Fmoc-N^{α} Amino Acids followed by Capping Reaction.

Steps 3, 4 and 5 were repeated for each protected amino acid residue assembled on the peptide chain according to the sequences of the target compound.

7-Boc-deprotection of N^{ε} Lysine residue of protected linear peptide on polymeric support.

The lyophilized fully protected linear peptide was dissolved in 3 mL of 50% (v/v) TFA / DCM mixture and was stirred at room temperature for 30 min. The reaction was monitored by TLC using the solvent system (Chloroform: Methanol: Acetic Acid 85/10/5) and positive ninhydrin test indicated the completeness of deprotection reaction. The solvent was evaporated under high vacuum, and the residue was then washed three times with ether and dried under vacuum.

8-Cleavage of the Synthesized Protected Linear peptide from the Polymeric Support

A mixture of 4 mL of 1M NaOH/dioxane (3:1) was cooled to 0 °C in an ice/water bath, was added to 10 mL reactor vial containing a pre-swelled peptide-resin and was left to stand at room temperature for about 20 min with occasional shaking. The resin was then filtered and an adequate volume of 1M HCL was dropwisely added while cooling in an ice bath until the pH of the filtrate was adjusted to 3. The filtrate was then washed with diethyl ether to get rid of any impurities and then precipitated and dried under vacuum or lyophilized. In order to measure cleavage efficiency, the same step of coupling capacity measurement was performed on

the dried unsubstituted solid support following the cleavage of the synthesized linear peptide [21].

9-C-terminal to N^{ε} -Side Chain Cyclization of Linear protected peptides in Solution

Analogues (I&II)

A solution of partially deprotected linear peptide, HOBt, EDC. HCl, in DMF/ DCM (1:3) was mixed with TEA in the respective ratio (1:2:2:1). The stirred mixture was cooled to 0°C. The temperature of mixture was left to rise to 25 °C. After stirring for 22 hours, the mixture was monitored by TLC till reaction completion, then concentrated in vacuo and washed with water. The obtained oily residue was triturated with dry ether and then lyophilized.

Analogue (III):

A solution of partially deprotected linear peptide, HOBt, BOP, in DMF/ DCM (1:3) was mixed with TEA in the respective ratio (1:2:2:1). The stirred mixture was cooled to 0°C then the temperature was left to rise to 25 °C. After stirring for 22 hours, the mixture was monitored by TLC till reaction completion, then concentrated in vacuo and washed with water. The obtained oily residue was triturated with dry ether and then lyophilized.

10-Removal of Side Chain Protecting Groups of partially protected cyclized peptide in solution

A cleavage cocktail of 4 mL of TFA: Thioanisole: EDT: H_2O (95:2.5: 1.25:1.25) was added to the partially protected cyclized peptide and the suspension was shacked at room temperature from one to four hours. The solution was washed several times with DCM, 10% DIPEA in DCM and then evaporated under reduced pressure. The residue was stored and dried under vacuum.

Synthesis of The Peptide Analogues (IV-V) with cyclization on polymeric support

The same steps (1-6) were applied using the required Fmoc-amino acids except in step 2 where Fmoc-Thr(tBu)-OH was replaced with Fmoc-Asp(OtBu)-OH.

7-Deprotection of Boc of N^{ε} of Lysine and OtBu protecting groups of Aspartic residues on polymeric support.

In 10 mL reactor vial, the pre-swelled full protected peptide attached to polymeric support was suspended in 3 mL 50% (v/v) TFA/DCM under shaking at room temperature for 30 min. The Boc-deprotection was monitored by the positive blue beads color of Kaiser Test, and then the suspension was filtered off, washed three times with DCM and three times with 5% (v/v) diisopropylethylamine (DIPEA)/DCM. Sample of polymer bound peptide was tested by Kaiser Reagent.

8-C^{α}-Side chain to N^{ε}-Side chain Cyclization of Linear partially protected peptide on the polymeric support.

A solution of (0.0174 gm, 0.114 mmol) HOBt, (0.0143 gm, 0.0176 mL, 0.114 mmol) DIC and (0.031 mL 0.11 mmol) Et_3N triethylamine were added to a 10 mL reactor vial containing a swelled (0.1g m, 0.026 mmol) of partial protected peptide attached to polymeric support. The mixture was subjected to oscillation mixing with microwave irradiation at 75°C for 10min. Then sample was tested by Kaiser reagent and washed several times with DMF, DCM, DMF, DCM, MeOH and ether finally dried and stored under vacuum.

9-Removal of Side Chain Protecting Groups of partially protected cyclized peptide on resin

A cleavage cocktail of 4 mL of TFA: Thioanisole: EDT: H_2O (95:2.5: 1.25:1.25) was added to partially protected cyclized peptide attached to polymeric support and was shacked at room temperature from one to four hours. The resin was filtered off and washed several times with DCM, 10% DIPEA in DCM and then evaporated under reduced pressure. Finally, the fully deprotected cyclic peptide was dried and stored under vacuum.

