



Treball Final de Grau

Synthesis of multifunctional peptides containing a fluorophore group as fluorescent probe.

Síntesis de péptidos multifuncionales que contienen un fluoróforo como sonda fluorescente.

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January 2016

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REPORT

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1. SUMMARY

Alzheimer Disease (AD) is the most common cause of dementia in the elderly population. Symptoms of the disease are: difficulty in remembering newly learned information, mood and behaviour changes, memory loss, judgment alteration and difficulties in daily and usual activities. Today Alzheimer disease affects more than 30 million people worldwide and it is estimated that more than 100 million people will be affected by 2050 mainly because of the fast growth of the elderly population.

Currently, it is generally accepted that Alzheimer disease originates from a process called amyloid cascade that occurs in the synaptic cleft, wherein A β aggregation due to its union with Cu(II), is a key event.

In this work, elaborated in collaboration with the group of Bioinorganic Chemistry led by Professor Patrick Gámez, the peptides with sequences Ac-His-Lys(Cm)-His-Lys-NH₂ and Ac-His-Lys(4-DMN)-His-NH₂ have been synthesized. These peptides encompass two amino acid derivatives containing two fluorescent probes: coumarin and 4-*N,N*-dimethylamino-1,8-naphthalimido. These peptide sequences will be studied as potential therapeutic agents due to its chelating properties of the Cu (II), as well as copper sensors for AD diagnosis.

The amino acid derivatives have been synthesized in aqueous phase while peptides were synthesized in solid phase through the Fmoc strategy, using as polymer support Rink Amide resin. The peptides prepared were functionalized with an acetyl group in the N-terminus and a carboxamide group in the C-terminus. To obtain the desired synthetic sequence, Fmoc amino acids, protected at the N-terminus with the 9-fluorenylmethoxycarbonyl group (Fmoc), which is labile to piperidine, were used. For generating the amide bonds, *N,N'*-diisopropylcarbodiimide (DIC) and Oxyma have been used. The cleavage of the peptide from the resin was performed under acidic conditions using trifluoroacetic acid (TFA) in the presence of triisopropylsilane (TIPS) as carbocation scavenger agent. The characterization of the products was carried out using different techniques such as ¹H and ¹³C NMR, HPLC and HPLC-MS.

Keywords: Alzheimer, amyloid- β , copper, fluorescent probes, peptides.

2. RESUMEN

El Alzheimer es una enfermedad neurodegenerativa y es la causa principal de la demencia en la población anciana. Sus principales síntomas son: la dificultad de recordar información recién aprendida, cambios de humor y comportamiento, pérdida de memoria, alteración en el juicio y dificultad de realizar las actividades cotidianas. Hoy en día la enfermedad del Alzheimer afecta a 30 millones de personas en todo el mundo y se estima que en el 2050 sea de 100 millones debido al incremento de la población anciana.

La hipótesis que actualmente se postula dice que el Alzheimer se produce a partir de un proceso llamado cascada amiloide, que tiene lugar en el hueco sináptico, en el que la agregación de la A β , debido a su unión con el Cu(II), es el evento principal.

En el presente trabajo, realizado con la colaboración del grupo de Química Bioinorgánica liderado por el Profesor Patrick Gámez, se ha estudiado las secuencias peptídicas Ac-His-Lys(Cm)-His-Lys-NH₂ y Ac-His-Lys(4-DMN)-His-NH₂ las cuales están formadas por dos derivados de aminoácidos que contienen dos sondas fluorescentes: coumarina y 4-N,N-dimetilamino-1,8-naftalamido. Estas secuencias peptídicas se estudiarán como agentes potenciales terapéuticos debido a sus propiedades quelantes sobre el Cu(II).

Los derivados de aminoácidos se han sintetizado en fase acuosa mientras que los péptidos se han sintetizado en fase sólida mediante la estrategia Fmoc, utilizando como soporte polimérico la resina Rink Amida. Los péptidos están formados por un grupo acetilo en el extremo N-terminal mientras que el extremo C-terminal estará en forma de grupo carboxiamida. Para obtener la secuencia sintética deseada, los aminoácidos están protegidos en el extremo N-terminal por grupos 9-fluorenimetoxicarbonil (Fmoc), lábiles a la piperidina. Para formar el enlace amida, se utilizaron *N,N'*-diisopropilcarbodiimida (DIC) y oxyma. La liberación del péptido de la resina se realizará en condiciones ácidas utilizando ácido trifluoroacético (TFA) en presencia de triisopropilsilano (TIPS) como agente captador de carbocationes. La caracterización de los productos se llevó a cabo mediante técnicas como ¹H y ¹³C NMR, HPLC y HPLC-MS.

Palabras clave: Alzheimer, β -amiloide, cobre, sondas fluorescentes, péptidos

3. INTRODUCTION.

3.1. ALZHEIMER DISEASE

Alzheimer Disease (AD) is the most common cause of dementia in the elderly population. Symptoms of the disease are: difficulty in remembering newly learned information (at the early stage), mood and behaviour changes, memory loss, judgment alteration and difficulties in daily and usual activities. Hence the patients need constant attention from their caregivers.

AD currently affects more than 30 million people worldwide and it is estimated that more than 100 million people will be affected by 2050 mainly because of the fast growth of the elderly population. Therefore, AD socioeconomical impacts are very important and the healthcare system is under increasing pressure.

AD is characterized by two pathological hallmarks in brains affected the disease: extracellular senile plaques (found in the synaptic cleft) and intracellular neurofibrillary tangles called NFTs¹. Senile plaques are mainly formed by aggregated amyloid- β peptide ($A\beta$) and NFTs are composed of the hyperphosphorylated forms of the microtubule associated protein, τ ².

The underlying mechanism of AD is not clear, but there is evidence and a relatively wide agreement that the so-called amyloid cascade is a key and early event in the development of AD (Figure 1). This hypothesis suggests that self-aggregation of the $A\beta$ peptides is the initiating event in AD pathogenesis and it occurs in the synaptic cleft³. In this process, several aggregation stages are likely involved: the formation of oligomers, protofibrils and fibrils which are found in the senile plaques. Soluble aggregation intermediates have been proposed as targets for further investigation since they are supposed to be involved in different pathological events, including formation of NFTs and disruption of synaptic connections, which would ultimately lead to neuronal cells death and dementia. However, the relationship between $A\beta$ protein accumulation and modification of τ protein, which lead to the formation of NFTs, is still little understood².

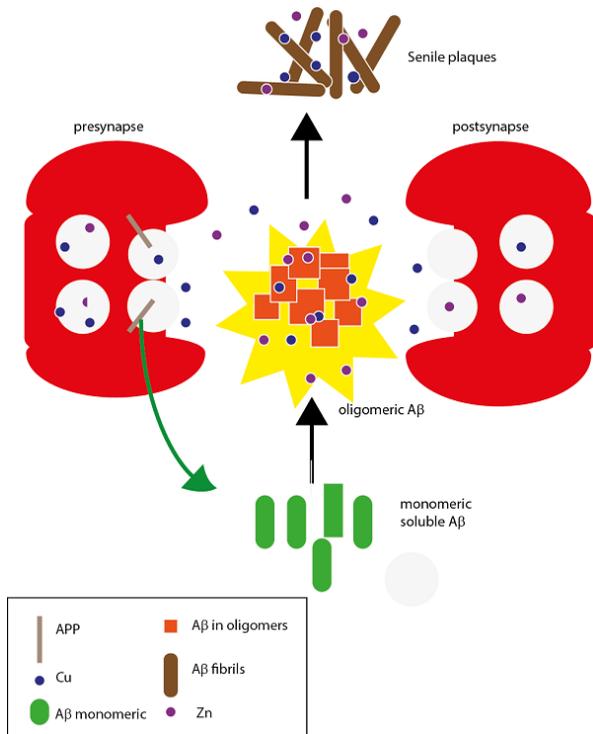


Figure 1. Scheme of synaptic cleft: The amyloid cascade

Amyloid β is a 39-43 residue peptide and is produced *via* cleavage of the amyloid precursor protein called APP. APP is multi-domain single pass transmembrane protein, which is mainly found in the synapses of neurons, with many different functional activities including a role in maintaining metal homeostasis. Treating neuronal cells with copper has shown an increase of the levels of APP at the cell surface, but a decrease in the concentration of intracellular copper promotes the generation of Amyloid β through the protease activity of β -secretase and γ -secretase leading to the fragments $A\beta$ -40 and $A\beta$ -42⁴.

In AD brains, amyloid plaques are enriched with mis-localized Cu^{2+} . The relationship between copper's dyshomeostasis and this neurodegenerative disease has become evident in the last years³. The Cu^{2+} - $A\beta$ binding event is proposed to be paramount in the cascade hypothesis, as Cu^{2+} has a high affinity for $A\beta$ -42, promoting its aggregation¹.

The toxicity of Cu-A β complexes is most likely due to their catalytic role in the formation of reactive oxygen species (ROS), which results in oxidative stress. Cu bound to A β is reduced with a concomitant production of H₂O₂, O₂⁻ and A β oxidised. These oxidative agents are involved in oxidative damage, including lipid peroxidation, protein oxidation and oxidative damage in mitochondria of AD brains².

Recent studies with small organic molecules and with moderate affinity for copper, called metal protein attenuating compounds (MPAC), have presented promising results. These compounds target mis-localized metals in senile plaques and bring them to their place of origin⁴.

The goal of this project is the synthesis of small peptides which act as MPAC and contain fluorophores groups for the detection of copper and, hence, for AD diagnosis.

3.2. FLUORESCENT PROBES

Fluorescent probes are the most commonly used tools for the diagnosis of AD. These probes are organic fluorophores groups that are introduced into peptides as amino acid derivatives. Among them, coumarin derivatives are extensively used as fluorescent probes in the peptides and other biomolecules due to their wide range of issue.

- They are used for diagnosis of different diseases because their emission decreases when copper is added (quenching), allowing its use as sensor of copper.
- They are used for the scavenging of reactive oxygen species (ROS) such as hydroxyl radicals and superoxide anions since many natural and synthetic coumarins with hydroxyl groups and other substituents have been found to inhibit lipid peroxidation and to influence the processes involving free radical-mediated injuries according to its antioxidant capacity.

On the other hand, a new fluorescent probe called 4-*N,N*-dimethylamino-1,8-naphthalimido (4-DMN) has been used in the study of protein-copper interactions. This fluorescent probe offers several distinct advantages over related fluorophores, including greater chemical stability under a wide range of conditions, a longer excitation wavelength (408nm) and a high quantum yield⁵.

3.3. COPPER BINDING PEPTIDES WITH FLUORESCENT PROBES

The histidine residues, through the imidazole rings, have the ability to capture mis-localized copper. Recent studies done in collaboration with the Bioinorganic Chemistry group, led by Professor Patrick Gámez, have shown that the -His-A_{AA}-His- sequence present greater affinity

for copper than those encompassing other sequences (namely, -His-His-AAA, -AAA-His-His-) studied previously and a greater affinity for copper than A β peptide.

In previous end of degree projects, studies with peptides containing coumarin as the fluorescent probe have been performed. First, H-His-Lys(Cm)-His-OH peptide was synthesized and the fluorescent properties of coumarin were studied. Then, Fmoc-His-Lys(Cm)-His-Lys-NH₂ peptide was synthesized for the study of its binding to the small gold particles (5-20 nm). Gold nanoparticles have been reported to cross the blood-brain barrier (BBB), which limits the treatment of disorders such as Alzheimer's disease and many others, and they are being extensively studied as drug-carriers. These studies are currently in progress.

