Tutor

Dr. Jaume Farràs Soler
Departament de Química Inorgànica i Orgànica

Grau de Química

Treball Final de Grau

Study of the full deprotection of a model peptide.
Estudi de la desprotecció total d'un pèptid model.

Júlia García Gros

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Science and everyday life can not and should not be separated.

Rosalind Franklin.

Aquest treball no s'hauria dut a terme sense l'ajuda de moltes persones. Gràcies,

Al Dr. Jaume Farràs, tutor d'aquest treball, per guiar-me i fer que estigui tan orgullosa d'aquest treball.

Al Dr. Ernesto Nicolás, que tot i no ser-ne el tutor, ha estat sempre present.

A la Macarena, no només per les hores de laboratori, sinó que també per deixar-me compartir tants bons moments amb ella.

A la família i als amics, per donar-me suport quan l'he necessitat.

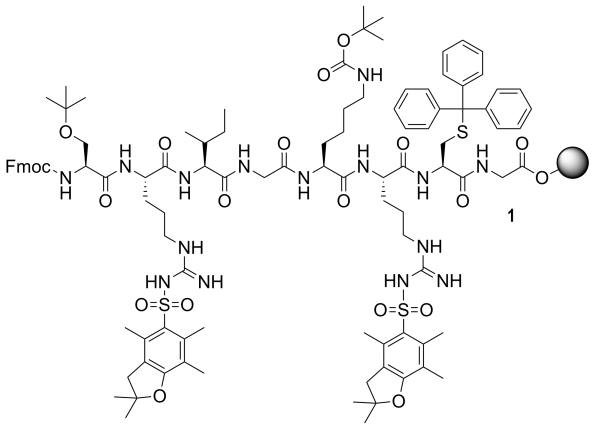
A en Joaquim, per estar sempre al meu costat.

REPORT

CONTENTS

1. SUMMARY

Fmoc chemistry has been used to synthesise the 2-chlorotrityl peptidyl-resin shown in the figure below, in order to study its cleavage conditions and the subsequent side products.



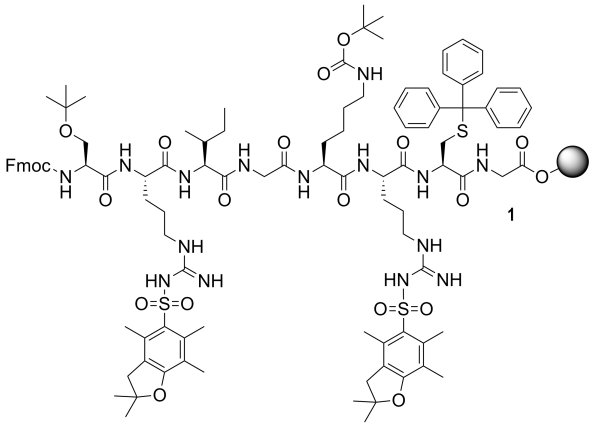
The cleavage of the peptide, as well as the deprotection of its side chains, are practically quantitative after 30 minutes of reaction time, using 95 % TFA, 2.5 % TIPS and 2.5 Milli-Q water. The main impurity belongs to a dimer that arises from the formation of a disulphide bond between the Cys residues of two identical peptide chains.

The addition of DTT to the cleavage mixture as a reducing agent does not prevent the formation of the dimer. On the other hand, using TCEP as a reducing agent minimises the formation of this side product in a very efficient way.

Keywords: SPPS, Fmoc chemistry, cleavage, deprotection, disulphide bond formation.

2. RESUM

S'ha utilitzat la química del Fmoc per tal de sintetitzar la 2-clorotritil peptidil resina model indicada a la figura i així poder-ne d'estudiar les condicions de desancorament i els subseqüents subproductes de reacció.



Tant el desancorament del pèptid com la desprotecció de les seves cadenes laterals són pràcticament quantitatives passats 30 minuts de reacció i utilitzant 95 % TFA, 2.5 % TIPS i 2.5 H_2O Milli-Q. La principal impuresa és un dímer que prové de la formació d'un pont disulfur, com a conseqüència de l'oxidació dels residus de Cys presents en el pèptid.

L'addició d'un agent reductor com el DTT no impedeix la formació del dímer. En canvi, l'ús de TCEP com a agent reductor sí que impedeix la formació d'aquest subproducte de manera molt efectiva.

Paraules clau: SPPS, Química del Fmoc, desancorament, desprotecció, formació de pont disulfur.

3. INTRODUCTION

Since the beginning of organic synthesis, the synthesis of peptides had been one of the largest challenges in this discipline. Emil Fischer described the chemistry of proteins for the first time in 1901, when he achieved the synthesis of the dipeptide glycyl-glycine in solution.[1,2] Synthesis of peptides in solution has many shortcomings such as difficult purification and isolation methods as a consequence of the increasing insolubility of the growing peptide chain. This insolubility may cause also difficulties in the sequential coupling of the amino acids to large peptide chains. It was not until the early 60s when Bruce Merrifield[3] introduced the solid phase peptide synthesis (SPPS), a more efficient way for synthesising these biomolecules.

SPPS presents many advantages such as the synthesized peptide is covalently bonded to an insoluble resin, which allows the easy separation of the growing peptide chain from any by-products or unused excess of amino acid by only washing the resin with the appropriate solvents. This is a major advantage when compared to the solution phase method, as the latter method requires harder purification steps such as chromatography or crystallisation, resulting in a decrease of the overall yield.

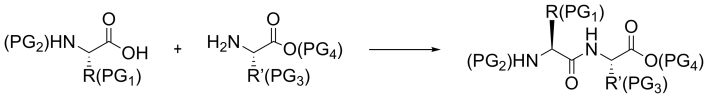
The development of new methodologies in solid-phase peptide synthesis has accelerated the discovery and synthesis of potential therapeutic compounds. Peptides are recognized for being highly selective and effective as they can bind to specific cell surface receptors or ion channels, where they trigger intracellular effects, which is why they are relatively safe and well tolerated. Consequently, there is an increased interest towards therapeutic peptides that leads to an increase of research and development. About 140 therapeutic peptides are currently being evaluated in clinical trials. In recent years, synthetic peptides are applied to treat several diseases such as autoimmune pathologies, viral or bacterial infections and cancer.[4,5]

Recently, our group has started a research line aimed to the development of synthetic methodology suitable for the large-scale preparation of peptides of pharmacological interest. In those studies, it was found that the cleavage and deprotection of certain peptides containing specific sequences of residues protected with Pbf, Trt, ^{t}Bu, and Boc lead to complex reaction

mixtures. In this context, the objective of the present work focuses on the study of the cleavage and deprotection steps in a model peptide in order to identify the problems associated to those processes as well as to stablish the appropriate reaction conditions.

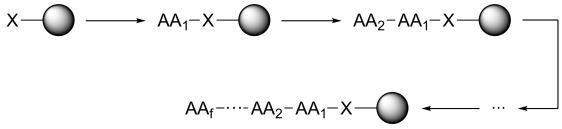
3.1. SOLID-PHASE PEPTIDE SYNTHESIS

A peptide is a sequence of amino acids covalently linked by a peptide bond, characterised for being a bond between the α-carboxyl group end from one amino acid with the α-amino group from another amino acid. To perform this reaction, amino acids and their side chains must be protected to avoid side reactions between the different functional groups of the present residues[6].



Scheme 1. Formation of a dipeptide.

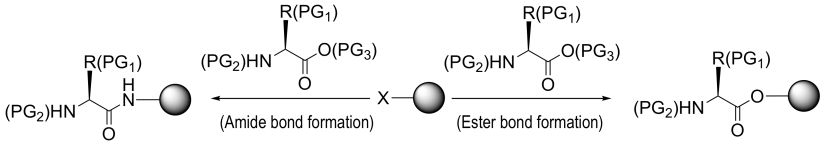
The first to introduce solid-phase peptide synthesis (SPPS) was Bruce Merrifield in 1963. It consists on attaching the carboxylic group of the first amino acid to an insoluble polymeric support, also known as resins, through an amide or ester bond. The peptide is then grown from the carboxyl group of the first amino acid to the amino group of the last amino acid.