10-Cleavage of the Synthesized Cyclized peptide from the Polymer

A mixture of 4 mL of 1M NaOH/dioxane (3:1) was cooled to 0 °C in an ice/water bath then was added to the pre-swelled free peptide attached to polymeric support and was left to stand at room temperature for about 20 min with shacking. The resin was filtered then an adequate volume of 1M HCL was added dropwisely with cooling in an ice bath until the pH of the filtrate was adjusted to 7. The filtrate was evaporated under vacuum and then lyophilized and stored under vacuum. The Solid Support Coupling Capacity was determined as mentioned previously.

The purity of the obtained peptide chains was investigated by HPLC (Fig 2) and mass spectroscopy (Fig 3). The functionality of the characterized peptides was characterized using IR spectroscopy.

Characterization Data for Analogues (I, II, III, IV, V)

Analogue I

Yellowish oily, R_f 0.28 with S1, Reversed HPLC retention time (t_r) 2.060 min, percentage area 63.23%, IR (KBr) cm^{-1}: 3421 (NH stretching, NH_2, OH alcohol), 2993 (CH aliphatic), 1672 (C=O), Mwt 1559.86, M/z 1559. 03, MS/MS 635.03 corresponding to (Fmoc-Arg-Thr-Arg $CO^{+)}$ fragment,1338.65 corresponding to analogue with Fmoc group fragment loss, 840.59 corresponding to heptapeptide ring with isopropyl fragment loss, 1478.32 corresponding to analogue with guanidino part fragment loss $(NH_2=C-NH)_2$ left Cpd with $NH_3^+)_2$

Analogue II

Yellowish oily, R_f 0.27 with S1, Reversed HPLC retention time (t_r) 2.058 min, percentage area 87.85 %, IR (KBr) cm^{-1} 3442 (NH stretching, NH_2, OH alcohol), 2993 (CH aliphatic), 1675 (C=O). Mwt 1403.68, M/z 1402.04, MS/MS 223.73 due to fragment (Fmoc CO^+), 1345.91 due to one guanidino group loss, 683.09 corresponding to heptapeptide ring with isopropyl fragment

Analogue III

Yellowish oily, R_f 0.63 with S1, Reversed HPLC retention time (t_r) 3.094 min, percentage area 93.86 %, IR (KBr) cm^{-1}, 3422 (NH stretching, NH_2, OH alcohol), 2997 (CH aliphatic), 1767(C=O ester), 1665 (C=O), 72 ($S-CH_3$), Mwt 1537.91, M/z 1535.99, MS/MS 614.14 corresponding to Fmoc Met-Leu-Phe-CO^+ fragment, 1451.75 corresponding to analogue with isopropyl loss, 1495.08 corresponding to (analogue-NH_3^+) due to guanidino part fragment loss (NH_2=C-NH)

Analogue IV

White solid, m.p 235-237, R_f 0.57 with S1, Reversed HPLC retention time (t_r) 2.197 min., percentage area 87%, IR (KBr) cm^{-1} 3423 (NH stretching , NH_2 , OH alcohol), 2926 (CH aliphatic), 2818.45(OH acid), 1605.45 (C=N), 1671, 1244 (C=O amide I, III) cm^{-1}, Mwt 1573.85, M/z 1572.08 MS/MS 1541.80 corresponding to (analogue-NH_3^+), 1409.72 corresponding to (analogue-NH_3^+), 1409.72 corresponding to analogue with guanidino part fragment loss $(NH_2=C-NH)_4$, 1095.33 corresponding to analogue-NH_3^+ due to fragment loss (Fmoc Arg Thr)

Analogue V

White solid, m.p 179-180, R_f 0.53 with S1, Reversed HPLC retention time (t_r) 2.448 min., percentage area 100% , IR (KBr) cm^{-1}, 3423 (NH stretching, NH_2, OH alcohol), 2923 (CH aliphatic), 1638, 1542 (C=O amide I,II) cm^{-1}, Mwt 1619.92, M/z 1619.31 MS/MS 224.84 due to fragment loss (Fmoc CO^+), 1575.03(analogue-CO_2), 1572.08 (analogue -$NH_3^+)_3$.

Results and Discussion

Peptides have diverse functions that range from hormones, neurotransmitters, growth factors to antibiotics and hence they are an optimal target for the pharmaceutical industry [22]. In fact, therapeutic peptides possess many valuable attributes compared to traditional small molecules, mainly high target potency and selectivity, lower side effects, and less toxic degradation products. Furthermore, they do not tend to interact with other drugs and show more in vivo predictability, making peptides a novel perspective for drug development [23]. Due to the chemical and biological diversity of peptides, several diseases can be treated with peptides and peptide-based drugs, such as cancer, diabetes, inflammation and some cardiovascular diseases [24]. Particularly, peptides have been introduced as alternative line of therapy to commonly used antimicrobial drugs, as a result of the growing threat of antibiotic resistance by Gram-negative bacteria. For instance, several antimicrobial peptides and peptide conjugates have been identified recently by members of our lab [25-29]. Well-known antibacterial polymyxins cause initial electrostatic interaction with the negatively charged phosphate groups of the outer membrane of Gram-negative bacteria, resulting in formation of destabilized areas through which polymyxin crosses the outer membrane and causes cell lysis [30].