In contrast to peptides previously synthesized in the group, the N-terminus of the peptides synthesized in this work was protected by an acetyl group because the Fmoc group and coumarin present interferences in the UV-Vis. The Ac-His-Lys(Cm)-His-Lys-NH₂ peptide is formed by two histidine residues responsible for the chelation of mis-localized copper, one lysine residue functionalized with a coumarin group that provides the sensor and antioxidant properties and one non-functionalized lysine residue that is responsible for the conjugation with the gold nanoparticles through amide bonds with polyethylene glycol (PEG) appendices, which also stabilize the nanoparticles (Figure 2.A).

On the other hand, a new fluorescent probe called 4-DMN, which exhibits a quantum yield higher than that of coumarin and an excitation wavelength in the visible range, is reported. This new fluorescent probe was introduced as an amino acid derivative in a peptide with Ac-His-Lys(4-DMN)-His-NH₂ sequence formed by two histidine residues whose imidazole rings are expected to bind mis-localized copper. (Figure 2.B).

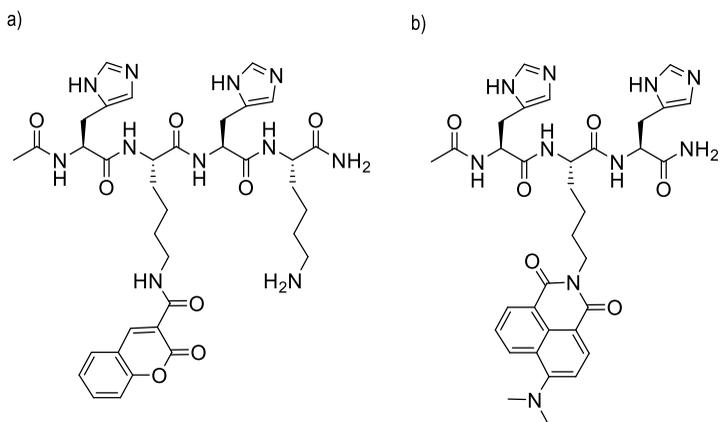


Figure 2. A) Ac-His-Lys(Cm)-His-Lys-NH₂ peptide. B) Ac-His-Lys(4-DMN)-His-Lys-NH₂

4. OBJECTIVES

- Synthesis of the two amino acid derivatives N^α -(9-fluorenylmethoxycarbonyl)- N^ϵ -(coumarin-3-ylcarbonyl)-L-Lysine and N^α -(9-fluorenylmethoxycarbonyl)- N^ϵ -(4-*N,N*-dimethylamino-1,8-naphthalimido)-L-Lysine.
- Solid phase synthesis of Ac-His-Lys(Cm)-His-Lys-NH₂ and Ac-His-Lys(4-DMN)-His-NH₂.

5. SYNTHESIS OF NON-NATURAL AMINO ACIDS WITH FLUORESCENT PROBES

In this part, the synthesis in solution of two non-natural amino acids using two different fluorescent probes is described. These amino acid derivatives were prepared for their potential use in the diagnosis of the disease.

5.1. SYNTHESIS OF THE NON-NATURAL AMINO ACID N^α -Fmoc- N^ϵ -(COUMARIN-3-ILCARBONYL)-L-LYSINE

This non-natural amino acid is prepared in two consecutive steps, described in the experimental part of the project. First, intermediate 3-(1*H*-benzotriazole-1-ylcarbonyl)-2-chromen-2-one, that will then react with commercial Fmoc-Lys-OH to finally yield the non-natural amino acid, was synthesized.

*Synthesis of 3-(1*H*-benzotriazole-1-ylcarbonyl)-2-chromen-2-one*

The intermediate is obtained by means of the reaction between 3-(2*H*-chromen-2-one)carboxylic acid, activated with an excess of SOCl_2 and 1*H*-benzotriazole in anhydrous THF as solvent at room temperature (Figure 3).

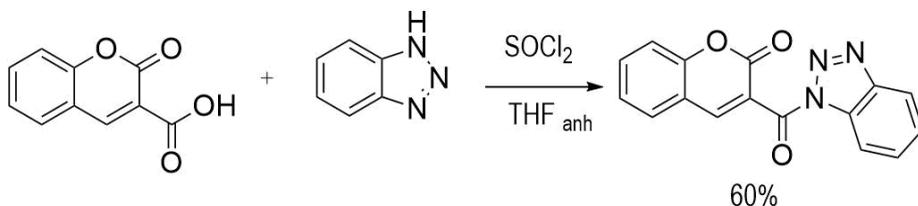


Figure 3. Synthesis of 3-(1*H*-benzotriazole-1-ylcarbonyl)-2-chromen-2-one

Synthesis of N^α -(9-fluorenylmethoxycarbonyl)- N^ϵ -(coumarin-3-ylcarbonyl)-L-Lysine

Next, the non-natural amino acid is formed from the above intermediate when reacted with commercially available Fmoc-L-Lys-OH along with Et_3N and using 1:1 mixture of MeCN and H_2O as solvent (Figure 4).

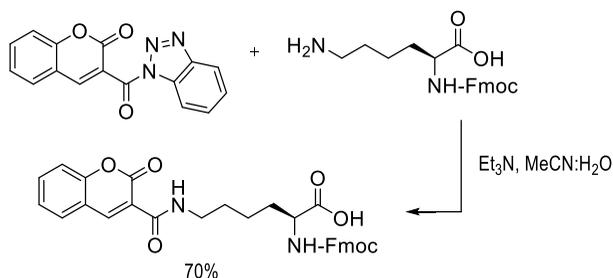


Figure 4. Synthesis of N^{α} -(9-fluorenylmethoxycarbonyl)- N^{ϵ} -(coumarin-3-ylcarbonyl)-L-Lysine

5.2. SYNTHESIS OF THE NON-NATURAL AMINO ACID N^{α} -FMOC- N^{ϵ} -(4-*N,N*-DIMETHYLAMINO-1,8-NAPHTHALIMIDO)-L-LYSINE

This synthesis is performed in three consecutive steps, as described in the experimental part. First, the 4-*N,N*-dimethylamino-1,8-naphthalic anhydride precursor is synthesized and subsequently reacted with commercial amino acid Boc-Lys-OH obtaining N^{α} -(*tert*-butoxycarbonyl)- N^{ϵ} -(4-*N,N*-dimethylamino-1,8-naphthalimido)-L-Lysine amino acid. Finally, the Boc protecting group is removed by treatment with TFA and the amino acid is reacted with Fmoc group thus yielding N^{α} -(9-fluorenylmethoxycarbonyl)- N^{ϵ} -(4-*N,N*-dimethylamino-1,8-naphthalimido)-L-Lysine amino acid.

Synthesis of 4-*N,N*-dimethylamino-1,8-naphthalic anhydride

4-Bromo-1,8-naphthalic anhydride and 3-dimethylamino-propionitrile are reacted using 3-methyl-1-butanol as solvent at reflux (Figure 5). The product is obtained in good yield as orange crystals which exhibit a strong fluorescent emission when dissolved in non-polar solvents such as CH_2Cl_2 .

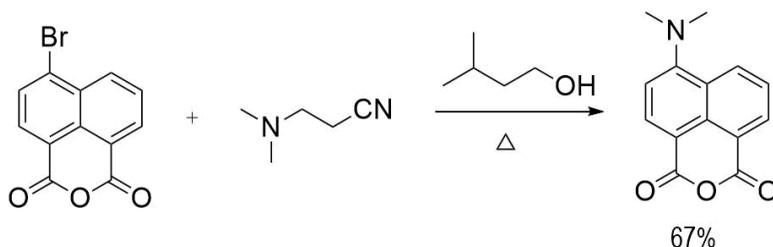


Figure 5. Synthesis of 4-*N,N*-dimethylamino-1,8-naphthalic anhydride

Synthesis of non-natural N^α -(*tert*-butoxycarbonyl)- N^ϵ -(4-*N,N*-dimethylamino-1,8-naphthalimido)-L-Lysine

This non-natural amino acid is formed by means of the reaction of the precursor synthesized as described above with commercial Boc-Lys-OH using dioxane as solvent. This reaction is carried out in basic conditions (NaHCO_3) and reflux. In this reaction it is important not to exceed the reflux temperature and slowly add the commercial amino acid to the precursor because it can undergo hydrolysis. Therefore, a higher yield and greater purity of the product is obtained and the reaction can proceed in larger scales.

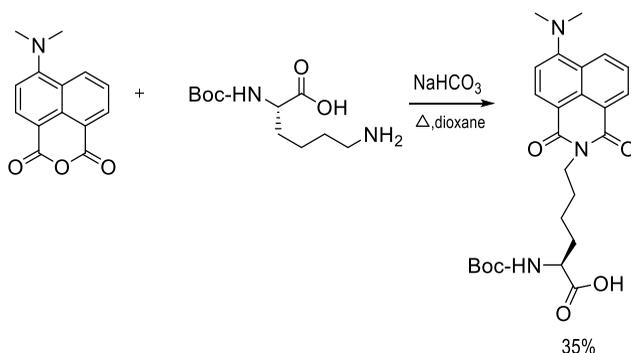


Figure 6. Synthesis of N^α -(Boc)- N^ϵ -(4-*N,N*-dimethylamino-1,8-naphthalimido)-L-Lysine

Synthesis of non-natural N^α -(9-fluorenylmethoxycarbonyl)- N^ϵ -(4-*N,N*-dimethylamino-1,8-naphthalimido)-L-Lysine

TFA is added to the non-natural N^α -(Boc)- N^ϵ -(4-*N,N*-dimethylamino-1,8-naphthalimido)-Lysine amino acid to remove the protecting group Boc using DCM as solvent. Subsequently, under basic conditions, the amino acid is reacted with *N*-(9-fluorenylmethoxycarbonyloxy)-Succinimide (Fmoc-Osu) using dioxane as solvent hence affording the Fmoc-protected fluorescent amino acid derivative in high yield (Figure 7).

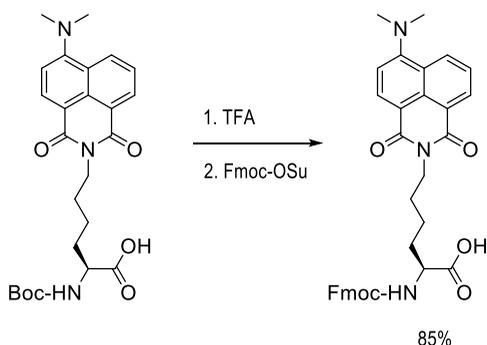


Figure 7. N^α -(Fmoc)- N^ϵ -(4-*N,N*-dimethylamino-1,8-naphthalimido)-L-Lysine

6. SOLID PHASE SYNTHESIS

The peptides are polymers of amino acids bound through amine bonds, called peptide bonds. Peptide bonds result from the condensation of the α -carboxyl group of one amino acid and the α -amino group of another one with concomitant loss of water. In the field of peptide synthesis, the amino acid residues bear several protecting groups in order to avoid side reactions (Figure 8).

