Tutor/s

Dr. Ernesto Nicolás Galindo
Departament de Química Inorgànica i Orgànica, Secció de Química Orgànica

Grau de Química

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Preparation of protected peptides by solid phase for a convergent synthesis.

Preparación de péptidos protegidos en fase sólida para una síntesis convergente.

Oriol Galeote Martin

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Quien nunca ha cometido un error nunca ha intentado nada nuevo.

Albert Einstein

Después de estos meses de trabajo, me gustaría agradecer a todas aquellas personas que me han apoyado durante el camino, sin ellos no hubiese sido posible llegar hasta el final.

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REPORT

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1. SUMMARY

Nowadays, the interest on the use of peptides as drugs is increasing due to their unique properties, such as high selectivity and low toxicity. As a result, pharmaceutical industry has more than 500 peptides that are currently in preclinical development and it is estimated that the peptides market involves 25.4 million dollars per year.[1]

This project was carried out in the *Smbiocom* research group, which collaborates with a pharmaceutical company which aims to synthetize a bioactive peptide with oncological properties and scale the method up to a pilot plant production. The strategy taken in the laboratory has consisted on a convergent synthesis of a fragment with eleven amino acids of the target peptide. In the first place, different peptide fragments have been synthetized in solid phase and finally they have been coupled in solution.

Five different protected peptide fragments of 4, 5, 6 and 9 amino acids have been synthetized by solid-phase methodology using the Fmoc/^{t}Bu strategy (1.2 mmol, 1.9 mmol, 1.1 mmol and 1.8 mmol scales) with a 2-CTC solid support resin. The reactions have been carried out using DIC as a coupling reagent, HOBt as an additive and finally each fragment has been characterized by HPLC-MS using reverse phase chromatography.

Furthermore, the C-terminal protected dipeptide fragment of the target peptide has been synthetized in solution and has been coupled to the nonapeptide in solution. The resulting full protected undecapeptide has been characterized by HPLC-MS.

Keywords: solid-phase synthesis, convergent synthesis, protected peptide, DIC, HOBt

2. RESUMEN

Hoy en día el interés en el uso de péptidos como medicamentos está aumentando debido a sus propiedades únicas, como por ejemplo su alta selectividad y baja toxicidad. Como resultado, la industria farmacéutica tiene más de 500 péptidos en desarrollo preclínico y se estima que el mercado de los péptidos involucra 25.4 millones de dólares anualmente.[1]

Este proyecto se ha desarrollado en el grupo de investigación *Smbiocom,* el cual colabora con una compañía farmacéutica que tiene el objetivo de sintetizar un péptido bioactivo con propiedades oncológicas y escalar la metodología hasta una producción de planta piloto. La estrategia seguida en el laboratorio ha consistido en una síntesis convergente de un fragmento de once aminoácidos del péptido final. En primer lugar, se han sintetizado diferentes fragmentos en fase sólida y finalmente se han acoplado en solución.

Cinco péptidos protegidos diferentes de 4, 5, 6 y 9 aminoácidos se han sintetizado mediante la metodología de fase sólida usando una estrategia Fmoc/^{t}Bu (en una escala de 1.2 mmol, 1.9 mmol, 1.1 mmol y 1.8 mmol) usando la resina 2-CTC como soporte sólido. Las reacciones se han llevado a cabo usando DIC como agente de acoplamiento, HOBt como aditivo y finalmente cada fragmento se ha caracterizado por HPLC-MS usando una cromatografía de fase inversa.

Además, se ha sintetizado en solución el dipéptido protegido C-terminal del péptido final y se ha acoplado con el nonapéptido en solución. El undecapéptido resultante completamente protegido se ha caracterizado por HPLC-MS.

Palabras clave: síntesis en fase sólida, síntesis convergente, péptido protegido, DIC, HOBt

3. INTRODUCTION

3.1. PEPTIDES AS DRUGS

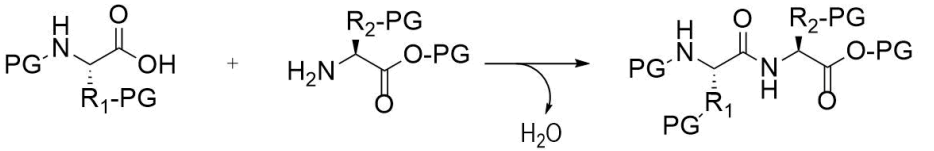
Peptides play many different roles in the organism. The most important ones are as intercellular communicators in the form of hormones, their participation in the immune system in the form of antibodies or as transport of molecules through membranes, amongst others.[2] Their relevance in a large number of biological processes makes their use as drugs an important field because of their potentially usefulness in many different diseases.

Nowadays, some peptides are commercially available and they can be applied therapeutically in cancer, HIV or osteoporosis, amongst other diseases.[3] The main drawbacks are their low oral availability, which leads to the need of injections for their administration, or their low lifetime in the body due to the peptide degradation system by enzymes. In contrast, the main advantages are their high specificity, so very low concentrations of the drug are required, and their low toxicity because peptides are easily metabolized by enzymes of our body.[4]

For a long time, attempts have been made to synthesize peptides by chemical methods. *Theodor Curtius* was the first one capable to synthetize and characterize a N-protected dipeptide in 1882. Some years later, *Emil Fisher* synthetized the first free dipeptide in 1901. *Curtius* and *Fisher* achieved their synthesis in traditional solution phase. However, the main problem of solution synthesis is the rapid decrease of yield due to not reaching quantitative conversions and the need of purification in each step. In 1963 *Merrifield* published the first SPPS and he was awarded with the Nobel prize for it.[2,5] This methodology avoids the need for purification of intermediate products and conversions are quantitative, so the yield does not decrease in each step. This is achieved by attaching the amino acid to a solid support and then adding excess of reagents to ensure quantitative reactions and different solvents to clean by-products of the reactions.

3.2 CHEMICAL SYNTHESIS OF PEPTIDES

Peptides are formed by amino acids that are bound by amide bonds between them. In traditional synthesis, the α-carboxylic acid reacts with the α-amino group of another amino acid by a condensation reaction to generate the peptide bond. This reaction is carried out at room temperature thanks to the use of coupling reagents, which activates the carboxylic acid and acts as a dehydrating agent. Therefore, the other functional groups of the amino acid should be protected to avoid side reactions (**Scheme 1**). In biological processes, the elongation is from N-terminal to C-terminal but by chemical synthesis the reverse process is usually preferred because it has less side reactions such as razemization.[2]



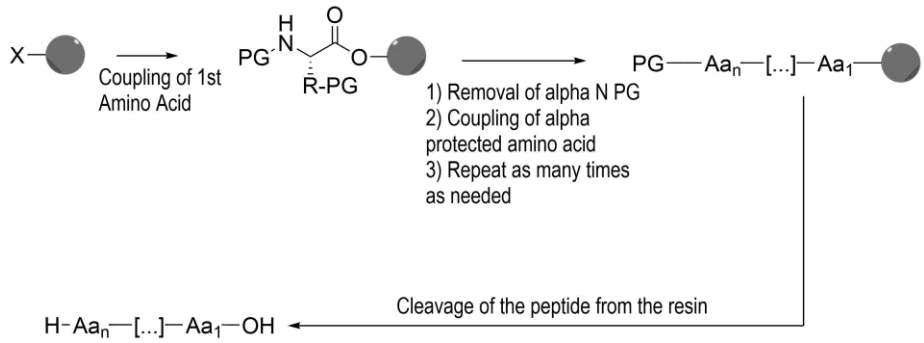
Scheme 1. Peptide bond formation of a dipeptide, where PG are protecting groups

The two main methodologies in peptide synthesis are the solid phase peptide synthesis (SPPS) and the solution phase peptide synthesis. The most used method is the SPPS and will be discussed later. Solution phase synthesis requires purification after each step, so it is only a good option in peptides of few amino acids such as dipeptides, or for couplings of peptide fragments in a convergent synthesis.

However, large scale peptide synthesis nowadays continues being a difficult field. Usually peptides can be synthetized in a SPPS method up to 50 Amino acids in small amounts,[6–8] but this method is not optimal if large quantities are required. A convergent synthesis which consists on synthetizing short fragments by SPPS and then coupling them in solution is usually the preferred method.

3.2.1 Solid-phase Peptide Synthesis (SPPS)

The general methodology of this type of synthesis is to attach the peptide chains to a solid support, grow the chains until the desired peptide is complete, and then cleave the peptide from the resin achieving a good purity and high yields. (**Scheme 2)**



Scheme 2. general SPPS synthesis

This type of synthesis is based in three different steps. In the first one, the first N-protected Amino acid is attached to the resin usually through an ester bond. In this sense, the solid support is considered a protecting group of the carboxyl group of this amino acid that will remain intact until the last step of the synthesis. (**Scheme 2**)

The second step is the elongation of the peptide chain on the resin. In order to do that, the N-terminal protecting group of the amino acid attached to the resin is removed and then, the next N-protected amino acid is coupled after activation of its carboxyl group. This step is repeated until the final assembly of the desired peptide chain. Finally, the third step consist on cleaving the peptide from the solid support under acidic conditions with concomitant removing of side chain protecting groups.[9]

This scheme is very general to most of the SPPS, but other strategies such as the N-terminal to C-terminal synthesis, partial deprotection to perform reactions in a peptide side chain or to cleave protected peptides from the resin can also be done by varying the protecting groups, the type of resins or the conditions of each step.[2]

3.2.2. Strategies in SPPS: Protecting groups

Molecules that have more than one functional group may involve several undesirable side-reactions if a reaction is carried out. To perform more selective reactions, the problematic functional groups are protected. In peptide chemistry, there are two main types of protecting groups: temporal and permanent. The former can be easily removed under mild conditions during peptide elongation (N^{α}-protecting groups), and the latter are stable in such conditions and they are removed during the final cleavage step (side chain protecting groups).

