Tutor/s

Dr. Vicente Marchán Sancho
Departament de Química Inorgànica i Orgànica

Dr. Carles Mas Moruno
Biomaterials, Biomechanics and Tissue Engineering (BBT), Universitat Politècnica de Catalunya (UPC)

Dra. Helena Martín Gómez
Biomaterials, Biomechanics and Tissue Engineering (BBT), Universitat Politècnica de Catalunya (UPC)

Grau de Química

Treball Final de Grau

New synthetic strategies for the synthesis of multifunctional peptidic platforms with osteoinductive potential.

Noves estratègies sintètiques per la síntesi de plataformes peptídiques multifuncionals amb potencial osteoinductiu.

Iván Sánchez Campillo

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To those who motivated me to keep pushing forward.

To the nice people who I met during these three months.

And to you ten, the number of perfection.

REPORT

CONTENTS

1. SUMMARY

In biomaterials science, peptides are widely used to functionalize material surfaces and confer biological potential (i.e. bioactivity) to otherwise inert substrates. The attachment of biomolecules to material surfaces is commonly achieved by using specific anchors with chemical affinity for the substrates. Well-known examples include the use of amines to bind polymers through amide bonds, thiols to bind gold substrates or catechol groups to bind titanium and other metallic oxides. However, this implies that each synthesized peptide can be used only for a narrow range of materials. Thus, in most cases, changing the material of study requires synthesizing the same peptide with a distinct anchor, resulting in time-consuming and repetitive procedures.

To solve this, this project aims to develop a novel and versatile click-based solid-phase synthetic strategy to prepare peptidic coatings for a variety of biomaterials. In detail, the project focuses on the solid-phase peptide synthesis of a branched peptidic structure (containing the RGD and DWIVA peptide sequences) and the optimization of the copper-catalysed azide-alkyne cycloaddition reaction to introduce three anchoring groups, namely an amine, a thiol and a catechol, to the peptidic backbone in solid phase.

By means of solid-phase synthetic methods and characterization by analytical HPLC and mass spectrometry, the feasibility of this strategy has been demonstrated. It is expected this new method will find applications to coat a wide range of biomaterials in a straightforward and cost-efficient fashion.

Keywords: peptide synthesis, click chemistry, multifunctionalization, chemical strategies

2. RESUM

En ciència de biomaterials, els pèptids són àmpliament utilitzats per funcionalitzar superfícies de materials i conferir potencial biològic a substrats inerts (procés de biofuncionalització). La unió de biomolècules a la superfície dels materials s'aconsegueix mitjançant diferents ancoratges amb afinitat química per el substrat. Alguns exemples coneguts són l'ús d'amines per polímers mitjançant enllaços amida, de tiols per or i de grups catecol per titani i altres òxids metàl·lics. No obstant, això implica que els pèptids sintetitzats poden ser utilitzats en un petit rang de materials. Així, en la majoria de casos, canviar el material d'estudi requereix repetir la síntesi del pèptid amb un nou ancoratge, resultant en processos llargs i repetitius.

Per solucionar-ho, aquest projecte busca desenvolupar una nova estratègia sintètica en fase sòlida basada en la química click que aporti versatilitat a l'hora de preparar recobriments peptídics per variats biomaterials. Detalladament, el projecte se centra en la síntesi de pèptids en fase sòlida d'una estructura ramificada (que conté les estructures peptídiques RGD i DWIVA) i la optimització de la reacció de cicloaddició d'azida i alquí catalitzada per coure per introduir tres grups d'ancoratge, consistents en una amina, un tiol i un catecol, a la matriu peptídica en fase sòlida.

Mitjançant mètodes de síntesi en fase sòlida i caracterització per HPLC analític i espectroscòpia de masses, la factibilitat d'aquesta estratègia ha estat demostrada. S'espera que aquest nou mètode pugui ser aplicat per recobrir una gran varietat de biomaterials de manera senzilla i sense sobrecostos.

Paraules clau: síntesi de pèptids, química click, multifuncionalització, estratègies químiques.

3. INTRODUCTION

In the field of medicine, regeneration and replacement of non-functional tissues has become a great challenge. To solve this problem, biomaterials have evolved from just biocompatible substrates to highly bioactive materials. That means that they no longer just support cell adhesion and growth, but they interact with cells and promote certain biological responses[1]. This is done by functionalizing the surface of the material in different ways. For example, they can be functionalized with peptides that mimic the extracellular matrix and interact with cell receptors. In the present work, the synthesized peptides are meant to functionalize biomaterials and promote bone regeneration.

3.1. SURFACE FUNCTIONALIZATION WITH PEPTIDES

When peptides are designed to interact with cells, the aim is to imitate the bioactive sequences of proteins. Once these sequences are isolated, they can be synthesized and put on a material, giving nearly the same effect as if the cell was interacting with the protein itself.

Proteins can consist of 150 amino acids or more, but only a few are active, meaning that most of them are structural. These amino acids are likely to be far in the sequence, but near in the 3D structure of the protein. Therefore, it is not a problem of sequence but conformation. When putting this amino acids in sequence, there are options to try to optimize the conformation and making them more active, like cycling the sequence or adding spacers between them[1].

To functionalize biomaterials, synthetic peptides commonly consist of an anchor group to specifically attach to the desired material, spacers and linear or cyclic bioactive sequences. These peptides can also be multifunctional, as they can have more than one bioactive sequence attached to a single anchor group. In this context, Lys can be used as a ramification branch thanks to the amine group in its lateral chain[2] (Figure 1). It is useful to add two motives with a different function each, commonly one of them being a cell-adhesion promoter and the other one the functional sequence.

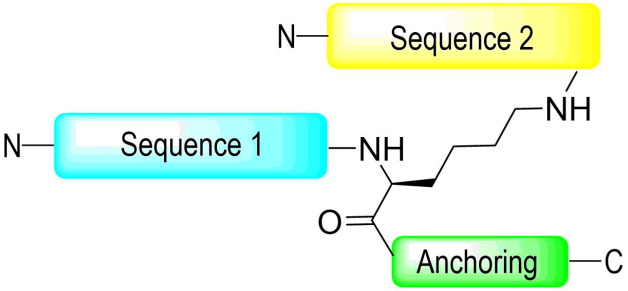


Figure 1: Representation of a dimeric peptide platform using Lys as branching unit. N and C represent N and C-terminal ends.

Regarding the anchor group, there are some functional groups that can be used (see Figure 2). However, some groups work better to bind a specific material than others. To give an example, polylactic acid (PLA) is functionalized through a process of hydrolysis which leaves free carboxylic acid groups. Here, an amine is the best option to attach the peptide to the surface, by means of an amide bond. The side chain of Lys is perfect to play that role.

A widely used biomaterial is titanium (Ti). It is widely used as prosthesis because its biocompatible properties, but it's not bioactive. Then, functionalization improves its properties as biomaterial. Its functionalization often goes by generation of hidroxyl groups, silanization and introduction of an electrophile linker[1] (see Scheme 1). For this type of system, what is needed is a good nucleophile, and the best option looking into amino acids is a thiol group, then Cys is the chosen one.

However, there is a novel option to functionalize Ti in which that previous treatment is not necessary, and it is the use of catechol groups. After the corresponding treatment, the phenolic oxygens can coordinate with Ti and then act as good and strong anchors. They can be introduced in the peptidic sequence as 3,4-dihydroxy-L-phenylalanine (L-DOPA). In this case, two of them are coupled instead of one to improve the anchoring[3].

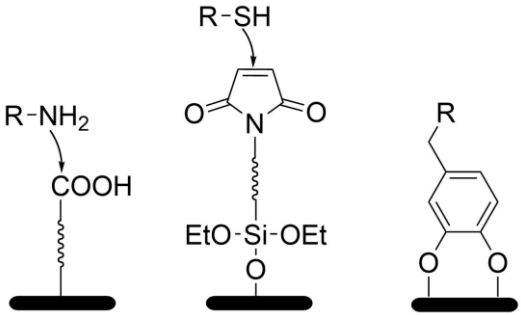
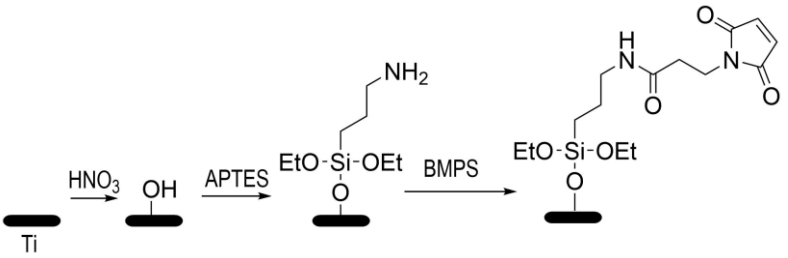


Figure 2. General scheme of functionalized surfaces.



Scheme 1. Schematic representation of Ti functionalization via silanization[4].

As previously shown, there are many options regarding the anchor group, but they depend on the target material, and the fact that these groups are introduced in the first steps of the synthesis, in the C-terminal end, supposes a problem. For example, if a 15 amino acids platform is synthesized with catechol groups, and it is decided later to functionalize PLA instead of Ti, all the synthesis needs to be repeated starting from the first amino acid. It would be more useful to add the anchor group in the end of the synthesis, once the peptide is finished and the material is selected. Also, that would allow to take samples of the obtained peptide and functionalize them with different groups. For example, one part with an amine, another one with a thiol and the last one with catechol, thus easily creating small libraries with capacity to functionalize a wide range of materials.

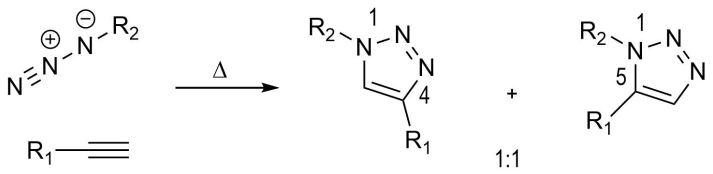
The study of this possibility is the main objective of this project. We aim to develop a strategy to introduce the desired functional groups after the synthesis of the peptide, introducing a functional group in the first amino acid that can react with a small unit containing the desired anchor and the group that makes possible the coupling with the peptide. This approach is studied using click chemistry.

3.2. CLICK CHEMISTRY AND CUAAC

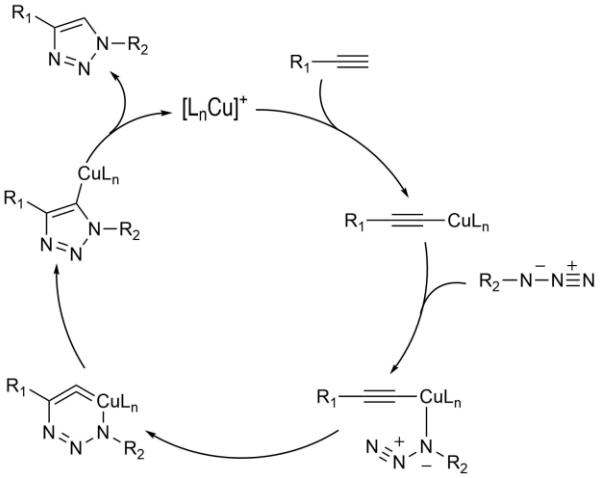
The term “click chemistry” was introduced by K. B. Sharpless et. al. in 2001[5], in parallel with the studies done by M. Meldal[6]. The philosophy is to imitate nature and generate substances by joining building blocks with heteroatom links (C-X-C). A click chemistry reaction must be ‘modular, wide in scope, give very high yields, generate only inoffensive by-products that can be removed by nonchromatographic methods, and be stereospecific (but not necessarily enantioselective)’[5]. If it is possible, it is better to have simple reaction conditions, stability to oxygen and water, and the use of a benign solvent (water) or easily removable.