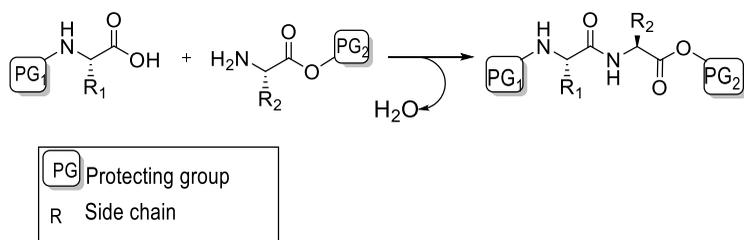


Figure 8. Example of reaction between amino acids

6.1. SOLID PHASE SYNTHESIS OF PEPTIDES

The solid phase synthesis of peptides (SPPS) is a methodology which consists on the sequential addition of the amino acids in different steps (Figure 9). Firstly, the first amino acid, with the amino group protected, is loaded to the resin through a covalent bond which can be ester or amide. Once the first amino acid is attached to the resin, the Fmoc protecting group at the α -amino group of the first amino acid is removed, allowing the reaction with the α -carboxylic acid of the next amino acid. The same procedure is repeated until the desired peptide is synthesized. Once the peptide is finished it is removed from the resin.

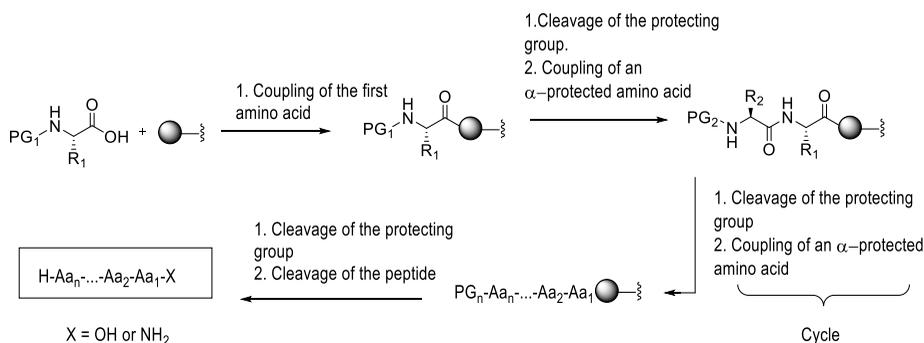


Figure 9. General scheme of solid phase peptide synthesis

The process of protection/deprotection of the amino acids extremes is very important because the amino acids have functional groups that must be protected to avoid side reactions. The different conditions needed for removing each protecting group allow a high control over the other protecting groups when one of them is being eliminated. Hence, the different protecting groups used to perform the synthesis of peptides have to be orthogonal to each other, that is, the other protecting groups must remain unaltered during the elimination process. For this reason, the Fmoc/*t*Bu strategy is widely used. It requires mild conditions and relies on the protection of α -amino groups of amino acids with the Fmoc group (9-fluorenylmethyloxycarbonyl), which is labile to basic conditions (piperidine), and the *t*Bu (*tert*-butyl) type groups to protect the side chains, which is removed in acidic conditions (trifluoroacetic acid, TFA). The side chains of the peptides prepared in this project are protected

with *t*Bu type groups like trityl or Boc, both labile to TFA. Figure 10 (see below) shows the synthetic strategy using histidine as amino acid.

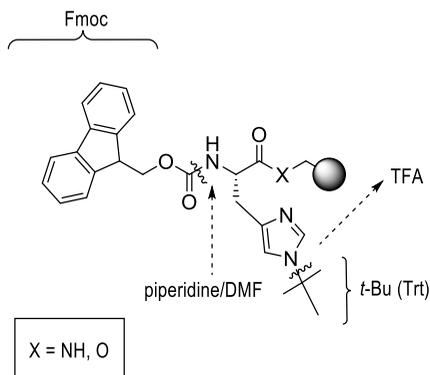


Figure 10. Amino acid protected with Fmoc and *t*Bu protecting groups

In SPPS, the reactions take place in a solid support wherein the amino acids are covalently bound. The polymer support has to meet certain requirements to work properly: it must allow the filtration of solvents that may contain reagents or by-products, must be physically stable and must be inert to those solvents and reagents used for the synthesis of peptides. To enhance the contact surface of the resin, some solvents can swell and shrink the polymer, which will be discussed later.

In the Fmoc strategy, in general, two types of resin are used: Rink Amide resin and 2-chlorotrityl chloride resin (CTC). The former is characterized by free amino groups, which are on the surface of the polymeric support and bind to the first amino acid through amide bonds, thus yielding a carboxamide bond in the C-terminus once the peptide is removed from the resin (Figure 11. A). The second one generates an ester bond with the amino acid in the C-terminus (Figure 11. B).

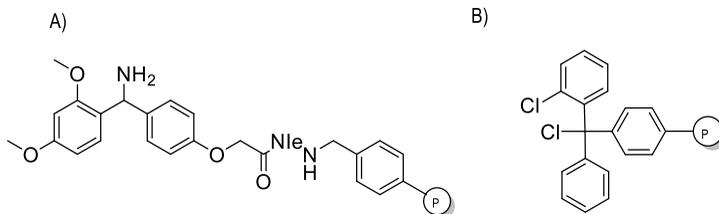


Figure 11. A) Rink Amide resin. B) CTC resin

In this project, Rink Amide resin was used in order to afford a carboxamide group in the C-terminus of the peptide. This is required since:

- Carboxypeptidases are enzymes whose function is hydrolyse peptide bonds of peptides and proteins with a carboxylic group in the C-terminus position, which thus lose their function. Peptides synthesized using Rink Amide resins present a carboxamide group at the C-terminus which makes them resistant to the action of these enzymes and therefore remain stable in biological fluids.

- The binding of the peptide to the gold nanoparticles is produced *via* the PEG appendices. The carboxylic group of the PEG chains needs to be activated to allow the ϵ -amino group of the lysine residue to attack and generate the amide bond. If the CTC resin was used, the carboxylic acid of the C-terminus position would be activated and may also be attacked by another α -amino group of another peptide, hence forming a dimer.

6.1.1. Synthetic strategy

In this section, the synthesis of the peptides will be described. As mentioned above, the Fmoc/*t*Bu strategy and the Rink Amide resin were used. Figure 12 shows the steps followed during the synthesis.

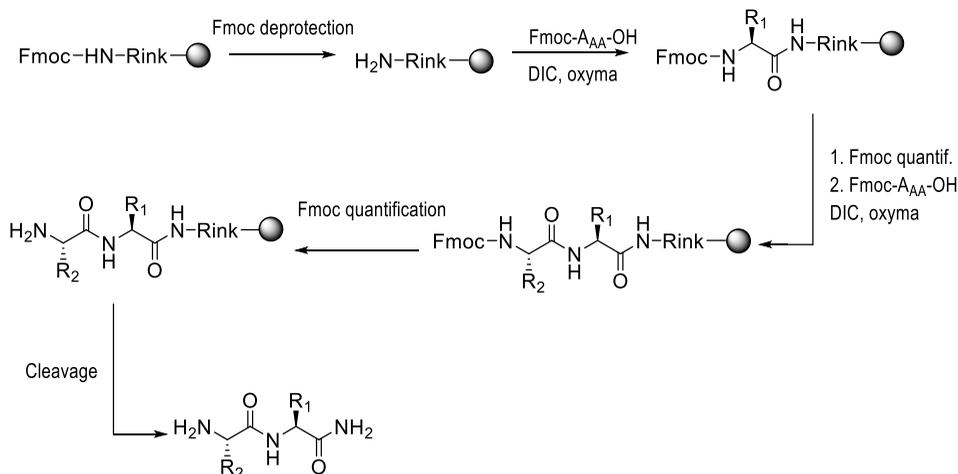


Figure 12. General scheme of the synthesis using Rink Amide resin

First, the resin was conditioned by washing with DMF (*N,N*-dimethylformamide) and DCM (dichloromethane) to swell and shrink the resin so that the interface between the resin and the amino acid is greater and to eliminate potential impurities. For optimal conditions of the resin, the conditioning cycle is performed after each stage. To bind the first amino acid to the resin, the Fmoc groups, which protect the binding points of the resin, are removed by washing with piperidine/DMF.

Coupling of the first amino acid

Certain conditions for binding the amino acid with the resin are required. First, the carboxylic group of the amino acid has to be activated under mild conditions using a carboiimide such as diisopropylcarbodiimide (DIC). The result of this reaction is the formation of an *O*-acylisourea, which is a good leaving group, and a symmetrical anhydride is formed through a nucleophilic attack of another amino acid. Subsequently, an additive such as oxyma is added to activate the ester group of the amino acid. These two differently activated groups, under a nucleophilic attack caused by α -amino group of the resin, bind to the polymeric support.

The role of oxyma is very important as it avoids side reactions, such as the irreversible formation of the *N*-acylurea and/or amino acid racemization (Figure 13).

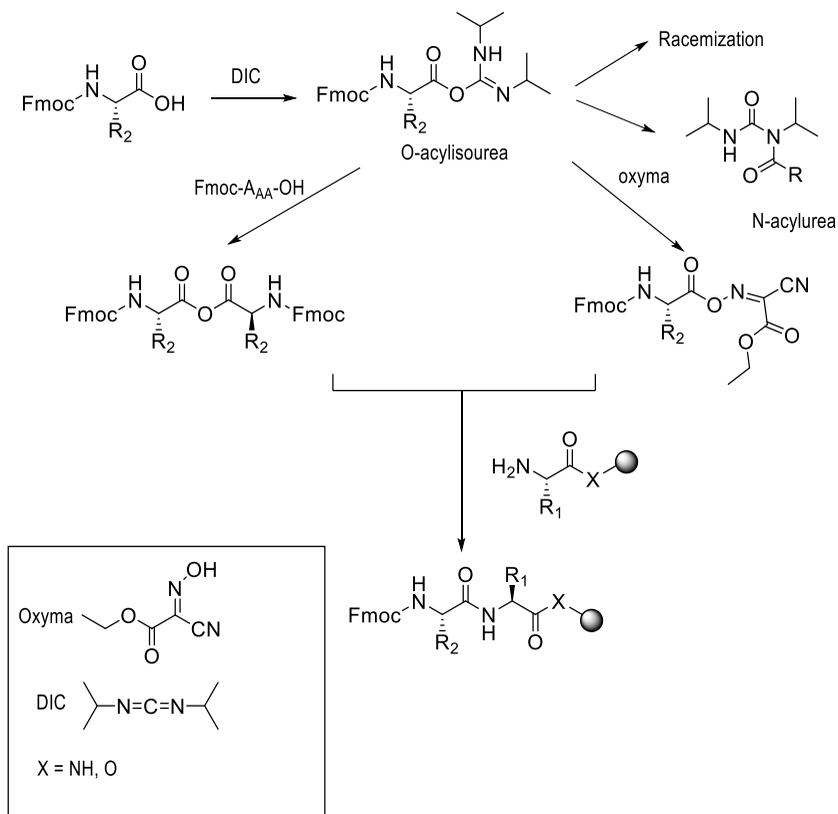


Figure 13. The role of DIC and oxyma in peptide synthesis

In order to achieve a large amount of final peptide it is important to ensure that as many amino acid molecules as possible are linked to the resin. Therefore, the process of coupling of the first amino acid is usually performed twice.

Resin capping

Once the first amino acid is linked, a capping step is performed through a treatment with Ac_2O and DIPEA (*N,N*-diisopropylethylamine), blocking the free amino functions that are in the resin to avoid the formation of deletion peptides.