According to the mentioned above, there are two main strategies in SPPS, the Fmoc/^{t}Bu and the Boc/Bzl. The Boc/Bzl strategy consists on the use of the *tert*-butyloxycarbonyl group (Boc) to protect the N$^{\alpha}$ of the amino acid, and benzyl-based groups (Bzl) to protect the side chains of trifunctional amino acids.[2] Both protecting groups are acid labile, so the strategy is partially-orthogonal. However, the deprotection conditions are milder for the Boc group (TFA) than for Bzl like groups (HF), so it is possible to obtain a good chemoselectivity. The main shortcoming of this strategy is the use of HF for the final full deprotection of the peptide chain due to its toxicity and difficulty to handle.

Regarding to the Fmoc/^{t}Bu strategy, it is based on the protection of N$^{\alpha}$ of the amino acid with the 9-fluorenylmethoxycarbonyl group (Fmoc), which is labile under basic conditions (piperidine), while the permanent side chain protecting groups are based on *tert*-butyl, *tert*-butoxy or trityl groups (**Figure 1**), which are acid labile (TFA). This strategy is fully orthogonal because the removal of temporal and permanent groups is carried out under basic and acid conditions respectively.

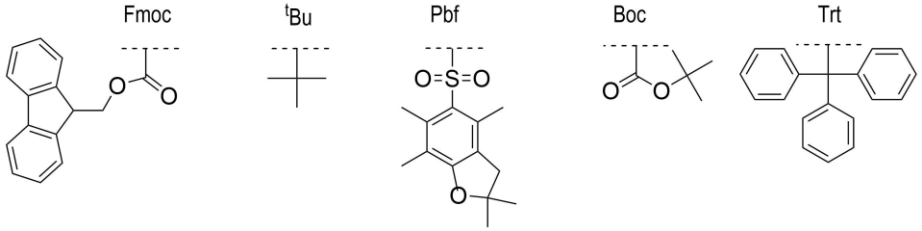


Figure 1. Protecting groups used in the Fmoc/tBu strategy

In this project the Fmoc/^{t}Bu strategy has been used because it requires deprotection conditions milder that those used with the Boc/Bzl strategy.[10] Therefore, this project is focused in Fmoc chemistry.

3.2.3. Solid support

Nowadays, different resins are used in Fmoc chemistry, and they vary in the introduction of the first amino acid, the cleavage conditions and the nature of the remaining functional group at the C-terminal after cleavage of the peptide from the resin. For example, 2-Chlorotrityl chloride (2-CTC), hypersensitive acid labile (HAL) and 2-(4-Hydroxymethil-phenoxy)-acetylaminomethyl (HMPA) resins provide a carboxylic acid group after cleavage while the super acid sensitive

resin (SASRIN-NH_2) and Sieber amide resin are used to obtain an amide in the C-terminal of the peptide[2,11] (**Figure 2**). Some polymeric supports allow cleavage of the peptide but not the other protecting groups, a propriety that is advantageous to perform a subsequent convergent synthesis.

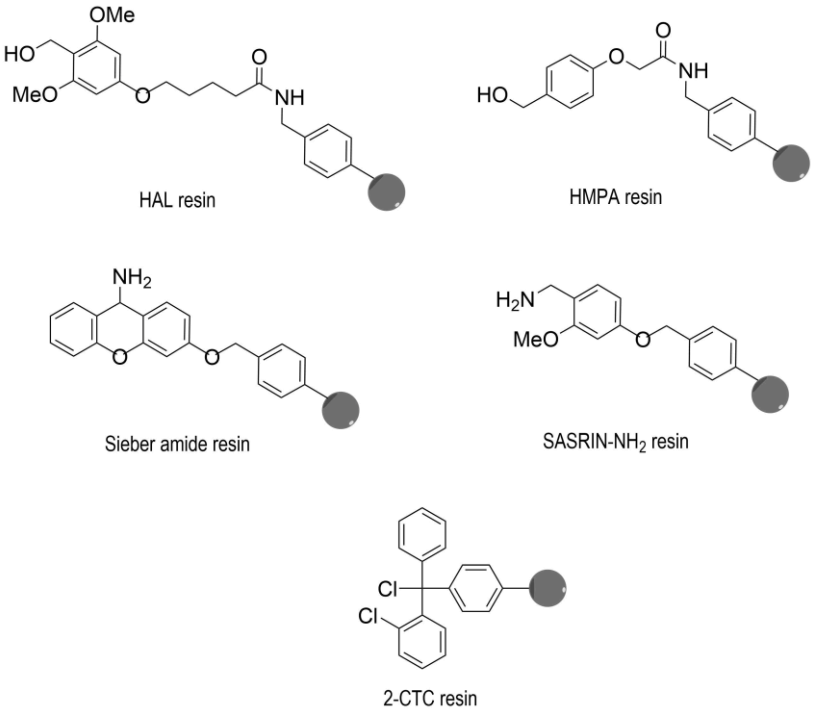
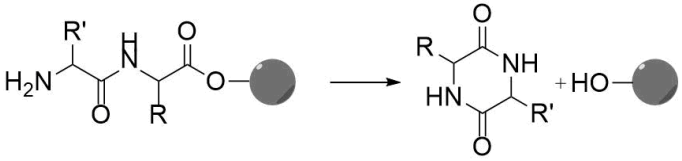


Figure 2. solid supports for SPPS

The resin must be physically and chemically stable and have a good swelling to the solvents. Most of the active points of the resin are inside beads, so a good swelling will determine the kinetics of the reaction, which is diffusion controlled, and therefore it will be crucial in the conversion achieved.[2,12]

In this project 2-CTC resin has been used (**Figure 2**). The peptide is attached to this polymeric support through an ester bond that is labile under acidic mild conditions (TFA) that allow to cleave the peptide chains from the resin while maintaining the protecting groups of side chains. Moreover, the 2-chloro group and the size of triphenylmethyl group provide enough stearic hindrance to avoid the formation of 2,5-diketopiperidines (DKP), one of the main side

reactions that occur when removing the Fmoc group of the second amino acid attached to the resin, as seen in the **Scheme 3**.



Scheme 3. formation of DKP under basic conditions.

3.2.4. Coupling reagents

Carboxylic acids are poorly reactant groups. The direct condensation reaction between a carboxylic acid and an amine is only possible at very high temperatures to remove the water formed and shift the equilibrium. At room temperature they will react by means of an acid-base reaction that inactivate the nucleophile character of amine group. In order to avoid this, the carboxylic acid must be activated by converting the hydroxyl group into a good leaving group before the reaction with the amine.

Traditionally, in organic synthesis, the carboxylic acid is activated in the form of acyl chloride, but this method is too harsh and usually many side reactions are involved.[13] That is why other activating agents such as carbodiimides are used in peptide synthesis to form the peptide bond under mild conditions. The most common carbodiimides are *N,N'*-dicyclohexylcarbodiimide (DCC), *N,N'*-diisopropylcarbodiimide (DIC) or *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC·HCl) (**Figure 3**).

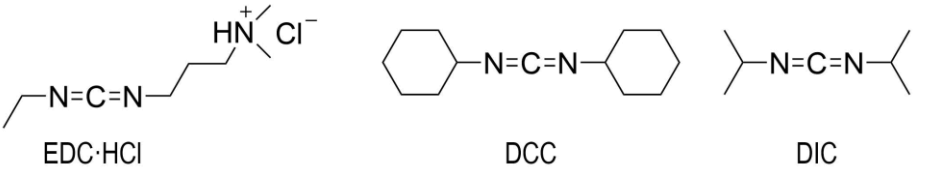
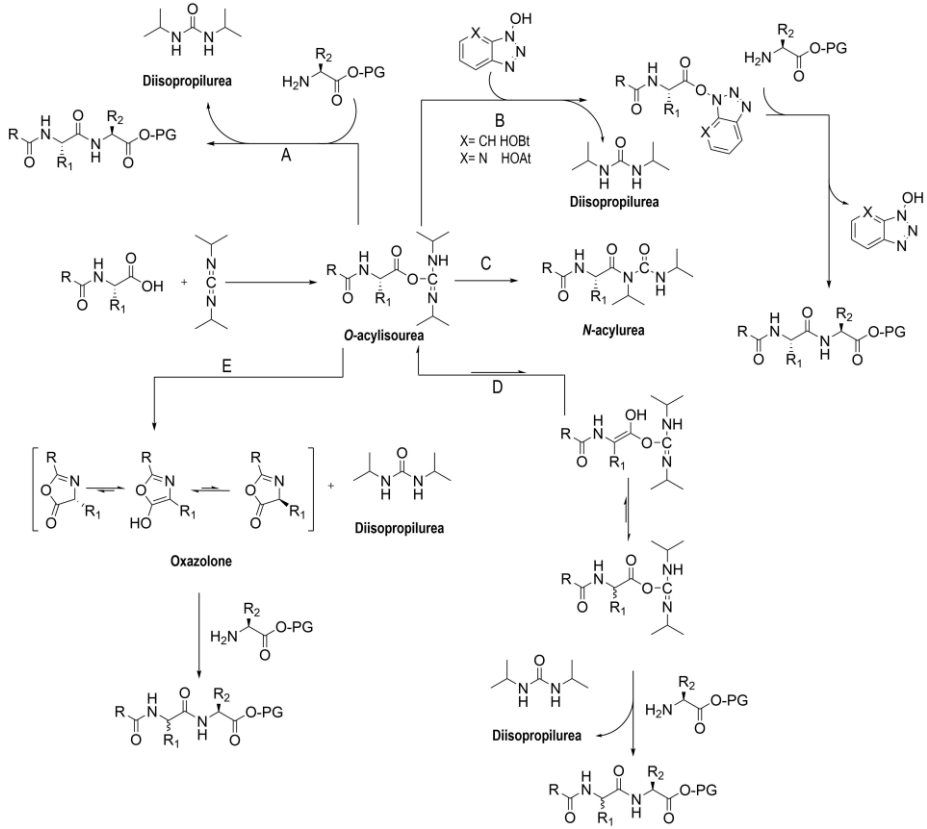


Figure 3. Common carbodiimides used in peptide synthesis.

As explained in **Scheme 4,** these reagents activate the carboxylic acid forming an active species (*O*-acylisourea) that reacts with the amine (reaction A) to form a urea as a by-product that can be easily removed by filtration or extraction methods. The *O*-acylisourea could provoque side reactions such as an irreversible process to form *N*-acylisourea (reaction C), and

racemization (reactions D and E). Usually additives such as 1-hydroxybenzotriazole (HOBt) or 1-hydroxy-7-azabenzotriazole (HOAt) are required (reaction B) to prevent racemization through oxazolone formation (reaction E) and keto-enol equilibrium (reaction D).