Among all the research papers discussing the synthesis of various analogues of polymyxin antibiotics, this work represents the first one focusing on the replacement of five Dab residues in both the cyclopeptide moiety of Polymyxin E_1 and in the tripeptide tail, with a natural non-substituted amino acid, Arg. The only Dab residue involved in lactam cyclization was substituted with another natural amino acid, Lys. Indeed, previous study confirmed the importance of the presence of two Arg residues in favoring

electrostatic interactions of polymyxin [9, 10]. On the other hand, several works from our lab studied the synthesis of Lys analogues of different types of polymyxins [13, 14]. It was previously shown that the antibacterial activity of small peptides is mainly governed by charge and lipophilic bulk. The charged moieties consisted of either the side chains of Arg or Lys, where the guanidinium group of Arg was preferred [31]. The current work represents a new approach in the synthesis of Arg and Lys analogues of Polymyxin E_1 using automated modified SPPS.

In this paper, two different modifications have been introduced to the tripeptide tail already containing Arg instead of Dab. In analogue V, Thr was replaced with Phe, while in analogue III, the whole tail has been replaced with Met-Leu-Phe. In fact, Phe imparts bulk hydrophobic part to the polymyxin analogue and presents the advantage of ease of synthesis as there is no requirement for additional protection of the side chain. Concerning analogue III, it was shown that the sequence Formyl-Met-Leu-Phe [11] conferred chemotactic and opsonic activities upon the conjugates, which were 8 to 10 times less toxic than the parent polymyxin antibiotic. The following five polymyxin analogues (I-V) were synthesized using Fmoc- SPPS with microwave technique application (Table 1).

Here, the Polymyxin E_1 decapeptide analogues (I-V) were synthesized using the Fmoc-SPPS strategy. In SPPS, the carboxylic acid terminus of the first amino acid is coupled to a polymeric support (solid resin with organic linker), while the N^{α}-amino group and the reactive side chain moieties are protected. The N-terminal protecting group is temporary, while the side-chain protecting groups and the resin remain till the end of the synthesis to prevent any unwanted side reactions. The deprotected N^{α}-amino group is allowed to couple with the next activated carboxy group of the second amino acid. Alternating steps of N^{α}-deprotection, activation and coupling are repeated till the desired peptide sequence is assembled on the polymeric support. Each coupling reaction is followed by a capping step. This step is crucial to block the unreacted amino groups and inhibit formation of deleted sequences of amino acids which decrease the purity of final product. Finally, the N-terminal amino acid is deprotected, the side-chain protecting groups are removed, and the peptide is cleaved from the polymeric support.

In this work, TentaGel™ was used as the resin, accounting for the modification of the original SPPS technique (named modified SPPS). TentaGel ™ resins are grafted copolymers consisting of a low crosslinked polystyrene matrix on which polyethylene glycol (PEG) is grafted. These graft copolymers contain about

TABLE 1. Structure of Synthetic Target Peptide Analogues (I-V) Using Modified Solid Phase Peptide Synthesis Assisted With Microwave Irradiation.

Parent	Fatty Acid	Linear tripeptide	Cyclic part
		1 2 3	4 5 6 7 8 9 10
	6-MOC	Dab^1-Thr^2-Dab^3	**Dab**-Dab-D-Leu-L-Leu-Dab-Dab-Thr
Synthesized Analogues			
I.	Fmoc	Arg^1-Thr^2-Arg^3	**Lys**^{4}-Arg^5-D-Leu^6-L-Leu^7-Arg^8-Arg^9-**Thr**^{10}
II.	Fmoc	Arg^1-Thr^2-Arg^3	**Lys**^{4}-D-Leu^5-L-Leu^6-Arg^7-Arg^8-**Thr**^{9}
III.	Fmoc	Met^1-Leu^2-Phe^3	**Lys**^{4}-Arg^5-D-Leu^6-L-Leu^7-Arg^8-Arg^9-**Thr**^{10}
IV.	Fmoc	Arg^1-Thr^2-Arg^3	**Lys**^{4}-Arg^5-D-Leu^6-L-Leu^7-Arg^8-Arg^9-**Asp**^{10}
V.	Fmoc	Arg^1-Phe^2-Arg^3	**Lys**^{4}-Arg^5-D-Leu^6-L-Leu^7-Arg^8-Arg^9-**Asp**^{10}

50-70% polyethyleneglycol attached to the matrix via an ethyl ether group and an X functional group end (in our case NH_2). TentaGel™ - NH_2 was chosen for its inertness towards the used chemical reagents, the improved physicochemical characteristics, good swelling properties, and withstanding mechanical properties. Thus, this modified technique combines the benefits of the soluble polyethylene glycol support and the insolubility characteristics of the polystyrene bead. It allows assembly of growing peptide chain onto solid polymer and provides good solvation requirement and penetration of reagents due to its liquid properties.