Fmoc removal

Whenever the coupling of an amino acid to another is required, the protecting groups attached to the N-terminus have to be removed to form the bond. After the addition of the first and the last amino acid, it is necessary to carry out a quantification of the Fmoc groups present

in the growing chain. The Fmoc quantification, which is described in the Analytical methods part (Section 8.2.2), is a process used to measure the amount of amino acid or peptide bonded with the resin.

Peptide elongation

To obtain the desired peptide, the remaining amino acids are added by the same procedure and the same reagents as in the process of coupling the first amino acid. Each time a new amino acid is added, the Fmoc protecting group attached to the N-terminus position has to be removed, so that it can react with the next amino acid. Once the last amino acid is bonded, the Fmoc quantification step is repeated to determine the final amount of peptide bonded to the polymeric support

Peptide cleavage

The cleavage process consists on releasing the peptide from the resin through a TFA treatment. During the process, triisopropylsilane (TIPS) is added as a carbocation scavenger to avoid side reactions due to the formation of highly reactive carbocations which can be attacked by nucleophilic functions of the peptide.

7. SOLID PHASE SYNTHESIS OF AC-HIS-LYS(CM)-HIS-LYS-NH₂ AND AC-HIS-LYS(4-DMN)-HIS-NH₂

7.1. SYNTHESIS OF AC-HIS-LYS(CM)-HIS-LYS-NH₂

The procedure followed in the synthesis of Ac-His-Lys(Cm)-His-Lys-NH₂ is analogous to that explained above except for two stages. As shown in figure 14, after the last Fmoc quantification, an acetyl group at the N-terminus was added by treatment with Ac₂O and DIPEA. After the attachment of the acetyl group it is important to wash the peptidyl-resin with piperidine/DMF because, according to previous results, a by-product appears. The reason why a by-product appears is unknown.

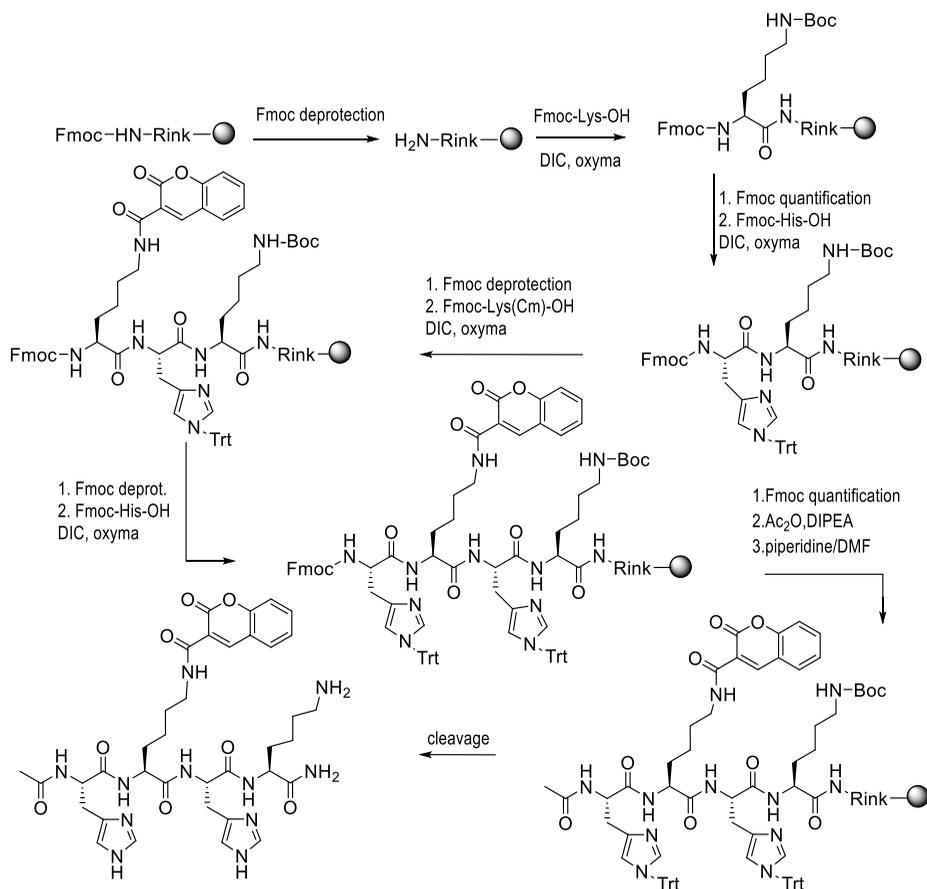


Figure 14. General scheme of the synthesis Ac-His-Lys(Cm)-His-Lys-NH₂ peptide

Results for Ac-His-Lys(Cm)-His-Lys-NH₂

The value obtained for the Fmoc quantification of the first amino acid was 73%, showing that the coupling of the first amino acid occurred in high yield. The second Fmoc quantification, once the last amino acid was added, was quantitative. The weight yield is 26%. Once the peptide was cleaved from the resin it was analysed by HPLC-MS analysis.

The chromatogram obtained for the product yielded after this process, as shown in figure 15, shows a single signal with a retention time of 8.37 min corresponding to the peptide Ac-His-Lys(Cm)-His-Lys-NH₂ ($m/z = 762 [M+H]^+$). A high chromatographic purity was obtained (94%).

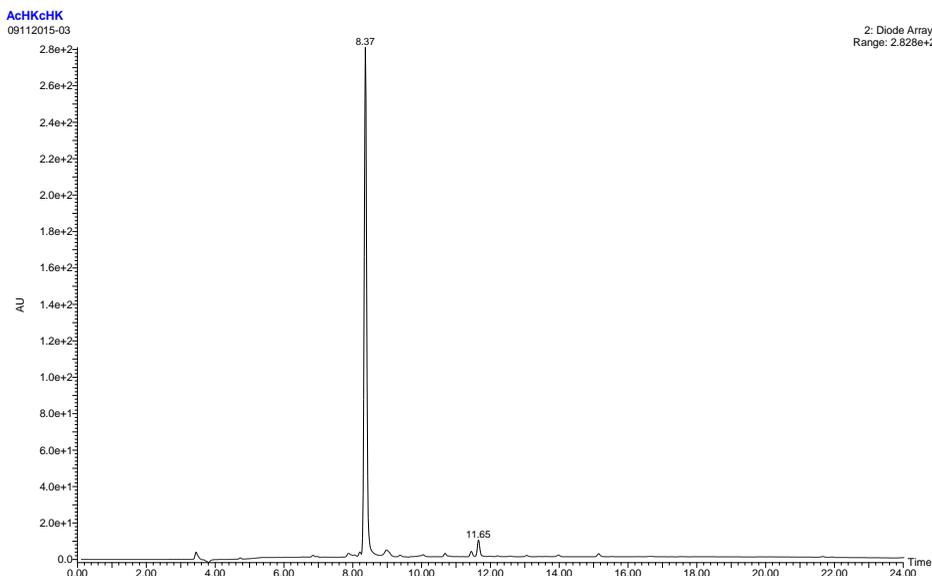


Figure 15. HPLC-MS of Ac-His-Lys(Cm)-His-Lys-NH₂

However, if the washings are not carried out, two signals appear. The first signal belongs to the peptide Ac-His-Lys(Cm)-His-Lys-NH₂ (retention time 8.72 min; $m/z = 762 [M+H]^+$), whereas the second corresponds to a by-product obtained after treatment with Ac₂O and DIPEA (retention time 9.95 min; $m/z = 889 [M+H]^+$) as shown in the figure 16. This m/z ratio fits that for the peptide with three acetyl groups instead of one. Analogous by-products with extra groups such as Fmoc or His residues have previously been observed in the group, although a satisfactory explanation has not been found. Nevertheless, the coumarin group seems to play an important role in the formation of such species since they are only formed when Lys(Cm) has already been added.

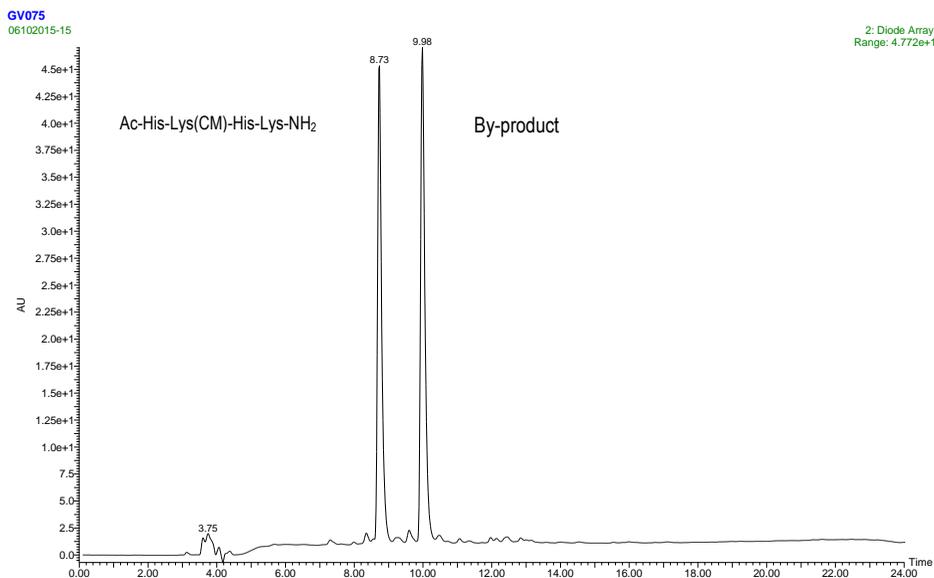


Figure 16. HPLC-MS of Ac-His-Lys(Cm)-His-Lys-NH₂ peptide and its by-product

7.2. SYNTHESIS OF AC-HIS-LYS(4-DMN)-HIS-NH₂

The procedure followed used in the synthesis of the Ac-His-Lys(4-DMN)-His-NH₂ is analogous to that explained above in the synthesis of the Ac-His-Lys(Cm)-His-Lys-NH₂ (Figure 17).

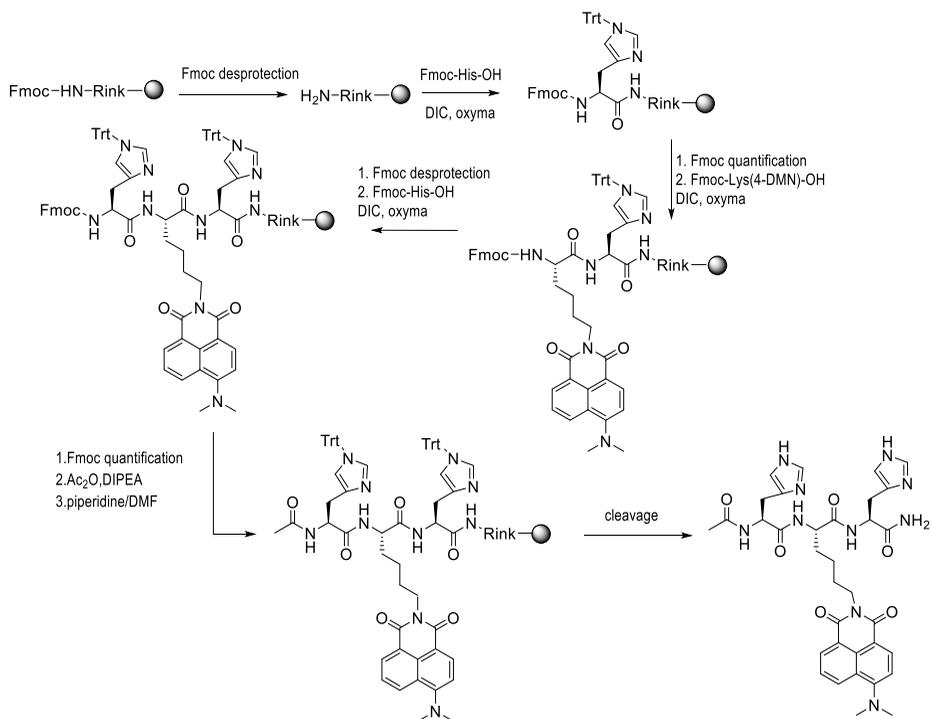


Figure 17. General scheme of the synthesis of Ac-His-Lys(4-DMN)-His-NH₂ peptide

Results for Ac-His-Lys(4-DMN)-His-NH₂

The value obtained for the Fmoc quantification of the first amino acid was 86%, showing that the coupling of the first amino acid took place in a high yield. The second Fmoc quantification, once the last amino acid was added, was quantitative. The weight yield, however, was a poor 17%, most likely due to an incomplete cleavage from the resin.