Some reactions have been described that follow these conditions and can be classified inside click chemistry, such as cycloadditions and Michael additions. The model reaction, and the one that is explored in the present work, is the copper-catalysed azide alkyne cycloaddition (CuAAC).

This reaction is a variant of the Huisgen 1,3-dipolar cycloaddition, named after R. Huisgen who described it[7]. It leads to a huge variety of disubstituted 1,2,3-triazoles, but it was not explored by synthetic chemists because it needs hard conditions that compromise the stability of azides (as high temperature) and the products are a mixture of 1,4 and 1,5-disubtituted triazoles (Scheme 2). However, in 2002 a variant that introduced a catalyst of copper (I) was described by Sharpless et. al.[8], which lead regiospecifically to 1,4-disubstituted product in simple experimental conditions, which means room temperature and common mixtures of water and organic solvents. Eventually, pure water also could be used. The proposed mechanism for this reaction can be seen in Scheme 3. Sharpless describes this reaction as “the cream of the crop” of click chemistry[4], and now it’s the first reaction that comes to example when click chemistry is named.



Scheme 2. General scheme of azide-alkyne 1,3-dipolar cycloaddition.



Scheme 3. Proposed mechanism for CuAAC.

Copper (I) is generally obtained in situ, from Cu (II) salts and a reducing agent (typically, $CuSO_4 \cdot 5\ H_2O$ and sodium ascorbate are used). Otherwise, a Cu (I) salt can be directly used, but it will need absence of oxygen and an equivalent of a nitrogen base as stabilizing agent, being TBTA the most commonly used[9].

In this work, the reaction will be carried on solid phase, which means that the substrate will be a resin-bounded peptide. This type of 'solid phase CuAAC' has been previously described[10], demonstrating a vast variety of uses, like couplings[11] and cyclizations[12]. The main advantages of working in solid phase are that the reaction can be made using excess of reagents, the easier isolation of the products and efficient removal of by-products. Also, all the functional groups will remain protected during the reaction, as all of them will be deprotected during cleavage after the reaction.

3.3. PEPTIDE PLATFORMS

Once the methodology is clear, what is needed is to define the desired product of the click reaction and the starting materials. They will be synthetic peptide-based reagents, containing azide and terminal alkyne groups. The final product of the reaction will be a platform containing two bioactive sequences and the anchoring group, linked by a triazole group (Figure 3).

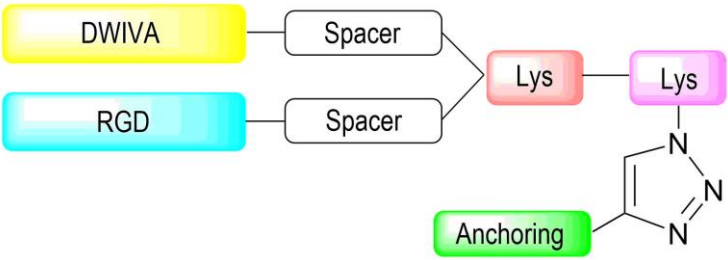


Figure 3: Schematic representation of the desired peptide-based platform.

The synthesis of this structure has been previously described and optimized by the co-workers in the BBT group[2]. It has two active sequences, one of them being Arg-Gly-Asp (RGD) and the other one being Asp-Trp-Ile-Val-Ala (DWIVA). RGD is an isolated sequence of fibronectin (FN), an extracellular matrix protein. It interacts with some types of integrins and promotes cell-adhesion[1]. DWIVA is a sequence derived from bone morphogenetic protein 2 (BMP-2), and it has been demonstrated that promotes cell-differentiation to osteoblasts[13]. The two units are bound to a single platform, by a Lys which is used as a ramification unit. Also, two 6-aminohexanoic acid (Ahx) are added between Lys and each sequence, just to minimize steric impediments between the anchoring unit and the material surface. N-terminal ends are acetylated to neutralize charges and inhibit their reactivity. The first amino acid is the one that contains the reactive group to which the anchoring units will be coupled. In this project a Lys containing an azide instead of an amine will be selected. Other options can be explored, or an alkyne could be added instead of an azide. Azide is chosen because its known low reactivity during the synthesis.

The anchoring units will consist of surface-specific anchor groups and a terminal alkyne. Therefore, structures like 'Alkyne-NH_2', 'Alkyne-SH' and 'Alkyne-DOPA' will be needed. The selected building blocks are shown in Figure 4.

When searching for the building block containing an amine, propargylamine represents a good starting point, as it contains a primary amine and is commercially available. Also, it has been proved that it can react via CuAAC[10]. The reaction will be carried out with the amine deprotected, as all the other groups in the peptide will be protected, but it can be protected easily (or also bought) if needed.

Analogues of 2-propyne-1-thiol and 'propargyl-cathecol' could not be found from commercial suppliers. Then, the best option is to synthesize the building blocks containing the two functional groups (thiol or catechol and alkyne) (Figure 4). An amino acid containing the desired group can

be coupled to an acid that has a terminal alkyne as capping (being 4-pentinoic acid a good and cheap option). For thiol, Cys, and for catechol, L-DOPA (in this case, two of them are used). They can be obtained easily by a stepwise solid phase peptide synthesis (SPPS) and then cleaved. The resin for this synthesis must meet two requirements: i) after cleavage, an amide must be left in the C-terminal end to ensure no extra charges are introduced into the peptide structure (e.g. carboxylic acid will introduce a negative charge) and ii) the protective groups should be maintained to avoid interfering with the click reaction afterwards. Thus, the peptide must be cleaved with a very low acid concentration. More details can be found in Experimental Section.

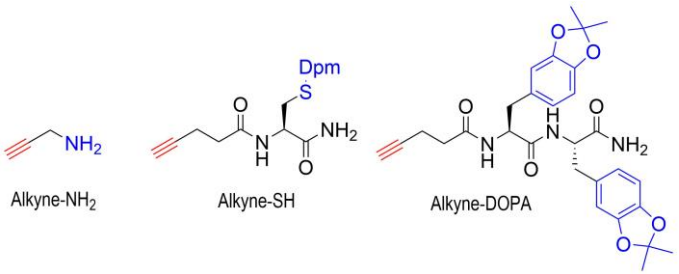


Figure 4. Selected alkyne building blocks.

4. OBJECTIVES

The objectives of this project are:

- Synthesis of the peptide-based platform containing the azido group.
- Synthesis of the anchoring building blocks (amino, thiol and catechol).
- Optimize the conditions for the CuAAC reaction.

5. MATERIALS AND METHODS

5.1. REAGENTS AND SOLVENTS

Source	Chemicals
Carlo Erba	DCM, DMF, piperidine, ACN
Iris Biotech GmbH	Fmoc-Rink Amide MBHA resin, Fmoc-Sieber PS resin, OxymaPure®, Fmoc-L-Lys(N_3)-OH, Fmoc-L-Lys(Alloc)-OH, Fmoc-6-Ahx-OH, Fmoc-L-Arg(Pbf)-OH, Fmoc-L-Trp(Boc)-OH
Merck KGaA	Fmoc-L-Ser(OtBu)-OH, Fmoc-L-Asp(OtBu)-OH, Fmoc-Gly-OH, Fmoc-L-Ala-OH, Fmoc-L-Val-OH, Fmoc-L-Ile-OH, Fmoc-L-Cys(Dpm)-OH, Fmoc-L-Cys(Trt)-OH
Sigma-Aldrich	DIC, DIEA, propargylamine, TFA, TIS, phenylsilane, $Pd(PPh_3)_4$, DEDTC, $CuSO_4 \cdot 5 H_2O$, sodium L-ascorbate
Bachem	Fmoc-L-DOPA(Acetonide)-OH

Table 1. Reagents and solvents

5.2. INSTRUMENTS AND EQUIPMENT

Intrument	Brand
HPLC	Shimadzu Prominence XR
HPLC-MS	Alliance Waters 2695
Orbital stirrer	Heidolph Rotamax 120
Centrifuge	Eppendorf Centrifuge 5430R
Freeze dryer	Telstar Cryodos -80

Table 2: Instruments and equipment

5.2.1. Analytical RP-HPLC

Analytical RP-HPLC was performed on a Shimadzu Prominence XR with an automatic injector and photodiode array. The column used was a Xbrigde BEH130 C_{18} 3.5 µm (4.6 mm x 100 mm) column run with linear gradients of H_2O with 0.045% TFA (A) and ACN with 0.036% TFA (B) over 8 min. UV detection was done at 220 nm, oven temperature was 25 ºC or 60 ºC and the system was run at a flow rate 1 mL/min.

5.2.2. HPLC-MS

HPLC-MS was performed on an Alliance Waters 2695 with an automatic injector, photodiode array and ESI. The column used was a Jupiter Proteo C_{18} column (250 mm × 4.6 mm, 90 Å, 4

µm, flow rate: 1 mL/min) with linear gradients of H_2O with formic acid 0.1% (A) and ACN with formic acid 0.1% (B) over 30 min. UV detection was done at 220 nm and 260 nm and ionization system is electrospray ionization (ESI). The system was run at a flow rate of 1mL/min.

5.3. GENERAL PROCEDURE OF SPPS

Peptides were synthesized using the standard Fmoc/tBu strategy and DIC/Oxyma as coupling reagents. The system used was an open syringe equipped with a filter and a pass key, connected to a vacuum system to remove solvents. It is a stepwise synthesis where amino acids were coupled one by one, starting from the C-terminus. Two resins have been used in this project: Fmoc-Rink Amide MBHA resin and Fmoc-Sieber PS resin (Figure 5).

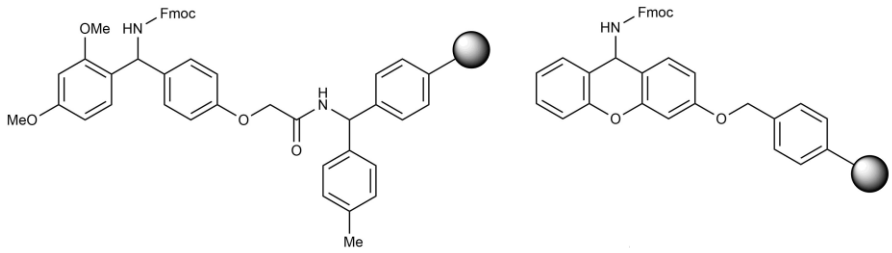


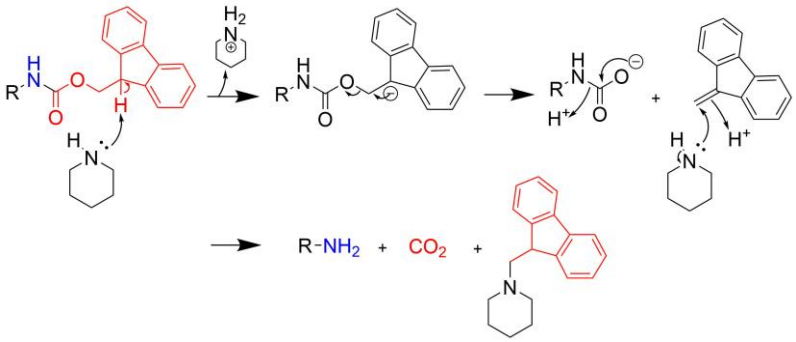
Figure 5: Fmoc-Rink Amide MBHA resin (left) and Fmoc-Sieber PS resin (right).

5.3.1. Conditioning and washing

The resin was conditioned with DCM (5 x 0.5 min), DMF (5 x 0.5 min) and DCM (5 x 0.5 min). Washings were done after each deprotection and coupling with DMF (5 x 0.5 min) and DCM (5 x 0.5 min).