The key step of SPPS is the attachment of the target assembled growing peptide to solid support via its C-terminal α-carboxyl group. 4-hydroxymethylbenzoic acid (HMBA) base labile bifunctional linker acts as a linkage between the assembled peptide chain and the solid support used through the synthesis of our compounds [32]. The linker binds to the Tentagel resin through amide bond formation between the amino group of Tentagel and the carboxy group of HMBA. One of the advantages of HMBA base labile linker is the ability to partial acid deprotection of the assembled peptide chain using TFA while keeping it still attached to the polymeric support. This enables the several modification reactions on the peptide chains to occur as well as the cyclization reactions on the resin. Another advantage of HMBA base labile linkers is the cleavage via nucleophilic substitution reaction. As the linker binds to the assembled growing peptide chain through ester bond formation, any applied nucleophile attacks the peptide carbonyl group of the ester bond, resulting in the release of the peptide chain from the polymeric support, and potential modification of the C-terminal group with different nucleophiles during the cleavage step. The completeness of HMBA linker coupling to TentaGel™ resin is simply indicated by the colorless white beads through Kaiser Test. The recoupling reaction could be repeated, if necessary, to get the polymeric support without any uncoupled amino group. The esterification coupling of activated C-terminal-N^{α}-protected amino acid residue with HMBA was carried out using DIC/ HOBT as coupling agent. 4-dimethylaminopyridine (DMAP) was added to catalyze the formation of benzotriazole active ester in situ under microwave irradiation for 10 min at 75°C [33].

The use of Fmoc group as N-terminal protecting group serves many purposes. Firstly, Fmoc can be removed easily with base such as piperidine in aprotic solvents (DMF), and therefore can be used in conjunction with acid-labile side chain protecting groups such as tBu and Boc achieving orthogonality approach. Secondly, it enables monitoring of coupling and deprotection reactions by UV spectroscopy [34]. Finally, as mentioned previously, it was shown that replacing the fatty acid in polymyxin with aromatic Fmoc molecules resulted in analogues with high antibacterial activity and significant reduced toxicity [8].

The time required for complete coupling and deprotection reactions for the first amino acids residues (either Fmoc-Thr(tBu)-OH in compounds I, II and II, or Fmoc Asp(OtBu)-OH in compounds IV and V) and percentage of the polymeric support capacity in the synthesized analogues by modified SPPS using microwave energy are indicated in Table 2. Coupling reactions were repeated when necessary.

All the coupling and deprotection reactions of the successive amino acids for the five analogues were designed to enhance coupling and deprotection efficiency in SPPS. It was done inside the microwave cavity of the fully automated Biotage® Initiator+ Alstra™ system. In addition, four-fold excess of Fmoc amino acid and DIC/HOBt coupling agents were used instead of eight-fold commonly used in conventional synthesis [35]. In such protocol, the in situ benzotriozyl active ester of the protected amino acid residue reacts with the amino group of the growing peptide chain and provides nonacidic, efficient leaving group that allows the peptide bond formation, in each coupling step through all the synthesized peptide chains. For each coupling reaction, washing solvents, capping, coupling and cleavage mixtures, and activated Fmoc amino acids were automatically inserted into the reaction vessel [19, 20]. Complete coupling and deprotection reactions were followed up by Kaiser Test [36] and spectroscopic loading capacity measurements [21]. Deprotection reactions of Fmoc protecting group were carried out at r.t. inside the reaction vessel but without microwave assistance with 20% piperdine / DMF. Indeed, the electron withdrawing fluorene ring system of the Fmoc group renders the lone hydrogen on the β-carbon very acidic and, therefore, susceptible to removal by weak bases [34]. The time required for complete coupling using microwave and deprotection reactions for in the synthesis for polymyxin E_1 decapeptide analogues is represented in Table 3.