Once the peptide was cleaved from the resin, it was analysed by HPLC-MS analysis (Figure 18).

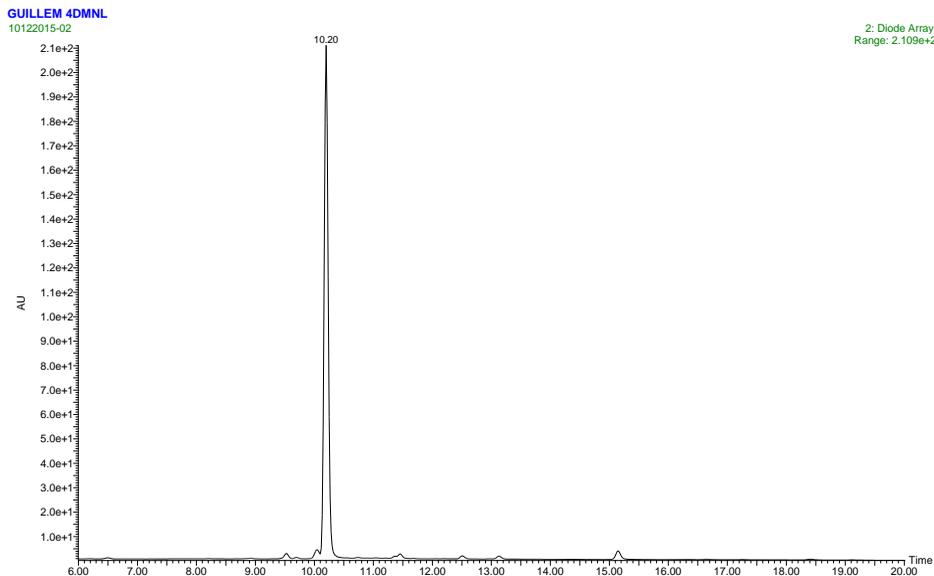


Figure 18. HPLC-MS of Ac-His-Lys(4-DMN)-His-NH₂ peptide

The chromatogram of the product showed a sole signal with a retention time of 10.36 minutes, corresponds to a product with $m/z=685$. This m/z ratio matches the expected value for the $[M+H]^+$ species. A high chromatographic purity was obtained (96%).

8. EXPERIMENTAL SECTION

8.1. MATERIALS AND METHODS

8.1.1. Reagents and solvents

Brand	Products
Acros	1H-benzotriazole, SOCl ₂ , coumarin-3-carboxylic acid
Aldrich	TIPS, Et ₃ N, piperidine, Et ₂ O, dioxane
Fisher scientific	TFA
Iris Biotech	Oxyma, Fmoc-Lys(Boc)-OH, Fmoc-L-His(Trt)-OH
Jescuder	Anhydrous MgSO ₄ , NaHCO ₃ , NaCl
Novabiochem [®]	Fmoc-Lys-OH, Fmoc-Osu, RA resin
Panreac	Pyridine
Scharlau	Acetone, AcOEt, DCM, HCl, MeOH, 3-methyl-1-butanol
Specialist des solvents	DMF
TCI	DIC, 4-bromo-1,8-naphthalic anhydride

Table 1. Reagents and solvents

- Anhydrous DCM was prepared in presence of calcium hydride (CaH₂) and N₂ stream being distilled before its use
- Anhydrous THF was prepared in the presence of Na and benzophenone and passed under a stream of N₂ and distilled before its use

- The dried Et₂O is kept over Na

8.1.2. Instruments

Instrument	Brand	Model
Analytical balance	Mettler	Toledo AB254
Centrifuge	Hettich	Rotofix 32A
HPLC	Shimadzu	LC-20AD
UV-Vis spectroscopy	Varian	Cary 100
NMR	Varian	Mercury 400 MHz
Rotatory evaporator	BUCHI	R-200
HPLC-MS	Waters	2695 separation module

Table 2. Instrumentation.

8.1.3. Chromatography

8.1.3.1. High performance liquid chromatography (HPLC)

A system constituted by a Shimadzu LC-20AD quaternary pump, an automatic injector SIL-10Advp, dual variable wavelength detector SPD-20A and an online degasser device DGU-20A5 was used.

A C18 reverse phase column supplied by Phenomenex[®] of 250x4mm with 5.15±microns of size particle and a pore size of 320±40Å was used. The eluents were H₂O with 0.1% of formic acid and acetonitrile with 0.1% of formic acid. Different gradients of H₂O/formic acid was used at a flow rate of 1 mL x min and double detection (λ) at 220 nm and 301 nm.

8.1.3.2. High resolution liquid chromatography coupled with electrospray mass spectrometry (HPLC-MS)

Waters system composed by a 2695 separation module, a PDA detector 2996 and a Micromass ZQ mass detector (ESI-MS) is used for HPLC-MS detections. The column and the eluents used are the same as mentioned in the previous section.

8.1.3.3. Thin layer Chromatography (TLC)

TLC was performed on silica gel 60 F254 plates purchased by Merk. UV light was used to reveal the thin films.

8.1.4. Nuclear magnetic resonance

^1H NMR spectra were recorded on a Varian Mercury 400 spectrometer. Chemical shifts (δ) are quoted in ppm and referenced to internal TMS (δ 0.00 for ^1H NMR). Data are reported as follows: s for singlet, d for doublet, t for triplet, q for quartet, m for multiplet and their corresponding combinations.

8.2. ANALYTICAL METHODS

8.2.1. Ninhydrin test^{6,7}

The Ninhydrin test or Kaiser is a qualitative test for the determination of the presence or absence of free primary amino groups, and it can be a useful indication about the completeness of a coupling step. The test is based on the reaction of Ninhydrin with primary amines, which gives a characteristic dark blue colour. The Ninhydrin reaction is shown in figure 19.

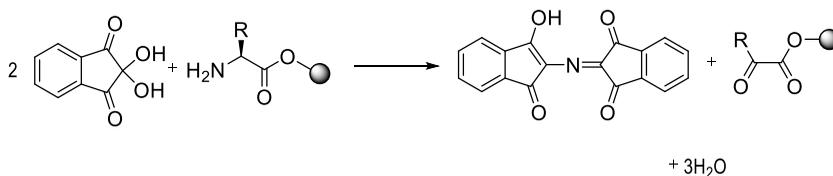


Figure 19. Ninhydrin test reaction

To perform the assay it is necessary to prepare two solutions:

- Reagent A: A warm solution of phenol (40 g) is prepared in absolute EtOH (10 mL). Independently, 2 mL of a solution of KCN (65 mg in 100 mL of H₂O) in pyridine (100 mL) freshly distilled over Ninhydrin, are prepared. The two solutions were stirred separately with 4g of Amberlite MB-3 for 45 min. The solutions were filtrated and the filtrates were mixed.

- Reagent B: a solution of Ninhydrin (2.5 g) was prepared in absolute EtOH (50 mL). This solution has to be protected from the light, preferably under N₂ atmosphere.

The analytical procedure is as follows: the peptide resin is previously washed with DCM to remove possible traces of DMF that may remain and can decompose giving primary amines, which would alter the results. A final wash with MeOH is needed for drying the resin. A small quantity of peptide-resin was introduced into a small tube and 6 drops of reagent A and 2 drops of reagent B are added. The tube with the solution is heated at 110 °C for 3 minutes. A blue colour of the solution indicates the presence of primary amines (positive test), demonstrating an incomplete amino acid coupling. Nevertheless, a yellow coloration indicates the absence of primary amines (negative test), demonstrating a complete coupling of the amino acid. A blank is performed in parallel to compare colours.

8.2.2. Fmoc quantification⁸

An exhaustive control of the resin during the peptide synthesis gives useful information about the progress of the peptide elongation. This can be easily done by quantifying the amount of Fmoc removal at each deprotection/coupling cycle by using UV-Vis spectroscopy, measuring the absorbance of the adduct which is formed after removing the protecting group (Figure 20).

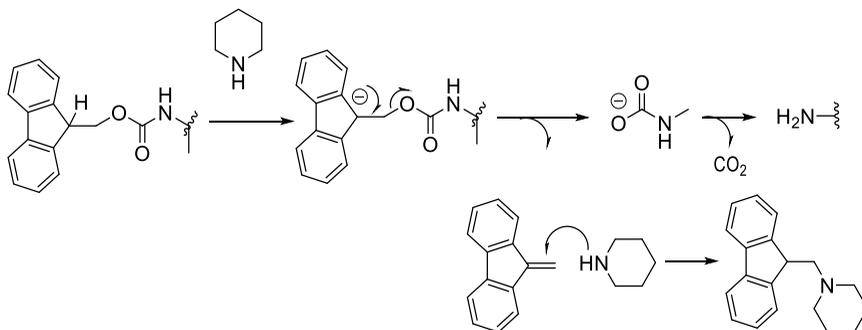


Figure 20. Reaction of the removal of Fmoc

To carry out this test, the resin is previously washed with DMF (3 x 30 s). After this, the resin is treated with 25 mL of 20% piperidine in DMF solution for 25 min (5 x 5 min). Washings with 25 mL DMF (5 x 30 s) are done after treating the resin.

The mixture of piperidine/DMF and the further washings with DMF are collected in 100 mL volumetric flask and diluted to the mark with DCM. In another 100 mL volumetric flask, 25 mL of

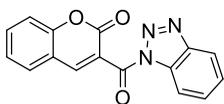
DMF and 25 mL of piperidine/DMF (20:80) are added to an aliquot of 2 mL from the first volumetric flask. The mixture is then diluted to the mark with DCM. In another 50 mL volumetric flask, is done the blank with 12.5 mL of piperidine/DMF (20:80) and 12.5 mL of DMF. The mixture is then diluted to the mark with DCM. Absorbance is measured and using the Lambert Beer Law and the concentration can be calculated ($\epsilon=7800 \text{ M}^{-1} \text{ cm}^{-1}$ at 301 nm with a $l=1\text{cm}$)

8.3. SYNTHETIC PART

8.3.1. Synthesis of N^α -Fmoc- N^ϵ -(coumarin-3ilcarbonyl)-L-Lysine⁹

8.3.1.1. Synthesis of the 3-(1H-benzotriazole-1-ilcarbonyl)-2-chromen-2-one intermediate

SOCl_2 (20 mmol, 2 eq) was added to a stirring solution of 1H-benzotriazole (50 mmol, 5 eq), previously purged with N_2 , using anhydrous THF (100 mL) as solvent at rt. After 20 min, 3-(2H-chromen-2-one)carboxylic acid (10 mmol, 1 eq) was added and the reaction mixture was stirred during 4h. During the reaction a white solid was formed. The solution was filtered and the filtrates were concentrated under reduced pressure to remove anhydrous THF and to obtain a white product in the round-bottom flask. The solid was dissolved in EtOAc (150 mL) and washed with a saturated aqueous solution of Na_2CO_3 (3 x 50 mL) and brine (50 mL). The resulting organic phase was dried with anhydrous MgSO_4 . The volatile solvents were removed under reduced pressure to obtain the desired product, which was recrystallized with a DCM-hexanes mixture. The product was obtained with a yield of 60%.