5.3.2. Fmoc removal

Fmoc removal was done with 20% (v/v) piperidine in DMF (1 x 1 min, 2 x 5 min). Fmoc group is removed by an alkali medium using piperidine, which creates an adduct with the Fmoc group and avoids side reactions (see Scheme 4 for the mechanism). If necessary, a ninhydrin test could be done after deprotection, giving a positive result if the deprotection was well-performed (see Ninhydrin test in section 5.3.4).



Scheme 4. Fmoc removal mechanism.

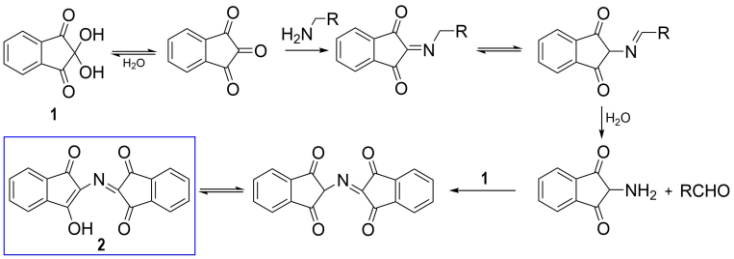
5.3.3. Loading

After conditioning and washing, the first amino acid was loaded into the resin using Oxyma (1 eq) and DIC (1 eq) in DMF and stirred for 1h. 0.1 mmol/g loading excess with respect to the desired one was used to ensure the complete loading. Afterwards, a capping step is needed to acetylate the unreacted amines and avoid undesired peptide chain elongation. For this step, a mixture of Ac_2O/DIEA (5:5) in DMF was added for 45 min.

5.3.4. Ninhydrin test

This test detects unprotected primary amines, and it's used as a standard method of synthesis control. The main reagent is ninhydrin (triketohydrindene hydrate) (1). Two molecules of ninhydrin react stoichiometrically with an unprotected amino acid to form a highly conjugated Schiff base, known as Ruhemann's purple (2), named after S. Ruhemann who described the test[14].

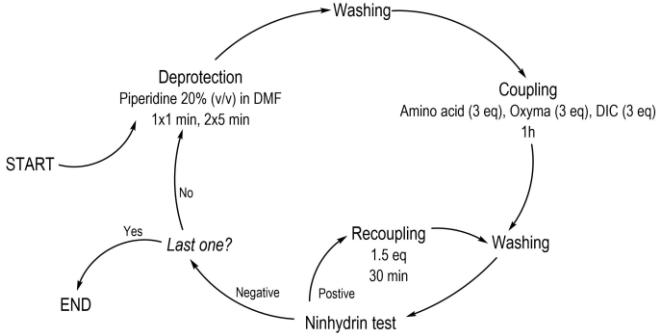
The reagents come as a commercially available kit of 3 solutions. A small amount of resin beads was transferred to a glass tube and 2 drops of each solution were added. Then, the tube was incubated at 110ºC for 3 min. A dark blue or purple color (in the solution and/or in the resin beads) reveals the presence of free primary amines (positive test), whereas a yellow coloration ensures 99.5% coupling rate (negative test). The mechanism is shown in Scheme 5.



Scheme 5. Ninhydrin test mechanism

5.3.5. Peptide chain elongation

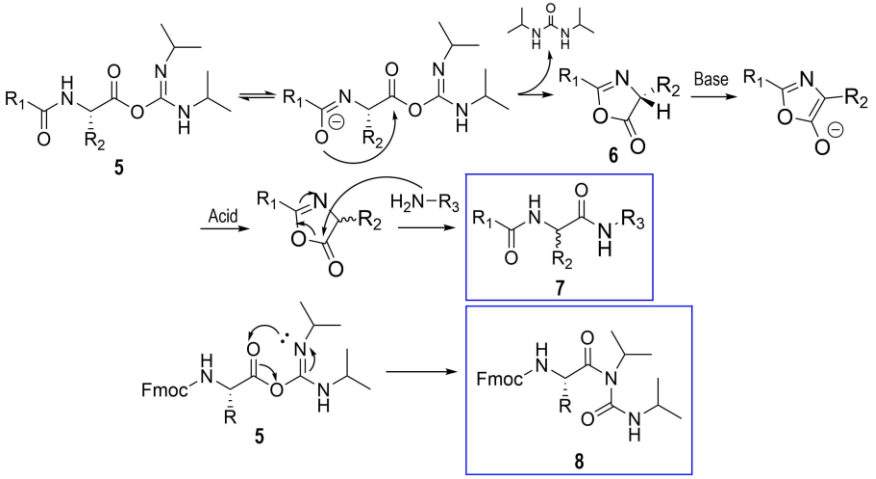
After deprotection and washing, a solution containing the amino acid (3 eq), Oxyma (3 eq) and DIC (3 eq) in DMF was added to the resin and stirred for 1 h. Then, the resin was washed, and a ninhydrin test was done. If it gave a positive result, a recoupling would be made with 1.5 eq of amino acid, Oxyma and DIC for 30 min. This is repeated for every new amino acid, following a cyclic synthetic route shown in Scheme 6.



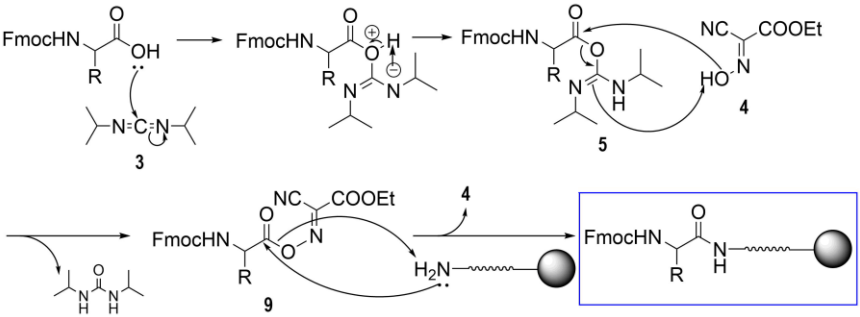
Scheme 6. General procedure for a peptide chain elongation.

DIC (3) and OxymaPure® (4) were used as coupling reagents. The direct coupling between the carboxylic acid and the amine is not possible in simple conditions, as they will act as an acid base pair. DIC is a carbodiimide, which reacts with the acid becoming an O-acylisourea (5), very reactive to a nucleophilic acyl substitution as the exiting group is a urea. This mechanism tend to show undesired side reactions such as the formation of 5(4H)-oxazolone (6) through cyclization, that can produce racemization (7) via enolization[15] or formation of inactive N-acylurea (8)

(Scheme 7). To avoid these reactions, Oxyma is added. It produces an active ester (9), which is less reactive but more stable[16]. The mechanism can be seen in Scheme 8.



Scheme 7. Side reactions of O-acylisourea.



Scheme 8: Coupling mechanism with DIC and Oxyma.

5.3.6. Acetylation

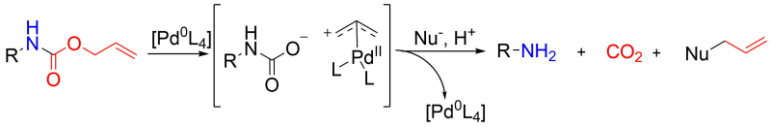
This step is needed for capping the N-terminus of the branches. It's not needed if there's not an amine in the end.

A solution of Ac_2O, DIEA and DMF (1:2:7, v/v) was used. The solution needs to be protected from light to prevent DIEA oxidation. Capping is done by adding this solution to the resin (1 x 5 min, 2 x 10 min).

5.3.7. Alloc removal

$Pd(PPh_3)_4$ (0.1 eq) and phenylsilane (10 eq) in DCM was added to the resin (3 x 15 min). Resin was washed with DCM (5 x 0.5 min) after each treatment.

$Pd(PPh_3)_4$ is easily oxidized by the oxygen present in the air, then the three times are needed to ensure full deprotection. The general mechanism can be seen in Scheme 9. Pd catalyst interacts with the allyl group and complexes it, and phenylsilane acts as a scavenger and as a hydride donor to accelerate the reaction.



Scheme 9. Alloc removal mechanism.

5.3.8. Cleavage

In this process, the peptide is released from the resin. Other effects are shown depending on the resin. It serves also as an analytical test to control the synthesis.

Acidic conditions are required to cleave the peptide from the resin. Trifluoroacetic acid (TFA) was used for this step because it is a strong acid which is soluble in organic solvents. The cleavage cocktail was chosen according the sensitiveness of the resin and the protecting groups of the residues present in the peptide sequence.

Harsh acidic conditions are required for the Rink Amide MBHA resin cleavege. A mixture of TFA, TIS and water (95:2.5:2.5, v/v/v) was added during 1.5 h. Triisopropyilsilane (TIS) is used as scavenger for cations, as in this acidic conditions TIS acts as a hydride donor. This resin has the particularity to leave an amide in the C-terminus. In this strong acid conditions, all other protective groups such as Boc, Pbf and tBu are removed (see their structure in Annex 1.2).

Peptides were cleaved from the Sieber resin using very mild TFA conditions. The resin was treated with TFA-DCM (3:97, v/v) (8 x 0.5 min) and washed with DCM (6 x 0.5 min). This resin also gives an amide in the C-terminus. However, in this weak acid conditions the protective groups are maintained.

6. EXPERIMENTAL SECTION

6.1. SOLID PHASE PEPTIDE SYNTHESIS

6.1.1. Synthesis of RGD-DWIVA peptides

The peptidic platform RGD-DWIVA was synthesized using the Fmoc-Rink Amide MBHA resin and a loading of 0.4 mmol/g. A general synthetic scheme can be consulted in Scheme 10. Firstly, the RGD containing chain was elongated and then, the second chain containing the DWIVA was elongated from the Alloc-protected amine. Two peptide batches were prepared, RGD-DWIVA-01 and RGD-DWIVA-02.

RGD-DWIVA-01

0.2128 g of Fmoc-Rink Amide MBHA resin were weighted. The resin was conditioned, Fmoc was removed using piperidine 20% (v/v) in DMF (1 x 1 min, 1 x 5 min, 1 x 10 min), and then the resin was washed. Fmoc-L-Lys(N_3)-OH (42.0 mg, 1.2 eq) was loaded into the resin using Oxyma (15.1 mg, 1.2 eq) and DIC (17 µL, 1.2 eq) for 1h in DMF. Then, the resin was washed, and the capping step was performed using Ac_2O (40 uL, 5 eq) and DIEA (74 uL, 5 eq) for 30 min in DMF. Fmoc was removed using piperidine 20% (v/v) in DMF (1 x 1 min, 2 x 5 min), and the resin was washed. Fmoc-L-Lys(Alloc)-OH (115.6 mg, 3 eq), Oxyma (36.3 mg, 3 eq) and of DIC (40 µL, 3 eq) in DMF were added to the resin and reacted for 1h. Ninhydrin test was done after the coupling.

The first peptide chain was elongated following the protocol from section 5.5.4. Two Ahx were coupled using Fmoc-6-Ahx-OH (90.2 mg, 3 eq), Oxyma (36.3 mg, 3 eq) and DIC (40 µL, 3 eq). Ser was coupled using Fmoc-L-Ser(OtBu)-OH (97.9 mg, 3 eq), Oxyma (36.3 mg, 3 eq) and DIC (40 µL, 3 eq). Asp was coupled using Fmoc- L-Asp(OtBu)-OH (105.1 mg, 3 eq), Oxyma (36.3 mg, 3 eq) and DIC (40 µL, 3 eq). Gly was coupled using Fmoc-Gly-OH (75.9 mg, 3 eq), Oxyma (36.3 mg, 3 eq) and DIC (40 µL, 3 eq). Arg was coupled using Fmoc- L-Arg(Pbf)-OH (165.7 mg, 3 eq), Oxyma (36.3 mg, 3 eq) and DIC (40 µL, 3 eq). After coupling Arg, Fmoc was removed (1 x 1 min, 1 x 5 min, 1 x 10 min), a ninhydrin test was done and the N-terminus from Arg was acetylated using a solution of Ac_2O/DIEA/DMF (1:2:7, v/v/v) (1 x 5 min, 2 x 10 min). Afterwards, the resin was washed, and a ninhydrin test was done.