Other usual coupling reagents are the uronium/aminium salts, which some of them are derivatives of HOBt or HOAt, such as *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium Hexafluorophosphate (HATU) or *O*-(benzotriazol-1-yl)-1,1,3,3–tetramethyl-uronium hexafluorophosphate (HBTU). They react with the amino acid under basic conditions through the formation of -OBt/-OAt active esters.



Scheme 4. Carbodiimide mediated amide bond formation

In this project, the coupling system DIC/HOBt has been used to assembly the peptide chains on solid phase since this carbodiimide generates a urea soluble in most common organic solvents, so it can be removed washing the resin.

4. OBJECTIVES

The research group *Smbiocom* has been working during the last three years on the scale up to a pilot plant of the synthesis of a 17 amino acid peptide. In this sense, a strategy had to be chosen to minimize the cost of the synthesis without losing purity of the final product. The first approach consisted in the linear synthesis of the full peptide in solid phase, but it was not a successful method and it was changed to a convergent strategy in which the peptide was divided into smaller fragments in order to synthetize them in solid phase and to further assembly the peptide chain in solution.

The selection of the fragments is critical to avoid undesirable side reactions. Optimally, the C-terminal residues of the fragments should be proline or glycine since their avoid the risk of racemization at the C-terminal position. *N*-acylamino acids, such as the C-terminal amino acid of a peptide, are more prone to racemize during activation than acyloxycarbonylamino acids (Fmoc-amino acids). As the target peptide does not have proline or glycine, arginine (Arg) and leucine (Leu) were the amino acids chosen for the C-terminal position of the fragments, as reported in the literature.[14] Considering this issue and the fact that sequences of short peptide lengths are preferred, the two strategies shown in **Figure 4** were chosen**.** In this project the different protected fragments required for both strategies were prepared.

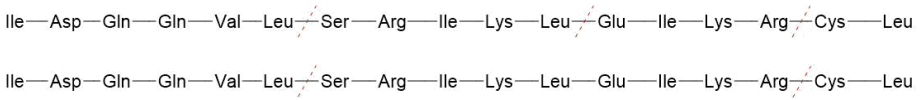


Figure 4. target peptide fragmentation strategies.

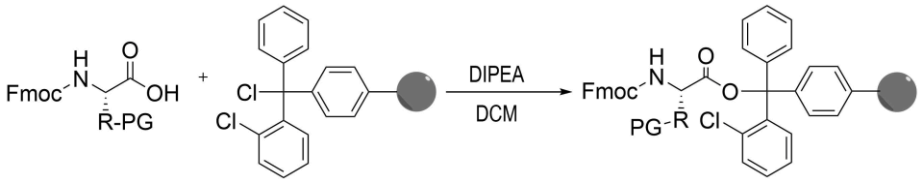
5. RESULTS AND DISCUSSION

5.1. SOLID PHASE SYNTHESIS

As mentioned above (**section 3.2.1.2**), all peptides were prepared using the Fmoc chemistry on a 2-CTC resin (2 g, 1.4 mmol/g). Protections for trifunctional amino acids were *tert*-Butyl (^{t}Bu) for Aspartic acid (Asp), Serine (Ser), Glutamic acid (Glu) and C-terminal Leucine (Leu), *tert*-butyloxycarbonyl (Boc) for Lysine (Lys) and N-terminal Isoleucine (Ile), Trityl (Trt) for Glutamine (Gln) and 2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-sulfonyl (Pbf) for Arg.

5.1.1. Coupling of the first amino acid

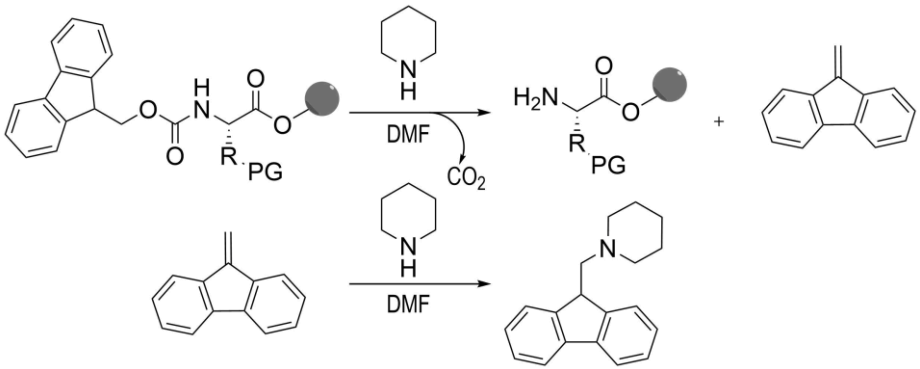
The first coupling was performed by a S_N1 reaction between the carboxylate anion generated with a non-nucleophile base such as *N,N'*-diisopropylethylamine (DIPEA) and the 2-chlorotrityl group (**Scheme 5**). The reaction time used was shortened for some fragments to avoid a high functionalization (over 1 mmol/g), which is undesirable because the peptide chains can form aggregates by hydrophobic interactions, which difficult the coupling reactions.[7,12]



Scheme 5. Attachment of the first amino acid to the 2-CTC solid support

After the coupling, the resin must be capped with methanol to block the unreacted chlorotrityl groups. This is done to avoid chain deletions that would yield peptides shorter than the desired product, thus hampering its final purification.

The removal of the Fmoc group is done with piperidine (20% in DMF) through a base-catalyzed decarboxylation process that affords dibenzofulvene. This by-product reacts with piperidine to form 1-(9H-fluoren-9-yl)methyl)piperidine that is used for quantification purposes (**Scheme 6. See experimental section 6.2.1**).



Scheme 6. Removal of the Fmoc group with *N,N*-dimethylformamide

It is important to reach this step to store the resin for a long time if required, because acid traces can cleave some peptide chains from the resin. However, any acid traces are neutralized with the free terminal amino group.

5.1.2. Elongation of the peptide chain

The coupling of the amino acid is performed using a large excess of DIC, HOBt and amino acid (3-4 eq) in the minimum quantity of DMF to increase the rate of the coupling reaction as much as possible and to assure the completion of the reaction (1 or 2 h). A ninhydrin test is performed (**see experimental section 6.2.2**) and, if the reaction is not complete (a positive blue test), a recoupling is performed under similar conditions, a protocol that has been proved by the research group to be more efficient than lengthening reaction times.

The resin is washed with different solvents to change the swelling of the resin depending on the polarity of the solvent.[12] Therefore, all possible reagents of the previous couplings and by-products are eliminated from inside the resin.

Ile derivatives were the most difficult amino acids to perform coupling reactions because recouplings and higher excess of reagents were usually required to complete the reaction. That is because this amino acid has a branched structure which provides more stearic hindrance than other amino acids do.

The second synthesis of nonapeptide was especially problematic because there were problems with ninhydrin tests. The coupling of second amino acid (Fmoc-Lys(Boc)-OH) was performed overnight to assure a complete coupling but the next day ninhydrin test was green instead of yellow. Therefore, an aliquot of the resin was treated with TFA and analysed the

resulting product by HPLC. Only one peak appeared in the chromatogram, so it was confirmed that the coupling was complete. After that period of time the polymeric support was affected, the filtration of solvents was slower and the remaining amino acids required recouplings or longer reaction times.

5.1.3. Cleavage of the peptide from the resin

Finally, the cleavage is done by a treatment of TFA 1% in DCM that affords the peptide fully protected because higher acid concentrations are required to remove side chain protecting groups.

Different variants of the same methodology were performed (**see experimental section 6.2.3.1**). The main issues encountered were that some fragments (pentapeptide and tetrapeptide) were not highly soluble in DCM and they precipitated easily before the extraction. Moreover, filtration was difficult and long times were required to isolate the product.

A qualitative assay of the solubility of tetrapeptide was studied in different solvents (20 mg in 0.7 mL) in order to modify the work-up. The results showed that tetrapeptide was not much soluble in most organic solvents and only THF formed a clear solution, but the peptide was unstable in this solution.

To solve these problems, the work-up was changed to a direct precipitation of the peptide in diethyl ether (Et_2O) that afforded a solid easier to filter, resulting in a common methodology for all fragments studied.

5.1.4. Results of peptide fragments synthesis in SPPS

Two batches of nonapeptide, one of tetrapeptide, one of pentapeptide and one of hexapeptide were synthetized starting from 2 g of resin of a functionalization of 1.4 mmol/g. The functionalization after the first coupling was determined by Fmoc quantification (**Table 1. See experimental section 6.2.1**), and the final yield was determined after cleavage by weight considering that all coupling reactions were quantitative by the ninhydrin test.

Peptide	Fmoc quantification (mmol) (a)	Yield (%) (b)	Chromatographic purity (%)
Nonapeptide batch 1	1.05	68.0	96.0
Nonapeptide batch 2	1.06	66.9	96.7
Tetrapeptide	1.18	72.8	93.4
Pentapeptide	1.95	92.1	95.1
Hexapeptide	1.83	94.3	98.3

(a) mmol of amino acid that were attached to the solid support after the coupling of the first amino acid

(b) Calculated from Fmoc quantification results

Table 1. Results of peptide fragments synthetized by SPPS

The purity of the protected peptides was determined by HPLC at λ = 220 nm and the identity of the product was confirmed by MS. Moreover, the protected tetrapeptide was analysed by NMR spectroscopy using the ^{1}H, ^{13}C, COSY and TOCSY. (**see experimental section 6.3.2.3, and Appendix 2, A.1.**) The first spectra were not clear due to heavy signals of protecting groups, so an aliquot of tetrapeptide was treated with a mixture of TFA, H_2O and TIPS (**see experimental section 6.3.2.4**) to remove side chain protecting groups. Then, the ^{1}H-NMR was recorded (**see Appendix 2, A.2.**) and were compared with the ^{1}H-NMR of the individual amino acids in order to facilitate the peak assignment and help at the same time to analyse the purity of the reagents used for the SPPS. Full assignment of the signals are presented in experimental section 6.2.3.3 and 6.2.3.4.