Scheme 2. Growing of the peptide chain.

3.1.1. General procedure

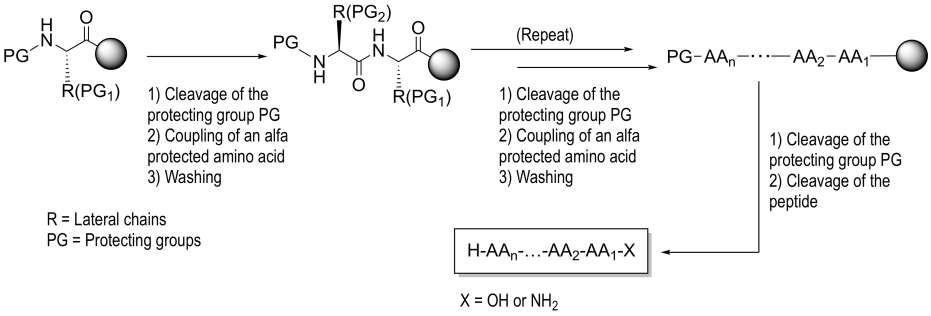
SPPS is performed in different steps. To start with, the first amino acid, side and α-amino protected, is attached to a polymeric support through an ester or amide bond.



Scheme 3. Attachment of an amino acid through an amide/ester bond.

The second step consists on the deprotection of the α-amino group followed by the addition of the second amino acid, which leads to the formation of an amide bond between the α-amino

group of the first amino acid and the α-carboxylic group of the second amino acid. This step is repeated as many times as necessary to obtain the desired chain. Once the peptide is synthesized, it is cleaved from the resin.



Scheme 4. General procedure in SPPS.

Amino acids must be orthogonally-protected, in order to allow the N-terminal protection removal without displacing the side chain protecting groups, and visa versa. This concept was introduced for the first time by George Barany in 1977[3].

3.1.2. Protection strategies

The strategies commonly used in SPPS are Boc/Bzl (tert-butoxycarbonyl groups for protecting the α-amino acid and benzyl groups for the lateral chains of the amino acids) and Fmoc/^{t}Bu (9-fluorenylmethoxycarbonyl for the α-amino protection and tert-butyl type for the lateral chains). The figure below, compares the cleavage behaviour of the amino acid Serine with Boc/Bzl strategy and Fmoc/^{t}Bu strategy[7].

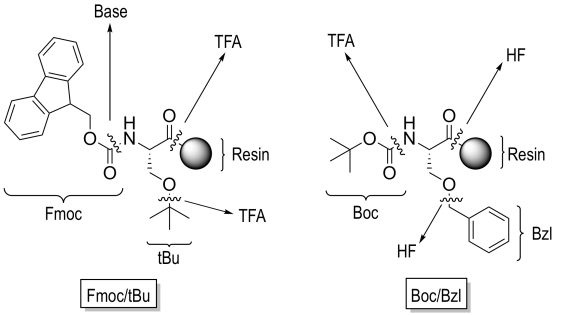
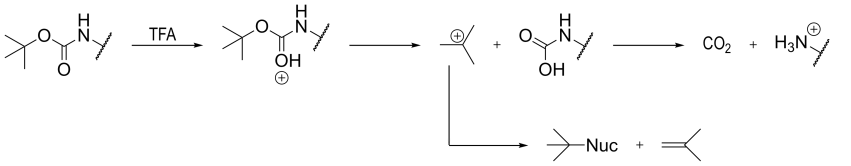


Figure 1. Comparison of the main protection strategies in SPPS.

3.1.2.1. Boc/Bzl strategy

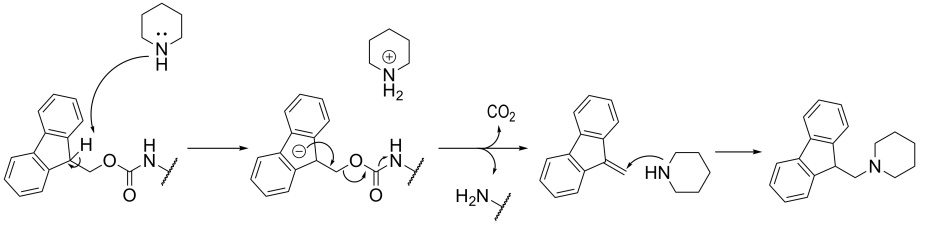
In the Boc/Bzl protection scheme, Boc protecting groups are used to temporally protect the α-amino group of the amino acids while benzyl-based protecting groups provide more permanent protection of side chains. Boc and benzyl-based protecting groups are both acid labile, so Boc/Bzl is not a true orthogonal protection scheme. It is practically used though, because the Boc group is removed under moderate conditions (50% TFA in DCM) while benzyl-based protection groups require very strong acids such as HF to remove them.



Scheme 5. Deprotection mechanism of Boc protecting group.

3.1.2.2. Fmoc/^{t}Bu strategy

Fmoc group[6] allows a selective coupling between the amino terminal group of the growing peptide sequence that is attached to the resin with the carboxylic acid of the amino acid that is being added. Every time a new residue is attached to the growing chain, it is necessary to remove the Fmoc. This protecting group is stable in front of acids, but not in front of bases such as piperidine. Conditions used for Fmoc group removal are piperidine/DMF (20:80, v/v).



Scheme 6. Deprotection mechanism of Fmoc protecting group.

The adduct formed between the piperidine and the dibenzofulvene is visible on the UV, allowing the quantification of amino acid that has been loaded.

In the present project, Fmoc strategy is used because it requires milder conditions, since HF is needed in Boc/Bzl strategy to perform the cleavage. All the amino acids used were already protected with Fmoc group.

It is worth mentioning that the ^{t}Bu group is not appropriate to protect all the side chains of different amino acids. In our case, we were interested in studying a peptide chain containing residues of Arg, Cys and Lys, frequently protected with Pbf, Trt and Boc, respectively.

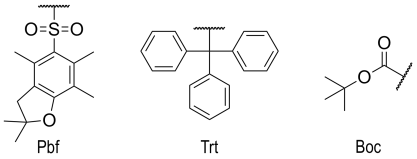


Figure 2. Pbf, Trt and Boc protecting groups of Arg, Cys and Lys, respectively.

The deprotection conditions of these protecting groups, as well as the conditions for ^{t}Bu group, are summarised in the Table 1. In all cases, the deprotection was performed in strong acidic conditions[8]. TIPS acts as scavenger and its function is to capture carbocations.

Entry	Amino acid	Protecting group	Removal conditions
1	Arginine	Pbf	90% TFA, 5%TIPS, 5%H_2O Milli-Q
2	Cysteine	Trt	95% TFA, 2.5%TIPS, 2.5%H_2O Milli-Q
3	Lysine	Boc	25-50%TFA-DCM
4	Serine	^{t}Bu	90% TFA-DCM

Table 1. Deprotection conditions of Pbf, Trt, Boc and ^{t}Bu protecting groups.

3.1.3. Polymeric suport

Reactions in SPPS take place on the surface of a solid polymeric support[3], where the first amino acid binds covalently to it. The properties that the polymeric support should have are:

- Good solvation, as it determines the efficiency of the synthesis.
- Physical stability.
- Allow the rapid filtration of solvents that may contain excess of reagents or by-products.
- An adequate loading (mmol of functional group per gram of resin).

There are some resins used in SPPS as polymeric support such as polystyrene, Merrifield Resin (chloromethylated polystyrene), hydroxymethyl Resin or trityl Resin, among others. In the present project, 2-chlorotrityl resin (2-CTC) is used. This resin is acid labile and can be cleaved with TFA.