Alloc was removed using $Pd(PPh_3)_4$ (9.8 mg, 0.1 eq) and $PhSiH_3$ (105 µl, 10 eq) in DCM (3 x 15 min), washing with DCM (5 x 0.5 min) after each step. Ninhydrin test was done after that. DWIVA branch was elongated and acetylated following the same protocols as for RGD branch.

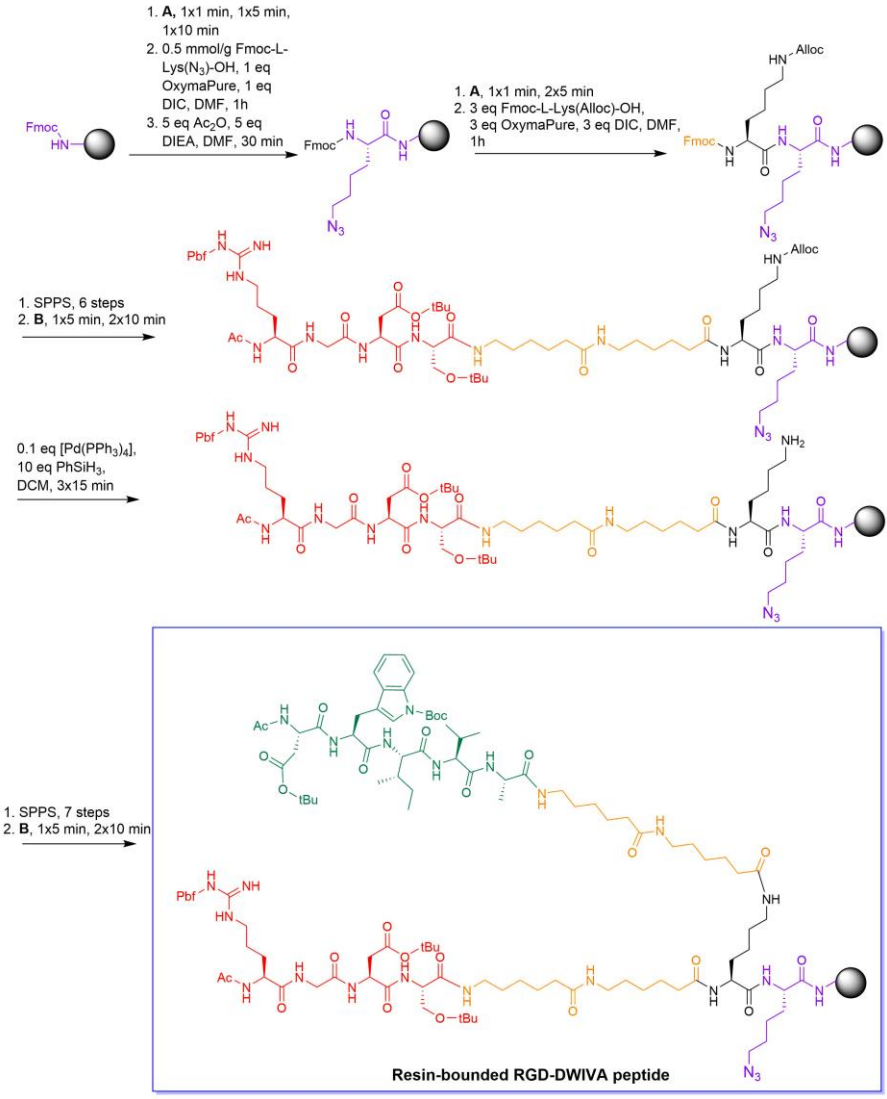
Two Ahx were coupled using Fmoc-6-Ahx-OH (90.2 mg, 3 eq), Oxyma (36.3 mg, 3 eq) and DIC (40 µL, 3 eq). Ala was coupled using Fmoc-L-Ala-OH (79.5 mg, 3 eq), Oxyma (36.3 mg, 3 eq) and DIC (40 µL, 3 eq). Val was coupled using Fmoc- L-Val-OH (86.7 mg, 3 eq), Oxyma (36.3 mg, 3 eq) and DIC (40 µL, 3 eq). Ile was coupled using Fmoc- L-Ile-OH (90.2 mg, 3 eq), Oxyma (36.3 mg, 3 eq) and DIC (40 µL, 3 eq). Trp was coupled using Fmoc- L-Trp(Boc)-OH (75.9 mg, 3 eq), Oxyma (36.3 mg, 3 eq) and DIC (40 µL, 3 eq). Asp was coupled using Fmoc- L-Asp(OtBu)-OH (105.1 mg, 3 eq), Oxyma (36.3 mg, 3 eq) and DIC (40 µL, 3 eq). After coupling Asp, Fmoc was removed (1 x 1 min, 1 x 5 min, 1 x 10 min), a ninhydrin test was done, and the N-terminus from Asp was acetylated using a solution of Ac_2O/DIEA/DMF (1:2:7, v/v/v) (1 x 5 min, 2 x 10 min). Afterwards, the resin was washed, and a ninhydrin test was done.

0.2480 g of peptidyl-resin were obtained.

RGD-DWIVA-02

0.2310 g of Fmoc-Rink Amide MBHA resin were weighted. The resin was conditioned, Fmoc was removed using piperidine 20% (v/v) in DMF (1 x 1 min, 1 x 5 min, 1 x 10 min), and then the resin was washed. Fmoc-L-Lys(N_3)-OH (45.6 mg, 1.2 eq) was loaded into the resin using Oxyma (16.4 mg, 1.2 eq) and DIC (18 µL, 1.2 eq) for 1h in DMF. Then, the resin was washed, and the capping step was performed using Ac_2O (43 uL, 5 eq) and DIEA (80 uL, 5 eq) for 30 min in DMF. Fmoc was removed using piperidine 20% (v/v) in DMF (1 x 1 min, 2 x 5 min), and the resin was washed. Fmoc-L-Lys(Alloc)-OH (125.4 mg, 3 eq), Oxyma (39.4 mg, 3 eq) and of DIC (43 µL, 3 eq) in DMF were added to the resin and reacted for 1h. Ninhydrin test was done after the coupling.

The first peptide chain was elongated following the protocol from section 5.5.4. Two Ahx were coupled using Fmoc-6-Ahx-OH (98.0 mg, 3 eq), Oxyma (39.4 mg, 3 eq) and DIC (43 µL, 3 eq). Ser was coupled using Fmoc-L-Ser(OtBu)-OH (106.3 mg, 3 eq), Oxyma (39.4 mg, 3 eq) and DIC (43 µL, 3 eq). Asp was coupled using Fmoc- L-Asp(OtBu)-OH (114.1 mg, 3 eq), Oxyma (39.4 mg, 3 eq) and DIC (43 µL, 3 eq). Gly was coupled using Fmoc-Gly-OH (82.4 mg, 3 eq), Oxyma (39.4 mg, 3 eq) and DIC (43 µL, 3 eq). Arg was coupled using Fmoc- L-Arg(Pbf)-OH (179.8 mg, 3 eq), Oxyma (39.4 mg, 3 eq) and DIC (43 µL, 3 eq). After coupling Arg, Fmoc was removed (1 x 1 min, 1 x 5 min, 1 x 10 min), a ninhydrin test was done and the N-terminus from Arg was acetylated using a solution of Ac_2O/DIEA/DMF (1:2:7, v/v/v) (1 x 5 min, 2 x 10 min). Afterwards, the resin was washed, and a ninhydrin test was done.



Scheme 10. Synthetic scheme for RGD-DWIVA peptide synthesis. A: Piperidine 20% (v/v) in DMF. B: Ac_2O/DIEA/DMF (10:20:70, v/v/v).

Alloc was removed using $Pd(PPh_3)_4$ (10.6 mg, 0.1 eq) and $PhSiH_3$ (114 µl, 10 eq) in DCM (3 x 15 min), washing with DCM (5 x 0.5 min) after each step. Ninhydrin test was done. After that, the resin was treated with sodium diethyldithiocarbamate (DEDTC) 0.02M in DMF (3 x 15 min) and washed. DWIVA branch was elongated and acetylated following the same protocols as for

RGD branch. Two Ahx were coupled using Fmoc-6-Ahx-OH (98.0 mg, 3 eq), Oxyma (39.4 mg, 3 eq) and DIC (43 µL, 3 eq). Ala was coupled using Fmoc-L-Ala-OH (86.3 mg, 3 eq), Oxyma (39.4 mg, 3 eq) and DIC (43 µL, 3 eq). Val was coupled using Fmoc- L-Val-OH (98.0 mg, 3 eq), Oxyma (39.4 mg, 3 eq) and DIC (43 µL, 3 eq). Ile was coupled using Fmoc- L-Ile-OH (98.0 mg, 3 eq), Oxyma (39.4 mg, 3 eq) and DIC (43 µL, 3 eq). Trp was coupled using Fmoc- L-Trp(Boc)-OH (146.0 mg, 3 eq), Oxyma (39.4 mg, 3 eq) and DIC (43 µL, 3 eq). Asp was coupled using Fmoc- L-Asp(OtBu)-OH (114.1 mg, 3 eq), Oxyma (39.4 mg, 3 eq) and DIC (43 µL, 3 eq). After coupling Asp, Fmoc was removed (1 x 1 min, 1 x 5 min, 1 x 10 min), a ninhydrin test was done, and the N-terminus from Asp was acetylated using a solution of Ac_2O/DIEA/DMF (1:2:7, v/v/v) (1 x 5 min, 2 x 10 min). Afterwards, the resin was washed, and a ninhydrin test was done.

0.3280 g of peptidyl-resin were obtained.

6.1.2. Characterization of RGD-DWIVA-01

To characterise this peptide, a mini-cleavage was made. The procedure is as follows:

A small aliquote of resin beds was transferred to an eppendorf and 0.5 ml of a solution of TFA/TIS/H_2O (95:2.5:2.5, v/v/v) were added and left to react for 1.5 h. After that, TFA solution was evaporated using an N_2 flow, and 1.5 ml of cold diethyl ether were added. The eppendorf was centrifuged during 3 min at 4ºC and 5000 rpm. After that, ether was removed, 100 µl of H_2O/ACN (1:1, v/v) were added, filtrated and transferred to an HPLC vial.

HPLC is done in 20-100% gradient of solvent B at 25ºC, and then in 10-100% at 60ºC. HPLC-MS was also performed in 10-100% gradient of solvent B.

25ºC chromatogram shows two major peaks at 6.0 and 6.1 min, and two minor peaks at 6.3 and 6.4 min (Appendix 2.1). 60ºC chromatogram shows one main peak at 6.9 min (Appendix 2.2). Its purity was 50%.

HPLC-MS shows one major peak at 10.7 min, that corresponds to the expected mass (Appendix 2.3).

6.1.3. Characterization of RGD-DWIVA-02

Mini-cleavage analysis was done in the same manner as RGD-DWIVA-01 (section 6.1.2). 25ºC and 60ºC HPLC were done in 20-100% gradient of B, and HPLC-MS in 0-100% of B.

Its chromatogram at 25ºC shows nearly the same peaks and shape of RGD-DWIVA-01, and retention times were also the same. 60ºC chromatogram shows a single major peak at 6.0 min with 70% purity (Figure 6 in page 30).