TABLE 2. The Solid Support Coupling Capacity in the synthesized sequences, the time required for complete coupling and deprotection reactions for [I,V]by Modified Solid Phase Peptide Synthesis Assisted With Microwave Irradiation

Fmoc-Amino Acid	% of maximum coupling capacity	Time required for maximum coupling in min	Time required for Maximum Fmoc deprotection in min
Fmoc-Thr(tBu)-OH	95	10	33
Fmoc-Asp(OtBu)-OH	94	10	35

TABLE 3. The time required for maximum coupling using microwave and deprotection reaction at r.t. for peptide sequences [I-V]

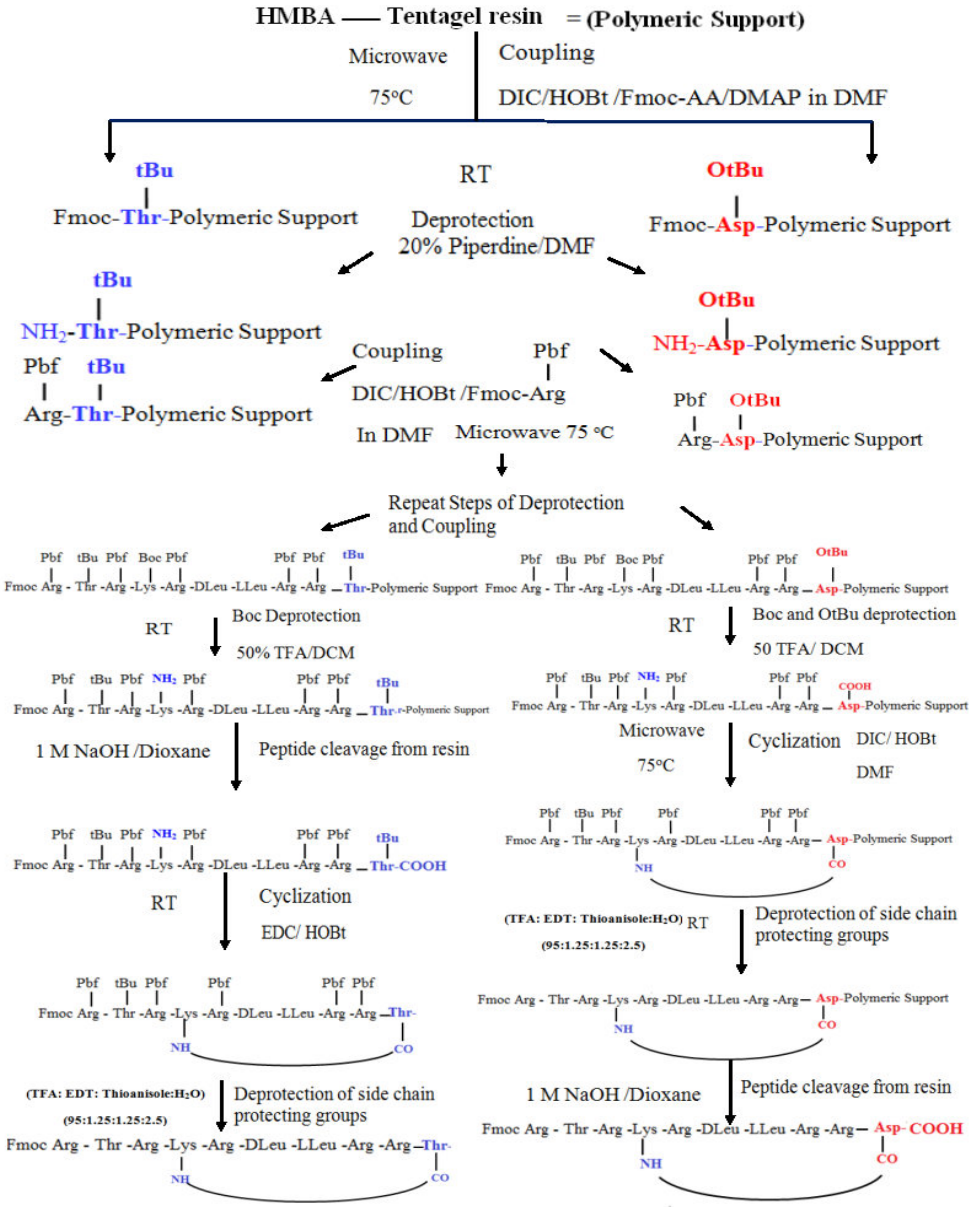
Fmoc AA	Analogue I		Analogue II		Analogue III		Analogue IV		Analogue V	
	Cyclized in solution						Cyclized on polymeric support			
	D/min	C/min	D/min	C/min	D/min	C/min	D/min	C/min	D/min	C/min
Fmoc-Thr residue **10**	30	10	33	10	25	10	-------	-------	-------	-------
Fmoc-Asp residue **10**	-------	-------	-------	-------	-------	-------	33	10	35	10
Fmoc-Arg residue **9**	20	10	40	10	30	10	29	10	40	10
Fmoc-Arg residue **8**	40	10	43	10	35	10	35	10	43	10
Fmoc-L-Leu residue **7**	40	10	45	10	47	10	40	10	40	10
Fmoc-D-Leu residue **6**	43	10	39	10	40	10	45	10	44	10
Fmoc-Arg residue **5**	36	10	-------	-------	45	10	47	10	47	10
Fmoc-Lys residue **4**	45	10	45	10	60	10	55	10	45	10
Fmoc-Arg residue **3**	47	10	39	10	47	10	35	10	47	10
Fmoc- Phe residue **3**	-------	-------	-------	------	50	10	------	-------	------	------
Fmoc-Thr residue **2**	47	10	50	10	-------	-------	42	10	------	-------
Fmoc-L-Leu residue **2**	-------	-------	-------	-------	47	10	--------	------	------	------
Fmoc- Phe residue **2**	-------	-------	-------	-------	-------	-------	-------	-------	50	10
Fmoc-Arg residue **1**	*****	10	*****	10	-------	-------	*****	10	*****	10
Fmoc-Met residue **1**	-------	-------	-------	-------	*****	10	------	------	------	------