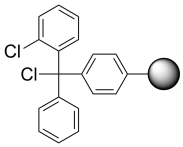
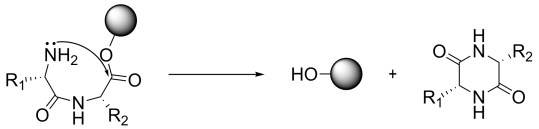


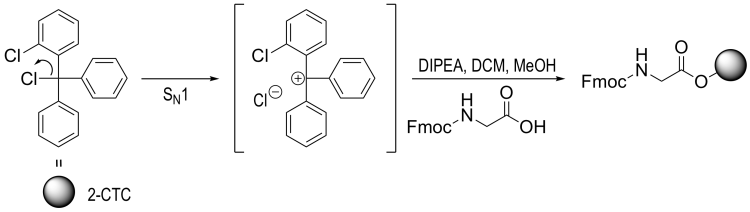
Figure 3. 2-CTC resin.

It is very used in SPPS since the bulky triphenylmethyl group prevents the cyclization on the anchoring linkage causing premature cleavage. Moreover, 2-CTC resin reduces the formation of 2,5-diketopiperazines, an important side reaction in Fmoc chemistry.



Scheme 7. Formation of DKP.

When attaching the first amino acid, an ester group is generated between the first amino acid and the polymeric support, yielding a carboxylic group in the C-terminal end of the peptide after the cleavage.



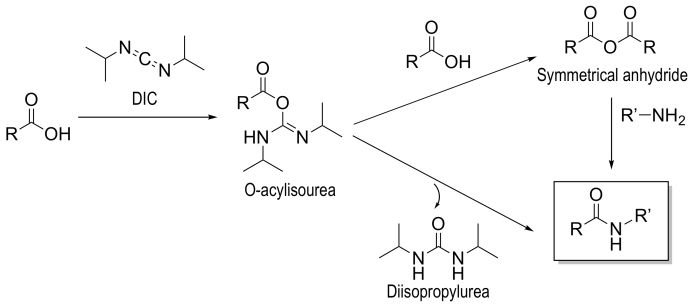
Scheme 8. Coupling of the first amino acid (Fmoc-Gly-OH) with 2-CTC resin

3.1.4. Coupling reagents

Dicyclohexylcarbodiimide (DCC) and diisopropylcarbodiimide (DIC)[9] are commonly used to construct amides, esters and acid anhydrides. Activation of a carboxylic acid with carbodiimides leads to the formation of ureas as by-products. DCC generates dicyclohexylurea, which its main shortcoming is its low solubility in organic solvents. This makes DCC a more appropriate coupling reagent for peptide synthesis in solution. On the other hand, DIC is commonly used in SPPS as the urea that is formed is soluble in organic solvents and can be easily removed by filtration.

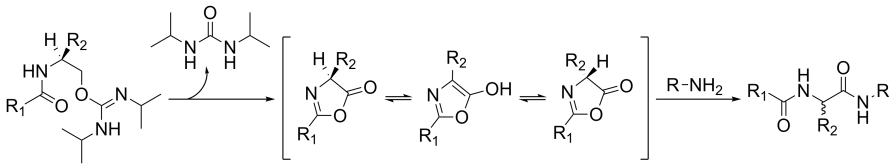
DIC activates a carboxylic acid to form an O-acylisourea as a reactive intermediate. This intermediate contains a good leaving group which can undergo aminolysis with another amino

acid leading to the formation of an amide bond and the diisopropylurea as a by-product. On the other hand, the reaction of the O-acylisourea with an unreacted carboxyl group yields to the formation of a symmetrical anhydride. This anhydride can react with the α-amino group from an amino acid of the growing chain leading to the formation of an amide bond[6] (Scheme 8).



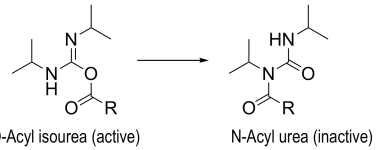
Scheme 9. Amide bond formation with DIC.

Activation of the carboxylic acid of amino acids via carbodiimides often leads to a partial racemization of the amino acid through the formation of an oxazolone (Scheme 10):



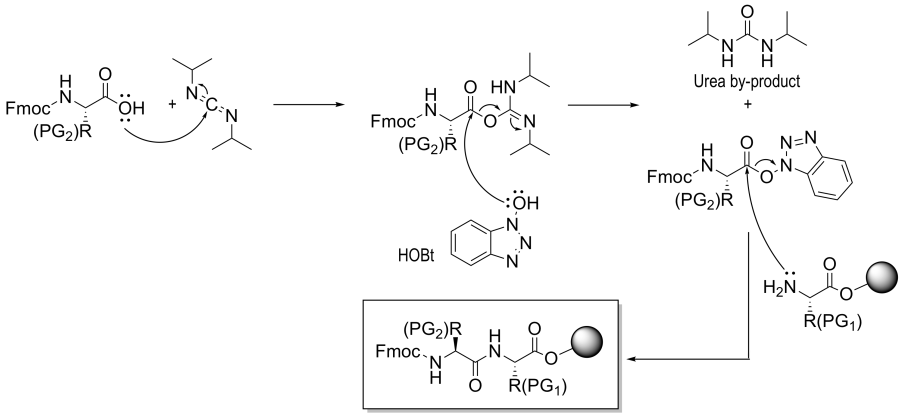
Scheme 10. Racemization via oxazolone formation.

Another side reaction is the formation of N-acylurea derivatives, which are not reactive (Scheme 11):



Scheme 11. O→N acyl transfer.

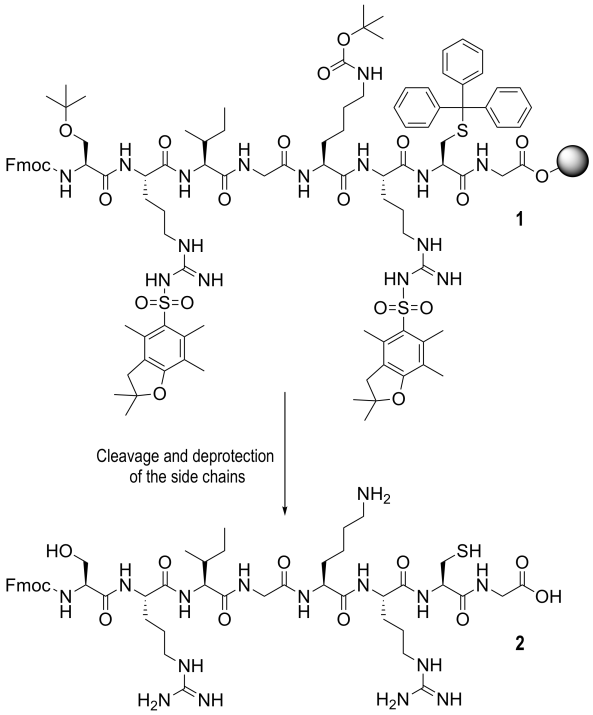
These side reactions can be avoided by introducing additives. These additives react with the highly reactive O-acylisourea leading to the formation of an active ester, which is more stable and less reactive. In the present project HOBt is used as an additive (Scheme 12).



Scheme 12. DIC/HOBt Coupling.

4. OBJECTIVES

The main objectives of this work are the synthesis a model peptidyl-resin of eight residues of different amino acids and to study its cleavage conditions.



Scheme 13. Cleavage and deprotection of the side chains of the model peptide.

This particular model peptidyl-resin was designed to mimic a sequence that in previous studies from our research group, lead to complex product chromatograms due to partial deprotection in the standard cleavage conditions. It was chosen in order to optimize the cleavage conditions and achieve full deprotection.

5. RESULTS AND DISCUSSION

The work described in this project can be divided in two parts:

1. Synthesis of the peptidyl-resin **1**.
2. Study of the cleavage conditions of peptidyl-resin **1**.

5.1 SYNTHESIS OF THE MODEL PEPTIDYL-RESIN

The model peptidyl resin has been synthetized using the Fmoc/tBu strategy as it requires milder conditions than Boc/Bzl (Section 3.1.2.2). The polymeric support used was 2-CTC resin (Section 3.1.3). DIC and HOBt were used as coupling reagent and additive, respectively. The protocol followed during the synthesis of the peptide and its cleavages is described in Section 6.3.1. The concentration in SPPS during the couplings of the amino acids is an important fact, as the molecules will react more if the concentration is higher. This is the reason why the minimum quantity of solvent was used.