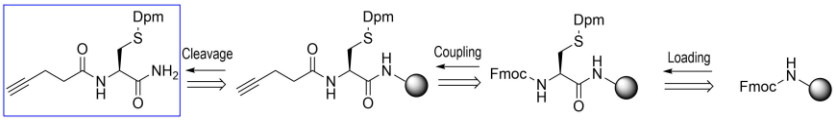
HPLC-MS spectrum show the same peaks as the ones in RGD-DWIVA-01 (Appendix 2.3).

6.1.4. Synthesis of 'Alkyne-SH'

Sieber resin was used to synthesize the 'Alkyne-SH' building block, that consist of a Cys protected with diphenylmethyl (Cys(Dpm)), and a 4-pentynoic acid (Pty) (Scheme 11). The loading was the maximum for this resin (0.59 mmol/g). The procedure that has been optimized to synthesize it is as follows:

0.3804 g of Fmoc-Sieber PS resin were weighted. Resin was conditioned and Fmoc was removed using piperidine 20% (v/v) in DMF (1 x 1 min, 1 x 5 min, 1 x 10 min). Fmoc-L-Cys(Dpm)-OH was loaded to the resin (343.1 mg, 3 eq) using Oxyma (95.7 mg, 3 eq) and DIC (104 µL, 3 eq) in DMF. The system was stirred for 1 h, washed, and a ninhydrin test was done. Fmoc was removed following the standard procedure described in section 5.3.2. After washing, Pty (33.0 mg, 1.5 eq) was coupled adding it alongside Oxyma (47.8 mg, 1.5 eq) and DIC (52 µl, 1.5 eq) in DMF. The system was stirred for 2 h and washed. Ninhydrin test was done.

Cleavage of the resin was performed in 5 extracts, collected on 20 ml of water inside falcons. These extracts were done by multiple washes with TFA-DCM (1.5:98.5, v/v) (6 x 0.5 min, 2 collects), DCM (6 x 0.5 min), TFA-DCM (1.5:98.5, v/v) (6 x 0.5 min) and TFA-DCM (3:97, v/v) (6 x 0.5 min). For each collect, DCM was evaporated using N_2 flow and 2 ml of ACN were added. 150 µl were transferred to an HPLC vial, the rest was freezed with liquid N_2 and taken to lyophilize. After freeze drying and unite the collects, 53.0 mg of peptide were obtained (66% yield).



Scheme 11: Retrosynthetic analysis of 'Alkyne-SH'

6.1.5. Characterization of 'Alkyne-SH'

HPLC of the extracts were done in a 30-100% gradient of solvent B at 25ºC. HPLC-MS was done in a 0-100% gradient of solvent B.

All the extracts show the same chromatogram, consisting of a single peak at 7.1 min (Appendixes 2.4 and 2.5) with >99.9 % purity.

HPLC-MS chromatogram shows this peak at 20.5 min. In its mass spectrum M+1 peak can be found, alongside with 2M+1H (dimer) and removed Dpm due to formic acid in mobile phase (Appendix 2.6).

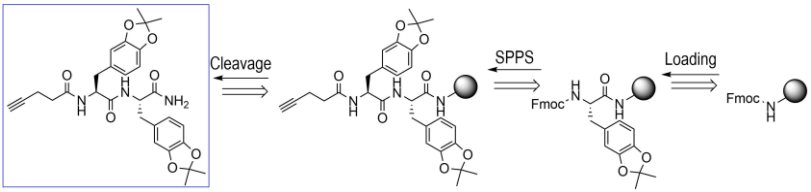
6.1.6. Synthesis of 'Alkyne-DOPA'

Sieber resin was used to synthesize the building block containing catechol groups, 'Alkyne-DOPA). It consists of two 3,4-dihydroxy-L-phenylalanine (L-DOPA) protected with acetonide and a 4-pentynoic acid (Pty) (Scheme 12). Its synthesis was done in the same manner as 'Alkyne-SH' with less equivalents in the loading step:

0.3000 g of Fmoc-Sieber PS resin were weighted. . Resin was conditioned and Fmoc was removed using piperidine 20% (v/v) in DMF (1 x 1 min, 1 x 5 min, 1 x 10 min). Fmoc-L-DOPA(Acetonide)-OH (162.7 mg, 2 eq) was loaded to the resin using Oxyma (50.3 mg, 2 eq) and DIC (55 µl, 2 eq) in DMF. The system was stirred for 1.5 h, washed, and a ninhydrin test was done. Fmoc was removed following the standard procedure described in section 5.3.2. After washing, Fmoc-L-DOPA(Acetonide)-OH (11.5 eq), Oxyma (37.7 mg, 1.5 eq) and DIC (41 µl, 1.5 eq) were added in DMF for the coupling of the second DOPA. The system was stirred for 2 h and a ninhydrin test was done. Fmoc was removed. After washing, Pty (26.0 mg, 1.5 eq), Oxyma (37.7 mg, 1.5 eq) and DIC (41 µL, 1.5 eq) were added in DMF. The system was stirred for 2 h. Ninhydrin test was done.

Cleavage of the resin was performed in 3 extracts, collected on 15 ml of water inside falcons. These extracts were done by multiple washes with TFA-DCM (3:7, v/v) (6 x 0.5 min, 2 collects),

DCM (6 x 0.5 min). For each collect, DCM was evaporated using N_2 flow and 2 ml of ACN were added. 150 µl were transferred to an HPLC vial, the rest was freezed with liquid N_2 and taken to lyophilize. After freeze drying and unite the collects, 33.8 mg of peptide were obtained (42% yield).



Scheme 12: Retrosynthetic analysis of 'Alkyne-DOPA'.

6.1.6. Characterization of 'Alkyne-DOPA'

HPLC of the extracts were done in a 40-100% gradient of solvent B at 25ºC. HPLC-MS was done in a 20-100% gradient of solvent B.

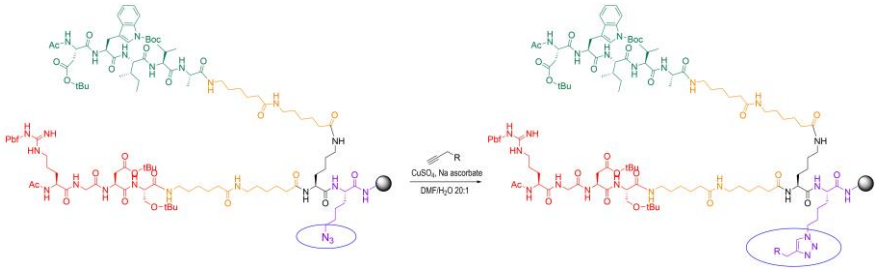
All the extracts showed the main peak at 5.5 min (Appendix 2.8), with a purity of 80%.

In HPLC-MS, this peak appeared at 17.3 min, showing a mass spectrum where $[M+1H]^+$ peak appeared as base peak (Appendix 2.9).

6.2. CLICK CHEMISTRY

Copper-catalysed azide alkyne cycloaddition (CuAAC) was done on solid phase, which means that azide-containing peptide was bounded to the resin and the alkyne-containing building blocks were added in solution. The reactions were followed by mini-cleavages of resin aliquots at certain times of reaction (the procedure for mini-cleavage is described in section 6.1.2). All HPLC runs were done in 20-100% gradient of solvent B at 25ºC or 60ºC, and HPLC-MS in 10-100%.

The conditions used for this reaction were based on the ones reported by V. Marchán et. al[11]. These conditions consist of the use of $CuSO_4$/ascorbate (3 eq:3 eq) as catalyst and DMF/H_2O 20:1 (v/v) as solvent under N_2 atmosphere, room temperature and overnight time of reaction. Each reaction has been optimized for each alkyne building block using RGDS-DWIVA peptides. To consult all the test reactions with their conditions and results, see Table 3. Tests reactions are coded with a letter that indicates which indicates what alkyne building block was used. A# tests are for 'Alkyne-NH_2' (propargylamine), S# for 'Alkyne-SH' and D# for 'Alkyne-DOPA'.



Test	RGD-DWIVA batch	Resin amount (mg)	Alkyne amount	$CuSO_4 \cdot 5\ H_2O$ (mg)	Sodium ascorbate (mg)	Reaction time	N_2 atm.	Notes	Product?	Yield
A1	01	20	1.5 µl (3 eq)	6.0 (3 eq)	4.8 (3 eq)	o.n.	Y	4 ml of solvent	Y	56%
A2	01	50	3.8 µl (3 eq)	15.0 (3 eq)	12 (3 eq)	o.n.	Y	Reduction to 1 ml of solvent.	Y	47%
A3	01	20	1.5 µl (3 eq)	6.0 (3 eq)	4.8 (3 eq)	o.n.	Y	Previous wash with DEDTC	N	-
A4	01	20	1.5 µl (3 eq)	6.0 (3 eq)	4.8 (3 eq)	o.n.	Y	50ºC	N	-
A5	01	20	1.5 µl (3 eq)	6.0 (3 eq)	4.8 (3 eq)	o.n.	Y	Repetition of 3	Y	Quant.
A6	01	20	1.5 µl (3 eq)	6.0 (3 eq)	4.8 (3 eq)	o.n.	Y	30ºC	N	-
A7	01	20	1.0 µl (2 eq)	6.0 (3 eq)	9.6 (6 eq)	o.n.	N		Y	Quant.
A8	02	15	0.8 µl (2 eq)	4.5 (3 eq)	9.6 (6 eq)	8 h	N	6 h of reaction + 2 h extra	Y	Quant.
S1	02	15	4.4 mg (2 eq)	4.5 (3 eq)	3.6 (3 eq)	o.n.	Y	1 ml of solvent	N	-
S2	02	15	6.6 mg (3 eq)	4.5 (3 eq)	3.6 (3 eq)	o.n.	Y	Substrate from test 1, cleavage with DTT additive	Y	Quant.
S3	02	15	4.4 mg (2 eq)	4.5 (3 eq)	3.6 (3 eq)	o.n.	Y		Y	Quant.

D1	02	15	6.8 mg (2 eq)	4.5 (3 eq)	3.6 (3 eq)	o.n.	Y			Y	Quant.

o.n.: overnight
DEDTC: sodium diethyldithiocarbate
DTT: dithiothreitol
Y: Yes
N: No

Table 3. List of test reactions.

6.2.1. Reaction with 'Alkyne-NH_2'

Two methods were used to perform the reaction with propargylamine. The main difference between the methods is that in one of them the reaction was put under inert gas (N_2).

Method 1: The desired amount of RGD-DWIVA resin was put in a glass vial and DMF was added. The mixture was purged and stirred 10 min under N_2 atmosphere. To purge, two needles were connected to the plug, one of them having N_2 flow and the other open to air. N_2 flow was maintained for 5 min and the open needle was retired as N_2 flow continued for 2 min. Propargylamine (desired eq), $CuSO_4 \cdot 5\ H_2O$ (3 eq) dissolved in water and sodium ascorbate (3 eq) were added to the pot, which was purged and left to reactthe desired time. After reaction, the system was opened, and the resin was transferred into a syringe and washed with DMF (5 x 0.5 min) and DCM (5 x 0.5 min).

Method 2: In a syringe, the desired amount of RGD-DWIVA resin was conditioned and DMF was added. Propargylamine (desired eq), $CuSO_4 \cdot 5\ H_2O$ (3 eq) dissolved in water and sodium ascorbate (6 eq) were added to the system and it was left stirring for the desired time. After reaction, the resin was washed with DMF (5 x 0.5 min) and DCM (5 x 0.5 min).

6.2.2. Reaction with 'Alkyne-SH'

Reactions with this compound follow the method 1 described for propargylamine.