C/min indicates the coupling time for each residue
D/min indicates the Fmoc deprotection time for each residue
***** indicates that the deprotection of Fmoc group was not applicable

The application of microwave energy with SPPS has been successfully used to increase the rate of peptide coupling reactions and not generate appreciable racemization. The increased rate of peptide coupling was reflected in our work as the completeness of reactions within only 10 mins. Alternatively, deprotection reactions that occurred at room temperature without microwave were time-consuming and varied between 20 to 50 mins according to the nature of the amino acid itself and the peptide chain length [37].

In this work, cyclization step of the different analogues represents an important factor in the synthesis protocol using solid phase methodology. Different techniques of cyclization were considered. We investigate the difference between the C-terminal of Thr to N^{ε}-terminal of Lys head to side chain cyclization in solution (analogues I-III) versus side chain to side chain cyclization (the N^{ε}-terminal Boc of Lys and the OtBu β-carboxylic side chain of Asp) on polymeric support using the fully automated Biotage® Initiator+ Alstra ™ system (analogues IV and V). In case of cyclization in solution (analogues I-III), the t-Boc protecting group of N^{ε}-terminal of Lys was easily removed by 50% TFA/DCM at room temperature for 30 min followed by cleavage of peptide chain attached to polymeric support by saponification using 0.1M NaOH/dioxane to produce linear fully protected peptides. The cyclization step was performed by reacting the amino N^{ε}-terminal of Lys^{4} residue and the free C-terminal carboxyl group of ($L\text{-}Thr^{10}$) in a head-to-tail cyclization step. On the other hand, replacing $L\text{-}Thr^{10}$ with Asp in compounds IV and V allowed the removal of both protecting groups, the N^{ε}-terminal Boc of Lys and the OtBu of β-carboxylic side chain of Asp in a single step. In this case, side chain to side chain cyclization occurred while the growing peptide chain was still attached to the polymeric support under heterogeneous conditions. Consequently, there is no requirement to cleave the resin before the cyclization, thus allowing the use of the fully automated Biotage® Initiator+ Alstra ™ system.

Scheme (1) shows the schematic synthetic steps performed for two analogues as a reference for each cyclization technique (analogue I for cyclization in solution (left) and analogue IV (right) for cyclization on polymeric support.



In Solution Cyclization
In Analogue I

On Polymeric Support Cyclization
In Analogue IV

Scheme1. Schematic synthetic steps performed during the cyclization in solution in analogue I (Left) and on polymeric support in analogue IV (Right).

In this work, the use of different coupling agents [38] was also investigated. Cyclization was performed using three different coupling reagents, EDC.HCl condensing agent in case of analogues (I-II), BOP in case of analogue (III) and DIC condensing agent in case of analogues (IV-V) under heterogenous conditions. In solution cyclization, EDC.HCl is a highly suitably used coupling agent because this reagent and its urea are soluble in aqueous solvents and can therefore be removed in the workup [39]. Cyclization using BOP coupling reagent proceeds more rapidly and to a greater degree of completion for peptide bond formations [40], which could be a factor explaining the higher purity obtained using BOP versus EDC. HCl where results show a 19.5% increase in purity and 13% increase in yield. On polymeric support cyclization, DIC is a highly processed coupling agent because its diisopropyl urea byproduct is more soluble in DCM, which therefore, renders it easier to wash off from the solid support [41]**.** Table 4 summarizes the different conditions for the cyclization reaction, in terms of temperature, time, coupling agent, yield, purity and consistency of the cyclized analogues **(I-V).**

The final step is the deprotection of all side chain protecting groups of Asp, Arg and Thr (OtBu), (Pbf), (tBu)respectively to obtain full free cyclized peptide using cleavage cocktail (TFA: Thioanisole: EDT: H_2O) in the ratios (95:2.5: 1.25:1.25). The most frequently used scavenger is water, which is moderately effective at scavenging t-butyl cations and the products of the cleavage of arylsulphonyl-based protecting groups. 1, 2-Ethanedithiol (EDT) is the best scavenger for t-butyl cations and suppression of methionine oxidation and accelerate removal of (Pbf) from Arg residues [42].