As the main objective of the synthesis was to study the deprotection steps of the model peptide, it was important to obtain it as pure as possible, free of deletion chains. After each coupling, a Ninhydrin test was performed in order to ensure that the coupling had occurred quantitatively (Section 6.2.1). Table 2 summarises the number of couplings of each amino acid that have been carried out for each of the three synthesis of **1** (Resins A, B and C, respectively). By following this protocol, quantitative couplings were achieved in each stage of the synthesis.

The first step of the synthesis consists on the attachment of the first amino acid to the resin, which has a loading of 30-40%. The quantification of the loading is performed by using UV-Vis spectrometry (Section 6.2.2). The values obtained are used to calculate the quantity of reactants needed in the posterior steps of the synthesis, as well as to ensure if the coupling has been quantitative. Unfortunately, the results obtained for the first synthesis (Synthesis A) were inconsistent with those obtained by Ninhydrin test (Table 3).

Entry	Amino acid	Number of couplings		
		Synthesis A	Synthesis B	Synthesis C
1	Fmoc-Cys-(Trt)-OH	1	1	3
2	Fmoc-Arg(Pbf)-OH	2	1	3
3	Fmoc-Lys(Boc)-OH	1	2	2
4	Fmoc-Gly-OH	1	1	1
5	Fmoc-Ile-OH	2	1	1
6	Fmoc-Arg(Pbf)-OH	1	2	2
7	Fmoc-Ser(tBu)-OH	1	3	1

Table 2. Number of couplings of each AA for each of the three synthesis.

Entry	Amino acid	Fmoc quantification	Ninhydrin test
1	Fmoc-Gly-OH	30 %	Quantitative
2	Fmoc-Cys-(Trt)-OH	95 %	Quantitative
3	Fmoc-Arg(Pbf)-OH	92 %	Quantitative
4	Fmoc-Lys(Boc)-OH	73 %	Quantitative
5	Fmoc-Gly-OH	65 %	Quantitative
6	Fmoc-Ile-OH	51 %	Quantitative
7	Fmoc-Arg(Pbf)-OH	92 %	Quantitative
8	Fmoc-Ser(tBu)-OH	-	Quantitative

Table 3. Fmoc quantification and Ninhydrin test for Synthesis A.

On the other hand, it is worth noting that the yields obtained after the cleavages (Table 4) suggest that they were quantitative.

Entry	Cleavage	Time of cleavage [min.]	Yield [%]
1	A1	120	99
2	A2	150	94

Table 4. Yields for cleavages A1 and A2.

Results were more surprising when the synthesis of the peptide was repeated (Synthesis B). As it can be observed in the table below, the results obtained by the Fmoc quantification were incongruous with those obtained by ninhydrin test.

Entry	Amino acid	Fmoc quantification	Ninhydrin test
1	Fmoc-Gly-OH	35 %	Quantitative
2	Fmoc-Cys-(Trt)-OH	57 %	Quantitative
3	Fmoc-Arg(Pbf)-OH	-	Quantitative
4	Fmoc-Lys(Boc)-OH	43 %	Quantitative
5	Fmoc-Gly-OH	31 %	Quantitative
6	Fmoc-Ile-OH	37 %	Quantitative
7	Fmoc-Arg(Pbf)-OH	21 %	Quantitative
8	Fmoc-Ser(tBu)-OH	-	Quantitative

Table 5. Fmoc quantification and Ninhydrin test for Synthesis B.

In this case, the yields obtained after the cleavages were notably lower than those obtained for synthesis A:

Entry	Cleavage	Time of cleavage [min.]	Yield [%]
1	B1	30	27
2	B2	60	21
3	B3	90	23

Table 6. Yields for cleavages B1, B2 and B3.

These results suggested that Fmoc quantification was not reliable. This could lead us to underestimate or overestimate the loading of the resin and to wrongly calculate the quantity of reactants and theoretical yields. In the case of underestimating the loading, the excess of reactants may not be enough and therefore, deletion products will be obtained, leading to complex mixtures of peptides after the cleavage.

We consider that this is one of the factors that may contribute to the appearance of complex mixtures in some of the experiments that were carried out by our research group. In our case, this shortcoming was partially balanced out by repeating the couplings as many times as necessary until the ninhydrin test was negative.

The Fmoc quantification (Section 6.2.2) proved difficult, even though it is an analytical method widely used in this field. In order to solve this problem, some calibration measures of the UV-Vis spectrophotometer were carried out, revealing that the system did not work correctly, yielding to a low sensibility below 301 nm. As there was not enough time to repair it during the project length, the methodology was changed and for a third synthesis (synthesis C), the loading was quantified by weight difference. Thus, a good drying of the resin was required before and after each reaction.

For synthesis C, it was assumed that the yield of each coupling was quantitative (as for each coupling, ninhydrin test confirmed it), so the same excess of reactants (calculated from the initial loading) was used. The overall yield was calculated and turned out to be quantitative.

5.2 STUDY OF THE CLEAVAGE CONDITIONS

Taking into account that previous experiments carried out by our research group suggested that the deprotection of the side chains was not totally completed when using standard times of cleavage (1-2 hours), it was decided to perform two first experiments of larger reaction times (2 hours 30 min. and 2 hours) (See Figures 4-5 and Scheme 13).

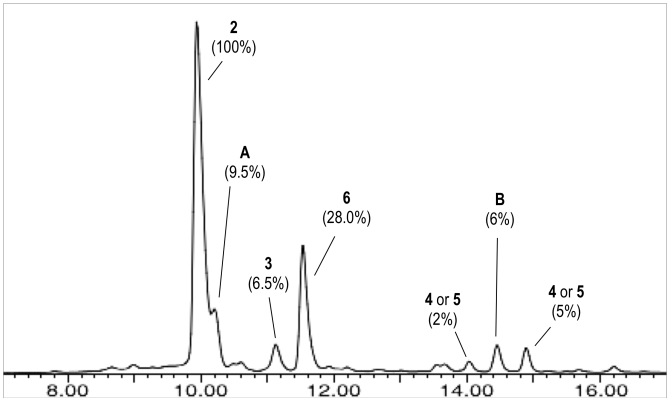


Figure 4. Chromatogram Table 7, entry 1.

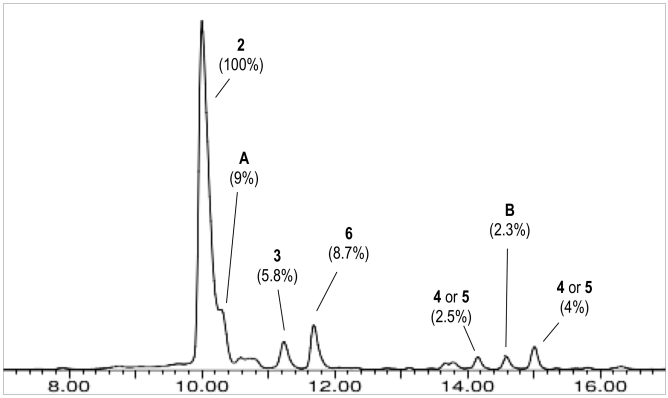


Figure 5. Chromatogram Table 7, entry 2.

It is worth mentioning that the products of these cleavages were obtained from Resin A corresponding to synthesis A (See Table 2). The chromatograms were recorded in a detection wavelength of 301 nm, which is the maximum absorption of Fmoc protecting group. The masses were obtained from the HPLC coupled with MS (Section 6.1.3). Peak percentages were obtained by integrating the peak areas of the chromatogram.

As it can be observed, aside from peaks belonging to partial protected peptide, some impurities of peptide nature can also be observed. Nevertheless, the impurities of partial protected peptide belong to peptide with tBu (impurity **3**), the peptide with one of the Pbf (impurity **4**) and the peptide with the other Pbf (impurity **5**) (Figure 9).