The desired amount of RGD-DWIVA resin was put in a glass vial and DMF was added. The mixture was purged and stirred 10 min under N_2 atmosphere. To purge, two needles were connected to the plug, one of them having N_2 flow and the other open to air. N_2 flow was maintained for 5 min and the open needle was retired as N_2 flow continued for 2 min. 'Alkyne-SH' (desired eq) dissolved in 150 µl of DMF, $CuSO_4 \cdot 5\ H_2O$ (3 eq) dissolved in water and sodium ascorbate (3 eq) were added to the pot, which was purged and left to react overnight. After

reaction, the system was opened, and the resin was transferred into a syringe and washed with DMF (5 x 0.5 min) and DCM (5 x 0.5 min).

6.2.3. Reaction with 'Alkyne-DOPA'

One reaction has been made with this building block, following the same method described for 'Alkyne-SH' (section 6.2.2).

The desired amount of RGD-DWIVA resin was put in a glass vial and DMF was added. The mixture was purged and stirred 10 min under N_2 atmosphere. To purge, two needles were connected to the plug, one of them having N_2 flow and the other open to air. N_2 flow was maintained for 5 min and the open needle was retired as N_2 flow continued for 2 min. 'Alkyne-DOPA' (desired eq) dissolved in 150 µl of DMF, $CuSO_4 \cdot 5\ H_2O$ (3 eq) dissolved in water and sodium ascorbate (3 eq) were added to the pot, which was purged and left to react overnight. After reaction, the system was opened, and the resin was transferred into a syringe and washed with DMF (5 x 0.5 min) and DCM (5 x 0.5 min).

7. RESULTS AND DISCUSSION

7.1. SYNTHESIS OF RGD-DWIVA PEPTIDES

Two batches of this peptide were synthesized, obtaining 50% and 70 % purity.

In the steps of loading and acetylation, last deprotection steps were carried during 10 min instead of 5 to ensure full removal of Fmoc. Before acetylation, ninhydrin test was also done to confirm it.

After removing Alloc, the resin was washed with a solution of sodium diethyldithiocarbamate (DEDTC) 0.02 M in DMF (3 x 15 min) to remove Pd that could remain in the resin. That was done after the full synthesis in RGD-DWIVA-01, and just after Alloc removal in RGD-DWIVA-02. That was found to be important for the CuAAC reaction (see Section 7.3.1).

There were some problems regarding the ninhydrin tests. As negative results are associated with yellow colour and positive to blue/violet, there were some tests that resulted in a green colour when the expected result was negative. It was decided to take green as negative, because resin

beads were transparent. Main hypothesis is that ninhydrin reagent could also interact with azide and give the green colour.

Another problem was that after the elongation and Fmoc removing from the last amino acid (aspartic acid) of the DWIVA branch, ninhydrin test was negative. After repeating the deprotection step with more piperidine treatments, the ninhydrin was still negative. The reason is that this amine is not as accessible as the previous ones, due to two branches of 6 and 7 amino acids, respectively. Then, this steric impediment does not allow ninhydrin reagents to react with the amine. It was decided to continue with acetylation because after that double treatment Fmoc was surely removed.

In their chromatograms at 25ºC, a specific pattern of four peaks can be seen. At 60ºC, these 4 peaks unify as one (Figure 6). This is due to the formation of two known by-products. One is an isomerization of the RGD branch. The other one is formed when Trp traps the CO_2 from the Boc protecting group once it is deprotected. At 60ºC, the RGD isomers get an equilibrium and after long time in solution, the CO_2 is removed. Note that this pattern is found in all the chromatograms of the click reactions if they are done at 25ºC.

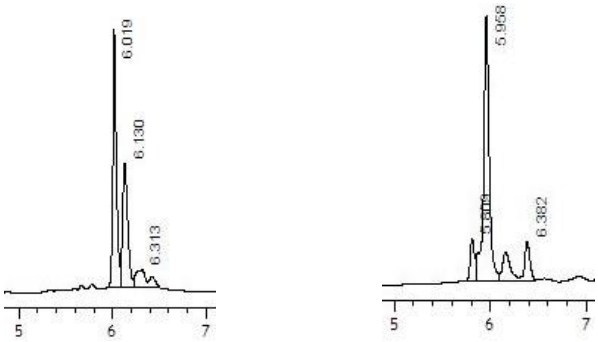


Figure 6. RGD-DWIVA-02 chromatograms at 25ºC (left) and 60ºC (right).

7.2. SYNTHESIS OF 'ALKYNE-SH'

For this compound, the obtained quantity was enough to perform the reactions and the purity was good. It was obtained as a white-yellow solid.

In order to find the loading conditions, 30 mg of Sieber resin were loaded with Fmoc-L-Cys(Trt)-OH (12.1 mg, 1.2 eq) for 1h and after a ninhydrin test, it was discovered that the loading step failed. As the maximum loading was wanted, it was found that adding stoichiometric amounts

for the loading did not result in the complete loading in 1h, and more time would be needed. However, with a 3x excess a complete loading was accomplished in 1h. This was demonstrated by ninhydrin tests. Once the conditions were optimized, the synthesis was repeated with the desired amino acid.

For cleavage, it was tried to be done with TFA 1.5% (v/v), as Sieber resin can be cleaved with 1% concentration[17,18] and in TFA 3% (v/v) it was possible that Dpm protective group could be released. However, after cleavage treatment with TFA 1.5% (as described in section 6.2.3), the peptide was not fully cleaved. When the concentration was raised to 3%, the resin was cleaved with a total of 6 washes of cleavage solution (6 x 0.5 min), but the extract became yellow. That was interpreted as the removal of Dpm from the thiol, but HPLC revealed that the group was maintained as all chromatograms were the same (Appendixes 2.4 and 2.5). Therefore, it was decided to cleave the resin with TFA 3% (v/v).

7.3. SYNTHESIS OF 'ALKYNE-DOPA'

The obtained quantity was enough to perform tests and with a decent purity. It was obtained as a white solid. The first collect was discarded as it showed yellow traces.

However, the obtained quantity was low. It was thought that more peptide could remain bounded to the resin, then the process of cleavage was repeated. The hypothesis was confirmed as more peptide was cleaved in the new collects, demonstrated by HPLC. Up to this date, there is not data about the amount of recovered peptide, then the shown amount is the one which was obtained in the first cleavage process. In the end, it was clear that more collects and more washes were needed to collect all the product.

7.4. CUAAC OPTIMIZATION

7.4.1. 'Alkyne-NH_2'

The first reagent to be tested was propargylamine. After optimizing the conditions with this reagent, the tests with other building blocks were based on the obtained results.

Before adding ascorbate to the mixture, the solution was blue due to copper (II). When it was added, the solution became green (copper (I)), and while the reaction took place the colour tended to yellow. When the resin was washed, it remained as green. It was found that dissolving copper and ascorbate in water together resulted in the formation of a brown solid which was not useful.

It hasn't been determined what compound is. However, the main theory is that is metallic Cu (excess of reduction) or Cu_2O (as it has the same colour). Adding $CuSO_4 \cdot 5\ H_2O$ dissolved in water and solid ascorbate avoided the formation of this by-product.

A1 test was monitored at 1 and 2 h of reaction. At 1 h there wasn't any product formed. At 2 h, a small peak appeared at 5.6 min, which increased after overnight (Figure 7). This peak was related to the new product, and showed the same pattern as the starting material, which shows the major peak at 6.1 min. The yield was 56%, calculated with the area relationship between these two major peaks.

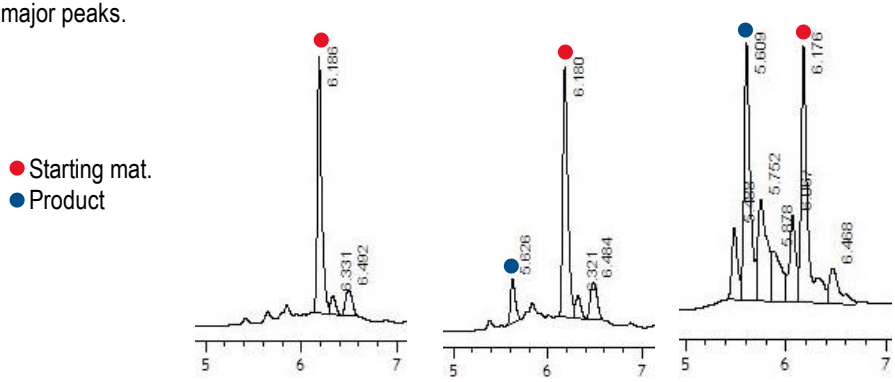


Figure 7. Evolution of A1 test at 1 h (left), 2 h (centre) and overnight (right). All done at 25ºC.

To try to improve the results obtained from A1, A2 test was performed, trying to concentrate the reagents reducing the amount of solvent and taking more resin. However, the obtained yield was nearly the same (47%), then it was found that the concentration was not the problem.

A3 and A4 tests didn't give any product due to manipulation problems.

A5 and A6 reactions were done in parallel. This time, the resin was washed with DEDTC to remove Pd, as it could be interfering with Cu in the catalytic process. It was also tested to accelerate the reaction by raising mildly the temperature, having reaction 6 at 30ºC. The result was that reaction 5 yield was 51% after 1 h, 83% after 2 h and quantitative after overnight (Figure 8). This was also demonstrated by HPLC-MS (Appendix 2.9). According to these data, it seemed that the time it took to be quantitative was 3-4h. A6 reaction solution and resin turned brown after overnight and it was decided that it was not necessary to analyse.

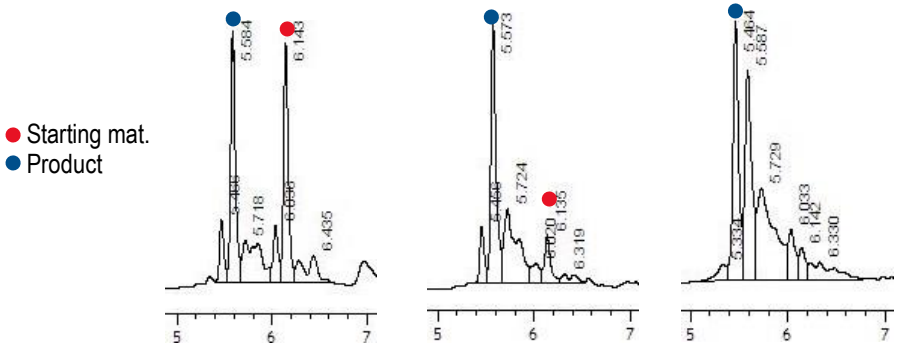


Figure 8. Evolution of A5 test at 1 h (left), 2 h (centre) and overnight (right). All done at 25ºC.

For A7 test, it was tried to avoid using N_2 atmosphere to facilitate the manipulation. For this reason, ascorbate ratio was doubled to prevent Cu(I) oxidation due to open air. Also, it was tried to reduce alkyne equivalents (from 3 to 2) to see if it worked, because the next reactions would be tested with synthetic reagents and it was better to use less quantity. At 3h, its yield was 38%, and at 4h it was 70%. (Figure 9). The reaction mixture showed its usual green colour. However, after overnight, the solution became brown as in A6 test. HPLC showed that the reaction was quantitative, but the reaction crude was very dirty (Figure 9). It was tried to wash the resin with DEDTC, but it was not effective.

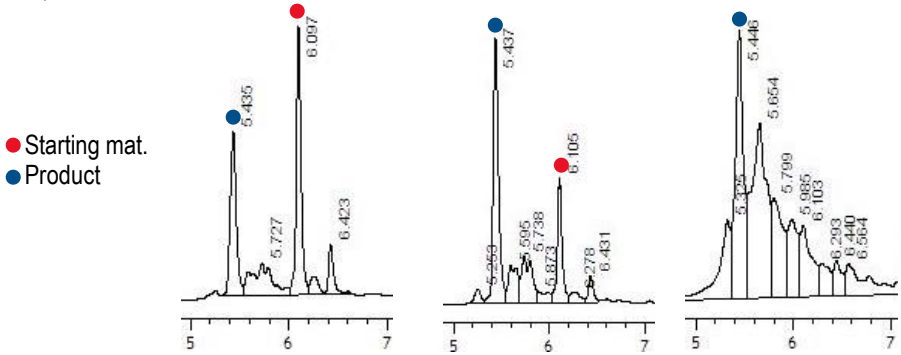


Figure 9. Evolution of A5 test at 3 h (left), 4 h (centre) and overnight (right). All done at 60ºC.