White solid. R_f ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 9:1): 0.85. $^1\text{H NMR}$ (CDCl_3 , 400 MHz): δ 7.35-7.39 (m, 1H), 7.39-7.43 (m, 1H), 7.52-7.56 (m, 1H), 7.63-7.65 (m, 1H), 7.66-7.68 (m, 1H), 7.68-7.72 (m, 1H), 8.12 (d, $J = 8.3 \text{ Hz}$, 1H), 8.30-8.33 (m, 2H). $^{13}\text{C NMR}$ (CDCl_3 , 100.6 MHz): δ 114.29, 117.08, 117.60, 120.37, 121.80, 125.21, 126.71, 129.56, 130.78, 131.13, 134.40, 146.13, 146.90, 154.78, 157.33, 162.57.

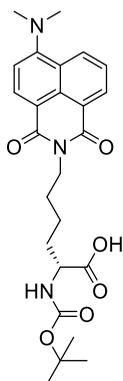
8.3.1.2. Synthesis of the N^α -Fmoc- N^ϵ -(coumarin-3ilcarbonyl)-L-Lysine

3-(1H-benzotriazole-1-ylcarbonyl)-2-chromen-2-one (4mmol, 1 eq) was added to a 100 mL round-bottom flask that contained Fmoc-L-Lys-OH (4 mmol, 1 eq) and Et_3N (8 mmol, 2 eq) in $\text{MeCN}:\text{H}_2\text{O}$ solution (40:20 mL). The reaction mixture was stirred at room temperature until the intermediate was not observed in TLC (2h). 4N HCl (4 mL) was then added and the reaction

8.3.2.2. Synthesis of non-natural N^α -(Boc)- N^ϵ -(4-N,N-dimethylamino-1,8-naphthalimido)-L-Lysine.

4-*N,N*-dimethylamino-1,8-naphthalic anhydride was added to a 250 mL three-necked round-bottom flask equipped with a magnetic stir bar and reflux condenser. The reaction vessel was purged with N_2 . Dioxane (120 mL) was then transferred to the reaction vessel *via* syringe through the rubber septum. The suspension was stirred vigorously as the temperature was raised to reflux (101°C). Once at reflux, Boc-Lys-OH (3.8 mmol, 1 eq) and $NaHCO_3$ (24 mmol, 6 eq) were dissolved in H_2O (25 mL) and transferred to a 60 mL addition funnel to finally be added dropwise. The reaction was allowed to proceed at reflux for 30 min before allowing cooling to room temperature.

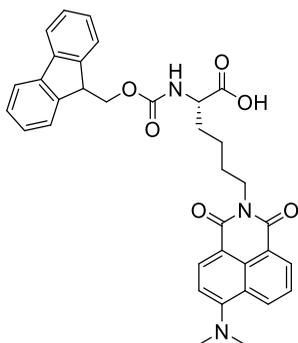
The reaction was then concentrated on the rotary evaporator to remove most of the dioxane before diluting to 200 mL with H_2O . The aqueous layer was then acidified with 4N HCl and extracted with DCM (3 x 100 mL). The organic layers were combined, dried with $MgSO_4$, filtered and concentrated. The crude was purified by flash column chromatography using ethyl acetate with 0.5% acetic acid as the solvent system. The fractions containing the desired product were combined and azeotroped in toluene (3 x 100 mL) to remove residual acetic acid. The product was isolated as a bright orange with a yield of 35%.



Orange solid. 1H NMR ($CDCl_3$, 400 MHz): δ 1.41 (s, 9H), 1.45-1.55 (m, 2H), 1.68-1.79 (m, 2H), 1.80-1.99 (m, 2H), 3.07 (s, 6H), 4.08-4.20 (m, 2H), 4.23-4.31 (m, 1H), 7.06 (d, $J = 8.24$, 1H), 7.62 (t_{app} , $J_{app} = 8.0$, 1H), 8.38 (d, 1H, $J = 8.40$), 8.43 (d, $J = 8.21$, 1H), 8.52 (d, $J = 7.21$, 1H). ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 22.68, 27.61, 28.32, 31.61, 39.48, 44.74, 53.48, 79.90, 113.25, 114.71, 122.87, 124.82, 125.11, 130.17, 131.14, 131.21, 132.83, 155.90, 156.97, 164.18, 164.70, 176.46.

8.3.2.3. Synthesis of non-natural N^α -(Fmoc)- N^ϵ -(4-N,N-dimethylamino-1,8-naphthalimido)-L-Lysine

Solid Boc-4DMNL (1.3mmol, 1 eq) was dissolved in DCM (14 mL) and stirred in a 100 mL round-bottom flask. Then, cold TFA (14 mL) was added by addition funnel over 5 min. The reaction was allowed to proceed at room temperature for 1.5h before concentrating to dryness and azeotroping with chloroform (3 x 15 mL) to remove residual TFA. The crude was then placed in a desiccator overnight. The crude was re-dissolved in H₂O (7 mL) with NaHCO₃ (6.7mmol, 5 eq) and the pH was tested to ensure the solution was basic. A solution of Fmoc-Osu (1.5 mmol, 1.1 eq) was then prepared in dioxane (34 mL) and slowly added to the stirring solution of the amino acid with an addition funnel. The reaction was allowed to proceed for 2h before concentrating to remove most of the dioxane and re-diluting to a total volume of 43 mL in H₂O. The aqueous layer was acidified with 4N HCl and the product was extracted in DCM (3 x 30 mL). The organic layers were combined, dried with MgSO₄, filtered and concentrated. The product was purified by flash column chromatography using DCM/EtOAc (1:1) to ethyl acetate with 0.5% acetic acid. The product was isolated as a bright orange with a yield of 85% (the final product contain small amount of residual toluene).



Orange solid. *R_f* (EtOAc with 0.5% AcOH): 0.50. **¹H NMR** (CDCl₃, 400 MHz): δ 1.42-1.56 (m, 2H), 1.67-1.85 (m, 2H), 1.86-2.05 (m, 2H), 3.01 (s, 6H), 4.08-4.20 (m, 4H), 4.25-4.35 (m, 2H), 4.35-4.44 (m, 2H), 6.96 (d, *J* = 8.2, 1H), 7.20-7.25 (m, 2H), 7.28-7.34 (m, 2H), 7.51-7.54 (m, 1H), 7.55 (d, *J* = 7.3, 2H), 7.67 (d, *J* = 7.5, 2H), 8.32 (d, *J* = 8.4, 1H), 8.41 (d, *J* = 8.2, 1H), 8.51 (d, *J* = 7.0, 1H) **¹³C NMR** (CDCl₃, 100.6 MHz): δ 22.59, 27.50, 31.44, 39.41, 44.68, 47.12, 53.82, 67.02, 113.21, 114.49, 119.83, 122.78, 124.80, 125.02, 127.04, 127.40, 130.19, 131.21, 131.31, 132.94, 141.18, 143.76, 156.36, 156.99, 164.35, 164.81, 175.84. HRMS (ESI): *m/z* calc. for C₃₅H₃₃N₃O₆ [M+H]⁺ 592.2448; found 592.2448.

8.3.3. Solid phase synthesis

The synthesis of each peptide was carried out manually and using solid phase chemistry. A system consisting of a 20 mL polypropylene syringe fitted with a filter was used in all cases to perform the synthesis. The stirring of the reaction mixture was performed with a Teflon stirring rod. Solvents and the excess of reagents were filtered under reduced pressure using a vacuum system.

Conditioning of the resin

Before the coupling of the first amino acid, the RA resin was washed with DMF (3 x 30 s), DCM (3 x 30 s) and finally, with DMF (3 x 30 s) again in order to swell and shrink the resin for enhances the contact surface and favour elimination of impurities.

Fmoc removal

In order to allow coupling between the resin and the first amino acid, it is necessary to remove the Fmoc groups attached to the resin. For this, five washes of 5 min each were performed with piperidine/DMF (20:80).

Coupling of the first amino acid

Once the Fmoc group was removed, 3 eq of the Fmoc-AAA-OH, 3 eq of oxyma and 3 eq of DIC were added to the resin with the minimum quantity of DMF that allowed the stirring of the mixture. The mixture was left reacting for 1h with occasional stirring. Once the reaction time is complete, the solution was filtered. The coupling of the amino acid was repeated using the same quantities of reagents. Finally, to see if the coupling is total, it is performed a Ninhydrin test. If the Ninhydrin test is positive after performing the coupling of the first amino acid, another coupling will be carried out. If the Ninhydrin test is negative, the coupling is complete and therefore can proceed to wash the resin with DMF (3 x 30 s), DCM (3 x 30 s) and finally, with DMF (3 x 30 s).

. Capping

After the coupling of the first amino acid it is necessary to cap the reactive points on the resin to avoid deletion peptides. The capping process was carried out through a short treatment (45 min) with Ac₂O (30 eq) and DIPEA (30 eq) with the minimum quantity of DMF that allowed the stirring of the mixture. Finally it is necessary to wash the resin with DMF (3 x 30 s), DCM (3 x 30 s) and finally, with DMF (3 x 30 s).

Fmoc quantification

After the first and the last coupling, it is necessary to quantify the Fmoc group to know how many amino acid or peptide molecules are attached to the resin. To do this, the procedure described in the section analytical methods was carried out.

Elongation of the peptide chain

Once the process of capping was finished, 3 eq of the Fmoc-AAA-OH, 3 eq of oxyma and 3 eq of DIC were added to the resin with the minimum quantity of DMF/DCM (1:1) that allowed the

stirring of the mixture. The mixture was left reacting for 1h with occasional stirring. Once the reaction time was complete, the solution was filtered. This procedure is repeated for each amino acid. After each coupling it is necessary to wash the resin with DMF (3 x 30 s), DCM (3 x 30 s) and finally, with DMF (3 x 30 s). A Ninhydrin test is necessary to determine if the coupling is complete. Once the peptide is finished, a quantification of Fmoc has to be performed.

Acetylation

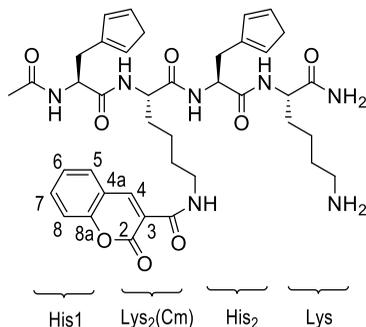
Once the peptide is formed and treated to remove the Fmoc groups from the last amino acid, the acetyl group was introduced at the N-terminus by treatment with 30 eq of Ac_2O and 30 eq of DIPEA for one hour. Once the reaction is complete, washings with piperidine/DMF (25 mL; 5 x 10 min) when necessary.