Surprisingly, significant differences between both chromatograms were not observed. Thus, it was decided to carry out 3 more experiments with the product obtained on the second synthesis (synthesis B, resin B) with reaction times of 1 h 30 min., 1 h and 30 min. (Table 7).

Entry	Resin	Time of cleavage [min.]	Conditions
1	A	150	95 % TFA; 2.5 % TIPS and 2.5 % Milli-Q water
2	A	120	
3	B	90	
4	B	60	
5	B	30	

Table 7. Cleavage conditions for experiments belonging to synthesis A and B.

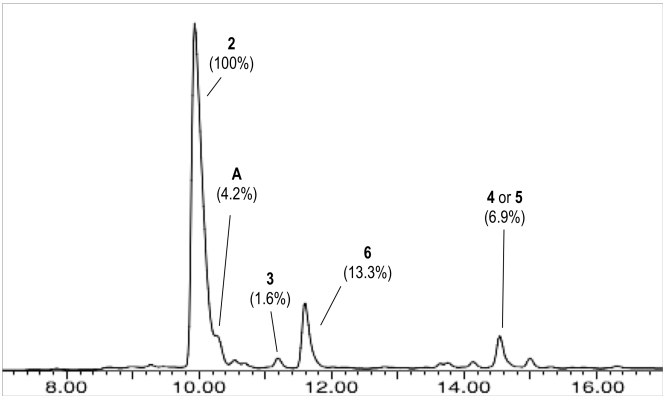


Figure 6. Chromatogram Table 7, entry 3.

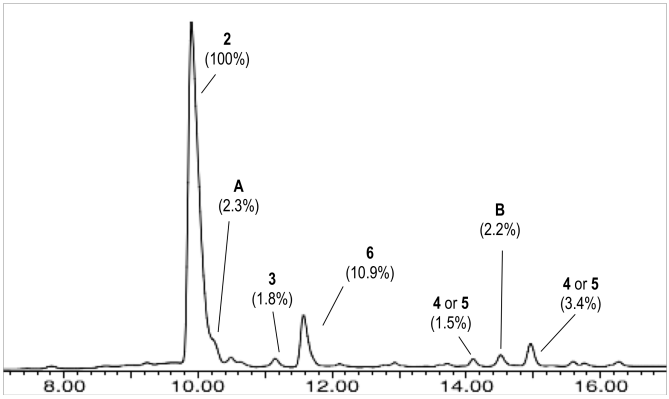


Figure 7. Chromatogram Table 7, entry 4.

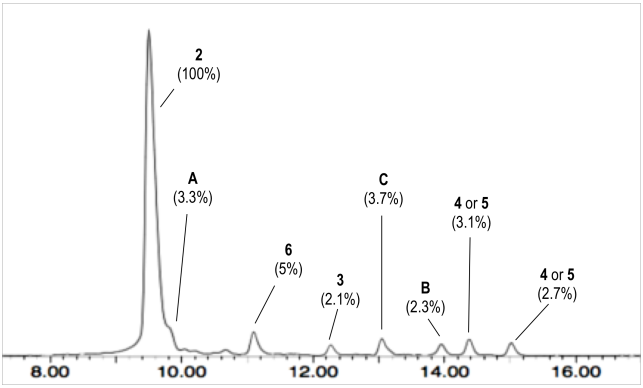


Figure 8. Chromatogram Table 7, entry 5.

Significant changes were neither observed in these experiments, and therefore, it was concluded that the deprotection was fast, even though small quantities were always present in the chromatograms, even at low reaction times.

On the other hand, it was observed that the main impurity did not belong to any of those protected peptide impurities. It belonged to a peak of m/z=732, which showed a rise of its intensity (from 5% to 28%) as the reaction time of cleavage increased (from 30 min. to 2 h 30 min., respectively).

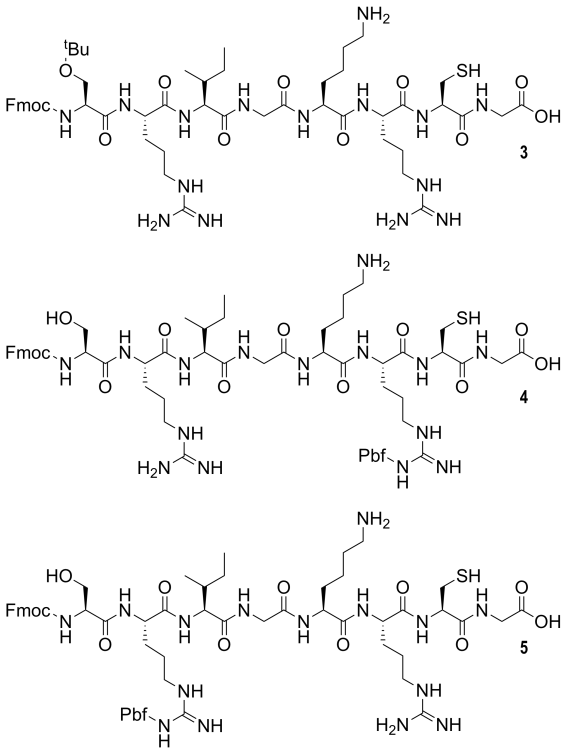
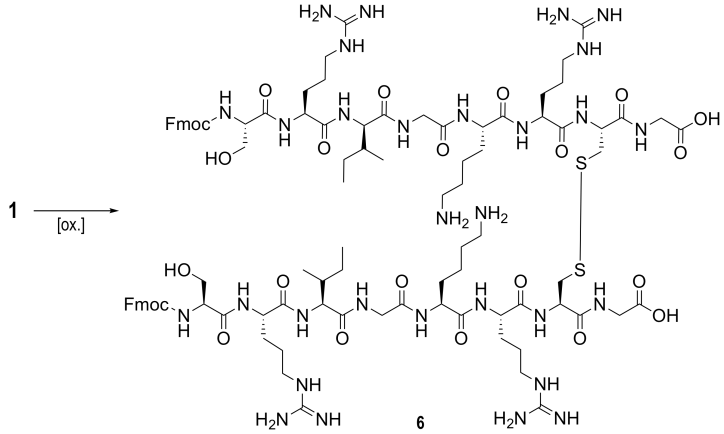


Figure 9. Peptide impurities.

The HPLC-MS analysis of peak **6**, showed a mass of 732, which belongs to the [M+3]/3 of a peptide dimer **6**. The dimer is obtained as a result of the oxidation of the Cys residue during the cleavage reaction (Scheme 14).

This result concluded that the main impurity of our model peptide belonged to the dimer. In order to minimize its formation, it was decided to introduce a reducing agent in the reaction mixture

instead of Milli-Q water for the next experiments with the product obtained on the last synthesis (synthesis C) belonging to resin C. (See Table 8)

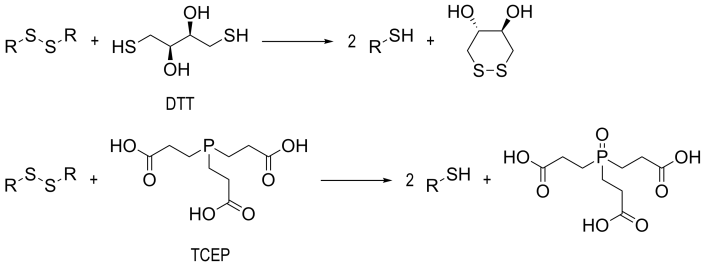


Scheme 14. Oxidation of the Cys, yielding to a dimer.

Entry	Resin	Time of cleavage [min.]	Conditions
1	C	90	95 % TFA; 2.5 % TIPS and 2,5 % DTT
2	C	90	95 % TFA; 2.5 % TIPS and 2,5 % TCEP
3	C	120	
4	C	60	
5	C	30	
6	C	5	
7	C	90	92.5 % TFA; 5 % TIPS and 2.5 % TCEP

Table 8. Cleavage conditions for experiments belonging to synthesis A and B.