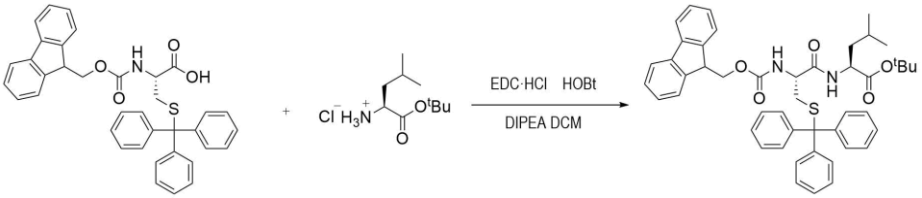
5.2. SOLUTION PHASE SYNTHESIS

In this project, the C-terminal dipeptide was synthetized in solution phase since only one coupling reaction and deprotection was required. Moreover, an assay of the convergent synthesis with the nonapeptide was performed in solution.

5.2.1. Synthesis of Fmoc-Cys(Trt)-Leu-O^{t}Bu

This synthesis is based in the reaction of the protected amino acids Fmoc-Cys(Trt)-OH and H_2N-Leu-O^{t}Bu in the presence of the carbodiimide EDC·HCl, which is very used in solution because it is soluble in water, so any excess or by-products are easily removed in the work-up, and HOBt to minimize racemization (**Scheme 7**). The Leu derivative was a hydrochloride salt,

so DIPEA was required to neutralize it in order to have the amino group free for the coupling reaction.

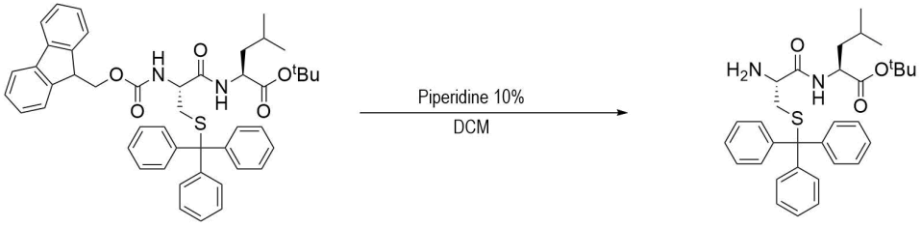


Scheme 7. coupling reaction of dipeptide

The synthesis was performed at 2 g scale and the desired product was obtained as a white solid (87.5%) that was used for the next reaction.

5.2.2. Synthesis of H-Cys(Trt)-Leu-O^{t}Bu

The Fmoc removal was carried out with 10% piperidine (3 eq) in DCM (**Scheme 8**) and the resulting product was purified using flash chromatography (78% yield).

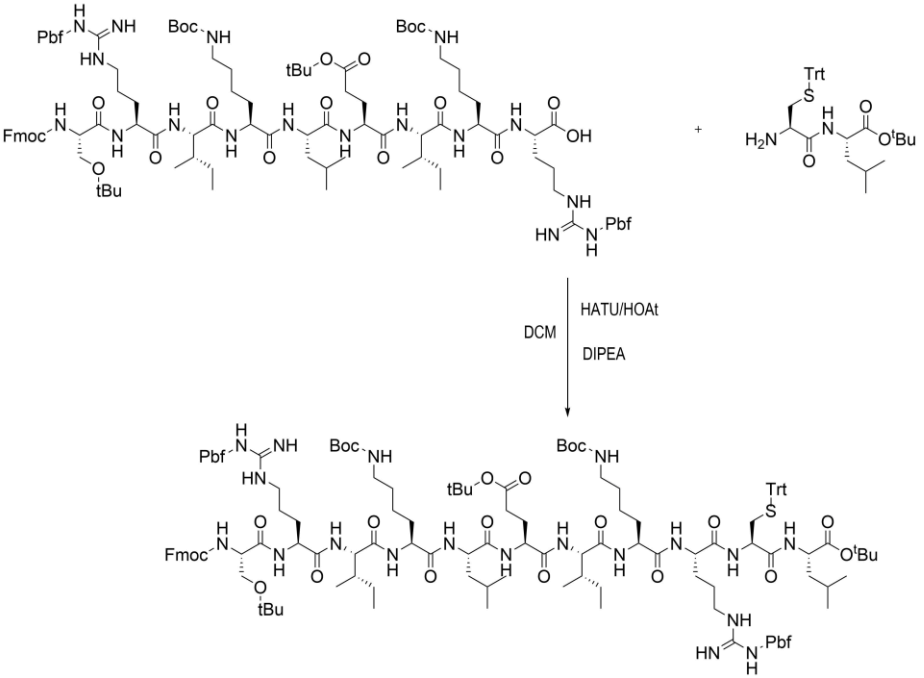


Scheme 8. deprotection of dipeptide

The final yellow product was characterized by HPLC (**See experimental section 6.1.3.1**) and only one peak at 22.4 min confirmed the presence of the desired unprotected product.

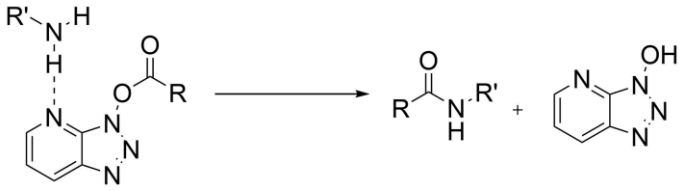
5.2.3. Convergent synthesis of the protected undecapeptide

The research group was studying different methodologies to optimize this particular coupling (**Scheme 9**). The main problem was the epimerization side reaction that is produced through the direct enolization and oxazolone mechanism (**Scheme 4, reactions D and E).**



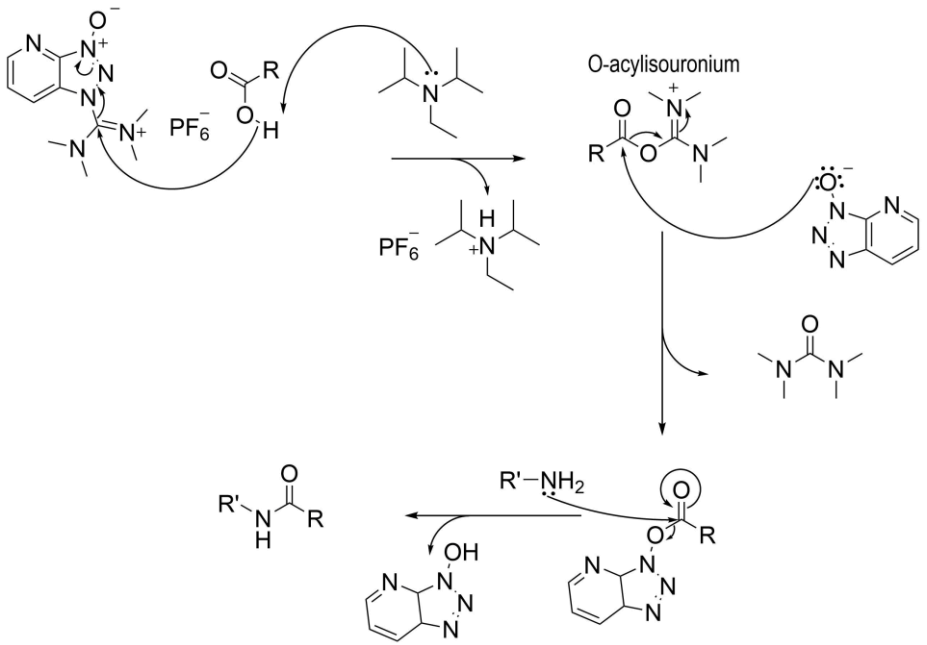
Scheme 9. Convergent synthesis of the protected undecapeptide

The reaction was carried out using the HATU/HOAt coupling system, which were the conditions that *a priori* minimize epimerization. Aminium/uronium salts are usually more efficient than carbodiimides,[14] and HOAt based compounds more effective than HOBt because of a neighbouring effect that facilitates the reaction with the amine with the active ester (**Scheme 10**).



Scheme 10. neighbouring effect of HOAt

HATU and DIPEA were added dropwise at the same time to prevent nonapeptide from remaining in the O-acylisouronium (**Scheme 11**) form and to have a low base concentration, which should avoid the epimerization reactions.



Scheme 11. Mechanism of HATU

These conditions were successful, and the final product was obtained as a white solid with a yield of 89.2% with a chromatographic purity of 89.5% with only 3% of epimer product.

6. EXPERIMENTAL SECTION

6.1. MATERIALS AND METHODS

6.1.1 Reagents and solvents

Entry	Product	Supplier
1	Amino acids	Esteve Química S.A
2	2-CTC resin	Irish-Biotech
3	DIPEA	Carlo Erba Reagents
4	TFA	Fisher Bioreagents
5	DIC	TCI
6	Piperidine	Panreac
7	DMF	Carlo Erba Reagents
8	DCM	Scharlau
9	MeOH	Carlo Erba Reagents
10	Acetone	Scharlau
11	HOBt	Esteve Química S.A
12	HATU	Esteve Química S.A
13	EDC·HCl	TCI

Table 2. Reagents and solvents used during the project

6.1.2 Instruments

Entry	Product	Brand	Model
1	HPLC-MS	Waters	LC-20AD
2	HPLC	Shimadzu	2695 separation module
3	UV-Vis spectroscopy	Varian	Cary 100
4	Analytical balance	Mettler	Toledo AB254
5	Water purification system	Milipore	Mili-Q Plus Water purification system
6	Centrifuge	Hettich	Rotofix 32
7	NMR spectroscopy	Bruker	400 Advance III with cryoprobe

Table 3. Instruments used in the project

6.1.3 Chromatography

6.1.3.1. High performance liquid chromatography (HPLC)

A C4 reverse phase column supplied by Aeris™ WIDEPORE was used (150x4.6 mm) with 3.6 μm of size particle and pore size of 200 Å. The eluents were H_2O with 0.045% TFA (A) and ACN with 0.036% TFA (B). The flow rate was settled at 1 mL/min with detection at 220 nm and 301 nm, being the last one the maximum of absorption of the 1-(9H-fluoren-9-yl)methyl)piperidine. The elution conditions were isocratic 5% of B for 2 min, from 5% to 100% of B for 30 min and isocratic of 100% of B for 10 min.