Table 4. The Cyclization Techniques of Synthetic Peptide Analogues (I-V)

Analogue	Cyclization Conditions				Yield	purity	Consistency
	Temperature	Time	Coupling agent	Condition			
I.	Room Temperature	22 hrs	EDC /HOBt	Manual, In solution	49%	63.23%	Yellowish, Oily
II.	Room Temperature	22 hrs	EDC /HOBt	Manual, In solution	55%	87.85%	Yellowish, Oily
III.	Room Temperature	22 hrs	BOP/ HOBt	Manual, In solution	60%	93.86%	Yellowish, Oily
IV.	Microwave 75°C	10 min	DIC/HOBt	Automated On polymeric support	72%	87%	Solid, White
V.	Microwave 75°C	10 min	DIC/HOBt	Automated On polymeric support	78%	100%	Solid, White

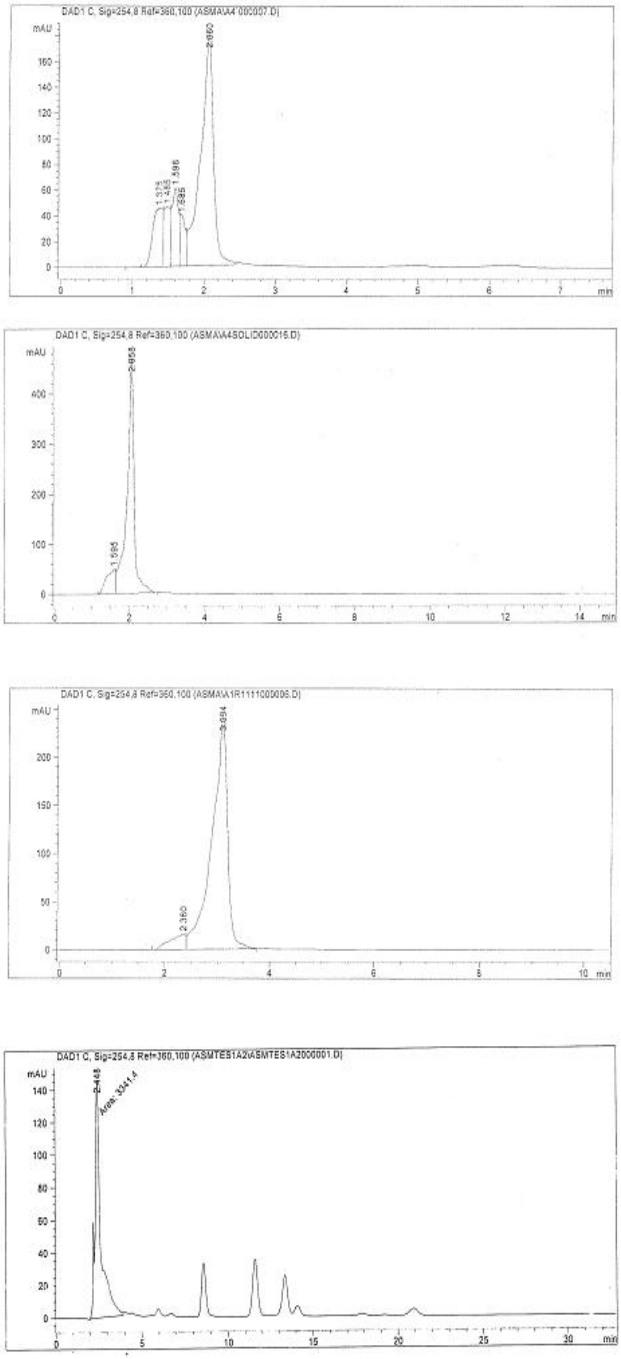


Fig.2. HPLC Charts of Analogues (I, II, III, V)