Two different reducing agents (DTT and TCEP)[10,11] were tested using the same reaction time of cleavage (Table 8, entries 1 and 2). The reducing reactions that these reactants suffer are shown in the following scheme.



Scheme 15. Reactions of DTT and TCEP, respectively.

Differences between these reagents were rapidly observed. The DTT did not minimize the impurity **6** as it can be observed (Figure 10). On the other hand, using the TCEP as reducing agent lead to a total reduction of the dimer **6**.

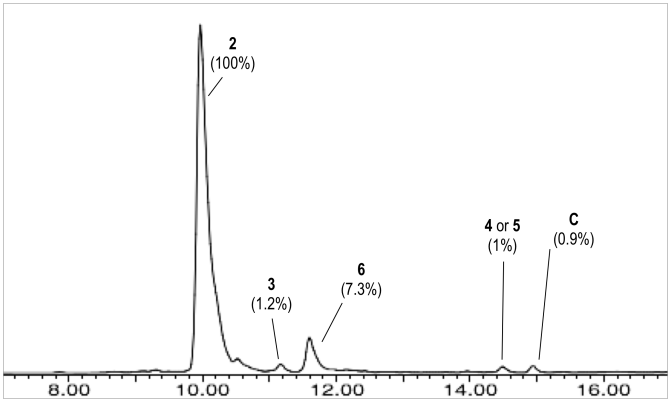


Figure 10. Chromatogram Table 8, entry 1.

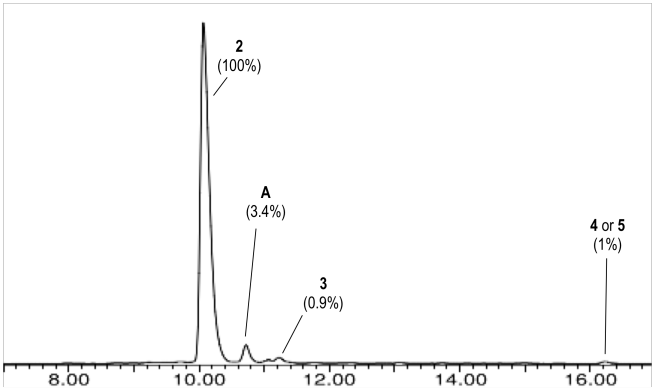


Figure 11. Chromatogram Table 8, entry 2.

Moreover, TCEP not only reduced the formation of dimer, but also minimised the presence of other impurities. At this point, it was decided to carry out the next experiments by only using TCEP as a reducing agent under the same conditions (Table 8, entries 3, 4, 5 and 6), only changing the reaction times of cleavage. The chromatograms below show the obtained results.

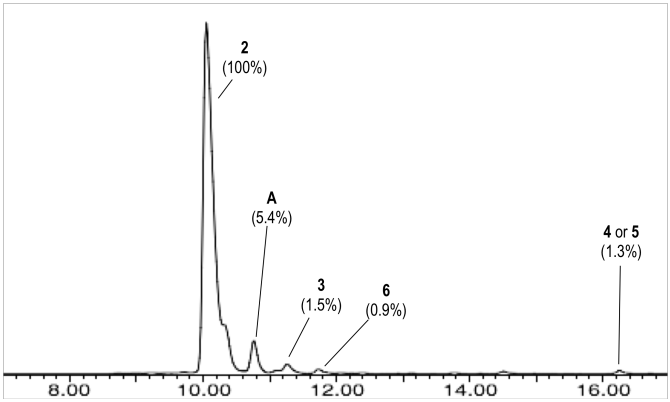


Figure 12. Chromatogram Table 8, entry 3.

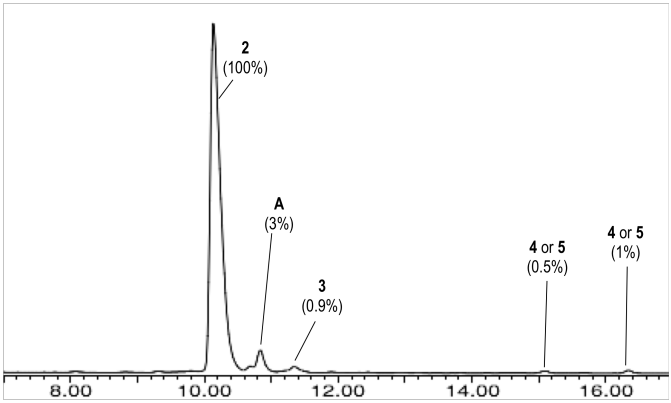


Figure 13. Chromatogram Table 8, entry 4.

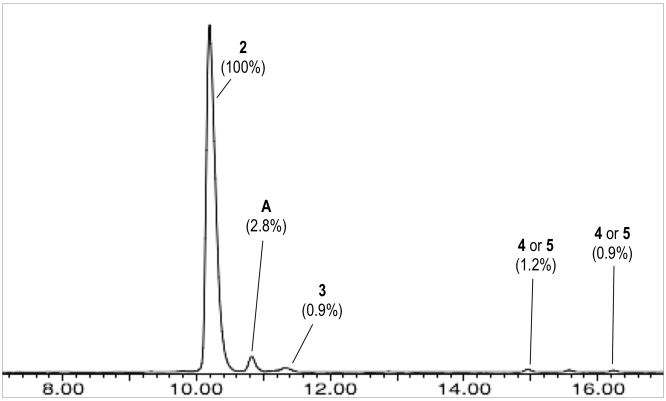


Figure 14. Chromatogram Table 8, entry 5.

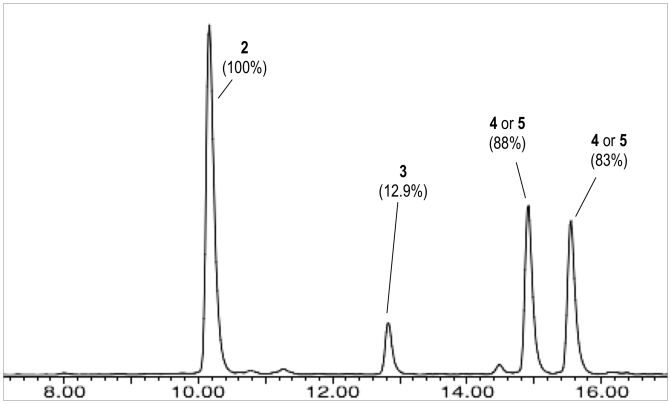


Figure 15. Chromatogram Table 8, entry 6.

Significant differences were not observed between the first three chromatograms. It is worth mentioning that in the chromatogram with the largest time of cleavage (Table 8, entry 3) appeared a small peak of impurity **6**, which belonged to the dimer. It was again concluded that the dimer showed a rise of its intensity as the reaction time of cleavage was increased.

However, significant differences were observed on the last chromatogram (Table 8, entry 6). At this time of cleavage, the peptide impurities **3**,**4** and **5** showed an increase, revealing that 5 minutes of cleavage were not enough to totally deprotect the side chains.

Finally, one last experiment was carried out (Table 8, entry 7) in order to test if it was possible to totally eliminate the impurities **A**, **3**, **4** and **5**. It was thought that these impurities could be

eliminated by increasing the percentage of scavenger (TIPS). Thus, the percentage of TIPS was increased from 2.5% to 5%.

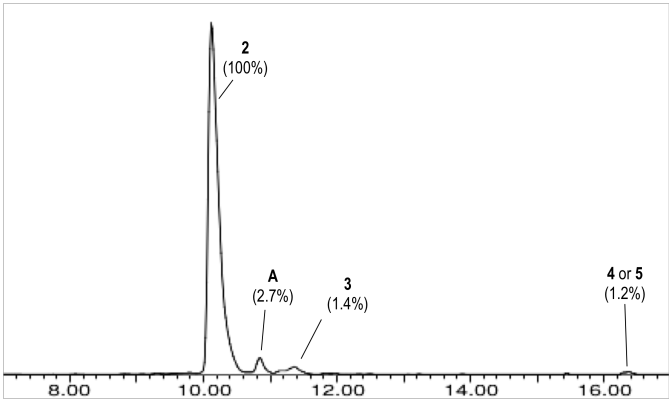


Figure 15. Chromatogram Table 8, entry 7.