6.1.3.2. High performance liquid chromatography coupled with electrospray mass spectrometry (HPLC-MS)

HPLC-MS was carried out with a Waters system equipped by a 2695 separation module, a PDA detector 2996 and a Micromass ZQ electrospray mass detector (ESI-MS). The eluent and column were the same as described in section 6.1.3.1

6.1.3.3. Thin layer chromatography (TLC)

TLC was performed on silica gel F254 plates supplied by Merck. 254 nm UV light was used in order to reveal the films.

6.2. ANALYTICAL METHODS

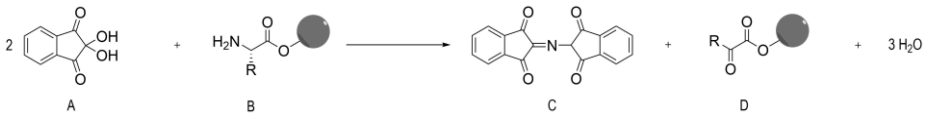
6.2.1 Fmoc Quantification

Quantification of the first amino acid was carried out by UV-Vis Spectrophotometry of the 1-(9H-fluoren-9-yl)methyl)piperidine formed during the removal of the Fmoc group.

To perform the quantification, the resin was washed 3 times with MeOH and an aliquot of 50 mg was taken and dried for 24 h. Afterwards, two replicates of 25 mg each were carried out by adding the resin to a solution of 20% piperidine in DMF using a 100 mL volumetric flask. The flasks were sonicated for 30 min to assure the complete removal of Fmoc and then it was diluted 1:10 in the same solution in order to perform the spectroscopy. A blank was also prepared. The concentration could be calculated using the Lambert Beer Law (ε = 7800 $M^{-1}cm^{-1}$ at λ = 301 nm and l = 1 cm)

6.2.2 Ninhydrin test

Known as Kaiser Test, this assay indicates the presence of primary amines.[15] The ninhydrin reacts with primary amines to form diketohydrindylidenediketohydrindamine (**Scheme 12, C**), also known as the Ruhemann's purple. If the coupling of the resin is not successful, the free N-terminal will react with ninhydrin and a deep blue colour will appear. However, if the coupling is complete the solution will remain yellow indicating that there are not free amines.



Scheme 12. ninhydrin reaction with primary amines

To perform the test three solutions previously prepared are needed: reagent A, 5 g of ninhydrin in 5 mL of EtOH; reagent B, 80 g of phenol in 20 mL of EtOH, and reagent C, 2 mL NaCN 0.01 M in 98 mL of pyridine distilled over ninhydrin. To carry out the test, an aliquot of resin is washed 3 times with MeOH and then 3 drops of every reagent are added and the mixture is heated to 100 ºC for 3 min in parallel with a blank. A yellow result is a negative test and blue is a positive test.

6.3. SYNTHETIC PART

6.3.1. Solid phase peptide synthesis (SPPS)

The reactions were carried out in 50 mL polypropylene syringes that contained a polyethylene filter. The stirring was done manually with a Teflon stick for the washes of the resin and the removal of Fmoc group while in the coupling reaction an automatic stirrer was used instead. The solvents and excess of reagents were filtered using a water vacuum pump system. **(Figure 5)**

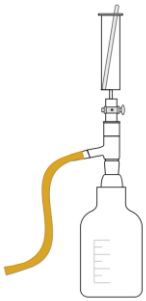


Figure 5. System used to perform SPPS

Different protocols were used to wash the resin in different situations (**Table 4**)

Treatment	Solvents sequence	Quantities
Protocol 1	DMF, DCM, iPrOH, DCM, DMF	20 mL of each, 2x30 s
Protocol 2	DMF, DCM, DMF, DCM, DMF, DMF	20 mL of each, 1x30 s
Protocol 3	DCM, DMF, DCM, DMF, DCM, DCM	20 mL of each, 1x30 s
Protocol 4	DCM	20 mL, 3x30 s

Table 4. Protocols used to wash the resin

6.3.1.1. Coupling of the first amino acid

The resin was washed (**Table 4, protocol 3**). Afterwards, 1.5 eq of amino acid were dissolved in the minimum quantity of DCM and 3 eq of DIPEA were added. After about 2 h of stirring, 1.6 mL of MeOH were added (0,8 mL/g resin) and the stirring was continued for 30 min.

Then, the mixture was filtered, and the resin was washed (**Table 4, protocol 1**) and an extra wash of iPrOH was performed (20 mL, 2x30 s). At this point an aliquot was taken for the Fmoc quantification (**see experimental section 6.2.1**). The resin was washed (**Table 4, protocol 2**) and it was treated two times with 20 mL of piperidine in DMF (20:80) for 5 min and 10 min. Then, the resin was washed (**Table 4, protocol 1**) and it was stored at this level if necessaire.

6.3.1.2. Elongation of peptide chain

The resin was washed (**Table 4, protocol 2**) and 3 eq of the N-protected amino acid and 3 eq of HOBt dissolved in the minimum quantity of DMF were added. Then, 3 eq of DIC were added and the resulting mixture was stirred for 1 h at rt. A ninhydrin test was performed (**see experimental part 6.2.1**) and, if the test was negative, the mixture was filtered and washed (**Table 4, protocol 1**). However, if the ninhydrin test was positive a recoupling was performed in the same conditions.

6.3.1.3. Cleavage of the peptide from the resin

The resin was washed (**Table 4, protocol 4**) and it was treated with a solution of 1% TFA in DCM (20 mL, 3x5 min) which was collected in a round bottomed flask containing 40 mL of water and 1.5 eq of pyridine. Then, washes of DCM (20 mL 3x1 min) were performed and they were collected in the same round-bottomed flask. Afterwards, the resultant organic phase was decanted, and it was dried using anhydrous Na_2SO_4. Finally, Et_2O was added until the peptide was completely precipitated, and it was recovered by filtration. Yields were determined by weight.

6.3.2 Synthesis of peptide fragments in SPPS

6.3.2.1. Nonapeptide: Batch 1

Polymeric support: 2.46 g of 2-CTC resin (1.4 mmol/g).

First coupling: 3.46 mg Fmoc-Arg(Pbf)-OH (1.55 eq) and 1.76 mL DIPEA in 20mL DCM for 120 min. Final loading of 0.35 mmol/g (1.05 mmol).

Coupling of amino acids:

Entry	Amino acid	Amino acid [g, eq]	HOBt·H_2O [g, eq]	DIC [g, eq]
1	Fmoc-Lys(Boc)-OH	3.41, 6.92	1.11, 6.89	1.20, 3
2	Fmoc-Ile-OH	1.24, 3.35	0.66, 4.12	0.52, 3
	recoupling	1.47, 3.96	0.60, 3.72	0.52, 3
3	Fmoc-Glu(^{t}Bu)-OH	1.54, 3.30	0.56, 3.46	0.52, 3
4	Fmoc-Leu-OH	1.17, 3.14	0.49, 3.06	0.52, 3
5	Fmoc-Lys(Boc)-OH	1.56, 3.17	0.53, 3.28	0.52, 3
	recoupling	1.52, 3.09	0.59, 3.65	0.52, 3
6	Fmoc-Ile-OH	1.29, 3.48	0.50, 3.09	0.52, 3
	recoupling	1.20, 3.22	0.54, 3.35	0.52, 3
7	Fmoc-Arg(Pbf)-OH	2.36, 3.46	0.65, 4.00	0.65, 4
8	Fmoc-Ser(^{t}Bu)-OH	1.63, 4.03	0.75, 4.68	0.65, 4

Table 5. Reagents used in coupling reactions for nonapeptide: batch 1

Cleavage of the peptide from the resin: 365 mL of Et_2O were required to precipitate the product. The final yield was 66.2% with a chromatographic purity of 96% at 220 nm.

6.3.2.2. Nonapeptide: Batch 2

Polymeric support: 2.03 g of 2-CTC resin (1.4 mmol/g).

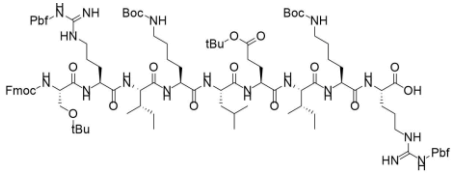
First coupling: 2.61 g of Fmoc-Arg(Pbf)-OH (1.41 eq) and 1.04 g DIPEA (2.83 eq) in 10 mL of DCM for 42 min. Final loading of 0.44 mmol/g (1.23 mmol).

Coupling of amino acids:

Entry	Amino acid	Amino acid [g, eq]	HOBt·H_2O [g, eq]	DIC [g, eq]
1	Fmoc-Lys(Boc)-OH	1.73, 2.99	0.55, 2.88	0.48, 3.11
	recoupling	1.66, 2.87	0.64, 3.41	0.49, 3.11
	recoupling	1.92, 3.31	0.56, 2.97	0.48, 3.09
2	Fmoc-Ile-OH	1.55, 4.15	0.72, 4.46	0.50, 3.77
	recoupling	1.42, 3.79	0.62, 3.83	0.53, 3.99
3	Fmoc-Glu(^{t}Bu)-OH	1.40, 3.00	0.51, 3.15	0.45, 3.41
4	Fmoc-Leu-OH	1.67, 4.47	0.67, 4.16	0.54, 4.05
	recoupling	1.48, 3.95	0.69, 4.27	0.54, 4.03
5	Fmoc-Lys(Boc)-OH	1.97, 3.97	0.68, 4.20	0.54, 4.07
	recoupling	1.95, 3.93	0.66, 4.07	0.53, 4.00
6	Fmoc-Ile-OH	1.65, 4.41	0.62, 3.80	0.55, 4.11
	recoupling	1.92, 5.13	0.70, 4.33	0.54, 4.02
7	Fmoc-Arg(Pbf)-OH	2.90, 4.23	0.66, 4.07	0.55, 4.12
	recoupling	2.82, 4.12	0.68, 4.17	0.55, 4.09
8	Fmoc-Ser(^{t}Bu)-OH	1.66, 4.10	0.64, 3.96	0.55, 4.10

Table 6. Reagents used in coupling reactions for nonapeptide: batch 2

Cleavage of the peptide from the resin: 300 mL of Et_2O were required to precipitate the product. The final yield was 66.9% with a chromatographic purity of 96.7% at 220 nm.