Then, it was decided to repeat the reaction stopping it in 6h (A8 test) to see if it was enough time to be quantitative and to avoid the formation of the brown precipitate, but the obtained yield was lower than the expected, 22%, so the reaction was resumed, and the mixture turned yellow first and started to get brown at 2h. In that moment it was stopped, and the chromatogram showed the same result as in reaction A7: quantitative, but dirty.

In the end, it was decided that the optimal conditions were the ones in A5. It was necessary to wash the resin with DEDTC before the reaction to avoid intermetallic interferences, and it was decided to use inert atmosphere and stoichiometric ratio of Cu (II) and ascorbate to avoid the formation of Cu precipitate and obtain a cleaner crude.

7.4.2. 'Alkyne-SH'

For S1 test, the colours progression was the same as the one seen with propargylamine. In its chromatogram a new peak appeared at 7.2 min, which was expected to be the product (Figure 10) with an apparent yield of 22%. However, the crude was very dirty, and it could not be assigned by HPLC-MS, as expected m/z could not be found, neither the product nor the starting material (Appendix 2.11).

S2 test was performed before obtaining HPLC-MS results of S1 test. As a low yield was calculated, it was decided to reuse that resin to try the same conditions but concentrating, and that was done raising the eq from 2 to 3 and reducing the solvent to the same proportion as it was for 20 mg of resin. It was also washed with DEDTC, as the crude was very dirty. After overnight, the chromatogram was almost the same as the obtained for S1. After some research, it was decided to do a mini-cleavage using dithiothreitol (DTT) as additive to the cleavage solution in small amount, as it prevents byproducts that come from thiol oxidation to disulphide[2]. The result was a new cleaner chromatogram where a major single peak appeared at 5.7 min (Figure 10). This peak was confirmed to be the product by HPLC-MS (Appendix 2.12).

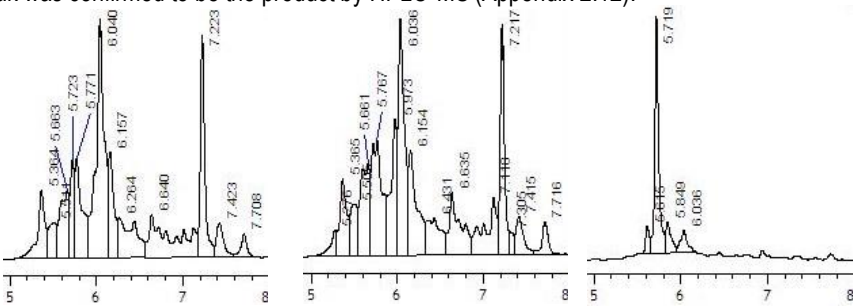


Figure 10. S1 mini-cleavage after overnight (left), S2 mini-cleavage without DTT (centre) and S2 mini-cleavage with DTT (right). All done at 60ºC.

After this result, a new test was performed (S3) using the same conditions for S1, to confirm that if it worked with 2 eq after overnight. After mini-cleavage with DDT and HPLC analysis, it was confirmed that it worked perfectly, as the same chromatogram as for S2 was obtained.

In the end, the same conditions that worked with propargylamine are good for this reaction, and it was demonstrated that alkyne equivalent could be reduced from 3 to 2. The only thing that needs to be changed is the cleavage with DTT due to the formation of numerous byproducts.

7.4.3. 'Alkyne-DOPA'

Only one reaction has been performed with this building block (D1 test). The conditions that were used worked with both propargylamine and 'Alkyne-SH', then it was expected to work well. The reaction progression was the expected one and nothing strange happened. The chromatogram of this reaction after overnight showed nearly the same profile as S3 test, including the main single peak at 5.7 min (Figure 11). Although this is surely the product, HPLC-MS analysis could not be done due to lack of time, then it needs further studies.

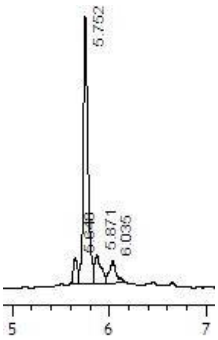


Figure 11. D1 test mini-cleavage after overnight. Done at 60ºC.

To sum up, it seems that the product has been obtained, but it needs confirmation by HPLC-MS.

8. CONCLUSIONS

The three proposed objectives were accomplished. All the target compounds were synthesized, and the three reactions were optimized to obtain the desired peptide platforms in good yields.

However, this is not the end for this study. Further studies in DOPA analogue should be done to confirm that the product is the desired one.

This project focused on the methodology, and now these methods are established to synthesize these moieties in larger scale. The next thing to do is produce them with more amounts of reagents, cleave them and purify them. Once they are obtained, they should be tested in surface functionalization studies to demonstrate their effectiveness on adhesion to the surface and on their function.

Also, it must be proved that this methodology is useful to synthesize other peptide platforms that contain other bioactive sequences, for example, a dimeric platform of RGD and lactoferrin (antibiotic properties), PHSRN (osteoblast response boost) or REDV (endothelialisation)[1]. Once this is proved to work with other structures, it will be considered as a solid methodology to prepare multifunctional platforms.

It is hoped that this project will serve as a starting point to develop new methodologies on SPPS of the peptide platforms destined to functionalize materials, introducing new ways of working that will be fast and efficient.

9. REFERENCES AND NOTES

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10. ACRONYMS

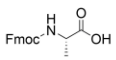
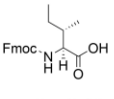
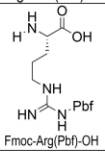
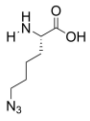
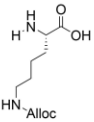
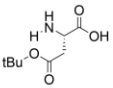
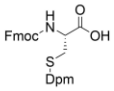
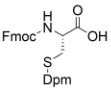
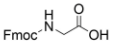
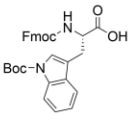
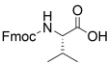
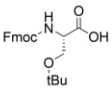
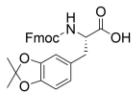
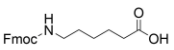
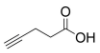
ACN:	acetonitrile
Ahx:	6-aminohexanoic acid
Alloc:	Allyloxycarboyl
APTES:	3-aminopropyltriethoxysilane
BMP:	Bone Morphogenetic Protein
BMPS:	3-(maleimido)propionic acid N-succinimidyl ester
Boc:	tert-butyloxycarbonyl
CuAAC:	Copper-catalysed Azide Alkyne Cycloaddition
DCM:	dichloromethane
DEDTC:	sodium diethyldithiocarbamate
DIC:	N,N'-diisopropylcarbodiimide
DIEA:	N,N-diispropylethilamine
DMF:	N,N-dimethylformamide
DOPA:	3,4-dihydroxyphenylalanine
Dpm:	diphenylmethyl
Dpm:	diphenylmethyl
DTT:	dithiothreitol
eq:	equivalent
Fmoc:	9-fluorenylmethoxycarbonyl
FN:	fibronectin
MS:	Mass Spectrometry
OxymaPure (Oxyma):	ethyl 2-cyano-2-(hydroxyimino) acetate
Pbf:	2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl

PLA:	polylactic acid
Pty:	4-Pentynoic acid
Rink Amide MBHA:	4-(2',4'-Dimethoxyphenylaminomethyl)-phenoxyacetamido-methylbenzhydrylamine
RP-HPLC:	Reversed Phase-High Performance Liquid Chromatography
Sieber PS:	9-amino-9H-xanthen-3-yl-oxymethyl polystyrene
SPPS:	Solid Phase Peptide Synthesis
TBTA:	tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine
^{t}Bu:	tert-butyl
TFA:	trifluoroacetic acid
TIS:	triisopropylsilane
Trt:	trityl

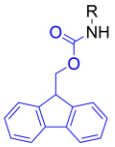
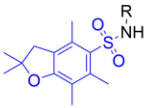
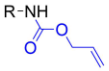
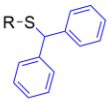
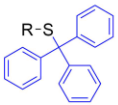
APPENDICES

APPENDIX 1: TABLES OF AMINO ACIDS AND PROTECTIVE GROUPS

1.1. Amino acids

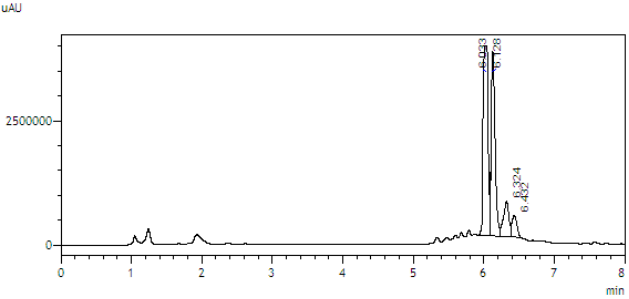
Alanine A	Isoleucine I	Arginine(Pbf) R
 Fmoc-Ala-OH	 Fmoc-Ile-OH	 Fmoc-Arg(Pbf)-OH
Lysine(N_3) K	**Lysine(Alloc) K**	**Aspartic acid D**
 Fmoc-Lys(N_3)-OH	 Fmoc-Lys(Alloc)-OH	 Fmoc-Asp(OtBu)-OH
Cysteine(Dpm) C	**Cysteine(Trt) C**	**Glycine G**
 Fmoc-Cys(Dpm)-OH	 Fmoc-Cys(Trt)-OH	 Fmoc-Gly-OH
Tryptophan(Boc) W	**Valine V**	**Serine S**
 Fmoc-Trp(Boc)-OH	 Fmoc-Val-OH	 Fmoc-Ser(OtBu)-OH
3,4-dimethoxyphenylalanine(Acetonide)	**6-Aminohexanoic acid**	**4-Pentynoic acid**
 Fmoc-L-DOPA(Acetonide)-OH	 Fmoc-6-Ahx-OH	 Pty

1.2. Protective groups

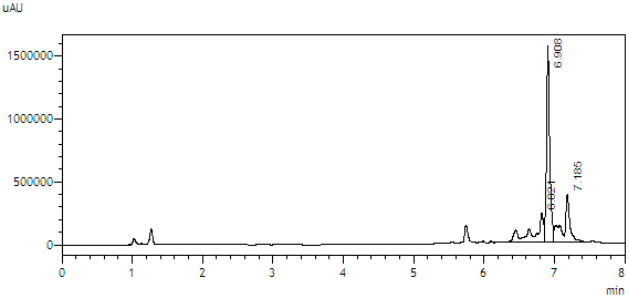
Fmoc	Boc	Pbf	Alloc
 9-Fluorenylmethoxycarbonyl	 tert-Butoxycarbonyl	 2,2,4,6,7-Petamethyldihydrobenzofuran-5-sulfonyl	 Allyloxycarbonyl
Dpm	**Trt**	**Acetonide**	**tBu**
 Diphenylmethyl	 Trityl (Triphenylmethyl)	 Isopropylidene ketal	 tert-Butyl