Unfortunately, significant differences were not observed as the impurities **A**, **3** and **4** or **5** were still present in the obtained chromatogram.

6. EXPERIMENTAL SECTION

6.1. MATERIALS AND METHODS

6.1.1. Reagents and solvents

Entry	Product	Brand
1	Amino acids	Irish-Biotech
2	2-CTC resin	
3	DIPEA	
4	TIPS	Sigma Aldrich
5	DTT	
6	TFA	Fisher Bioreagents
7	DIC	TCI
8	Piperidine	Panreac
9	DMF	Carlo Erba Reagents
10	DCM	Scharlau
11	MeOH	
12	Acetone	
13	TCEP[a]	-
14	HOBt[b]	-

a) TCEP reactant was obtained from the Parc Científic de Barcelona.
b) HOBt reagent was provided by a pharmaceutical company.

Table 9. Reagents and solvents used during the synthesis and the cleavages.

6.1.2. Instruments

Entry	Instrument	Brand	Model
1	UV-Vis spectroscopy	Varian	Cary 100
2	HPLC-MS	Shimadzu[a]	LC-20AD
3	HPLC	Waters[b]	2695 separation module
4	Analytical Balance	Mettler	Toledo AB254
5	Centrifuge	Hettich	Rotofix 32A
6	Water purification system	Millipore	Milli-Q Plus Water Purification System

a) Shimadzu system composed by a LC-20AD quaternary pump, an automatic injector SIL-Dvp, a dual variable wavelength detector SPD-20AD and an online degasser device DGU-20A5.
b) Waters system composed by a 2695 separation module, a PDA detector 2996 and a Micromass ZQ mass detector (ESI_MS).

Table 10. Instrumentation used.

6.1.3. Chromatography

A C18 reverse phase column supplied by Grace Vydac of 250x4.6 mm with 5 microns of size particle and a pore size of 300 Å was used. The eluents were H_2O with 0.045 % TFA (A) and ACN with 0.036 % TFA (B). Gradient of H_2O/TFA and ACN/TFA is described in the following table, at a flow rate of 1 mL/min. with detection (λ) at 301 nm, which is the maximum of absorption of Fmoc protecting group.

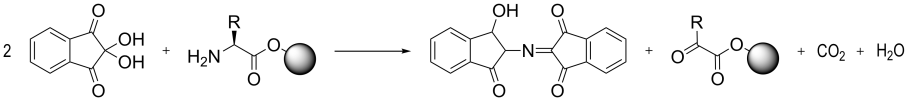
Entry	Time (min)	%A	%B
1	0	80	20
2	2	80	20
3	30	0	100
4	35	0	100
5	36	80	20
6	45	80	20

Table 11. Gradient of eluents H_2O/TFA (A) and ACN/TFA (B).

6.2. ANALYTICAL METHODS

6.2.1. Ninhydrin Test

Ninhydrin or Kaiser Test is a qualitative test used to detect free primary amino groups. It is used in SPPS as an indicator of the completeness of a coupling step. It is based on the reaction between ninhydrin and a primary amine (Scheme 16).



Scheme 16. Ninhydrin test reaction.

The product obtained has a characteristic dark blue colour. If the amino acid has been successfully coupled, there will not be any free amino group, and the ninhydrin test will give a colourless or yellow result. On the other hand, if the couple has not been successful, the ninhydrin will give a dark blue colour.

To perform the assay, three solutions (already prepared in the laboratory) are needed:

Reagent A: 5 g of ninhydrin in 5 mL of EtOH.

Reagent B: 80 g of phenol in 20 mL of EtOH.

Reagent C: 2 mL of NaCN 0.01 M in 98 mL of pyridine distilled over ninhydrin.

The analytical procedure is as follows: the peptide resin is previously washed with NMP to remove the excess of reagents and then with MeOH, in order to dry the resin. A small quantity of the peptide resin is introduced into a small tube and 2 drops of each reagent are added. The tube with the solution is heated at 110 ºC for 3 minutes. A blank is performed in parallel to compare colours. A light-yellow coloration (negative test) indicates the absence of primary amines, and so a complete coupling of the amino acid. A dark blue coloration (positive test) indicates the presence of primary amines and so an incomplete coupling of the amino acid.

Figure 16. On the left, ninhydrin negative test. On the right, ninhydrin positive test.

6.2.2. Fmoc quantification

The quantification of Fmoc group gives useful information about the progress of the peptide elongation. This can be easily done by quantifying the Fmoc removed before coupling a new amino acid by using UV-Vis spectroscopy and measuring the absorbance of the adduct formed (see Scheme 16).

To carry out the quantification, the resin is previously washed 3 times consecutively with about 4 mL of DMF/DCM/DMF (1 min. per wash). Then, the resin is treated with 20 % piperidine in DMF (1 mL per 200 mg of resin) during 5 minutes. After this, the same treatment with 20 % piperidine in DMF is added and left reacting during 10 minutes. Washings with 1 mL/200 mg of DMF (3x1 min.) are carried out after treating the resin.

The mixture of piperidine in DMF and further washings with DMF are collected in a 100 mL volumetric flask and diluted in DCM. The solution is diluted in a 10 mL volumetric flask in a proportion of 2:10. A blank is prepared in a 25 mL volumetric flask with 0.5 mL of 20 % piperidine in DMF, 0.75 mL of DMF and diluted in DCM. Finally, the absorbance is measured and the concentration can be calculated using the Lambert Beer Law (ε=7800 M^{-1} at 301 nm with a l=1 cm).

NOTE: As mentioned in Section 5.1, these measures did not provide useful information because of a hardware malfunction of the Varian Cary 100 UV-Vis spectrometer.

6.3. SYNTHETIC PART

6.3.1. General procedures

6.3.1.1 Solid phase synthesis

The synthesis of the peptide was performed manually using solid phase chemistry and Fmoc strategy (Sections 3.1.1 and 3.1.2.2)[12,13].

A 20 mL syringe fitted with a filter was used in order to carry out the reaction and occasional stirring was done manually with a Teflon stirring rod. The solvents and the excess of reactants were removed using a vacuum system.

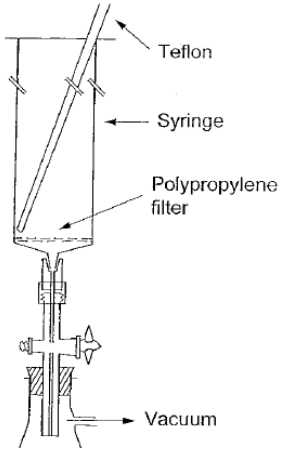


Figure 17. Synthesis system.
(Image obtained from ref. 13)

6.3.1.2. Conditioning of the resin

Resin was weighted in a syringe. Once the syringe was connected to the system shown in figure 16, the resin was then washed 3 times consecutively with about 4 mL of DCM, DMF, and DCM (1 min. per wash) to swell and deflate it, in order to increase the contact surface and favour the elimination of impurities.

6.3.1.3. Coupling of the first amino acid: preparation of Fmoc-Gly-resins

Fmoc-Gly-OH amino acid, DIPEA and a minimum volume of DCM were added to the resin and occasionally stirred during 1.5 hours. Then, an excess of MeOH was added, and the reaction was left for 30 additional minutes. The purpose of this step (capping) is to link a small nucleophile (MeOH) to the unreacted carbocations of the 2-CTC resin. Finally, the solvents and the excess of reactants were filtered and the resin was then washed 3 times consecutively with DCM (1x4 mL, 1 min.). A last wash with MeOH (1x4 mL, 1 min.) was carried out and the resin was dried at reduced pressure.