White solid. MS (ESI) m/z calc. for $C_{109}H_{169}N_{17}O_{25}S_2^{2+}$ $[M+2H]^{2+}$ 1091.89; found 1092.0

6.3.2.3. Tetrapeptide

Polymeric support: 2.11 g of 2-CTC resin (1.4 mmol/g).

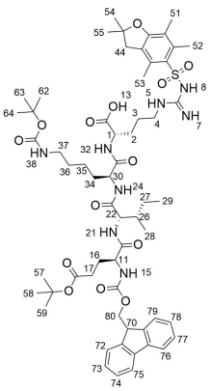
First coupling: 2.82 g of Fmoc-Arg(Pbf)-OH (1.47 eq) and 1.16 g of DIPEA (3.05 eq) in 10 mL of DCM for 145 min. Final loading of 0.42 mmol/g (1.18 mmol).

Coupling of amino acids:

Entry	Amino acid	Amino acid [g, eq]	HOBt·H_2O [g, eq]	DIC [g, eq]
1	Fmoc-Lys(Boc)-OH	1.64, 2.97	0.54, 2.99	0.54, 3.67
2	Fmoc-Ile-OH	1.22, 2.93	0.60, 3.36	0.50, 3.38
3	Fmoc-Glu(^{t}Bu)-OH	1.56, 3.00	0.59, 3.30	0.48, 3.27

Table 7. Reagents used in coupling reactions for tetrapeptide

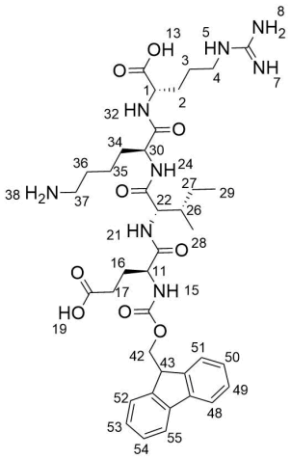
Cleavage of the peptide from the resin: Each treatment was collected in a different round-bottomed flask that cointained 40 mL of water and 1.5 eq of pyridine. The peptide precipitated in the flask with the water and it was redissolved with DCM (125 mL, 50 mL and 25 mL for each fraction respectively). Then, the organic phases were combined, and it was concentrated under vacuum. Finally, it was precipitated with 300 mL of hexane. The final yield was of 72.8% with a chromatographic purity of 93.4% at λ = 220 nm.



White solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.99 (t, J = 7.5 Hz, 2H, H_{24},H_{32}), 7.88 (d, J = 7.5 Hz, 2H, H_{76}, H_{75}), 7.75 (s, 1H, H_{21}), 7.70 (t, J = 7.1 Hz, 2H, H_{79}, H_{72}), 7.53 (d, J = 8.1 Hz, 1H, H_{15}), 7.41 (t, J = 7.4 Hz, 2H, H_{77}, H_{74}), 7.31 (t, J = 7.4 Hz, 2H, H_{73}, H_{78}), 6.70 (s, 2H, H_{5}, H_{8}), 6.41 (s, 1H, H_7), 4.33 – 3.98 (m, 7H, H_{70}, H_{80}, H_{11}, H_{22}, H_{30}, H_1), 3.06 – 2.98 (m, 2H, H_4), 2.94 (s, 2H, H_{44}), 2.90 – 2.81 (m, 2H, H_{37}), 2.47 (s, 3H, H_{53}), 2.41 (s, 3H, H_{52}), 2.20 (p, J = 10.0 Hz, 2H, H_{17}), 1.99 (s, 3H, H_{51}), 1.91 – 1.46 (m, 9H, H_{16}, H_{26}, H_{34}, H_2, H_3), 1.39 (s, 3H, H_{55}), 1.37 (s, 3H, H_{54}), 1.35 (s, 18H, H_{57}, H_{58}, H_{59}, H_{62} H_{63}, H_{64}), 1.27 – 0.96 (m, 2H, H_{27}), 0.83 (dd, J = 6.9, 1.7 Hz, 3H, H_{28}), 0.80 – 0.72 (m, 3H, H_{29}). ^{13}C NMR (101 MHz, DMSO) δ 11.42, 11.72, 12.73, 15.69, 18.05, 19.40, 22.95, 23.03, 24.66, 25.87, 27.83, 28.19, 28.73, 28.94, 29.74, 31.90, 32.11, 37.17, 42.91, 47.10, 52.05, 52.82, 54.27, 57.11, 66.14, 77.85, 80.17, 86.78, 116.75, 120.57, 124.80, 125.73, 127.54, 128.12, 131.90, 134.58, 137.73, 141.16, 144.14, 144.31, 150.04, 156.00, 156.37, 156.53, 157.92, 171.19, 171.62, 171.87, 172.19, 173.83. HPLC-MS (ESI): *m/z* calc. for $C_{60}H_{86}N_8O_{14}S^+$ $[M+H]^+$ 1175.60; found 1175.91. MS (ESI): *m/z* calc. for $C_{60}H_{86}N_8O_{14}S^+$ $[M+H]^+$ 1175.60; found 1176.61. MS (TOF): *m/z* calc. for $C_{60}H_{86}N_8O_{14}S^+$ $[M+Na]^+$ 1197.6; found 1197.6.

6.3.2.4. Assay of full deprotection of the tetrapeptide

A full deprotection of tetrapeptide fragment was performed in order to characterize the product by NMR. The tetrapeptide (87.1 mg) was placed in a glass vial and a mixture of TFA/H_2O/TIPS 90:5:5 (1 mL) previously prepared in a different glass vial was added using a syringe. The mixture was left at rt for 1 h and was transferred to a 15 mL falcon tube with 3 mL of Et_2O. The resulting mixture was left in the refrigerator overnight, was centrifugated and was decanted. To the white solid formed, Et_2O (5 mL) was added and the resulting suspension was sonicated for 5 min followed by a centrifugation of 6 min and decantation. This procedure was repeated twice with Et_2O and twice with EtOAc. The peptide was dried under vacuum and was analysed by NMR (**See Appendix 2, A.2**). The software MestReNova and Chemdraw was used to analyse all NMR spectra.



White solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.42-814 (m, 3H, H_8,H_5), 7.89 (d, J = 7.5 Hz, 2H, H_{48}, H_{55}), 7.84 (dd, J = 8.0, 2.6 Hz, 1H, H_{21}), 7.73 (s, 1H, H), 7.72 – 7.68 (m, 2H, H_{51}, H_{52}), 7.61 (d, J = 8.1 Hz, 1H, H_{15}), 7.42 (t, 4H, H_7, H_{49}, H_{54}), 7.33 (t, J = 7.5, 1.2 Hz, 2H, H_{50}, H_{53}), 4.32 – 4.24 (m, 2H, H_{42}), 4.24 – 4.18 (m, 2H, H_1, H_{43}), 4.15 – 3.96 (m, 3H, H_{11}, H_{22}, H_{30}), 3.07 (s, 2H,), 2.76 (t, J = 7.2 Hz, 2H, H_{37}), 2.27 – 2.20 (m, 2H, H_{17}), 1.95 – 1.84 (m, 2H, H_{16}), 1.80 – 1.28 (m, 11H, H_{26}, H_2, H_{34}, H_{36}, H_3, H_{35}), 1.12 – 0.96 (m, 2H, H_{27}), 0.81 (d, J = 6.7 Hz, 3H, H_{28}), 0.76 (t, J = 7.4 Hz, 3H, H_{29}). HPLC-MS (ESI): *m/z* calc. for $C_{38}H_{54}N_8O_9^+$ $[M+H]^+$ 767.4; found 767.6. *m/z* calc. for $C_{38}H_{54}N_8O_9^+$ $[M+2H]^{2+}$ 384.2; found 384.4.

6.3.2.5. Pentapeptide

Polymeric support: 2.14 g of 2-CTC resin (1.4 mmol).

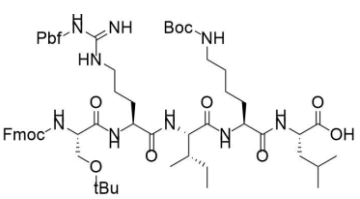
First coupling: 1.59 g of Fmoc-Leu-OH (1.50 eq) and 1.17 g DIPEA (3.02 eq) in 8 mL DCM for 129 min. Final loading of 0.71 mmol/g (1.95 mmol).

Coupling of amino acids:

Entry	Amino acid	Amino acid [g, eq]	HOBt·H_2O [g, eq]	DIC [g, eq]
1	Fmoc-Lys(Boc)-OH	2.71, 2.97	0.95, 3.19	0.78, 3.19
2	Fmoc-Ile-OH	2.04, 2.96	1.02, 3.41	0.71, 2.90
3	Fmoc-Arg(Pbf)-OH	3.74, 2.95	0.96, 3.20	0.73, 2.95
4	Fmoc-Ser(^{t}Bu)-OH	2.35, 3.14	0.91, 3.05	0.74, 2.99

Table 8. Reagents used in coupling reactions for pentapeptide

Cleavage of the peptide from the resin: The peptide precipitated directly when it was collected in the water and pyridine containing round-bottomed flask. It was tried to redissolve with 460 mL of DCM but it was not possible, so the volatiles were removed under vacuum. Finally, the resultant solid was filtered and cleaned with water until neutral pH. The final yield was 92.1% with a chromatographic purity of 95.1%.



White solid. HPLC-MS (ESI): *m/z* calc. for $C_{64}H_{95}N_9O_{14}S^+$ $[M+H]^+$ 1246.7; found 1247.0. MS (ESI): *m/z* calc. for $C_{64}H_{95}N_9O_{14}S^+$ $[M+H]^+$ 1246.7; found 1246.8. *m/z* calc. for $C_{64}H_{95}N_9O_{14}S^+$ $[M+Na]^+$ 1268.7; found 1268.9.