APPENDIX 2: CHROMATOGRAMS AND SPECTRA

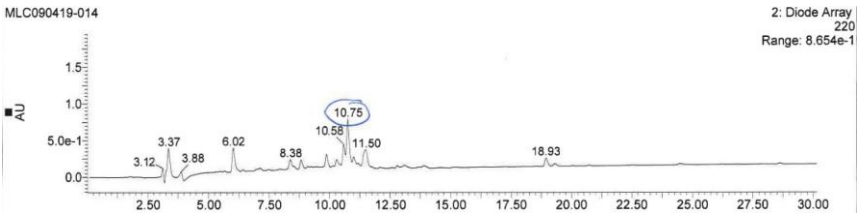
2.1. RGD-DWIVA-01, 25ºC

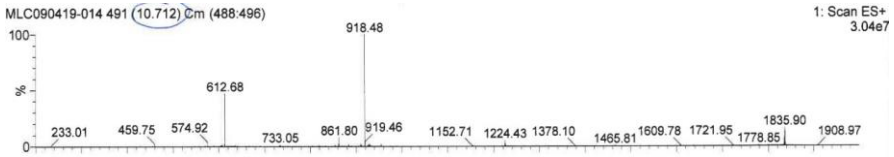


2.2. RGD-DWIVA-01, 60ºC



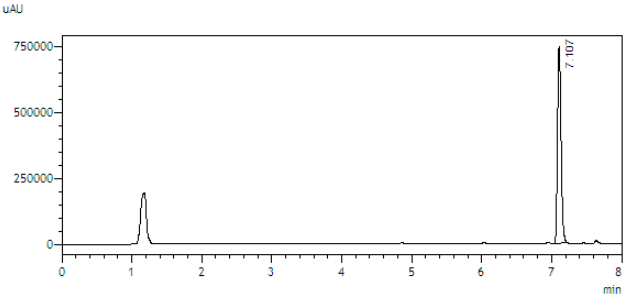
2.3. RGD-DWIVA-01, HPLC-MS



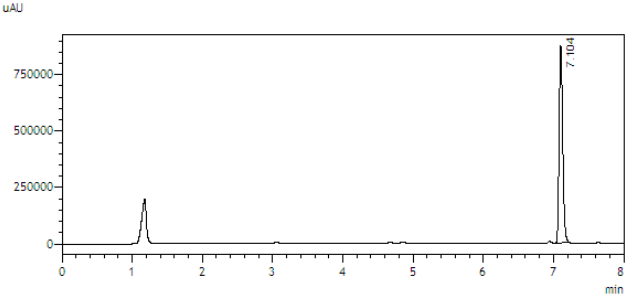


M=1835.04. $[(M+3H)/3]^{3+}$: 612.68 (612.68). $[(M+2H)/2]^{2+}$: 918.48 (918.52). $[M+1H]^{+}$: 1835.90 (1836.04).

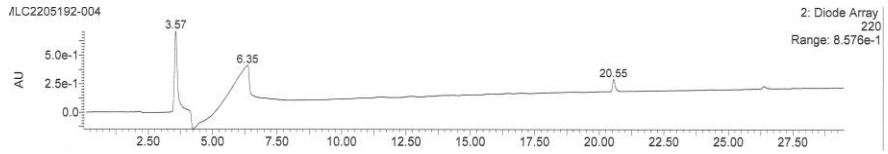
2.4. 'Alkyne-SH' (cleavage with TFA 1.5% v/v)

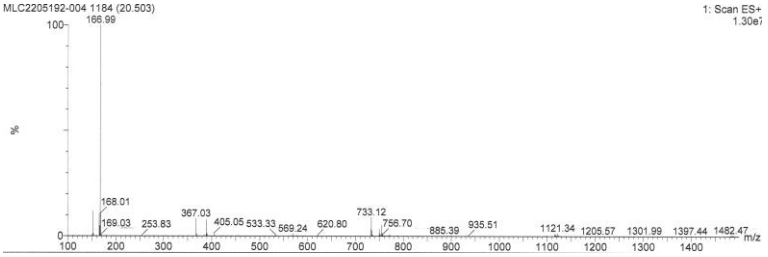


2.5. 'Alkyne-SH' (cleavage with TFA 3% v/v)



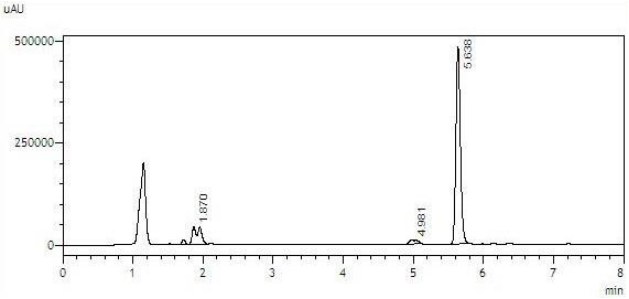
2.6. 'Alkyne-SH', HPLC-MS



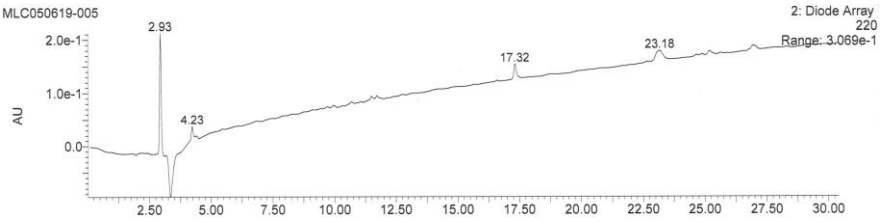


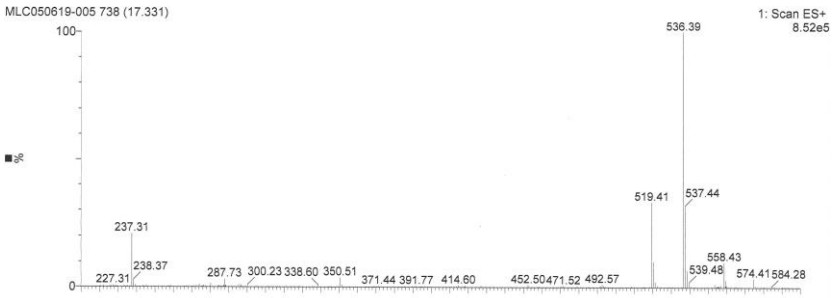
M=366.48. $[Dpm]^+$: 166.99 (167.09). $[M+1H]^+$: 367.03 (367.48). $[2M+1H]^+$: 733.12 (733.96).

2.7. 'Alkyne-DOPA'



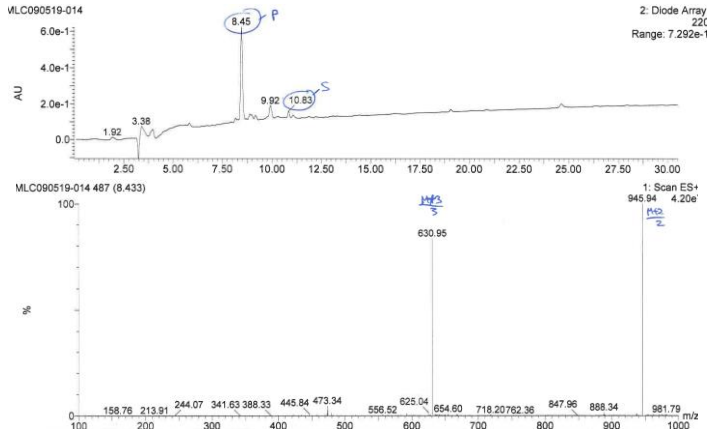
2.8. 'Alkyne-DOPA', HPLC-MS



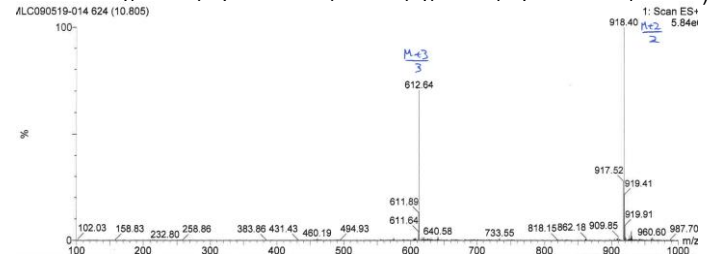


M=535.60. $[M+1H]^+$: 536.39 (536.60).

2.9. A5 test, HPLC-MS

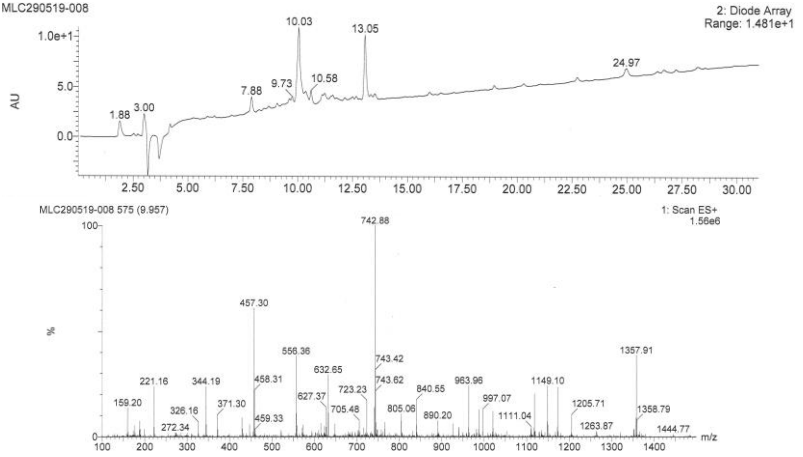


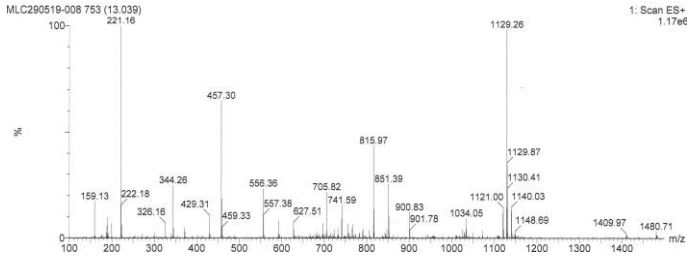
M=1890.08. $[(M+3H)/3]^{3+}$: 630.95 (631.03). $[(M+2H)/2]^{2+}$: 945.94 (946.04).



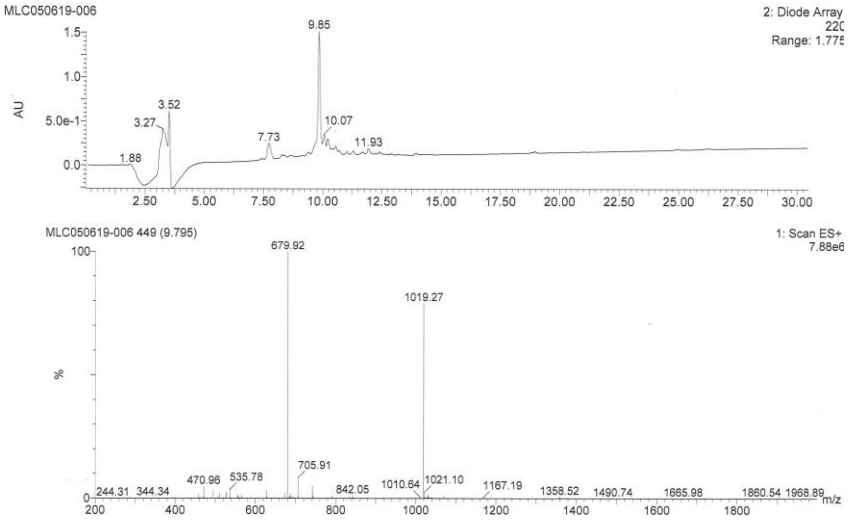
M=1835.04. $[(M+3H]/3]^{3+}$: 612.64 (612.68). $[(M+2H]/2]^{2+}$: 918.40 (918.52).

2.10. S1 test, HPLC-MS





2.11. S3 test, HPLC-MS



M=2035.10. $[(M+3H]/3]^{3+}$: 679.92 (676.37). $[(M+2H]/2]^{2+}$: 1019.27 (1018.55).