Cleavage of the peptide from the resin

Previous washings with DMF (1 x 30 s), DCM (3 x 30 s), MeOH (3 x 30 s) and finally with DCM (3 x 30 s) were performed before carrying out the cleavage of the peptide. A mixture of 95% TFA and 5% TIPS was added and occasionally stirred for 1 hour. Then, the resin was washed with a 1:1 TFA/DCM mixture (2 x 2 mL). The filtrates were collected in a round-bottom flask with anhydrous Et_2O (60 mL) for precipitating the peptide. The precipitate was centrifuged and dried on a vacuum.

8.4. SYNTHESIS OF PEPTIDES

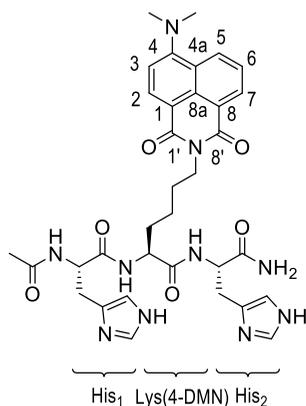
8.4.1. Synthesis of Ac-His-Lys(Cm)-His-Lys-NH₂



White solid. HPLC-MS: $t_R = 8.37$ min. $^1\text{H NMR}$ (D_2O , pH = 7.4, 400 MHz): δ 1.20-1.30 (m, 4H, $\text{H}_\delta\text{-Lys}_2 + \text{H}_\delta\text{-Lys}$), 1.49-1.59 (m, 4H, $\text{H}_\gamma\text{-Lys}_2 + \text{H}_\gamma\text{-Lys}$), 1.60-1.72 (m, 4H, $\text{H}_\beta\text{-Lys}_2 + \text{H}_\beta\text{-Lys}$), 1.84 (s, 3H, Me-Ac), 2.74-2.84 (m, 2H, $\text{H}_\beta\text{-His}_1$), 2.84-2.90 (m, 2H, $\text{H}_\epsilon\text{-Lys}$), 2.91-3.02 (m, 2H, $\text{H}_\beta\text{-His}_2$), 3.28-3.41 (m, 2H, $\text{H}_\epsilon\text{-Lys}_2$), 4.08-4.13 (m, 1H, $\text{H}_\alpha\text{-Lys}$), 4.15-4.20 (m, 1H, $\text{H}_\alpha\text{-Lys}_2$), 4.37 (dd, 1H, $J = 8.4$, $J = 5.8$, $\text{H}_\alpha\text{-His}_1$), 4.46 (t, 1H, $J = 7.4$, $\text{H}_\alpha\text{-His}_2$), 6.75 (s, 1H, $\text{H}_5\text{-His}_1$), 6.81 (s, 1H, $\text{H}_5\text{-His}_2$), 7.32 (d, 1H, $J = 8.7$, $\text{H}_8\text{-Lys(Cum)}$), 7.36 (t_{app} , 1H, $J_{\text{app}} = 7.6$, $\text{H}_6\text{-Lys(Cum)}$), 7.49 (s, 1H, $\text{H}_2\text{-His}_1$), 7.56 (s, 1H, $\text{H}_2\text{-His}_2$), 7.65-7.66 (m, 1H, $\text{H}_7\text{-Lys(Cum)}$), 7.67-7.68 (m, 1H, $\text{H}_5\text{-Lys(Cum)}$), 8.64 (s, 1H, $\text{H}_4\text{-Lys(Cum)}$). $^{13}\text{C NMR}$ (D_2O , pH = 7.4, 100.6 MHz): δ 21.58 (Me-Ac), 21.91 ($\text{C}_\delta\text{-Lys}$), 22.09 ($\text{C}_\delta\text{-Lys}_2$), 26.14 ($\text{C}_\gamma\text{-Lys}$), 27.83 ($\text{C}_\gamma\text{-Lys}_2$), 28.45 ($\text{C}_\beta\text{-His}_2$), 28.89 ($\text{C}_\beta\text{-His}_1$), 30.14 ($\text{C}_\beta\text{-Lys}_2 + \text{C}_\beta\text{-Lys}$), 39.10 ($\text{C}_\epsilon\text{-Lys}$), 39.29 ($\text{C}_\epsilon\text{-Lys}_2$), 53.07 ($\text{C}_\alpha\text{-Lys}_2$), 53.57 ($\text{C}_\alpha\text{-Lys}$), 53.82 ($\text{C}_\alpha\text{-His}_2$), 54.17 ($\text{C}_\alpha\text{-His}_1$), 116.27 ($\text{C}_8\text{-Lys(Cum)}$), 116.68 ($\text{C}_5\text{-His}_1 + \text{C}_5\text{-His}_2$), 117.35 ($\text{C}_3\text{-Lys(Cum)}$), 118.13 ($\text{C}_{4a}\text{-Lys(Cum)}$), 125.63 ($\text{C}_6\text{-Lys(Cum)}$), 130.14 ($\text{C}_5\text{-Lys(Cum)}$), 132.72 ($\text{C}_4\text{-His}_1 + \text{C}_4\text{-His}_2$), 134.86 ($\text{C}_7\text{-Lys(Cum)}$), 135.85 ($\text{C}_2\text{-His}_1$), 135.91 ($\text{C}_2\text{-His}_2$), 149.01 ($\text{C}_4\text{-Lys(Cum)}$), 153.79 ($\text{C}_{8a}\text{-Lys(Cum)}$), 162.23 ($\text{C}_2\text{-Lys(Cum)}$), 163.75 ($\text{CONH}_2\text{-Lys(Cum)}$), 172.62 (CO-His_2), 173.37 (CO-Lys_2), 173.52 (CO-His_1), 174.06 (CO-Ac), 176.07 (CO-Lys). m/z calc. for $([\text{C}_{36}\text{H}_{47}\text{N}_{11}\text{O}_8 + \text{H}]^+)$: 762.36; found: 762.25

Synthetic step	Reagents
Coupling of the first amino acid	498 mg of Rink Amide resin
	482 mg of Fmoc-Lys(Boc)-OH
	146 mg of oxyma
	158 μ L of DIC
Acetylation	975 μ L of Ac ₂ O
	1.80 mL of DIPEA
Yield	73%
Coupling of the second amino acid	465 mg of Fmoc-L-His(Trt)-OH
	107 mg of oxyma
	116 μ L of DIC
Coupling of the third amino acid	405 mg of Fmoc-Lys(Cm)-OH
	107 mg of oxyma
	116 μ L of DIC
Coupling of the fourth amino acid	465 mg of Fmoc-L-His(Trt)-OH
	107 mg of oxyma
	116 μ L DIC
Yield	Quantitative
Acetylation	700 μ L Ac ₂ O
	1.30 mL DIPEA
Weight yield	26% (70 mg)
Chromatographic purity	94%

Table 4. Synthesis of Ac-His-Lys(Cm)-His-Lys-NH₂

8.4.2 Synthesis of Ac-His-Lys(4-DMN)-His-NH₂

Yellow solid. HPLC-MS: $R_t = 10.20$ min; $^1\text{H NMR}$ (D_2O , pH = 4.5, 400 MHz): δ 1.22-1.38 (m, 2H, H $_{\beta}$ -Lys₂), 1.50-1.63 (m, 2H, H $_{\gamma}$ -Lys₂), 1.67-1.83 (m, 2H, H $_{\beta}$ -Lys₂), 1.88 (s, 3H, Ac), 2.79-3.01 (m, 2H, H $_{\beta}$ -His₁), 3.04 (s, 6H, NMe₂), 3.08-3.26 (m, 2H, His₃), 3.79 (t, 2H, $J = 6.5$, H $_{\epsilon}$ -Lys₂), 4.23-4.29 (m, 1H, H $_{\alpha}$ -Lys₂), 4.51-4.56 (m, 1H, H $_{\alpha}$ -His₁), 4.60-4.65 (m, 1H, H $_{\alpha}$ -His₃), 6.74 (d, 1H, $J = 8.5$, H_{3-4DMNL}), 7.12 (s, 1H, H_{5-His1}), 7.25 (s, 1H, H_{5-His3}), 7.36 (t_{app}, 1H, $J_{app} = 7.9$, H_{6-4DMNL}), 7.78 (d, 1H, $J = 8.4$, H_{2-4DMNL}), 8.00 (d, 1H, $J = 7.3$, H_{7-4DMNL}), 8.09 (d, 1H, $J = 8.4$, H_{5-4DMNL}), 8.52 (s, 1H, H_{2-His1}), 8.56 (s, 1H, H_{2-His3}). $^{13}\text{C NMR}$ (D_2O , pH = 4.5, 100.6 MHz): δ 24.43 (Me-Ac), 25.31 (C $_{\delta}$ -Lys₂), 29.26-29.33 (C $_{\beta}$ -His₁ + C $_{\beta}$ -His₃), 29.53 (C $_{\gamma}$ -Lys₂), 33.35 (C $_{\beta}$ -Lys₂), 42.76 (C $_{\epsilon}$ -Lys₂), 46.95 (NMe₂), 55.15 (C $_{\alpha}$ -His₃), 55.40 (C $_{\alpha}$ -His₁), 56.49 (C $_{\alpha}$ -Lys₂), 113.52 (C_{1-4DMNL}), 115.09 (C_{3-4DMNL}), 119.98-120.04 (C_{5-His1} + C_{5-His3}), 123.30 (C $_{\delta}$ -4DMNL), 125.74 (C_{4a-4DMNL}), 127.15 (C $_{\delta}$ -4DMNL), 131.48-131.59 (C_{4-His1} + C_{4-His3}), 132.02 (C_{8a-4DMNL}), 134.03 (C_{7-4DMNL}), 135.30 (C_{5-4DMNL}), 135.91 (C_{2-4DMNL}), 136.43-136.50 (C_{2-His1} + C_{2-His3}), 159.78 (C_{4-4DMNL}), 167.35 (C_{1-4DMNL}), 168.19 (C $_{\delta}$ -4DMNL), 174.80 (CO-His₁), 176.53 (CO-Lys₂), 176.73 (CO-His₃), 176.80 (CO-Ac). ; m/z calc. for $([\text{C}_{34}\text{H}_{40}\text{N}_{10}\text{O}_6 + \text{H}]^+)$: 685.32 ; found: 685.20

Synthetic step	Reagents
Coupling of the first amino acid	220 mg of Rink Amide resin 282 mg of Fmoc-L-His(Trt)-OH 65 mg of oxyma 70 μ L of DIC
Acetylation	430 μ L of Ac ₂ O 800 μ L of DIPEA
Yield	86%
Coupling of the third amino acid	233 mg Fmoc-Lys(4-DMN)-OH 56 mg of oxyma 61 μ L of DIC
Coupling of the fourth amino acid	244 mg of Fmoc-L-His(Trt)-OH 60 μ L of DIC
Yield	quantitative
Acetylation	370 μ L Ac ₂ O 690 μ L DIPEA
Weight yield	17% (20 mg)
Chromatographic purity	96%

Table 5. Synthesis of Ac-His-Lys(4-DMN)-His-NH₂

9. CONCLUSIONS

- The non-natural amino acid Lys(Cm) was synthesized for its study as copper sensor and its antioxidant capacity. The synthesis of this amino acid derivative was satisfactory, achieving a high yield and high purity.
- The non-natural amino acid Lys(4-DMN), which has a higher quantum yield than that of the coumarin chromophore and an excitation wavelength in the visible range, thus avoiding degradation of the molecule, was synthesized. The synthesis of this amino acid presented problems due to its low yield of production. After several attempts, it was observed that it is important to control the reflux temperature of the reaction and add the commercial amino acid slowly over the anhydride precursor because the latter undergoes hydrolysis.
- The Ac-His-Lys(Cm)-His-Lys-NH₂ peptide was synthesized. The binding of this peptide to gold nanoparticles will be studied. Contrary to the peptide sequences studied previously, our peptide presents an acetyl group in the N-terminus to avoid interferences between the Fmoc group and the coumarin in UV-Vis. The introduction of this group showed difficulties because a by-product was formed by a process which is unknown. Finally, it was observed that washings with piperidine / DMF eliminated this by-product thus obtaining the desired peptide in high purity.
- The Ac-His-Lys-(4-DMN)-His-NH₂ peptide was synthesized. The copper chelating capacity and the copper sensor properties of this peptide containing the new amino acid derivative will be studied. The peptide synthesis was successful, a high purity being achieved.