6.3.2.6. Hexapeptide

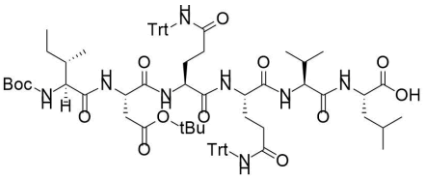
Polymeric support: 2.01 g of 2-CTC resin (1.4 mmol/g)

First coupling: 1.49g of Fmoc-Leu-OH (1.43 eq) and 1.01 g DIPEA (2.78 eq) in 8 mL DCM for 72 min. Final loading of 0.71 mmol/g (1.83 mmol).

Entry	Amino acid	Amino acid [g, eq]	HOBt·H2O [g, eq]	DIC [g, eq]
1	Fmoc-Val-OH	1.87, 3.00	0.84, 2.98	0.74, 3.20
2	Fmoc-Gln(Trt)-OH	1.98, 2.92	0.97, 3.45	0.71, 3.06
3	Fmoc-Gln(Trt)-OH	1.97, 2.91	0.95, 3.37	0.71, 3.07
4	Fmoc-Asp(^{t}Bu)-OH	2.41, 3.19	0.93, 3.30	0.72, 3.10
5	Boc-Ile-OH	1.70, 3.99	1.18, 4.19	0.91, 3.91
	recoupling	1.78, 4.20	1.28, 4.55	0.90, 3.88
	recoupling	1.75, 4.12	1.09, 3.89	0.87, 3.74

Table 9. Reagents used in coupling reactions for hexapeptide

Cleavage of the peptide from the resin: Instead of water, Et_2O was used to collect the treatments of TFA (1%) in DCM and DCM washes. A ratio of 4:1 Et_2O/DCM was maintained during the treatments, so 480 mL of Et_2O were required. The resultant solid was filtered and cleaned with water and Et_2O. The final yield was 94.3% with a chromatographic purity of 98.3%.



White solid. HPLC-MS (ESI): *m/z* calc. for $C_{78}H_{98}N_8O_{13}^+$ $[M+H]^+$ 1355.73; found 1356.74.

6.3.3. Solution phase synthesis

6.3.3.1. Synthesis of Fmoc-Cys(Trt)-Leu-O^{t}Bu

Fmoc-Cys(Trt)-OH (1.55 g, 1 eq) and Leu-O^{t}Bu (0.65 g, 1.1 eq) were added to a 25 mL round bottomed flask and were dissolved in 6 mL of DCM. The solution was cooled in an ice bath and 0.38 g of DIPEA were added within 30 min with an automatic injector. Then, 20 mL of AcOEt were added and the organic solution was washed with aqueous sat. citric acid and aqueous sat. $NaHCO_3$ (3x20 mL each). The resultant organic phase was dried with anhydrous Na_2SO_4 and volatiles were removed under vacuum to yield 1.75 g of the desired product as a white solid (87.5%)

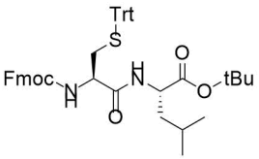


Figure 6. Structure of the dipeptide

6.3.3.2. Synthesis of H-Cys(Trt)-Leu-O^{t}Bu

Fmoc-Cys(Trt)-Leu-O^{t}Bu (1.39 g, 1 eq) and 10% piperidine in DCM (5.3 mL, 2.9 eq) were mixed in a 25 mL round bottomed flask and left for 3.3 h. Then, 15 mL of DCM were added and the organic solution was washed with H_2O (3x20 mL). The resultant aqueous phase was extracted with 10 mL of DCM, and organic phases were combined and dried with anhydrous Na_2SO_4. Finally, volatiles were removed under vacuum and the resulting yellow solid was purified by flash chromatography (dry loading in silica) using hexanes/AcOEt 20:80 and DCM/MeOH 90:10 to afford 0.768 g of the desired peptide as a yellow solid (78%).

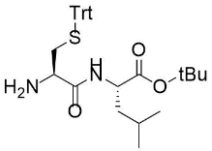
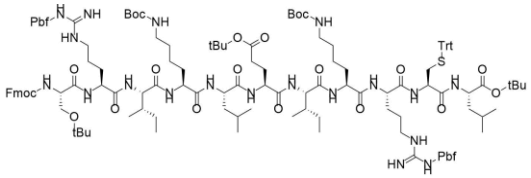


Figure 7. Structure of unprotected dipeptide

6.3.3.3. Protected undecapeptide

Nonapeptide from batch 2 (368.5 mg, 1 eq), H-Cys-Leu-O^{t}Bu (102.3 mg, 1.1 eq) and HOAt (25.4 mg, 1.1 eq) were added to a 25 mL round-bottomed flask and they were dissolved in 8 mL of DMF. HATU (72 mg, 1.1 eq) and DIPEA (43.7 mg, 2.0 eq) were dissolved separately in 1 mL of DMF and both solutions were transferred to two different syringes. Then, the round-bottomed flask was cooled in an ice/MeOH bath and DIPEA and HATU were added with an automatic injector at the same time for 30 min. Finally, an aliquot was injected in the HPLC and the undecapeptide was precipitated with 180 mL of water and filtered. Finally, 408.2 mg were obtained as a white solid (89.2%). The final product was characterized by HPLC-MS.



White solid. HPLC-MS (ESI): *m/z* calc. for $C_{60}H_{86}N_8O_{14}S^+$ $[M+2H]^{2+}$ 1348.2; found 1348.3.

7. CONCLUSIONS

- Full protected fragments of a seventeen amino acids peptide have been successfully synthetized using Fmoc chemistry and a 2-CTC resin, except of dipeptide which was synthetized in solution. These peptides have been prepared at a scale of 2 g of polymeric support, coupling the first amino acid with DIPEA in DCM and using DIC/HOBt in DMF as a coupling system to assemble the peptide chain. TFA (1% in DCM) has been used to cleave the peptide from the resin.
- The protected nonapeptide has been obtained with overall yields of 68 and 67%, and a chromatographic purities of 96 and 97% respectively. The protected tetrapeptide, pentapeptide and hexapeptide have been obtained with overall yields of 73, 92 and 94% respectively, and a chromatographic purities of 93, 95 and 98%.
- Finally, the protected dipeptide has been synthetized in solution using EDC·HCl/HOBt in DCM as the coupling system, with a yield of 88%.
- The coupling of the protected nonapeptide to the dipeptide has been assayed previous deprotection of the latter with 10% of piperidine in DCM (78% yield), using HATU/HOAt and DIPEA in DCM as the coupling system. The desired undecapeptide has been obtained with a yield of 89.2%, a chromatographic purity of 89.5% and 3% of epimerization.

8. REFERENCES AND NOTES

1. Fosgerau, K. & Hoffmann, T. Peptide therapeutics: current status and future directions. *Drug Discov. Today* **20**, 122–8 (2015).
2. Jaradat, D. M. M. *Thirteen decades of peptide synthesis: key developments in solid phase peptide synthesis and amide bond formation utilized in peptide ligation*. *Amino Acids* **50**, (Springer Vienna, 2018).
3. Loffet, A. Peptides as Drugs: Is There a Market? *Pept. Wave Futur.* **7**, 214–216 (2014).
4. Vlieghe, P., Lisowski, V., Martinez, J. & Khrestchatisky, M. Synthetic therapeutic peptides: science and market. *Drug Discov. Today* **15**, 40–56 (2010).
5. Chandrudu, S., Simerska, P. & Toth, I. Chemical methods for peptide and protein production. *Molecules* **18**, 4373–4388 (2013).
6. Montalbetti, C. A. G. N. & Falque, V. Amide bond formation and peptide coupling. *Tetrahedron* **61**, 10827–10852 (2005).
7. Bray, B. L. Online Mendelian Inheritance in Man : Large-scale manufacture of peptide therapeutics by chemical synthesis. *Discovery* **2**, 5–9 (2003).
8. Kelley, W. S. Therapeutic Peptides: The Devil is in the Details. *Nat. Biotechnol.* **14**, 28–31 (1996).
9. Bulletin, T. Cleavage , Deprotection , and Isolation of Peptides after Fmoc Synthesis. *Tech. Bull. from Appl. Biosyst.* 11–14 (2008).
10. Albericio, F., Isidro-llobet, A. & Mercedes, A. Amino Acid-Protecting Groups. *Chem. Rev.* **109**, 2455–2504 (2009).
11. Bachem group. Tips and Tricks for Solid Phase Peptide Synthesis from the Experts at Bachem. *Solid Phase Pept. Synth.* 1–55 (2016).
12. Merrifield, B. The role of the support in solid phase peptide synthesis. *Br. Polym. J.* **16**, 173–178 (2007).
13. El-Faham, A. & Albericio, F. Peptide Coupling Reagents, More than a Letter Soup. *Chem. Rev.* **111**, 6557–6602 (2011).
14. Andersson, L. *et al.* Large-scale synthesis of peptides. *Biopolym. - Pept. Sci. Sect.* **55**, 227–250 (2000).
15. Kaiser, E.; Colescott, R. L. ; B. C. D. . C. P. L. Color test for Detection of Free Terminal Amino Groups in the Solid-Phase Synthesis of Peptides. *Anal Biochem* **34**, 595–598 (1970).