6.3.1.4. Elongation of the peptide chain: preparation of peptidyl-resins

The Fmoc-Gly-resin was weighted in a syringe and washed 3 times consecutively with about 4 mL DMF/DCM/DMF (1 min. per wash). Once the Fmoc was removed and quantified by UV-Vis (as described in Section 6.2.2), the resin was washed 3 times consecutively with about 4 mL NMP/DCM/NMP (1 min. per wash). After that, 3 eq of the new amino acid, 3 eq of the coupling reagent (DIC) and the minimum quantity of solvent (NMP) were added into the syringe. The reaction was left for an hour. Before filtering the excess of solvent and reactants, an aliquot was taken and ninhydrin test was performed. If the test was positive, the reaction was left for 30 additional minutes. Finally, the solvent and the excess of reactants were filtered, and the resin was washed with NMP and MeOH. In order to ensure the reaction was complete, the ninhydrin test (described in Section 6.2.1) was carried out. If ninhydrin test was still partially positive, the coupling was repeated again by following the same steps.

After coupling the last amino acid, the Fmoc group was not removed, in order to facilitate its characterisation by HPLC-MS.

6.3.1.5. Cleavage of the peptide from the resin

The peptidyl-resin was washed 3 times consecutively with about 4 mL of DMF, DCM, and DMF (1 min. per wash). Then, 1 mL of the cleavage mixture was added to 100 mg of the resin

(the mixtures used for the cleavages are described in Tables 7 and 8). The reaction time varied for each cleavage (see Section 5.2). The filtrate was collected in a round-bottomed flask with 30 mL of methyl *tert*-butyl ether to precipitate the peptide. The suspension was trespassed onto a Falcon tube and centrifuged, the supernatant liquid was decanted and the precipitate was dried on a vacuum.

6.3.1.6. Preparation of the HPLC-MS sample

An aliquot of 1 mg of the peptide was weighed in a HPLC vial and dissolved in 1 mL of MeOH (HPLC grade), using the ultrasonic bath if needed. Finally, the solution was filtered through a 0.45 u PTFE hydrophobic filter in another vial.

6.4 SYNTHESIS OF THE PEPTIDE

6.4.1. Preparation of Fmoc-Gly-resins

Two Fmoc-Gly-resin batches where prepared according to the procedure described in section 6.3.1.2 using the quantities summarised in the table below.

Entry	Resin [mg]	Fmoc-Gly-OH [mg]	DIPEA [mL]	Loading [%]
1[a]	807	529	1.53	30-35[c]
2[b]	811.25	400	0.466	25[d]

a) For the first synthesis of the Fmoc-Gly-resin, 2 eq of Fmoc-Gly-OH and 10 eq of DIPEA were used.
b) For the second synthesis of the Fmoc-Gly-resin, 1 eq of Fmoc-Gly-OH and 3 eq of DIPEA were used.
c) Loading was calculated by Fmoc quantification. As mentioned in Section 5.1, it is not reliable.
d) Loading was calculated by difference of weight, as decribed in Section 5.1.

Table 12. Quantities of reactants used for synthesis A.

6.4.2. Synthesis of peptidyl-resin 1

Three batches of 1 were prepared according to the general procedure described in Section 6.3.1 using the quantities summarised in Tables 12, 13, 14, and 15.

6.4.2.1 Synthesis A

This batch was performed using 201 mg or Fmoc-Gly-resin (Table 12, entry 1). Quantification of the Fmoc was not reliable (Section 5.1) so the yield could not be properly determined.

Entry	Amino acid	Amino acid [mg]	HOBt [mg]	DIC [µL]
1	Fmoc-Cys(Trt)-OH	183	32	
2	Fmoc-Arg(Pbf)-OH	135	36	
3	Fmoc-L-Lys(Boc)-OH	96	33	
4	Fmoc-Gly-OH	62	32	31.37
5	Fmoc-L-Ile-OH	75	32	
6	Fmoc-Arg(Pbf)-OH	132	32	
7	Fmoc-L-Ser(tBu)-OH	78	32	

Table 13. Quantities of reactants used for synthesis A.

6.4.2.2 Synthesis B

This batch was performed using 403 mg or Fmoc-Gly-resin (Table 12, entry 1). Quantification of the Fmoc was not reliable (Section 5.1) so the yield could not be properly determined.

Entry	Amino acid	Amino acid [mg]	HOBt [mg]	DIC [µL]
1	Fmoc-Cys(Trt)-OH	279	2	
2	Fmoc-Arg(Pbf)-OH	301	77	
3	Fmoc-L-Lys(Boc)-OH	217	70	
4	Fmoc-Gly-OH	137	71	71.66
5	Fmoc-L-Ile-OH	164	74	
6	Fmoc-Arg(Pbf)-OH	303	74	
7	Fmoc-L-Ser(tBu)-OH	177	72	

Table 14. Quantities of reactants used for synthesis B.

6.4.2.3 Synthesis C

This batch was performed using 811,25 mg or Fmoc-Gly-resin (Table 12, entry 2). The yield was 96% (determined by difference of weight, see Section 5.1).

Entry	Amino acid	Amino acid [mg]	HOBt [mg]	DIC [µL]
1	Fmoc-Cys(Trt)-OH	400	105	
2	Fmoc-Arg(Pbf)-OH	444	108	
3	Fmoc-L-Lys(Boc)-OH	321	106	
4	Fmoc-Gly-OH	207	106	106
5	Fmoc-L-Ile-OH	242	107	
6	Fmoc-Arg(Pbf)-OH	449	106	
7	Fmoc-L-Ser(tBu)-OH	264	107	

Table 15. Quantities of reactants used for synthesis C.

6.4.3. Cleavage

Peptidyl-resins synthesised in Section 6.4.2 were cleaved according to general procedure described in Section 6.3.1.5. The reagents and conditions used in these experiments are summarised in Tables 7 and 8.

7. CONCLUSIONS

The model peptidyl-resin **1** has been synthesised successfully using standard Fmoc chemistry. This model peptidyl-resin was used to study the impurities appearing in its cleavage.

Cleavage of **1** with 95 % TFA, 2.5 % TIPS and 2.5 % Milli-Q water provided the peptide **2** as the main product (HPLC-MS). Partially deprotected peptides **3**, **4** and **5** were detected in small percentages. The main impurity corresponded to the dimer **6**. Other impurities were also present in small percentages but could not be identified.

Cleavage of **1** was tested at different reaction times and it was observed that cleavage of the peptide from the resin was very quick (about 5 min.).

Deprotection of the side chains was also fast. No significant differences were appreciated when changing the reaction time from 30 minutes to 2 hours and 30 minutes. Nevertheless, the chromatograms always showed residual quantities of **3**, **4** and **5**.

On the other hand, the percentage of dimer **6**, the main impurity of the cleavage, showed a rise in its intensity (from 5 % to 28 %) as the reaction time increased. This result was interpreted assuming that the formation of **6** arose from the oxidation of the Cys residues of the free peptide, because of the oxygen and other oxidants remaining in the reaction mixture. At short times of cleavage, the percentage of dimer is very small.

The addition of DTT as a reducing agent in the reaction mixture did not prevent the formation of **6**. However, using TCEP as a reducing agent reduced the formation of dimer.

8. REFERENCES AND NOTES

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9. ACRONYMS

Arg	Arginine
Boc	tert-butoxycarbonyl
Bzl	benzyl
2-CTC	2-chlorotrityl resin
Cys	Cysteine
DCC	N,N'-Dicyclohexylcarbodiimide
DCM	dichloromethane
DMF	N,N-dimethylformamide
DIC	N,N'-diisopropylcarbodiimide
DIPEA	N,N'-diisopropylethylamine
DTT	Dithiothreitol
eq	molar equivalent
Fmoc	9-fluorenylmethyloxycarbonyl
Gly	Glycine
HOBt	hydroxybenzotriazole
HPLC	high performance liquid chromatography
HPLC-MS	high performance liquid chromatography-mass spectrometry
Ile	Isoleucine
Lys	Lysine
NMP	N-methylpyrrolidine
Pbf	2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl
TCEP	tris(2-carboxyethyl)phosphine
TFA	trifluoroacetic acid

TIPS	triisopropylsilane
Trt	trityl
Ser	Serine
SPPS	solid-phase peptide synthesis