10. REFERENCES AND NOTES

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11. ACRONYMS

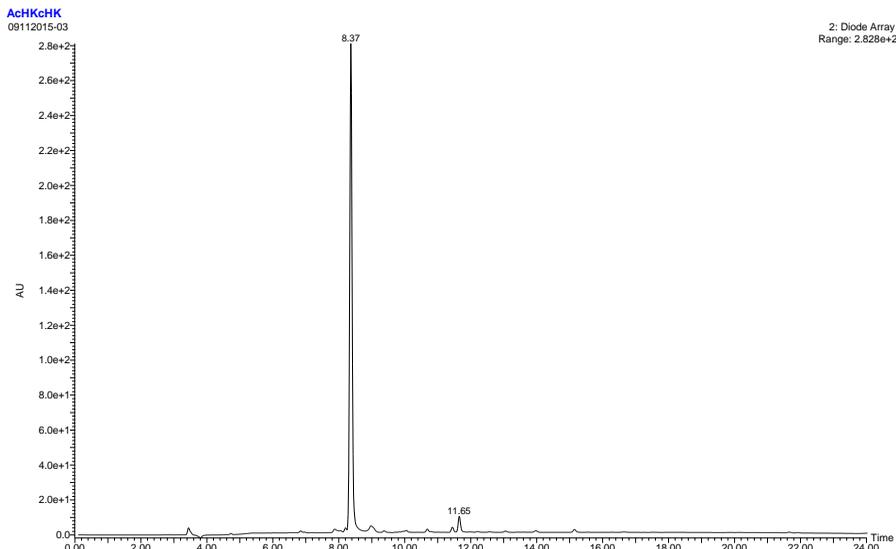
A β	Amyloid B
AD	Alzheimer disease
APP	Amyloid precursor protein
BBB	Blood-brain barrier
Cm	coumarin
CTC	2-chlorotriyl chloride resin
DCM	Dichloromethane
DIC	Diisopropylcarbodiimide
DIPEA	<i>N,N</i> -diisopropylethylamine
DMF	<i>N,N</i> -dimethylformamide
DMN	<i>N,N</i> -dimethylamino-1,8-naphthalimido
Eq	Equivalent
Fmoc	9-fluorenylmethoxycarbonyl
Fmoc-OSu	<i>N</i> -(9-fluorenylmethoxycarbonyloxy) succinimide
HPLC-MS	High resolution liquid chromatography coupled with electrospray mass spectrometry
Lys	Lysine
MPAC	Metal protein attenuating compounds
NFTs	Neurofibrillary tangles
PEG	Polyethylene glycol molecules
Rt	Room temperature
ROS	Reactive oxygen species
SPPS	Solid phase synthesis

TLC	Thin layer chromatography
TFA	Trifluoroacetic acid
TIPS	Triisopropylsilane
	Tryl

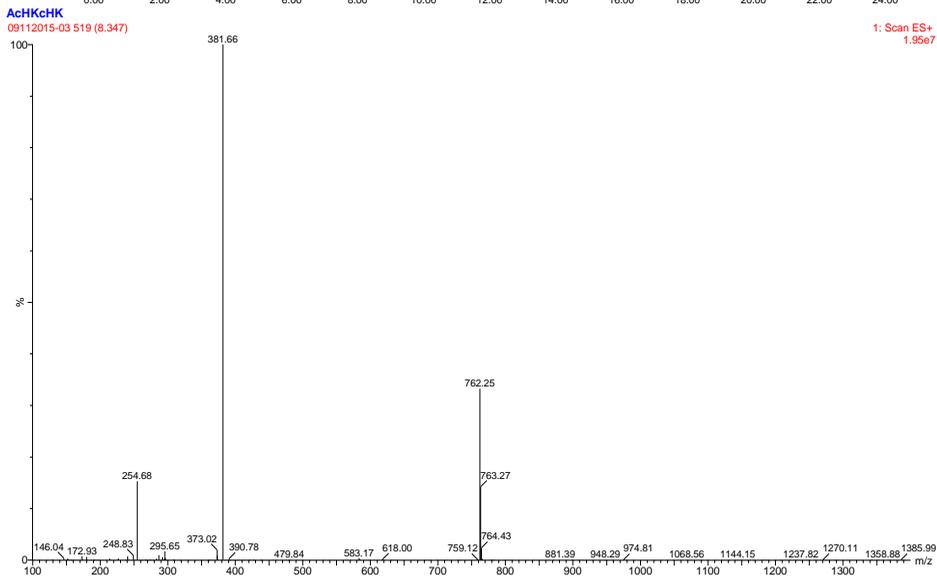
APPENDICES

APPENDIX 1: HPLC-MS OF EACH PEPTIDE

A.1.1. Ac-His-LYS(CM)-His-LYS-NH₂



2: Diode Array
Range: 2.828e+2

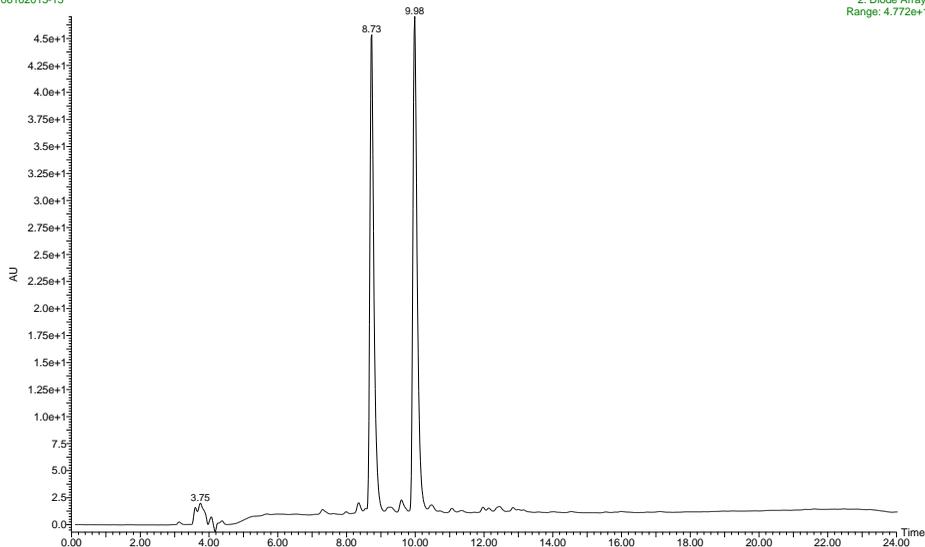


1: Scan ES+
1.95e7

A.1.2. AC-HIS-LYS(CM)-HIS-LYS-NH₂ AND BY-PRODUCT

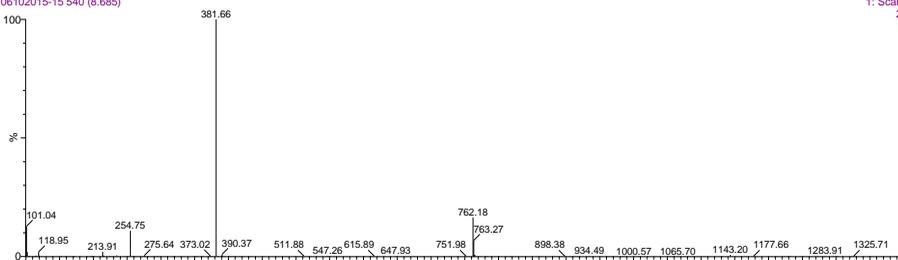
GV075

06102015-15

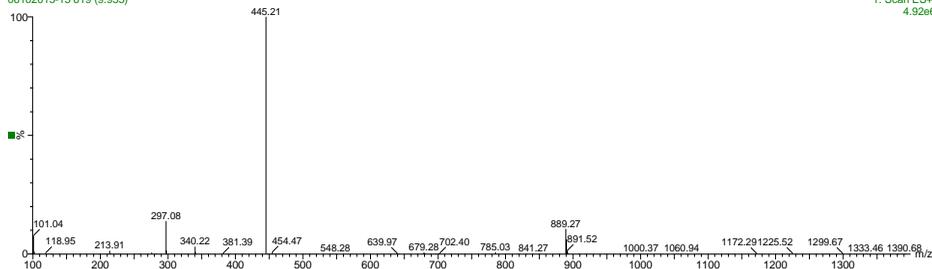
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GV075

06102015-15 540 (8.685)

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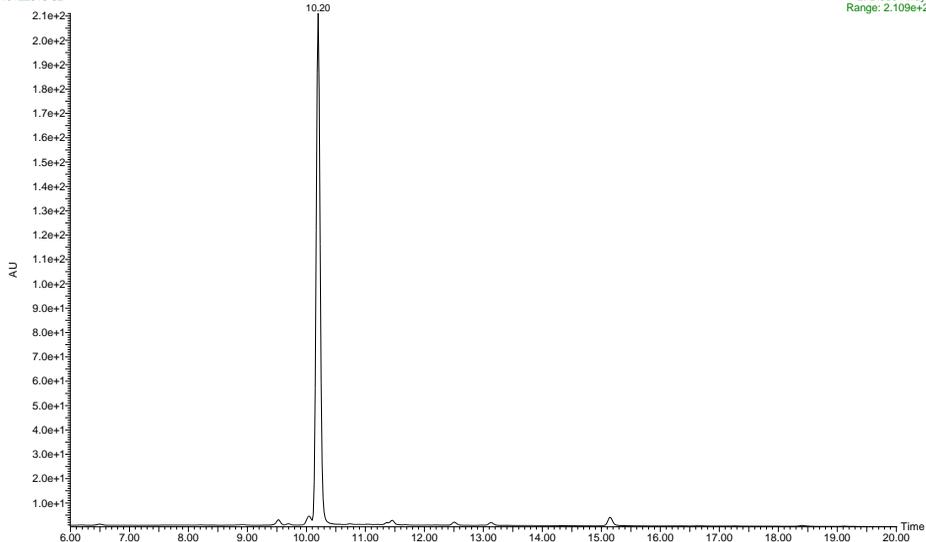
06102015-15 619 (9.955)

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4.92e6

A.2.1. Ac-His-Lys(4-DMN)-His-NH₂

GUILLEM 4DMNL

10122015-02



GUILLEM 4DMNL

10122015-02 633 (10.181)