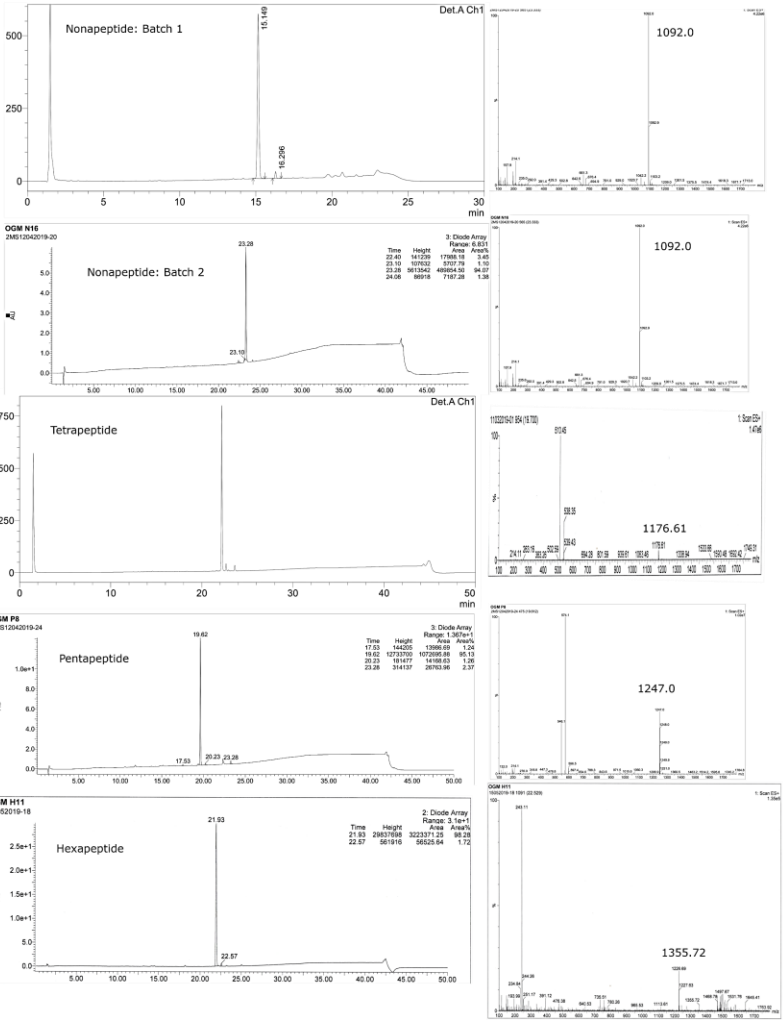
9. ACRONYMS/ABBREVIATIONS

2-CTC	2-Chlorotrityl chloride
ACN	Acetonitrile
AcOEt	Ethyl acetate
Arg	Arginine
Boc	*tert*-Butyloxycarbonyl
Bzl	Benzyl
Cys	Cysteine residue
DIPEA	*N,N*'-diisopropylethylamine
DCC	*N,N*'-dicyclohexylcarbodiimide
DIC	*N,N*'-diisopropylcarbodiimide
DCM	Dichloromethane
DMF	*N,N*-dimethylformamide
EDC	*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide
Eq	Equivalent
ESI-MS	Electrospray Ionization – Mass Spectroscopy
Et_2O	Diethyl ether
Fmoc	9-fluorenylmethyloxycarbonyl
Glu	Glutamic acid residue
HATU	*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium Hexafluorophosphate
HBTU	*O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	1-hydroxybenzotriazole
Ile	Isoleucine residue
Leu	Leucine residue
Lys	Lysine residue
MeOH	Methanol
Pbf	2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
PG	Protecting group
rt	Room temperature
sat	Saturated solution
Ser	Serine residue
SPPS	Solid phase peptide synthesis
^{t}Bu	*tert*-Butyl
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
Trt	Trityl

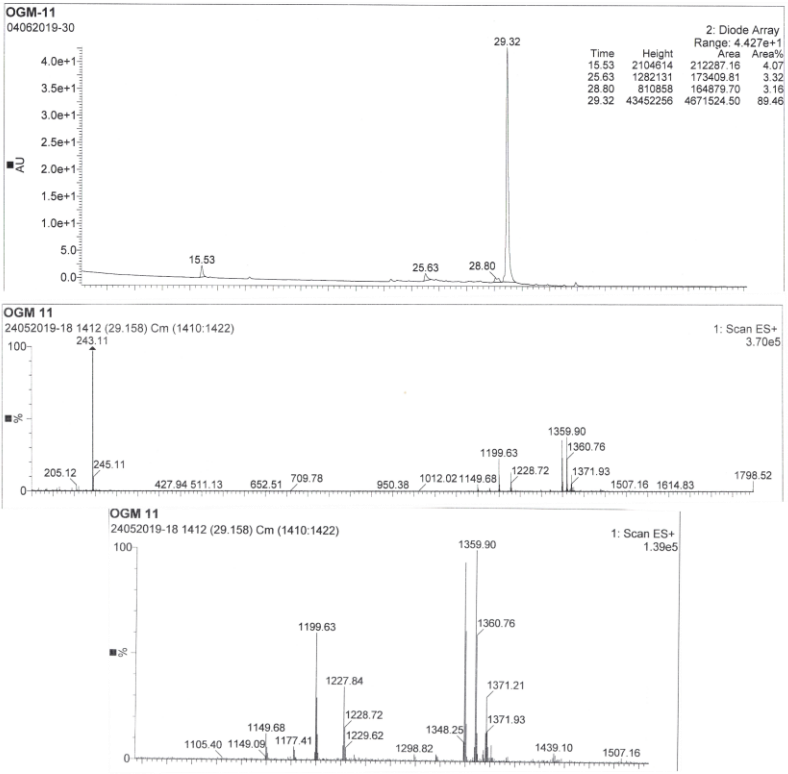
APPENDICES

APPENDIX 1: CHROMATOGRAMS AND MASS SPECTRA

A.1 HPLC and MS of Fragments synthetized by SPPS methodology

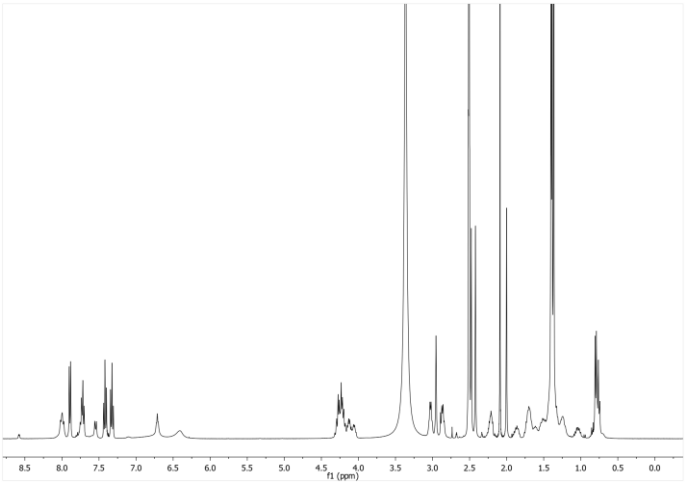


A.2 HPLC and MS of Undecapeptide.

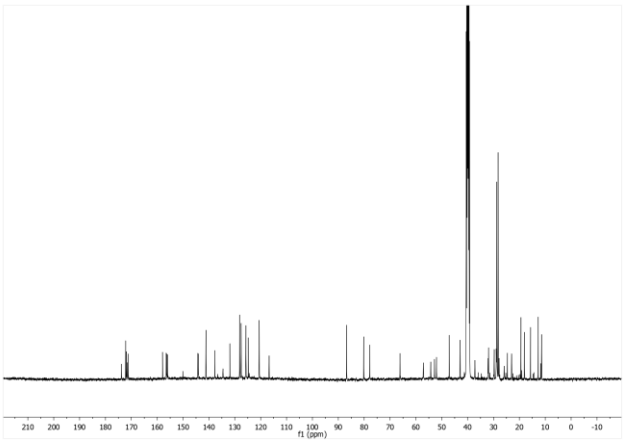


APPENDIX 2: NMR DATA OF TETRAPEPTIDE

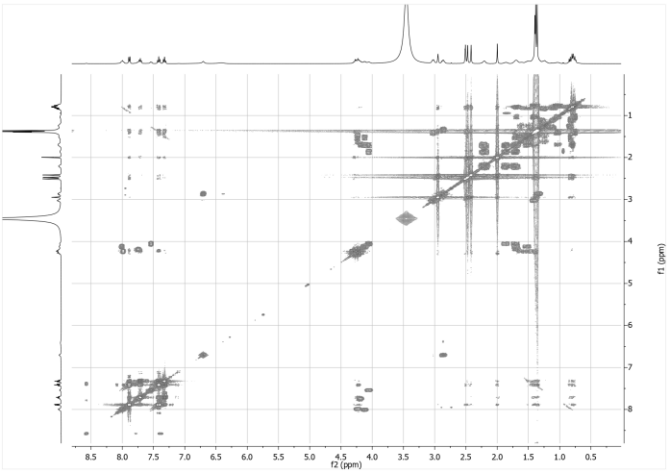
A.1 Protected tetrapeptide NMR spectra



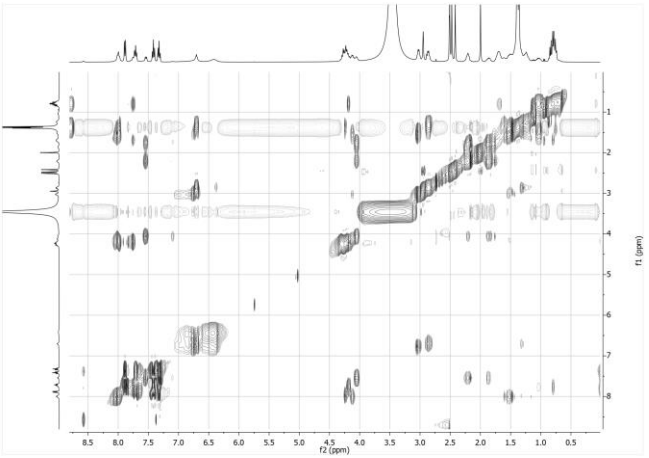
Experimental ^{1}H NMR of protected tetrapeptide



Experimental ^{13}C NMR of protected tetrapeptide

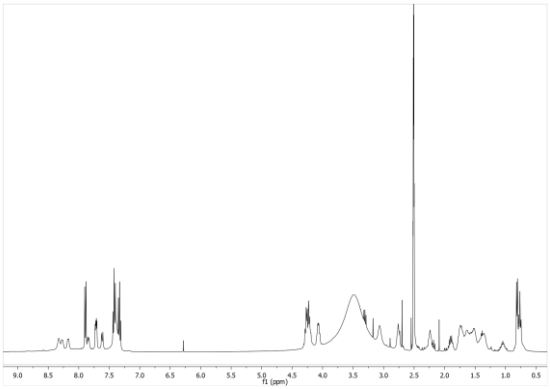


Experimental COSY spectra of protected tetrapeptide



Experimental TOCSY spectra of protected tetrapeptide

A.2 Unprotected tetrapeptide NMR spectra



Experimental ^{1}H NMR of unprotected tetrapeptide