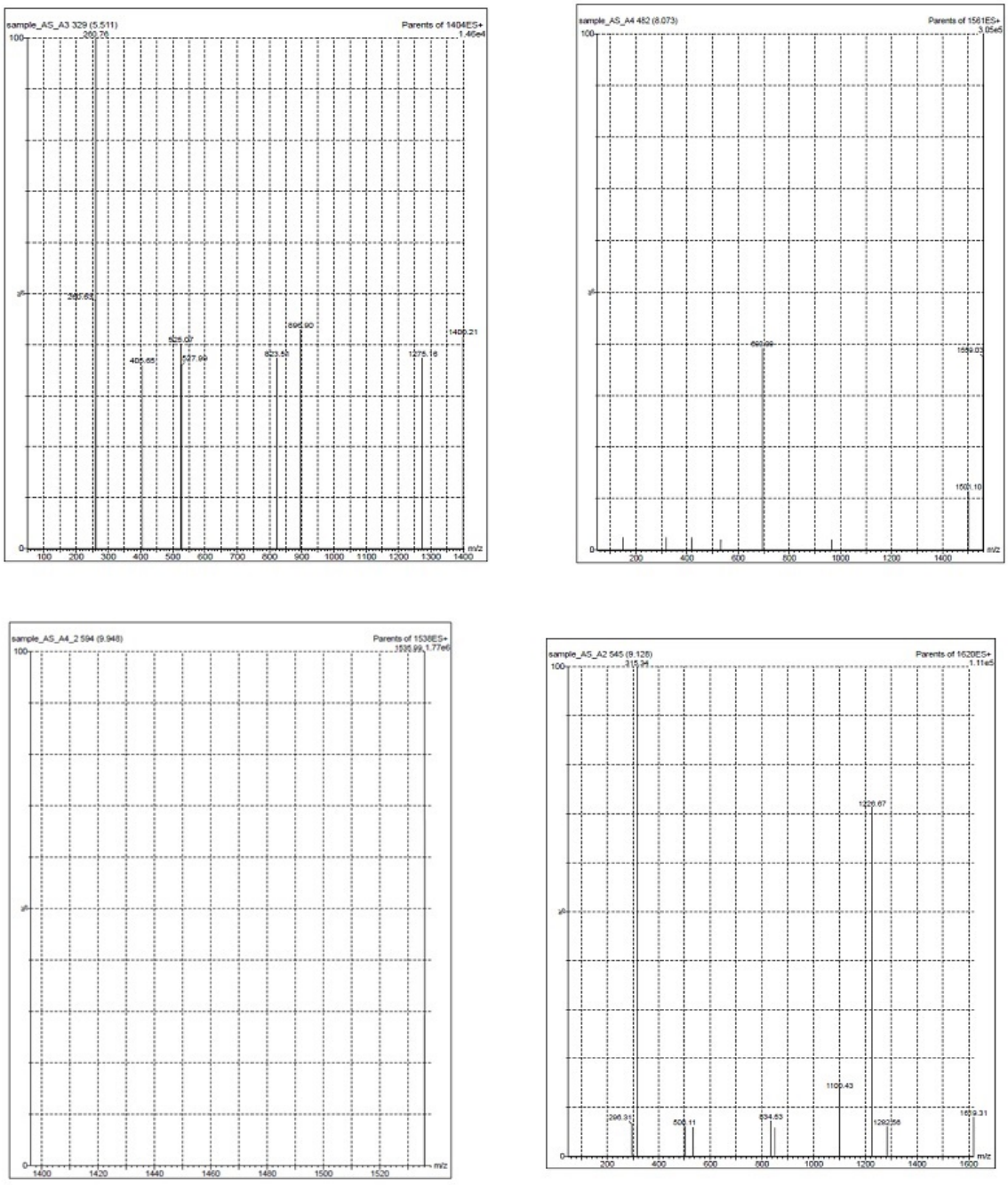


Fig. 3. Mass Spectra of Analogues (I, II, III and V)

Conclusion

The present study describes the synthesis and characterizes five different analogues of polymyxin E_1, using fully automated Biotage® Initiator+ Alstra ™ microwave peptide synthesizer. Solid support cyclization of analogues IV and V using the fully automated system resulted in a 27% increase in the yield and an average 13% increase in purity, as shown in the HPLC diagrams, also demonstrated by the better consistency of the compounds. The automated procedure is clearly less time-consuming than the manual conventional cyclization.

The application of microwave energy with SPPS has been successfully used to increase the rate of peptide coupling reactions of our compounds. Whenever cylization in solution would be the only option available, the coupling agent of choice would be BOP as its use improved the purity and yield of the synthesized compounds.

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تم تشييد خمسة متشابهات من المضاد الحيوي بوليمكسين E_1 بطريقة السطح الصلب والحماية الأمينية بمجموعة - Fmoc باستخدام جهاز التشييد الاتوماتيكى المزود بأشعة الميكروويف Biotage® Initiator+Alstra ™. فالهدف من العمل هو دراسة تأثير استبدال الحمض الأميني ٤,٢- ثنائي امينو بيوترك الموجود بالموضع-رقم ٥ و٨ و٩ بالتسلسل البيتيدي بالحمض الامينى ارجنين. و أيضا استبدال نفس الحمض الأميني الموجود بالموضع-رقم ٤ بالتسلسل والمسئول عن تكوين الرأس الحلقية البيتيدية السباعية بالحمض الامينى ليسين. وقد تم أستبدال الحمض الامينى رقم عشرة الثريونين فى بعض المتشابهات بالحمض الامينى الاسبرتك للحصول على الرأس البيتيدية الحلقية بطرق متنوعة. وقد تم تغيير تسلسل الأحماض الأمينية بالسلسلة الطرفية الثلاثية فى متشابه اخر وذلك لقياس الفاعلية البيولوجية لتلك المركبات. وللتأكد من صحة التراكيب الكيمائية للسلاسل البيتيدية المطلوبة فقد تم عمل التحاليل الطيفية و الكروماتوجرافية اللازمة